GENETIC ENGINEERING OF TNF FAMILY PROTEIN-BASED VACCINES FOR ANTITUMOR IMMUNOTHERAPY

by

Zoya R. Yurkovetsky

BS, University of Pittsburgh, 1996

Submitted to the Graduate Faculty of

School of Engineering in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2003

UNIVERSITY OF PITTSBURGH

SCHOOL OF ENGINEERING

This dissertation was presented

by

Zoya R. Yurkovetsky

It was defended on

May 23, 2003

and approved by

Daniel L. Farkas, Vice Chairman and Professor, Department of Surgery, and Bioengineering

William J. Federspiel, Associate Professor, Departments of Chemical Engineering, Surgery, and Bioengineering

Johnny Huard, Associate Professor, Departments of Orthopaedic Surgery, and Biochemistry and Molecular Genetics

Dissertation Director: Paul D. Robbins, Full Professor, Departments of Molecular Genetics and Biochemistry, and Orthopaedic Surgery

© Copyright by Zoya R. Yurkovetsky 2003

GENETIC ENGINEERING OF TNF FAMILY PROTEIN-BASED VACCINES FOR ANTITUMOR IMMUNOTHERAPY

Zoya R. Yurkovetsky, PhD

University of Pittsburgh, 2003

The interaction between tumor cells and dendritic cells (DC) is a critical event for initiation and regulation of specific antitumor immune responses. Clinical data demonstrate that tumor-derived factors influence DC generation, maturation, activity, and survival both *in vitro* and *in vivo*. Therefore, DC dysfunction in the tumor-bearing hosts could be responsible for the ability of tumor cells to escape from immune recognition. However, understanding the suppressive effect of tumor on DC, and the protection of DC from tumor-induced dysfunction has not been studied. The Tumor Necrosis Factor Ligand (TNFL) family is a group of cytokines, which regulate cell functions and activities. CD40L, RANKL and 4-1BBL, TNFL proteins, were shown to influence DC activation and cytokine secretion. However, the mechanisms of action of these cytokines on immune effectors are not fully understood. Here, we have evaluated the ability of local adenoviral gene transfer of CD40L, RANKL and 4-1BBL to elicit an antitumor immune response to established tumors in mice. Our results demonstrate that intratumoral administration of these vectors resulted in a significant inhibition of murine MC38 colon and TS/A breast adenocarcinoma growth when compared with control groups. In addition, a single intratumoral injection of DC transduced with the adenoviral vectors also resulted in a significant inhibition of tumor growth. Furthermore, treatment of TS/A tumors with DC transduced with Ad-CD40L

induced a complete tumor rejection with the generation of a tumor-specific immune memory, demonstrating the strongest antitumor effect compare to Ad-CD40L or RANKL- and 4-1BBL-based therapeutic approaches. Next, we have observed that DC generated or isolated from tumor-bearing mice express low levels of CD40, CD80 and CD86 molecules, produce decreased amounts of IL-12, and demonstrate down-regulated antitumor potential. Subsequently, we have shown that CD40L, in addition to stimulating and up-regulating many DC functional activities, was able to restore co-stimulatory molecules and IL-12 protein levels, suppressed by tumor. In summary, our data demonstrate that CD40L-based immunotherapy is an effective approach for inducing antitumor immunity and rescuing DC from tumor-induced dysfunction. These results should guide the development of novel therapies for prevention of immunosuppression in cancer patients and design of novel effective immunotherapeutic strategies for cancer.

TABLE OF CONTENTS

LIST OF TABLES xii

LIST OF FIGURES xiii

1.0 INTRODUCTION 1

1.1 ANTITUMOR IMMUNE RESPONSES 1

1.1.1 Adaptive antitumor immunity 1

1.1.2 Mechanisms of tumor escape from immune recognition 2

1.1.3 Inhibition of the DC system by tumors 5

1.2 THE TUMOR NECROSIS FACTOR SUPERFAMILY 8

1.2.1 The TNF family ligands 8

1.2.2 The TNF receptor family 9

1.2.3 Regulation of DC maturation and function by CD40L 10

1.2.4 CD40/CD40L in tumor immunology 11

1.2.5 RANK Ligand (RANKL) 13

1.2.6 4-1BB Ligand (4-1BBL) 14

1.3 IMMUNE GENE THERAPY 16

1.3.1 Immunotherapy 16

1.3.2 Gene Therapy 17

1.3.3 Experimental therapeutic strategies 18

1.3.4 Limitations of modern DC-based immunotherapies ... 19
1.4 STATEMENT OF THE PROBLEM ... 21
1.5 SIGNIFICANCE ... 24
2.0 TUMOR-INDUCED DC DYSFUNCTION AND DOWN-REGULATION OF CD40 EXPRESSION ... 26
2.1 INTRODUCTION ... 26
2.2 MATERIALS AND METHODS ... 29
2.2.1 Animals ... 29
2.2.2 Tumor cell lines ... 29
2.2.3 DC generation ... 30
2.2.4 Flow cytometry ... 31
2.2.5 IL-12 production ... 31
2.2.6 Evaluation of CD40 mRNA expression ... 31
2.2.7 Mixed Leukocyte Reaction (MLR) Assay ... 32
2.2.8 Data Analysis ... 33
2.3 RESULTS ... 33
2.3.1 DC generated from tumor-bearing mice display a decreased antitumor potential in vivo ... 33
2.3.2 Tumors induce down-regulation of expression of co-stimulatory molecules on DC ... 34
2.3.3 Tumors inhibit antigen-presenting function of DC ... 36
2.3.4 DC generated from MC38 and TS/A bearing mice display a decreased ability to produce IL-12 ... 37

2.3.5 DC generated from tumor-bearing mice express low levels of CD40 molecules.... 38

2.3.6 DC generated from tumor-bearing mice express lower levels of CD40 mRNA 39

2.3.7 Tumor inhibits expression of CD40, CD80 and CD86 on DC in vivo 41

2.4 CONCLUSIONS.. 43

3.0 CD40L IN ANTITUMOR IMMUNITY: ROLE OF DC 46

3.1 INTRODUCTION 46

3.2 MATERIALS AND METHODS... 49

3.2.1 Animals.. 49

3.2.2 Tumor cell lines 49

3.2.3 Preparation of adenoviral vectors 49

3.2.4 DC generation.. 50

3.2.7 Data Analysis.. 52

3.3 RESULTS 53

3.3.1 CD40L protein 53

3.3.2 Preparation of Ad-CD40L.. 54

3.3.3 Examination of efficacy of Ad-CD40L transduction... 56

3.3.4 Intratumoral injection of Ad-CD40L inhibits tumor growth in vivo 57

3.3.5 Transduction of DC with Ad-CD40L in vitro.. 59

2.3.6 Transduction of DC with Ad-CD40L increases their antitumor activity in vivo 60

3.3.7 CD40L rescues DC from tumor-induced suppression of antitumor activity 63

3.4 CONCLUSIONS.. 65

4.0 MECHANISM OF ANTITUMOR ACTIVITY OF LOCAL DELIVERY OF CD40L *IN VIVO* 67

4.1 INTRODUCTION 67
4.2 MATERIALS AND METHODS 70
4.2.1 Animals 70
4.2.2 Tumor cell lines 70
4.2.3 DC generation 70
4.2.4 DC survival 71
4.2.5 Direct effect of DC on tumor cells 72
4.2.6 Flow cytometry 72
4.2.7 IL-12 productio 72
4.2.8 MLR Assay 72
4.2.9 Presentation of TAA by DC in vitro 73
4.2.10 Cytotoxic T lymphocyte assay 74
4.2.12 DC trafficking studies 75
4.2.13 Data Analysis 76
4.3 RESULTS 76
4.3.1 Transduction of DC with CD40L induces significant IL-12 production 76
4.3.2 CD40L transduction protects DC from tumor-induced suppression of IL-12 production 78
4.3.3 CD40L-transduced DC express high levels of co-stimulatory molecules 79
4.3.4 CD40L induces clustering of transduced DC 83
4.3.5 DC/CD40L do not have a direct antitumor effect in vitro 84
4.3.6 CD40L increases survival of DC in growth factor-depleted cultures 85

4.3.7 Transduction of DC with CD40L significantly augments their ability to present TAA to T cells 86

4.3.8 Transduction of DC with CD40L induces DC ability to efficiently stimulate T cell proliferation........ 88

4.3.9 Treatment of tumor-bearing mice with Ad-CD40L elicits CTL response........ 89

4.3.10 Increased accumulation of intratumoral CD11c+ DC in TS/A tumors following the DC-based immunotherapy 91

4.3.11 Developing methods to evaluate trafficking of DC in vivo........ 93

4.3.12 CD40L stimulates DC trafficking to the lymphatic system in DC-based immunotherapy 95

4.4 CONCLUSIONS........ 101

5.0 COMPARATIVE ANALYSIS OF CD40L WITH OTHER TNF FAMILY LIGANDS: RANKL AND 4-1BBL 103

5.1 INTRODUCTION 103

5.2 MATERIALS AND METHODS........ 106

5.2.1 Animals........ 106

5.2.2 Tumor cell lines 107

5.2.3 Tumor therapy model........ 107

5.2.4 Preparation of adenoviral vectors 107

5.2.5 DC generation 108

5.2.6 Flow cytometry 108

5.2.7 IL-12 production........ 108

5.2.8 Data Analysis........ 109

5.3 RESULTS 109

5.3.1 Intratumoral injection of Ad-CD40L, Ad-RANKL and Ad-4-1BBL inhibits tumor growth in vivo 109

5.3.2 Transduction of DC with Ad-CD40L, Ad-RANKL and Ad-4-1BBL 112

5.3.3 Ad-CD40L, Ad-RANKL and Ad-4-1BBL-infected DC do not have a direct effect on tumor growth in vitro 115

5.3.4 Induction of IL-12 production by transduced DC 116

5.3.5 Evaluation of the effect of DC transduced with Ad-CD40L, Ad-RANKL and Ad-4-1BBL on expression of co-stimulatory molecules 117

5.3.6 CD40L, RANKL and 4-1BBL increase survival of DC in growth factor-depleted cultures 120

5.4 CONCLUSIONS 121

6.0 DISCUSSION 124

FUTURE DIRECTIONS 139

APPENDIX 141

BIBLIOGRAPHY 144

LIST OF TABLES

Table 1 DC isolated from spleen of tumor-bearing mice express low levels of co- stimulatory molecules………………………………………..…………………………...…….44

Table 2 Transduction of DC with Ad-CD40L stimulates expression of CD40, CD80, CD86 and class I molecules……………………………………………………………..…..85

Table 3 Summary of immunohistochemical analysis…………………………………...95

Table 4 The effect of the TNF family ligands on the expression of co-stimulatory molecules on transduced DC………………………………………………………...……..…….123

Table 5 Summary of antitumor effect of the TNF family ligands……………………………..126

Table 6 Comparison analysis of properties of the tested TNF family ligands…………………129

LIST OF FIGURES

Figure 1.1 Tumor can actively suppress immune cells………………………………………..…3

Figure 1.2 Tumor induces DC dysfunction……………………………………………..……...6

Figure 1.3 Maturation of murine bone marrow-derived DC *in vitro* is accompanied by an increased expression of CD40 molecules……………………………………………..11

Figure 1.4 CD40 ligation plays a critical role in immunobiology…………………...……….13

Figure 2.0 Interactions between DC, T cells and Tumor cells……………………..………29

Figure 2.1 DC generated from tumor-bearing mice display a decreased antitumor potential *in vivo*……………………………………………………………………..…..………36

Figure 2.2 DC generated from tumor-bearing mice demonstrate down-regulated expression of CD80 and CD86 molecules……………………………………..……………….38

Figure 2.3 DC generated from tumor bearers demonstrate a suppressed ability to stimulate T cell proliferation……………………………………………………………….….…..39

Figure 2.4 DC generated from tumor-bearing animals produce a decreased levels of CD40L-induced IL-12……………………………………………….............................40

Figure 2.5 DC generated from tumor-bearing mice express low levels of CD40 molecules……………………………………………...………………………42

Figure 2.6 DC generated from tumor-bearing mice exhibit a suppressed production of CD40 mRNA……………………………………………………………….....…..……43

Figure 2.7 Inhibition of co-stimulatory molecules on DC isolated from tumor-bearing mice……………………………………………………………………………………45

Figure 3.0 Three approaches of delivery of CD40L into the tumor site……………………...55

Figure 3.1 Systemic administration of soluble CD40L protein has no significant effect on tumor growth in MC38 tumor model……………………………………………..…….57

Figure 3.2 Preparation of Ad-CD40L vector……………………………………………………58

Figure 3.3 Evaluation of Ad-CD40L transduction efficiency…………………………………...60

Figure 3.4 Intratumoral administration of Ad-CD40L induces strong antitumor effect………...61

Figure 3.5 Evaluation of efficacy of DC transduced with Ad-CD40L…………………………64

Figure 3.6 Antitumor effect of DC transduced with Ad-CD40L in two tumor models…………66

Figure 3.7 Transduction with CD40L rescues DC from tumor-induced suppression…………..69

Figure 4.1 CD40L transduction stimulates IL-12 production ………………………………..82

Figure 4.2 CD40L transduction rescues DC from tumor-suppressed IL-12 secretion…………83

Figure 4.3 Transduction with Ad-CD40L up-regulates expression of co-stimulatory molecules on DC and rescues DC from tumor-mediated down-regulation of CD80, CD86 and CD40 expression……………………………………………………...……………………...87

Figure 4.4 Cluster formation by CD40L-transduced DC ………………………………………89

Figure 4.5 DC transduced with Ad-CD40L do not have a direct effect on TS/A tumor growth *in vitro*…………………………………………………………….…………………......90

Figure 4.6 Transduction with CD40L prolongs survival of DC in growth factor-depleted cultures……………………………………………………………………………...91

Figure 4.7 CD40L-transduction significantly amplifies the ability of DC to present tumor antigen to T cells…………………………………………………………………92

Figure 4.8 Transduction with Ad-CD40L induces DC ability to stimulate T cell proliferation…………………………………………………………………………93

Figure 4.9 Intratumoral DC/CD40L-based therapy induces Cytotoxic T lymphocyte (CTL) response……………………………………………………………………………..94

Figure 4.10 DC/CD40L-based therapy stimulates accumulation of CD11c+ DC at the tumor site………………………………………………………………...………...96

Figure 4.11 Development of method to evaluate trafficking of DC injected into the tumor site……………………………………………………………………………..98

Figure 4.12 CD40L-transduction stimulates trafficking of DC injected into the tumor site to lymphoid organs…………………………………………………….……………..101

Figure 5.1 Antitumor effect of intratumoral administration of adenoviral vectors encoding the CD40L, RANKL and 4-1BBL genes…………………………....................................115

Figure 5.2 Effect of DC transduced with Ad-CD40L, Ad-RANKL, or Ad-4-1BBL on tumor growth *in vivo*……………………………………………………………..…...118

Figure 5.3 Lack of direct effect of DC transduced with Ad-CD40L, RANKL or Ad-4-1BBL on TS/A tumor growth *in vitro*…………………………………………………….....119

Figure 5.4 Transduction of DC with different TNF family member genes modulates IL-12 production……………………………………………………………………....121

Figure 5.5 Phenotyping of DC transduced with Ad-CD40L, Ad-RANKL, and Ad-4-1BBL…………………………………………………………………………....122

Figure 5.6 The TNF ligands prolong DC survival in cultures…………………………………124

Figure 6.1 Summary of anticancer effect generated by DC/CD40L-based immunotherapy…………………………………………………………………....135

Figure 6.2 CD40L-based immunotherapy stimulates strong antitumor immunity and protects DC from tumor-mediated suppression of their functional activity……………………………………………………………………..…141

1.0 INTRODUCTION

1.1 ANTITUMOR IMMUNE RESPONSES

1.1.1 Adaptive antitumor immunity

An effective immune response induced against tumor cells should discriminate between malignant and non-cancerous cells. Cytotoxic T cells are the critical effectors of antitumor immunity responsible for elimination of tumor cells (1). Naïve T cells are not capable of directly recognizing antigens, including tumor-associated antigen (TAA). They require help from specialized professional antigen-presenting cells (APC), which first interact with tumors and then present processed tumor antigens to T cells in the context of MHC class I and II complexes. Contact-dependent interactions between naïve T cells and APC are required for initiation of an efficient immune response mediated by activated T cells. Several different cell types perform an APC function, including B cells, macrophages, and dendritic cells (DC). The interaction between T cells and APC provides bidirectional stimulatory signals that are important in activating specific T cells and in the regulation of functional activity of APC. The model of T cell activation postulates the requirement of at least two distinct signals for T cell activation (2); the first signal is believed to be the interaction of the T cell receptor (TCR) with MHC/peptide

complex on the surface of the APC, and the second signal comes from co-stimulatory molecules such as CD80 and CD86 on the surface of APC binding CD28 receptor on the surface of T cells.

The most potent and powerful APC are DC. They play a key role in antigen recognition, processing and presentation, since they are the only APC able to activate naïve T lymphocytes. Therefore, DC perform an essential role in the generation and regulation of antitumor immune responses. In the immature stage, DC are widely distributed throughout the body and occupy sentinel positions in non-lymphoid tissues. They constantly sample their environment for antigens by phagocytosis, macropinocytosis, and highly efficient receptor-mediated pinocytosis. In the presence of appropriate inflammatory 'danger' signals, such as Tumor Necrosis Factor α (TNFα), immature DC undergo maturation characterized by the up-regulation of surface MHC and co-stimulatory molecules (3). Subsequent migration to lymphoid tissue results in efficient presentation of optimally processed antigens to T cells. These specialized functions of DC permit the generation and maintenance of adaptive immune responses to tumor antigens.

1.1.2 Mechanisms of tumor escape from immune recognition

The immune system plays a critical part in early detection and destruction of malignant cells. One of the essential characteristics of all malignant tumors is an active or passive ability to avoid immune surveillance (**Figure 1.1**). Active resistance is achieved by secretion of factors that disable the key components of the host's immune response, allowing tumor cells to survive in spite of their apparent antigenicity. Passive avoidance involves incomplete or defective expression of tumor antigens or MHC I molecules which otherwise would flag malignant cells as targets for immune-mediated destruction.

Escape from immune surveillance is a fundamental feature of tumors, which contributes to their uncontrolled growth, leading to death of the host (4, 5). The immune system plays a critical part in the early detection and destruction of malignant cells.

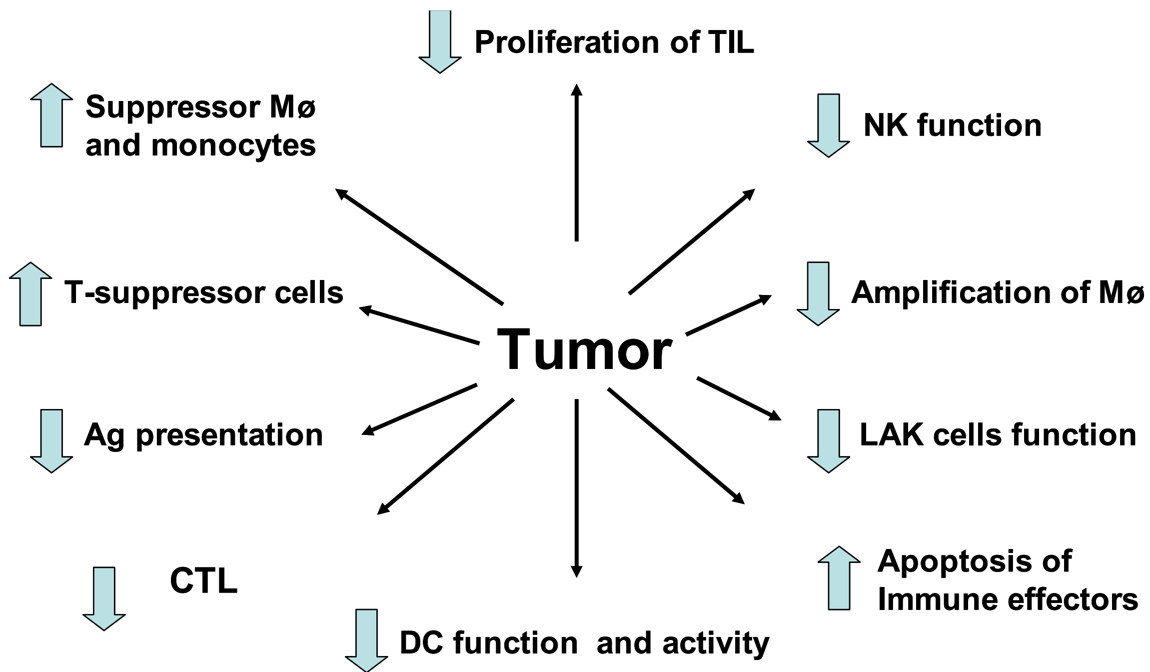


Figure 1. 1 Tumor could actively suppress immune cells. TIL, tumor-infiltrating lymphocytes; NK, natural killer cells; Mø, macrophages; LAK, lymphokine-activated killer cells; CTL, Cytotoxic T lymphocytes; Ag, antigen.

T lymphocytes play an important role in the development of a host immune response, including antitumor immunity. Multiple studies have demonstrated that progressive growth of tumors is accompanied by suppression of specific T cell responses (6, 7). In addition, it has been shown that the immunosuppression is displayed in inhibition of the mitogen- and antigen-stimulated proliferation of T cells (8), NK cell function and activity (9-11) and Lymphokine-

activated killer (LAK) cell function (12). Different mechanisms have been proposed to be responsible for tumor-induced immunosuppression: direct activation of T-suppressor cells (13), suppressive monocytes and macrophages (14), suppressive mediators, like transforming growth factor ß (TGF-ß) (5, 15), prostoglandins (16), gangliosides (17), IL-10 (18) and other suppressor molecules derived from tumors.

A large number of cytokines, hormones, and other molecules secreted by tumors were demonstrated to have immunomodulating properties. The most extensively studied immunosuppressive molecules secreted by tumors are TGF-ß, interleukin 10 (IL-10), gangliosides, and prostaglandin E_2 (PGE_2). TGF-ß, in particular, may play a key role in tumor-induced immunosuppression for many tumors. It is consistently secreted from a variety of tumor cell lines and is detected in plasma of tumor-bearing hosts (19-22). Moreover, the level of TGF-ß production by tumor cells correlates with their metastatic potential. In addition, it has been demonstrated that TGF-ß suppressed CTL and NK activity, enhancing tumor ability to escape immune surveillance (23). IL-10 is an important immunoregulatory cytokine which is thought to negatively affect both T cells and APC (18). For example, Haase et al. have shown that IL-10 can prevent DC maturation, as measured by cytokine production and T-cell priming capacity *in vitro* (24). Tumor gangliosides are highly immunosuppressive membrane glycosphingolipids that are shed into the tumor cell microenvironment. It has been demonstrated that tumor-derived gangliosides inhibit syngeneic antitumor immune responses by inhibiting the function of tumor-infiltrating leukocytes, suppressing the generation of tumor-specific CTLs, and inhibiting the increase of draining lymph node T cells, B cells, and dendritic cells/macrophages (17, 25). These data suggest that gangliosides are potent factors in promoting tumor formation and progression. PGE_2 may play a significant role in early stages of tumor development, promoting the process of

tumorigenesis in some tumors. For instance, melanoma growth leads to increased production of PGE_2, which impairs macrophage cytotoxic mechanisms (26, 27).

The tumor-secreted cytokines have a strong suppressive effect on helper T cell amplification (28, 29). Several studies have examined the phenotype of these cells found at the tumor site, and detected that they are not functional. These T cells are incapable of generating a Th1 or Th2 response, and secrete low levels of cytokines (30-32). Antigen-presenting cells, critical to stimulating T cell activity, are also not functional (33).

1.1.3 Inhibition of the DC system by tumors

A major contributor to host immunity and immune surveillance against infection and malignancy is the DC system. Therefore tumor might develop mechanisms suppressing the activity of the DC system in order to evade immune recognition and elimination. In fact, we and others have recently demonstrated that different tumors effectively inhibit DC generation (dendropoiesis), maturation, functional activation, and survival (34-36).

The escape of malignant cells from immune recognition results from the defective function and activity of cells of the immune system, including DC, which play a key role in the induction and regulation of specific immune responses. Impairment of the number and activity of DC results in deficient expansion of specific cytotoxic T lymphocytes and failure to eliminate tumor cells (37). In fact, it has been noted that alterations in the density and distribution of epidermal DC in the peritumoral infiltrate of malignant melanoma may be responsible for determining the degree of T cell activation (38). Several studies reported tumor-induced inhibition of DC function or differentiation (**Figure 1.2**). For instance, human DC, obtained from progressing, chemotherapy non-responsive melanomas, showed a marked depression of CD86 expression and induced anergy of syngeneic CD4+ T cells (39). Gabrilovich et al. showed that

the ability of DC isolated from breast cancer patients to stimulate allogeneic T lymphocytes was profoundly suppressed (40). Similar data were recently reported for human basal cell carcinoma DC which were deficient in CD80 and CD86 expression as well as in their ability to stimulate T lymphocyte proliferation (41).

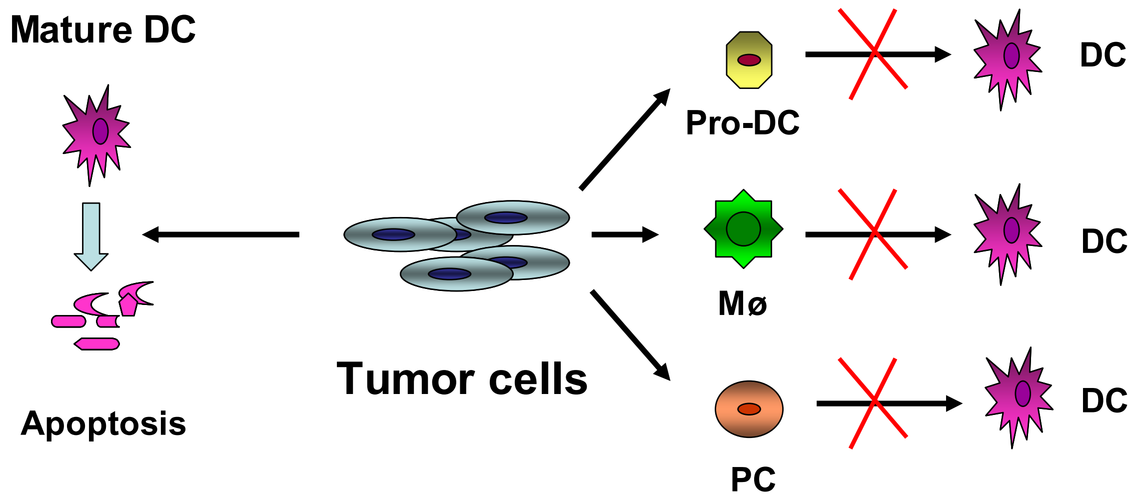


Figure 1. 2 Tumor induces DC dysfunction. Tumor inhibits DC generation by blocking: (i) Pro-DC, DC precursors; (ii) Mø, macrophages; (iii) PC, hematopoietic progenitors, thus, suppressing trafficking of DC to tissues, recognizing, processing and presenting antigens to T cells and producing cytokines. In addition, tumor induces DC apoptosis.

Consequently, a variety of tumors are involved in the induction of immunosuppression responsible for the failure to generate an effective antitumor immune response in cancer patients. We have demonstrated here that DC generated from mice bearing a tumor were characterized by a lower cytokine production, inability to stimulate proliferation of T cells, and decreased expression of co-stimulatory molecules (18). Importantly, DC obtained from tumor-bearers produce and express decreased levels of CD40 molecules. These and other data allowed us to

hypothesize that tumor-induced inhibition of DC maturation and activation resulted from reduced expression of CD40, which, in turn, blocks the development of effective antitumor immune responses.

The effect of tumor-released factors on the differentiation and generation of DC from hematopoietic precursors was not demonstrated until recently. Gabrilovich et al. first reported that tumor cells released factors which were able to inhibit the production of DC (42). These results were confirmed by others in *in vitro* studies. For instance, it was shown that when the supernatant from murine sarcoma cell line C3 was added to DC cultures derived from murine bone marrow cells, the proliferation of these cells and their differentiation into functionally active DC were significantly suppressed (43). Similar data were obtained when human CD34+ hematopoietic precursor cells were cultured with human tumor cells (17, 36), suggesting that most of the tested tumor cell lines markedly inhibit or block DC generation and differentiation in *in vitro* experiments. Results of *in vivo* studies have confirmed this conclusion.

It is well known that if antigen-presenting cells do not express CD80 or CD86, and thereby fail to provide an appropriate second signal for T cells, tolerance or anergy may develop (44, 45). In fact, DC derived from colon cancer are not only much less potent inducers of T cell proliferation in an allogeneic MLR, but indeed induce T cell anergy (46). A similar toleragenic effect of melanoma-associated DC was also reported (39). Thus, tumor-derived DC from progressing metastases might actively induce tolerance in the immune system against tumor tissue. This process might ultimately lead to acceptance of metastatic tumor growth with a "silenced" immune system incapable of rejecting the tumor (39).

Furthermore, it has recently been reported that other functions of DC may also be inhibited by tumor-derived factors (47, 48). For instance, Tas et al. reported a decreased ability

of peripheral blood DC to form clusters with T cells in patients with head and neck cancer (49). Thurnher et al. observed that DC harvested from renal-cell carcinomas had a reduced potential to capture soluble antigen, as shown by the exclusion of FITC-labeled dextran molecules (50). Taken together, all these data demonstrate that tumor significantly suppresses DC functional activity. Therefore, new immunotherapeutic approaches should be focused on stimulating DC in the tumor microenvironment and protecting from tumor-mediated induction of DC dysfunction.

1.2 THE TUMOR NECROSIS FACTOR SUPERFAMILY

1.2.1 The TNF family ligands

The Tumor Necrosis Factor Ligand (TNFL) superfamily comprises a group of cytokines which regulate proliferation, activation, differentiation, and maturation of lymphoid, myeloid and other hematopoietic cells, as well as various non-hematopoietic cell types including tumor cells (51-53). Members of this family include TNF-α, lyphotoxin α (LT-α, TNF-β), lyphotaxin β (LT-β), OX-40L (tax-transcriptionally activated glycoprotein 1), CD40L (TRAP, CD154), Fas ligand (FasL), CD70 (CD27L), CD30L (CD153), 4-1BBL, TRAIL (TNF-related apoptosis inducing ligand, APO-2L), TRANCE (RANKL, OPGL), TWEAK (APO3L), APRIL, THANK (TALL-1), LIGHT, TL1 (VEG1), AITRL. Some new members of this group were recently cloned but are not well characterized yet. TNFL molecules mediate the interaction between T and B cells, T cells and monocytes, DC and T cells, and communications between T cells. They are also involved in the regulation of cytokine secretion and expression of adhesion and co-stimulatory molecules, all known to amplify stimulatory and regulatory signaling. However, differences in

the kinetics of induction, distribution and stimulation of cells support a defined role for each of the ligands for cell-mediated immune responses. For example, TNF-α, LT-α, CD30L, CD95L, and FasL (54-56) are responsible for induction of cytotoxic cell death. Different members of the TNF family of proteins, like TNF-α, TRAIL, CD40L, and 4-1BBL have demonstrated a significant antitumor potential in a number of preclinical murine and human tumor models (55, 57-60). However, the mechanisms of their action as well as the sensitivity of malignant cells were different. In addition, CD40L, RANKL and 4-1BBL were shown to induce DC activation and cytokine production (61-66). Furthermore, CD40L and RANKL play a critical role in DC survival (67, 68). Since CD40L, RANKL and 4-1BBL play an important role in DC function, in this study, we have focused on evaluation of whether expression of CD40L, RANKL, 4-1BBL, or DC which overexpress these proteins at the site of the tumor, elicits an immune response to established tumors in mice, and on studying mechanisms of action of these molecules.

1.2.2 The TNF receptor family

The TNF receptor superfamily include TNFR1 (CD120a), TNFR2 (CD120b), LTBR (CD18), OX40, CD40, Fas (CD95, APO-1), DcR3, CD27, CD30, 4-1BB (CD137), DR4 (Apo2, TRAILR-1), DR5 (TRAILR-2), DcR1 (TRAILR-3), DcR2 (TRAILR-4), osteoprotegerin (OPG), activator of NFκB (RANK), DR3 (TRAMP, APO-3), DR3L, herpes virus entry mediator (HVEM), TNFRSF16, BCM (BCMA), and AITR (GITR). All receptors are type I transmembrane glycoprotein with a common homology in the extracellular domain of variable numbers of cystein-rich repeats. The TNF receptors are present on almost all known cell types with few exceptions, such as erythrocytes and unstimulated T lymphocytes. Of the TNF ligands, TNF-α, LT-α, and LT-β and their receptors interact in a complex fashion of cross-binding. However, the other family members presently have a one-ligand-one-receptor binding principal.

In general, the signals can be transduced not only through the receptors but also through at least some of the ligands and can be stimulatory or inhibitory depending on the target cell or the state of activation.

1.2.3 Regulation of DC maturation and function by CD40L

CD40, a 48-50 kD glycoprotein, was first identified in 1985 (69) and is a member of the TNF receptor superfamily (70). CD40-related studies were initially focused on its role in the regulation of humoral immune responses because CD40 was first identified on B cells. Later, it was demonstrated that CD40 is also expressed on endothelial cells, fibroblasts, and epithelial cells, where CD40 is involved in regulating inflammatory responses (71-74). It was also shown that CD40 is expressed on professional APC, such as DC and macrophages (75, 76). Expression of CD40 on monocytes/macrophages is low, but can be induced by cytokines, including interferon-γ (IFN-γ) and GM-CSF (75). Expression of CD40 is constitutive on freshly isolated Langerhans cells and splenic DC and can be further up-regulated after a short-term culture *in vitro* (77).

CD40 ligation plays a crucial role in DC development and maturation (76). CD40 ligation on DC promotes their differentiation and maturation into effective inducers of cell-mediated immunity, as demonstrated by enhanced production of cytokines, chemokines and expression of co-stimulatory molecules (78). In fact, CD40 ligand (CD40L, CD154) has been shown to enhance survival of monocytes and DC, stimulate secretion of different cytokines, including IL-1, IL-6, IL-8, IL-12, and TNF-α, enhance tumoricidal activity of macrophages and NO synthesis (79, 80).

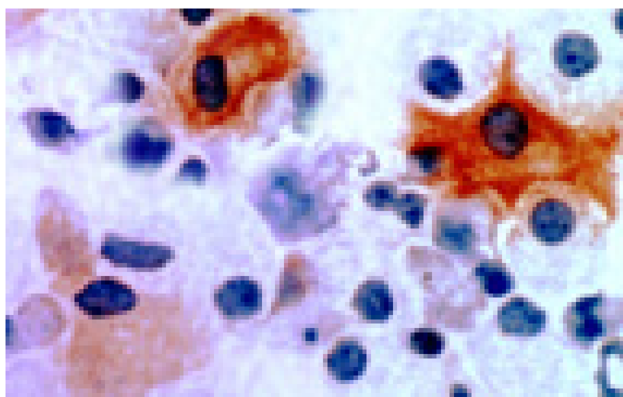


Figure 1. 3 Maturation of murine bone marrow-derived DC *in vitro* is accompanied by an increased expression of CD40 molecules. Immunopositive CD40 was detected in cytospin DC preparation by immunocytochemical staining (red-brown color).

Furthermore, CD40 ligation enhances the efficiency of DC to present antigen to T cells by up-regulating the expression of CD80, CD86 and adhesion molecules CD54/ICAM, CD58/LFA-3 on DC (3, 81, 82). Additionally, CD40/CD40L interaction is a key trigger for IL-12 production by DC, which is essential for induction of Th1 responses *in vivo* (80) and regulation of Th1/Th2 balance (83, 84). It has been also shown that CD40-deficient DC spontaneously produce IL-10, but not IL-12, and induce T cell hyporesponsiveness both *in vitro* and *in vivo* (85, 86). However, the role of elevated CD40 signaling on DC function in the tumor microenvironment has not yet been studied.

1.2.4 CD40/CD40L in tumor immunology

A growing body of evidence suggests that the interaction between CD40 and its ligand, is an important element in the development of antitumor immunity (**Figure 1.4**). DC require CD40-mediated maturation in order to generate protective antitumor immunity (76). In contrast, T cell responses are also dependent on CD40/CD40L interaction (80). Thus, CD40/CD40L interaction

is crucial for both APC and T cell function. Interruption of the CD40/CD40L interaction during T cell/DC dialogue results in reduced T cell proliferation, altered CD40 signaling in APC, inhibited expression of co-stimulatory molecules, blocked cytokine production and impaired CD40L signaling in T cells (87). Taken together, these data suggest a key role of CD40/CD40L signaling in both inductive and effector phases of immune responses, including antitumor immune responses.

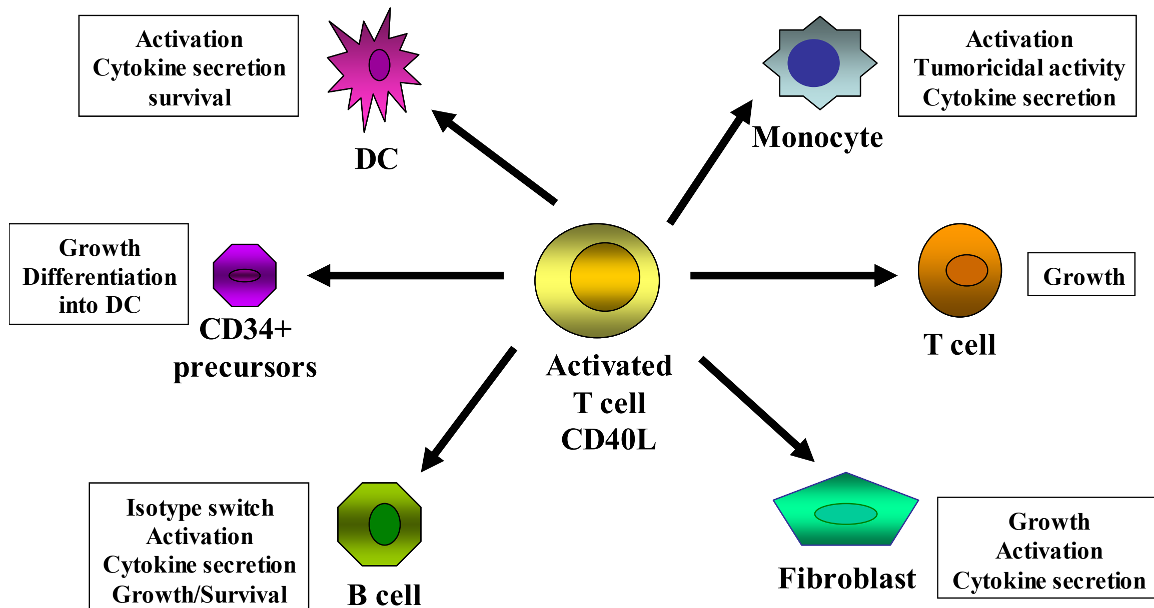


Figure 1. 4 CD40 ligation plays a critical role in immunobiology. Interaction of T cells with many different immune cells plays an important role in activation and stimulation of functional activities of these cells. DC – dendritic cells

It has been recently shown that administration of anti-CD40L antibody completely abrogates antitumor immune responses induced by different vaccines in mice (88). Mice treated with anti-CD40L antibody demonstrated an inhibited Th1 type cytokine production and were unable to produce IL-12 and IFN-γ by lymph node cells re-stimulated with tumor antigen *in vitro*. However, tumor vaccine engineered to produce IL-12 could effectively protect CD40L-deficient mice or anti-CD40L-treated mice from parental tumor challenge (88). Importantly, CD40-deficient DC failed to produce IL-12 during their interaction with tumor-specific CD4+ T cells suggesting that CD40-deficient DC are unable to generate protective tumor immunity and antitumor immune responses (76, 79). However, due to lesion in APC function, antitumor response in CD40-deficient mice could be restored by the co-administration of CD40+/+, but not CD40-/- DC pulsed with tumor antigen. Thus, it is possible that inability to generate protective tumor immunity in the absence of CD40/CD40L interactions is in part due to a failure of DC to produce IL-12 following CD40 ligation and lack of the Th1 type responses. Our preliminary data support this hypothesis, which we propose to test using both *in vitro* and *in vivo* models. It is also possible that the deficient CD40-mediated signaling in DC causes reduced survival of DC in circulation and in different tissues, especially in the tumor environment.

1.2.5 RANK Ligand (RANKL)

TNF-related activation-induced cytokine (TRANCE or RANKL) is a member of the TNF superfamily of proteins recently identified on activated T cells. Josien et al. reported that RANKL mRNA was constitutively expressed in memory but not naive T cells (61). High levels of RANK receptor expression were found on mature DC while activated T and B cells express low levels of RANK receptor. However, RANKL does not exert any significant effect on the proliferation, activation or survival of T and B lymphocytes (61). In DC, RANKL induces the

expression of proinflammatory cytokines IL-6 and IL-1, and T cell growth and differentiation factors IL-12 and IL-15, in addition to enhancing DC survival. Furthermore, RANKL synergizes with CD40L in increasing viability of DC, suggesting that several TNF-related molecules on activated T cells may cooperatively regulate the function and survival of DC (61). Josien et al. have also reported that treatment of antigen-pulsed mature DC with RANKL before the immunization enhances their adjuvant capacity and elicits improved T cell priming *in vivo* in a way that both primary and memory T cell immune responses are enhanced (62). Enumerating migratory DC in the draining lymph nodes and evaluating their ability to stimulate naive T cells, the authors have demonstrated that one of the underlying mechanisms for the enhanced T cell responses is an increase in the number of *ex vivo* antigen-pulsed DC that are found in the T cell areas of lymph nodes (62). These results lead to the suggestion that the longevity and abundance of mature DC at the site of T cell priming influence the strength of DC-initiated T cell immunity *in situ*. RANKL inhibits apoptosis of mouse bone marrow-derived DC and human monocyte-derived DC *in vitro*. The resulting increase in DC survival is accompanied by a proportional increase in DC-mediated T cell proliferation (68). Therefore, RANKL is a new DC-restricted survival factor that mediates T cell/DC communication and may provide a tool to a selective enhancement of DC activity. However, there are no data available regarding the role of inducible RANK signaling for antitumor activity of DC at the tumor site.

1.2.6 4-1BB Ligand (4-1BBL)

4-1BB ligand (4-1BBL) is another member of the TNF family expressed on activated APC. 4-1BBL binds to 4-1BB receptor (CD137) on activated CD4 and CD8 T cells and in conjunction with a T cell receptor (TCR) signal provides a CD28-independent co-stimulation leading to high levels of IL-2 production by T cells. Co-stimulatory molecules, including 4-1BBL, play an

important role in initiating antitumor immune responses. Salih et al. have reported that 4-1BBL is expressed on various human carcinoma cell lines, cells of solid tumors derived from these cell lines, and cells obtained from human tumors (89). They also showed that incubation of tumor cells with a 4-1BB-Ig fusion protein led to the production of IL-8 by the cells, demonstrating that the 4-1BBL is functionally active and signals back into the tumor cells. Furthermore, 4-1BBL expressed on the carcinoma cells functioned as a co-stimulatory molecule for the production of cytokines (most notably IFN-γ) in co-cultures of T cells and tumor cells suggesting that expression of 4-1BBL at the site of the tumor might influence the outcome of a CTL activation (89). In fact, engineered tumor cells expressing co-stimulatory molecules have been used as cancer vaccines in both experimental tumor models and pre-clinical trials. For instance, it has been reported that expression of 4-1BBL on EL-4 lymphoma cells induces tumor regression in syngeneic BALB/c mice and leads to the protective immunity against the parental EL-4 tumor (90). These results indicate the potential utility of engineered tumor cells expressing co-stimulatory molecule 4-1BBL, especially in combination with other co-stimulatory molecules such as CD80 in a cancer vaccine (90). Merelo et al. have shown that mice bearing P815 tumor expressing 4-1BBL develop a strong cytotoxic T lymphocyte response and a long-term immunity against the wild-type tumor (91). In addition, co-expression of 4-1BBL and B7-1 in the poorly immunogenic AG104A sarcoma enhanced the induction of effector CTL and the rejection of the wild-type tumor while neither 4-1BBL nor B7-1 single infections were effective, suggesting a synergistic effect between the 4-1BB and the CD28 co-stimulatory pathways (92).

Taken together, these data demonstrate that increased 4-1BB-mediated signaling in the tumor microenvironment leads to the induction of antitumor immunity. However, the role of 4-1BBL in DC vaccination in cancer has not been yet investigated. We hypothesize that a local

delivery of 4-1BBL into the tumor by means of DC, known to play a key role in tumor antigen recognition and CTL survival within the tumor mass, might significantly improve the induction of specific antitumor immune responses.

1.3 IMMUNE GENE THERAPY

1.3.1 Immunotherapy

The concept of immunotherapy is based on the body's natural defense system, which protects against a variety of diseases. Immunotherapy seems to offer a great promise as a new dimension in cancer treatment, but it is still very much in its infancy. Immunotherapy involving certain cytokines and antibodies has now become a part of standard cancer treatment of choice. Other examples of immunotherapy remain experimental. Although many clinical trials of new forms of immunotherapy are in progress, an enormous amount of research remains to be done before the findings can be widely applied.

Immunotherapy, also referred to as biological therapy, is based on the theory that it is possible to mobilize the body's own immune defenses against cancerous cells. One of the important uses of immunotherapy is a stimulation of the immune system to treat cancer. Immunotherapy for cancer is essentially the stimulation of the immune system via a variety of reagents such as vaccines, adaptive transfer of immune cells, or cytokines. These reagents act through one of several mechanisms: 1) by stimulating the antitumor response, either by increasing the number of effector cells or by producing one or more soluble mediators such as cytokines; 2) by decreasing suppressor mechanisms; 3) by altering tumor cells to increase their

immunogenicity and make them more susceptible to immunologic defenses, and 4) by improving tolerance to cytotoxic drugs or radiotherapy, such as stimulating bone marrow function with granulocyte colony-stimulating factor (G-CSF). Immunotherapy is generally thought of as conferring either passive or active immunity. Passive immunity supplies the immune response with antibodies or cytotoxic T cells, rather than activating the immune system directly. These approaches have met with some success, however it is short-lived. Active immunity may be a more successful immunotherapy. Active immunity induces an endogenous immune response, where the immune system is primed to recognize the tumor as foreign.

Cancer vaccines typically consist of a source of cancer-associated antigen, along with other components, to further stimulate the immune response against the antigen. Cancer vaccines often utilize specialized immune cells such as DC, T lymphocytes (T cells) or Natural Killer cells (NK cells), in combination with tumor cell extracts to produce enhanced antitumor immune responses. Cancer vaccines have shown remarkable efficacy in some advanced stage cancers, and appear to be far less toxic than conventional therapies such as chemotherapy and radiation. The challenge has been to find better antigens, as well as to package the antigen in such a way as to enhance the patient's immune system to fight cancer cells. Cancer vaccines are poised to become a major method of cancer treatment in the near future.

1.3.2 Gene Therapy

Gene therapy is a treatment or prevention of a disease by gene transfer. There are two different approaches that have been used. One is an indirect (*ex-vivo*) method which involves injection of cell modified in cultures. The second method is a direct (*in vivo*) gene transfer by injecting a specific vector. There are two types of vectors: viral and non-viral. Viral vectors include

retroviruses, adenoviruses, adeno-associated viruses, herpes simples viruses and others. Non-viral vectors are liposome, gene gun, DNA conjugates and non-viral hybrids.

1.3.3 Experimental therapeutic strategies

The absence of effective conventional therapy for most cancer patients justifies the application of novel, experimental approaches. One alternative to conventional cytotoxic and hormonal agents is to promote the ability of the immune system specifically to target and eliminate tumor cells on the basis of expression of tumor associated antigen (TAA). The recognition of class I restricted T lymphocytes (CTL) as a critical component of the antigen-specific immune response to tumors and identification of several TAA have allowed a more defined molecular approach for cancer treatment. Initial attempts to enhance immune responses to tumor employed foreign carrier proteins, adjuvants, cytokines, and genetically engineered viruses (93). Vaccination using these approaches has not generally been successful (94, 95). Alternatively, TAA could be presented to T cells by professional antigen presenting cells that generate a more efficient and effective antitumor immune response. In fact, it has been well documented that DC, the most immunologically potent APC, are capable of recognizing, processing and presenting TAA, in turn initiating a specific antitumor immune response (96-99). DC have been shown to activate tumor specific T cells both *in vitro* and *in vivo*, induce both protective and therapeutic immunity, and cause tumor rejection in several animal tumor models. However, results from several laboratories and clinical trials (100-103) suggested a significant, but still limited efficacy of TAA-pulsed DC administered to tumor-bearing hosts. This raises the question of how to improve or develop new DC-based immunotherapies for cancer. For instance, intratumoral administration of DC has been recently suggested as a novel experimental approach to treat cancer and demonstrated a surprising efficiency in a first clinical trial (104). This approach allows avoiding

many limitations of TAA-pulsed DC, including HLA restriction and identification of TAA. Thus, understanding the mechanism of action of intratumoral DC vaccines as well as the immunobiology of DC in tumor-bearing hosts will be important to enhance the efficacy of this novel approach.

1.3.4 Limitations of modern DC-based immunotherapies

Identification of TAA and their respective CTL epitopes stimulated interest in peptide-based vaccinations, including DC-based vaccines (105, 106). While murine models display promise in vaccine studies, human clinical trials have been somewhat disappointing (107, 108). Systemic CTL responses to the vaccines most often do not lead to clinical regression leading investigators with paradoxical observation of identifiable CTL activity that is not capable of destroying the targeted tissue (109). Among the questions raised by this problem stands the enigma of whether tumors are resistant to immunotherapy because the immune response elicited is insufficient or because tumor cells rapidly adapt to immune pressure by switching into less immunogenic phenotype (110, 111). Animal models support either point of view. Considering the fact that escape variants expanded again after nearly complete rejection, it is important to determine how to prevent the tumor escape mechanisms to obtain durable remission. Since this dichotomy is far from been solved in humans, an alternative approach to peptide-pulsed DC in clinical trials is the intratumoral administration of DC without addition of TAA. Intratumoral administration of DC may have several advantages: It ensures the contact between APC and tumor cells leading to the generation of MHC class I restricted CTL, which are considered to be of special importance in the effector's arm of cell mediated immune responses against tumors. The use of DC protected from tumor-mediated immunosuppression or apoptosis should result in high-level antigen presentation and T cell activation. Finally, intratumoral DC may present previously unidentified

CTL and T helper epitopes of the tumor antigen in association with different MHC molecules, whereas peptide vaccines are restricted by the HLA haplotype of the patient. In fact, intratumoral injection of DC has been recently shown to induce marked antitumor immune responses *in vivo* both in mice and humans (104, 112, 113).

In recent studies on immunological antitumor mechanisms, it has been concluded that CD8+ CTL are critical for causing tumor regression, but quantity of CTL alone is not sufficient: rather, qualitatively different CTL that produce more IFN-γ and remain activated *in vivo* may be critical (114). These results further support the idea that the functional activity of administered DC that regulate both CD8+ and CD4+ cells, play a key role for the therapeutic efficacy of DC-based vaccines. In addition, it has been recently reported that multiple applications of DC or the application of not optimally matured DC may decrease antitumor immunity (115, 116). Further studies of DC regulation in the tumor environment will be important for designing optimal immunotherapy.

The discovery of an increasing number of genes regulating DC activity and differentiation and a better understanding of the molecular interactions between immune cells and tumor cells has provided the molecular basis for specific approaches of active immunotherapy in cancer patients. Concurrent abrogation of the inhibitory effects of tumor cells without compromising normal host cell-mediated immunity may provide a successful concerted approach to cancer immunotherapy.

Given the ability of CD40L to stimulate immunity *in vivo*, we were interested in determining the antitumor effects of intratumoral delivery of CD40L, RANKL and 4-1BBL. Previously it has been demonstrated that genetic modification of tumor cells to express CD40L by retroviral infection resulted in an effective antitumor response during tumor establishment

(117, 118). In this project we compared different delivery approaches (protein, adenoviral vector or transduced DC), diverse therapies (CD40L, RANKL or 4-1BBL), and the efficacy of these approaches in a various tumor models.

1.4 STATEMENT OF THE PROBLEM

A growing body of evidence demonstrates that tumor-derived factors suppress DC generation, function, and resistance to an apoptotic death. Thus, there is a real chance of faulty antigen presentation by DC derived from tumor-bearing host, which could result in tolerance induction to the antigen contained within the vaccine and subsequent rapid tumor progression. This raises the possibility that defects in the DC system in patients with cancer may be a main contributor to both immune-escape and immune tolerance pathways. However, mechanisms responsible for the sensitivity of DC precursors to tumor-mediated inhibition were not yet determined. Knowledge of mechanisms involved in suppression of DC function in cancer is essential for the improved therapeutic efficacy of DC vaccines, which are currently under consideration for development in several clinical trials. Genetically modified DC overexpressing ligands of the TNF superfamily of proteins may serve as a basis for DC-based clinical protocols, including the development of therapies for patients with cancer, HIV and infectious diseases.

Tumor-induced inhibition of DC results in sufficient suppression of antigen recognition, processing and presentation to cytotoxic T lymphocytes and, in turn, failure to develop effective antitumor immune responses. Cytokines belonging to the TNF family will be used directly or by gene transduction to improve DC activation and function at the site of the tumor which otherwise

are impaired because of cancer-induced inhibition. Protection of DC function within the tumor tissue is likely to improve immune status of cancer patients and result in induction of efficient antitumor immunity and inhibition of tumor growth and progression.

We recently have demonstrated that DC obtained from tumor-bearing mice are deficient in CD40 expression and functionally immature. However, little is known about the role of CD40 ligation on DC in vivo in a tumor-bearing host. We determined the means of effective protection of DC from tumor-induced inhibition by genetic engineering technique and evaluated the primarily mechanisms of antitumor immunity induced by genetically modified DC in animal tumor models. It was important to investigate the antitumor properties of adenoviral vectors encoding CD40 ligand, RANK ligand, and 4-1BB ligand, as well as genetically engineered DC overexpressing these ligands, using murine colon and breast adenocarcinoma tumor cells both *in vitro* and *in vivo*. Receptors for these ligands are found on DC and the ligation leads to activation and/or stimulation of DC function. Therefore, overexpression of CD40L, RANKL or 4-1BBL at the tumor site or on DC injected intratumorally, could generate a strong antitumor immunity.

The central hypothesis was that tumor-induced inhibition of CD40 expression and signaling on DC results in abnormal DC maturation and function and, in turn, insufficient antitumor immune responses. Furthermore, we hypothesize that overexpression of DC-related ligands of the TNF superfamily on DC in the tumor microenvironment is likely to lead to the generation of effective antitumor immunity and improvement of existing immunotherapy of cancer. We also believed that identification of primary mechanisms responsible for the induction of antitumor immune responses by genetically modified DC at the site of the tumor might force the expansion of novel protective therapeutic strategies allowing for increased efficacy of immunotherapy for cancer. The rationale for the proposed study was that the number and

function of DC in cancer patients are likely to play an important role in the regulation of tumor growth. To be able to control the tumor progression, it is necessary to define the relationship between DC and tumor cells and increase the functional activity of DC in the tumor microenvironment.

Furthermore, a better understanding of the regulation of DC trafficking might offer new opportunities for therapeutic interventions designed to suppress or stimulate the immune response. Improving the delivery and presentation of the appropriate signals, including CD40L, RANKL or 4-1BBL, to initiate antitumor immune response using DC may allow for more aggressive treatment in tumor therapy. Ultimately, the knowledge gained from this study can be applied to clinical situations, where dysfunction of the immune system is quite likely involved in the etiology and/or pathogenesis of cancer. Finally, elucidation of cellular mechanisms responsible for stimulation of antitumor immunity by a local administration of genetically engineered DC, might also allow us to reveal novel approaches to restore deregulated antitumor immunity.

This research project contributes to a better understanding of CD40/CD40L, as well as RANK/RANKL and 4-1BB/4-1BBL pathways in DC function in tumor-bearing hosts through (i) identifying new autocrine mechanisms involved in the regulation of DC activity in cancer and (ii) the exploration of novel approaches of effective modifications of DC activity for vaccinations in cancer. Immunotherapy with modified DC playing a key role in the induction of specific antitumor immune responses seems to be one of the promising alternative approaches in the treatment of cancer. Finally, our results will serve as a basis for development of effective strategies designed to improve the immune responses and induce their recovery from tumor-induced dysfunction.

To accomplish the objectives in this research, we pursued the following Specific Aims:

1. Determine the role of tumor-induced inhibition of CD40 expression in abnormal DC function both *in vivo* and *in vitro.*
2. Demonstrate the extent to which a genetically engineered DC overexpressing CD40L-based vaccine augments antitumor immunity in murine tumor models.
3. Characterize primary mechanisms responsible for the antitumor immunity induced by genetically engineered DC modified to express CD40L.
4. Examine other TNF family proteins, RANKL and 4-1BBL, as antitumor vaccines in comparisons with CD40L.

1.5 SIGNIFICANCE

This research project has several appealing features. It allows for improved understanding of the immunobiology of DC in cancer and the development of novel effective methods for cancer treatment. Knowledge of mechanisms involved in suppression of DC function in cancer is essential for the enhanced therapeutic efficacy of DC vaccines, which are currently under consideration for development in several clinical trials. The described studies consent to a rapid translation of basic knowledge of DC/tumor interactions into protocols for efficient modifications of DC used in clinical trials for cancer. In addition, use of genetically engineered intratumoral DC to induce a specific antitumor immune response is a novel treatment strategy, which may result in reduced treatment costs, duration of therapy and improved efficacy.

Genetically modified DC overexpressing ligand of the TNF superfamily of proteins may serve as a basis for all DC-based clinical protocols, including the development of therapies for patients with cancer, HIV and infectious diseases.

The significance of this research is that it contributes to a better understanding of CD40/CD40L, as well as RANK/RANKL and 4-1BB/4-1BBL pathways in DC function in tumor-bearing hosts through (i) identifying new autocrine mechanisms involved in the regulation of DC activity in cancer and (ii) the exploration of novel approaches of effective modifications of DC activity for vaccinations in cancer. Immunotherapy with modified DC playing a key role in the induction of specific antitumor immune responses seems to be one of the promising alternative approaches in the treatment of cancer. In these studies, we developed methods for the effective activation of DC, which have been recently demonstrated to be strongly inhibited in tumor bearers. Tumor-induced inhibition of DC results in sufficient suppression of antigen recognition, processing and presentation to cytotoxic T lymphocytes and, in turn, failure to develop effective antitumor immune responses. Cytokines belonging to the TNF family were used directly or by gene transduction to improve DC activation and function at the site of the tumor which otherwise are impaired because of cancer-induced inhibition. Protection of DC function within the tumor tissue is likely to improve immune status of cancer patients and result in induction of efficient antitumor immunity and inhibition of tumor growth and progression. Finally, our results served as a basis for development of effective strategies designed to improve the immune responses and induce their recovery from tumor-induced dysfunction.

2.0 TUMOR-INDUCED DC DYSFUNCTION AND DOWN-REGULATION OF CD40 EXPRESSION

2.1 INTRODUCTION

The induction of antitumor immune responses is a complex sequence of carefully orchestrated events leading to the subsequent activation, migration and proliferation of different immune cells. DC interact with tumors, uptake tumor cells/proteins, process them and present antigenic peptides to T cells in the contest of MHC class I/II. The contact between DC and tumor cells in the tumor microenvironment is critical for the stimulation of specific antitumor immune responses (**Figure 2**). However, a great number of preclinical and clinical studies provide evidence demonstrating that the immune system is significantly disbalanced in the presence of the tumor. A variety of different processes involved in the immune response has been impaired in the tumor-bearing hosts. For example, multiple tumor-produced factors exhibit immunomodulatory properties and play an important role in the regulation of tumor growth and tumor escape from immune recognition and elimination. Some of these molecules inhibit activity of natural killer cells and cytotoxic T lymphocytes (119, 120), and impair macrophage and DC functions (17); other induce apoptosis of NK and T cells (121, 122), and cause apoptotic death of DC (118, 123).

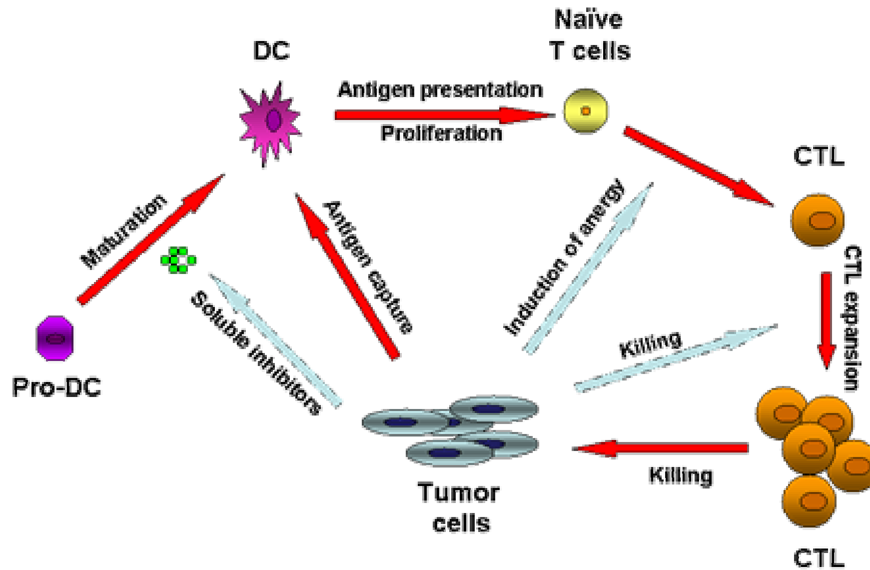


Figure 2 Interactions between DC, T cells and Tumor cells. Pro-DC, DC progenitors; CTL, Cytotoxic T lymphocytes

A major contributor to host immunity and immune surveillance against infection and malignancy is the DC system. Therefore tumor develops mechanisms that suppress the activity of the DC system in order to evade immune recognition and elimination. A growing body of evidence demonstrates that tumor-derived factors suppress DC generation, function, and resistance to cell death (34, 36, 43, 124). In fact, numerous clinical data have demonstrated that the reduced number of DC infiltrating tumor correlates with a poor prognosis for patients (125, 126). For instance, analysis of the correlation between the infiltration of DC and lymphocytes in hepatocellular carcinoma (HCC) tissue and postoperative tumor recurrence and survival rate demonstrated that tumor recurrence was markedly late in patients with DC count ≥20 and positive lymphocyte infiltration (group A) as compared with those who did not meet both criteria simultaneously (group B), with a median interval of 21.6 months for group A and 4.1 months for group B (127). The 1, 3, and 4-year survival rates were significantly greater in group A than in

group B: 83.5% vs. 42.2%, 61.8% vs. 28.4% and 48.7% vs. 23.0%, respectively, suggesting that the infiltration of HCC mass by DC and lymphocytes is closely related to postoperative prognosis. A similar conclusion was made after immunohistochemical analysis of gastric carcinoma tissues: The survival curves show that the prognosis for patients with a low density of DC was significantly poorer than that for patients with high DC density (126).

The CD40/CD40L interaction is critical for the generation of protective cell-mediated tumor immunity and is an essential regulator of DC generation, maturation and function both *in vivo* and *in vitro*. (70, 76, 128). Engaging CD40 on DC promotes their differentiation and maturation into effective inducers of cell-mediated immunity, as demonstrated by enhanced production of cytokines, chemokines and expression of co-stimulatory molecules (70, 76). In addition, CD40 ligation enhances the efficiency of DC to present antigen to T cells by up-regulation of expression of CD80, CD86 and the adhesion molecules ICAM and LFA-3 on DC (3). Furthermore, it has been demonstrated that CD40/CD40L interaction on DC increases their resistance to tumor-induced apoptosis and, thus, promotes the induction of antitumor immune responses (118). However, the role of CD40 in the regulation of DC generation, maturation and function in the tumor microenvironment has not been studied.

In this chapter, we studied the effect of tumor cells on DC maturation, differentiation and function, and the expression of CD40 on DC and DC precursors. We hypothesized that the expression of CD40, as well as of other co-stimulatory molecules might be down-regulated on DC generated from tumor-bearing mice. In addition, we speculated that some functions of DC may be also impaired in the tumor microenvironment. The rationale for these hypotheses was based on previous studies demonstrating an essential role of functionally active DC in initiating effective antitumor immunity.

To examine longevity of DC in cancer, DC generated from tumor bearers were compared to control DC generated from tumor-free animals. DC functions such as IL-12 secretion, expression of co-stimulatory molecules, antigen presentation, and antitumor effect were all evaluated. To summarize our data, we have demonstrated a decrease in expression and function of CD40 on DC isolated or generated from tumor-bearing mice, suggesting a new mechanism of inhibition of DC maturation and function in cancer.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Female 6-8 week-old C57BL/6 (H-2^b) and Balb/c (H-2^d) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in groups of five in a 12:12 hour light:dark cycle with standard mice chow and water as libitum. All animals were acclimatized at least 2 weeks prior to experimentations. Animals were handled in accordance with IACUC protocols.

2.2.2 Tumor cell lines

MC38 colon and TS/A breast adenocarcinoma cells were maintained in RPMI 1640 medium (GIBCO BRL-Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (GIBCO BRL-Life Technologies, Grand Island, NY).

A single-cell suspension of tumor cells (0.2 ml) was injected s.c. into the right flank or i.v. into the tail vein. Injection of HBSS served as a control. C57BL/6 mice received s.c. or i.v. injection of 5 X 10^5 MC38 cells. Balb/c mice received s.c. injection of 2 X 10^5 TS/A cells.

2.2.3 DC generation

DC were generated from hematopoietic progenitors isolated from bone marrow obtained from control (tumor-free) or tumor-bearing mice. Femur and tibia marrow cells from C57BL/6 or Balb/c mice were passed through a nylon cell strainer to remove pieces of bones and debris and depleted of erythrocytes by incubating with lysing buffer for 2 min. Then cell suspensions were incubated with anti-mouse B220, CD4 and CD8 antibodies for 1 h at 4ºC. This was followed by incubation with rabbit complement for 30 min at 37ºC to deplete B and T lymphocytes. The cells were then plated in 6-well plates (0.2x10^6 cells/ml) in a complete medium (RPMI 1640, 10% heat-inactivated FBS, 2mM L-glutamine, 10mM Hepes, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate) overnight. Then the non-adherent cells were collected and resuspended in a complete medium with addition of 1000U/ml mGM-CSF and mIL-4 (ENDOGEN, Woburn, MA). On Days 3 and 5, an additional dose of cytokines was added to all cell cultures. On Day 7, DC were collected and used for intratumoral injections (10^6 DC/mouse) or other assays.

CD11c+ cells, presumably DC, were isolated from single-cell suspensions of splenocytes of experimental or control mice using a MACS paramagnetic cell sorting procedure (Miltenyi Biotec, Auburn, CA). Cells were washed in PBS/BSA (PBS + 1% BSA, 5mM EDTA (pH 7.2) at 4°C), followed by incubation with CD11c microbeads (20 µl/10^7 cells) and PBS/BSA (80 µl/10^7 cells) at 4°C for 25 min. Unbound beads were removed by washing with PBS/BSA. Cells were re-suspended in PBS/BSA and passed twice through a MACS MS+ positive selection column on a MACS separation system. CD11c+ cells were removed from columns by washing with 4 or 5

volumes of PBS/BSA. Columns were then removed from the MACS separation system and CD11c+ cells eluted by washing with 4 or 5 volumes of PBS/BSA.

2.2.4 Flow cytometry

For phenotypic analysis of DC (Day 7), FITC- or PE-conjugated monoclonal antibodies recognizing murine CD11c, CD80, CD86, MHC class I and class II, and CD40 molecules were used (Pharmingen, San Diego, CA). After incubation with antibodies for 30 min at 4°C, cells were washed with PBS and analyzed on a FACStar using Cellquest FACS analysis software (Becton Dickinson, San Diego, CA).

2.2.5 IL-12 production

IL-12 production by DC was determined by p70 IL-12 ELISA. A standard curve was generated using recombinant murine IL-12 (R & D Systems, Minneapolis, MN). To induce IL-12 expression, DC cultures were activated with either CD40-transfected fibroblasts or inactivated *Staphylococcus aureus* (0.1% v/v of essentially non-viable cell suspension) (Sigma, St Louis, MO) 20 µl/well per $1x10^6$ DC cells/24 h.

2.2.6 Evaluation of CD40 mRNA expression

Expression of CD40 mRNA in murine DC was analyzed by RT-PCR. RNA was extracted from cells using the RNAzol reagent (Life Technologies, Gaithersburg, MD). cDNA was synthesized from 2µg of RNA in 20 µl reactions using random primers (Bioehringer-Mammheim, Mannheim, Germany) and AMV reverse transcriptase (GIBCO BRL, Gaithersburg, MD). cDNA (2 µl) was amplified using Taq polymerase in a 25 µl reaction volume. Each cDNA sample within the linear range was amplified by 30-35 cycles using a Perkin-Elmer Cetus DNA thermal cycler (Norwalk, CT) under optimized conditions for each set of primers: for mCD40, 35 cycles of denaturation at 94°C for 60 sec, annealing at 55°C for 60 sec and extension at 72°C for 60 sec;

and for the housekeeping gene (β-actin), 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 60 sec and extension at 72°C for 60 sec; for detection of mCD40, the following primers were used: 5'-GTTTAAAGTCCCGGATGCGA-3' and that of the antisense primer 5'-CTCAAGGCTATGCTGTCTGT-3'. Due to alternative splicing of mRNA, CD40 cDNA amplified with these primers is 307 and 408 bp. The sequence of sense and antisense primers for β-actin was 5'-TTCTACAATGAGCTGCGTTG-3' and 5'-CACTGTGTTGGCATAGAGGTC-3', respectively. Amplification with these primers was expected to yield a 560 bp product. PCR products were separated on 1% agarose gels containing 1mg/ml ethidium bromide together with an appropriate marker ladder and viewed under UV light. mRNA expression was compared and analyzed by volume quantitation using a Bio-Rad (Richmond, CA) densitometer. The sample/β-actin ratio was first calculated for each sample and used for comparison. The expression of mRNA is presented as a percentage of control (nonstimulated value).

2.2.7 Mixed Leukocyte Reaction (MLR) Assay

Functional activity of mouse DC was determined in a one-way allogeneic MLR using T lymphocytes. Allogeneic T cells were obtained from spleen cell suspensions by passage through the nylon wool columns after lysing of red blood cells. Cultured DC generated from control or tumor-bearing mice were added to T cells (3 X 10^5 cells/well) at different ratios (10^2-10^6 cells per well) in final volume of 200 µl per well. Cells were mixed in 96-well round-bottom plates and incubated for 3 days. Cell cultures were pulsed with 1 µCi of ^{3}H-thymidine/well (DuPont-NEN, Boston, MA) for 16-18 h and harvested using a Skatron (Skatron, Lier, Norway) cell harvester. Proliferation of T cells was determined by measuring uptake of ^{3}H-thymidine on a MicroBeta TRILUX liquid scintillation counter (WALLAC, Gaithersburg, MD). Tests were

performed in triplicates and results were expressed as the mean counts per minute (cpm) ± standard deviation (SD).

2.2.8 Data Analysis

Statistical analysis of experimental data was performed with a software package SigmaStat (STSS). For all analysis, the level of significance was set at a probability of 0.05 with results that had a p value less then the 0.05 considered significant. Student t-test was used for comparison between two groups after evaluation for normality. Two-way repeated measurements ANOVA was used to compare tumor growth for multiple groups of mice. All experiments were conducted with 5-7 mice per group receiving identical treatments. All experiments were repeated at least twice.

2.3 RESULTS

2.3.1 DC generated from tumor-bearing mice display a decreased antitumor potential *in vivo*

To demonstrate that tumor cells affect DC, DC were generated from control and TS/A tumor-bearing mice in cultures for 7 days in the presence of mIL-4 and mGM-CSF. Then, cells were collected and 10^6 DC/mouse were injected directly into the tumor site of tumor-bearing mice 7 days after TS/A cell inoculation. HBSS (100 µl/mouse) was used as a control. Cells generated from tumor-bearing mice showed a decreased antitumor effect when compare with DC generated from tumor-free animals.

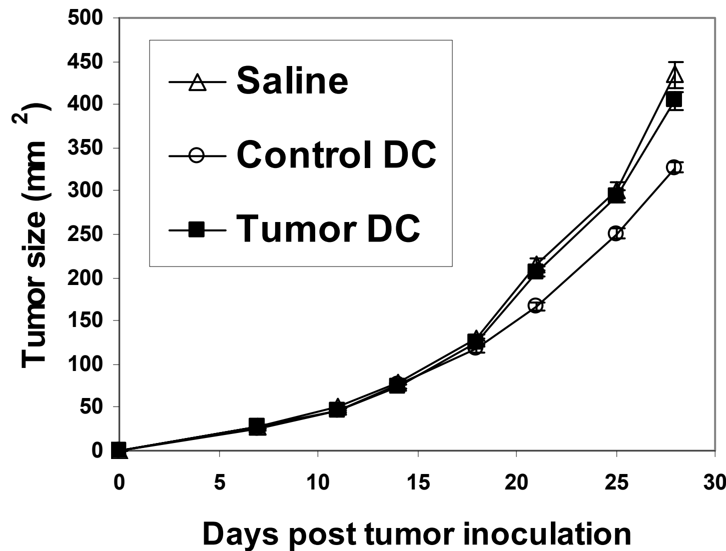


Figure 2. 1 DC generated from tumor-bearing mice display a decreased antitumor potential *in vivo*. DC were generated from control tumor-free and TS/A breast adenocarcinoma-bearing Balb/c mice. DC were generated from bone marrow precursors for 7 days in a complete medium supplemented with GM-SCF and IL-4. DC were collected, washed and injected (10^6 cell/animal) intratumorally 7 days after tumor cell inoculation. Tumor growth was measured twice a week and plotted as a function of tumor size over time. (N=5-7 animals/group). The results of representative experiment are shown. Three independent experiments demonstrated similar results.

As shown on **Figure 2.1**, HBSS control and DC/Tu have a very similar growth curves. In contrast, DC generated from control mice demonstrate a significant induction of antitumor immunity *in vivo* ($p<0.05$). These data raise the question of whether DC generated from tumor-bearing mice are phenotypically and functionally different from control cells.

2.3.2 Tumors induce down-regulation of expression of co-stimulatory molecules on DC

To examine the expression of co-stimulatory molecules on the surface of DC, we generated cells from control and tumor-bearing mice and cultured them in complete medium with IL-4 and GM-CSF. FACScan analysis of DC on Day 7 revealed that the presence of tumor caused a significant

down-regulation of the expression of B7 molecules on DC. We have demonstrated that CD80 and CD86 were down-regulated on CD11c+ DC (**Figure 2.2 A**). For example, in C57BL/6 mice the percentage of CD86 was decreased from 73.2 ± 5.2% on control DC to 26.5 ± 1.8% on DC generated from MC38–bearing mice (p<0.01); CD80 was lowered from 69.9% on control cells to 50.6% on DC from tumor-bearing animals (p<0.05). A similar pattern was detected for expression of CD80 and CD86 on Balb/c-derived DC (**Figure 2.2 B**). The total number of recovered DC was similar in cells generated from control or tumor-bearing animals. These results suggest that DC generated from tumor-bearing mice express lower levels of CD80 and CD86, compare to controls, and could be considered immature.

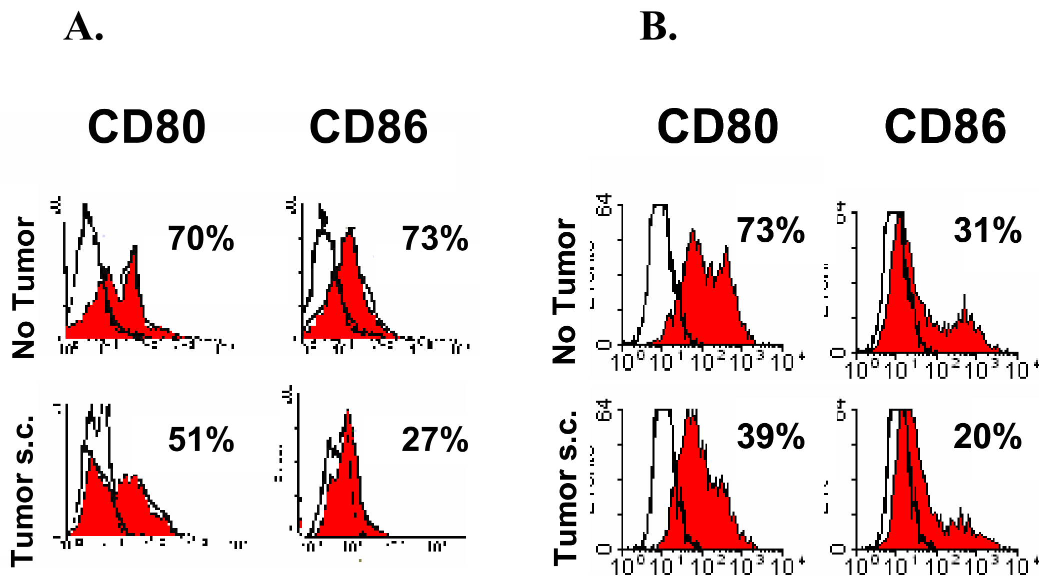


Figure 2.2 DC generated from tumor-bearing mice demonstrate down-regulated expression of CD80 and CD86 molecules. DC were generated from tumor-bearing and tumor-free mice. Cells were cultured for 7 days in complete DC medium with IL-4 and GM-CSF. On Day 7 cells were collected and levels of co-stimulatory molecules were measured by FACScan analysis. Set of 5 independent experiments showed reproducible results. A. DC generated from C57BL/6 mice. B. DC generated from Balb/c mice.

2.3.3 Tumors inhibit antigen-presenting function of DC

To determine whether tumor affects the functional activity of DC to induce T cell proliferation, we evaluated the stimulatory capacity of control DC and DC generated from TS/A-bearing animals to induce proliferation of allogeneic T cells in an MLR assay. As shown on **Figure 2.3,** TS/A tumor significantly decreased the ability of DC to stimulate T cells. The maximum inhibition of T cell proliferation was observed at Effector : Target ratio 1:30, where control DC induce T cell proliferation as 98808.8 ± 10636.4 cpm versus 70947.3 ± 10733.5 cpm for DC/Tu ($p<0.05$). These results conform that tumor has a strong influence on DC functional activity which was assessed as a decreased APC ability of DC generated from tumor-bearing animals compare to DC from tumor-free mice.

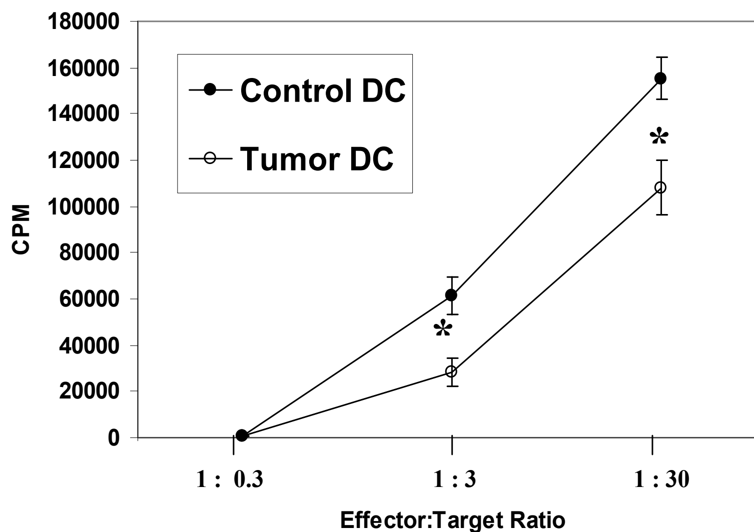


Figure 2.3 DC generated from tumor bearers demonstrate a suppressed ability to stimulate T cell proliferation. DC progenitors were generated from control and TS/A-bearing mice. T cells were isolated from spleens of C57BL/6 mice and co-incubated with DC at

different ratios. T cell proliferation was measured by MLR assay. Two independent experiments demonstrated similar results. Data represent mean ± SD.

2.3.4 DC generated from MC38 and TS/A bearing mice display a decreased ability to produce IL-12

To examine whether the functional activity of DC could be affected by the tumor, we have measured the IL-12 production by DC generated from tumor-bearing mice, since it has been shown that IL-12 is the most important cytokine produced by these cells (129-131). C57BL/6 and Balb/c mice were injected s.c. with $5x10^5$ murine MC38 colon or TS/A breast adenocarcinoma cells, respectively, and DC were generated from the bone marrow precursors 3 weeks later. We have demonstrated that upon stimulation of DC with either CD40-transfected fibroblasts or *Staphylococcus aureus*, DC generated from tumor-bearers produce significantly lower levels of IL-12 compared with DC obtained from control animals, as determined by p70 IL-12 ELISA (up to 61 % of inhibition; p<0.05) (**Figure 2.4 A**).

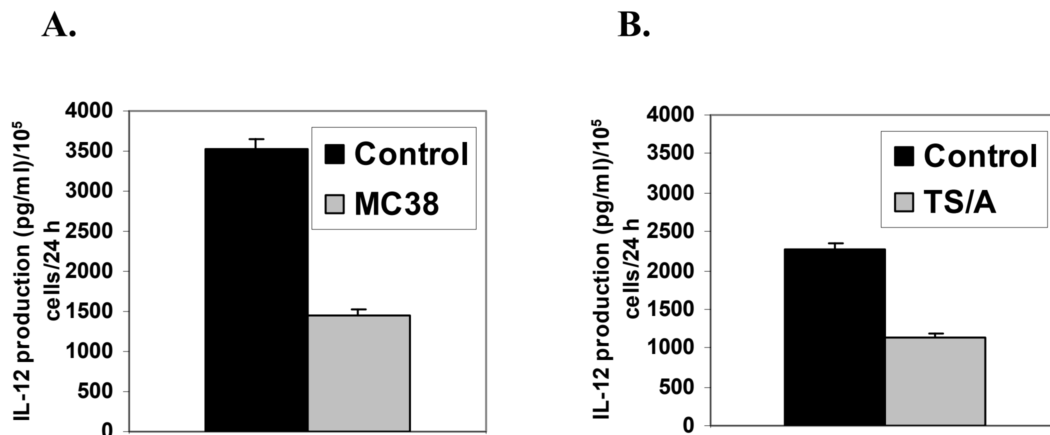


Figure 2.4 DC generated from tumor-bearing animals produce a decreased levels of CD40L-induced IL-12. DC progenitors were

generated from MC38 (**A**) or TS/A (B) bearers. Cell-free supernatants were collected on Day 7 and IL-12 production was measured by p70 IL-12 ELISA 24 hr later. Averages of two independent experiments are shown (mean ± SD).

For instance, DC generated from control C57BL/6 mice released 3527 ± 65 pg/ml of IL-12 upon CD40L stimulation, whereas DC generated from MC38-bearers and stimulated with CD40L secreted 1438 ± 43 pg/ml of IL-12. Similar results were observed in Balb/c mice with TS/A tumors (**Figure 2.4 B**). These data allowed us to hypothesize a possible mechanism of tumor-induced inhibition of IL-12 synthesis in DC: decreased expression of CD40 receptors on DC.

2.3.5 DC generated from tumor-bearing mice express low levels of CD40 molecules

Since CD40 is an important receptor involved in cellular signaling and activation and plays a key role in DC biology, we have evaluated whether CD40 expression on DC is regulated by tumor. We have determined whether cultured DC generated from tumor-bearing mice express lower levels of surface CD40 receptors by the FACScan analysis. MC38 and TS/A cells were injected s.c. into mice. Three weeks after the tumor inoculation, bone marrow progenitors were isolated and cultured with *m*GM-CSF and *m*IL-4 (1000 U/ml). A FACScan analysis of cultured DC, assessed as CD11c positive cells demonstrated a significantly decreased (up to 60%, $p<0.001$) expression of CD40 on DC prepared from tumor-bearing animals. For instance, CD40 expression on CD11c + DC decreased from 61% on control DC to 40% ($p<0.01$) on DC generated from MC38-bearing mice (**Figure 2.5**). Similarly, in TS/A-bearing animals, the expression of CD40 decreased from 33% on control DC to 22% on DC generated from tumor-bearers. Since CD40 is an important maturation signal, DC generated from tumor-bearers could be functionally immature. These results explain why a tumor-bearing mouse-derived DC did not induce IL-12 production upon CD40 ligation and a down-regulation of B7 molecules on the surface of these

cells. Thus, taken together these data suggest that DC generated *in vitro* from bone marrow precursors obtained from tumor-bearing animals expressed low levels of CD40.

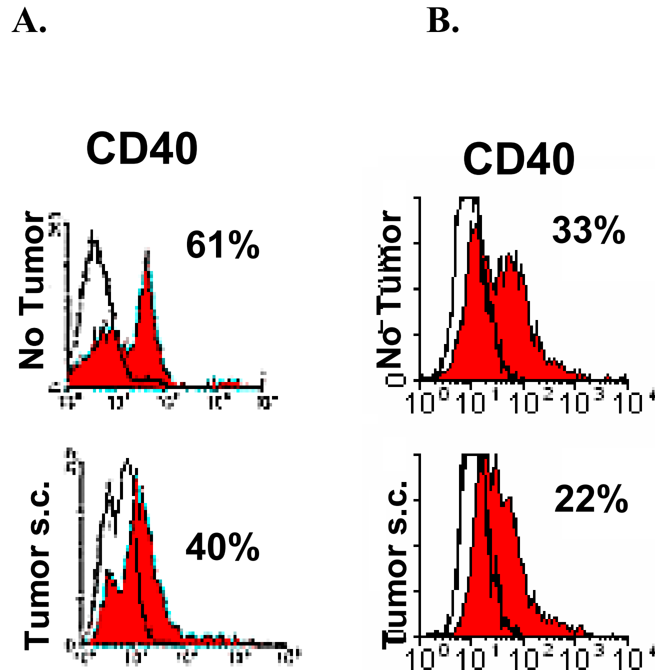


Figure 2.5 DC generated from tumor-bearing mice express low levels of CD40 molecules. DC precursors generated from control and tumor-bearing mice were cultured in a complete DC medium with addition of IL-4 and GM-CSF. On Day 7 DC were collected and CD40 expression was measured by FACScan analysis. Representative data of three independent experiments are shown. **A.** DC generated from MC38-bearing and tumor-free C57BL/6 mice **B.** DC generated from TS/A-bearing or tumor-free Balb/c mice.

2.3.6 DC generated from tumor-bearing mice express lower levels of CD40 mRNA

Next, we determined whether CD40 expression was down-regulated at the level of translation. To study tumor effect on CD40 mRNA in DC, we generated DC from MC38-bearing and control

mice and cultured them as described above. Expression of CD40 mRNA was detected by RT-PCR. β-actin mRNA was used as a housekeeping marker.

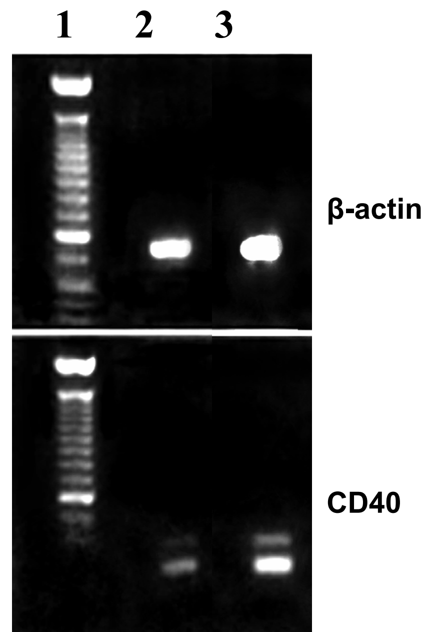


Figure 2.6 DC generated from tumor-bearing mice exhibit a suppressed production of CD40 mRNA. DC were generated from control and MC38-bearing mice. Expression of CD40 mRNA was detected by RT-PCR in DC. Lanes: 1, marker; 2, DC from tumor-bearing mice; 3, DC from tumor-free mice. Upper panel, β-actin; lower panel, CD40. The results of a representative experiment are shown. Two separate experiments demonstrated similar data.

As expected, expression of CD40 mRNA was markedly suppressed on DC generated from tumor-bearing mice compared to DC generated from tumor-free animals (**Figure 2.6**). These data confer that tumor induces inhibition of DC maturation by down-regulating CD40 expression and also suggest that DC generated form tumor bearers could be functionally immature.

2.3.7 Tumor inhibits expression of CD40, CD80 and CD86 on DC *in vivo*

To verify that endogenous DC could also be affected by tumor *in vivo*, CD11c+ DC were isolated from the spleen of tumor-free or MC38-bearing mice by positive selection using a MACS paramagnetic cell sorting procedure. Isolated CD11c+ DC, that is DC generated *in vivo*, were stained with CD86, CD80 and CD40 FITC or PE-conjugated antibodies. We found that CD40, CD86 and CD80 expression on DC isolated from spleen of tumor-bearing mice was significantly reduced compared to control DC (**Table 1**).

Table 1 DC isolated from spleen of tumor-bearing mice express low levels of co-stimulatory molecules

	CD40	**CD80**	**CD86**
No tumor	**356.8 ± 42.4**	**159.1 ± 21.8**	**49.7 ± 7.7**
MC38 tumor s.c	**185.7 ± 22.4***	**74.4 ± 9.4***	**18.0 ± 3.4***
MC38 tumor i.v.	**92.7 ± 12.6***	**44.9 ± 6.6***	**11.1 ± 2.3***

C57BL/6 mice were injected with 5 X 10^5 MC38 tumor cells (s.c. in the right flank or i.v. in the tail vain). On Day 21 spleens were collected and pooled (n=3), and CD11c+ DC were isolated by MACS cell sorting. FACScan detected expression of CD40, CD80 and CD86 molecules on DC isolated from tumor-bearing and tumor-free animals. The data represents MFI values after subtraction of the control ± SEM and the results of 3 independent experiments. (* p<0.01 vs tumor-free group).

Importantly, this effect was more profound in the group of animals receiving i.v. injection of tumor cells. These mice had visible metastasis in the liver and lungs. For instance, CD40 expression in this group decreased up to 60%, while in the s.c.-injected group CD40 expression was reduced by 30%. Furthermore, expression of CD86 on splenic DC isolated from the i.v.-injected mice was completely abrogated (<1%, p<0.001), although it was down-regulated in the s.c.-injected group from 73.2% (control) to 26.5% (p<0.01) (**Figure 2.7**).

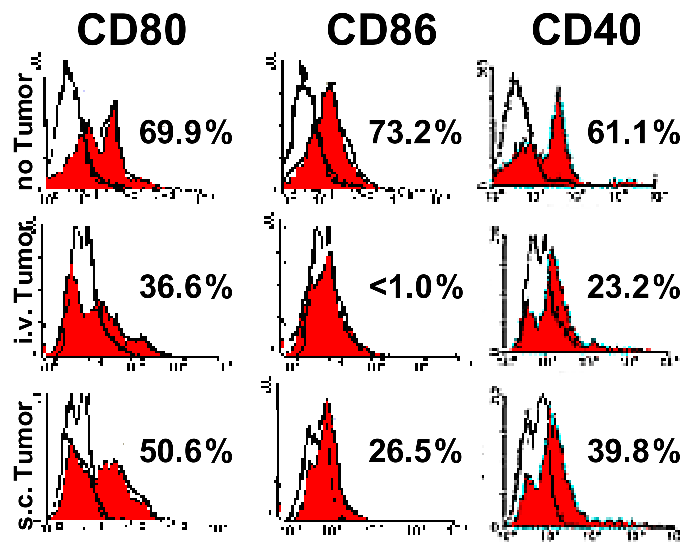


Figure 2.7 Inhibition of co-stimulatory molecules on DC isolated from tumor-bearing mice. Spleens were collected from MC38-bearing mice 21 day after s.c or i.v. administration of tumor cells. CD11c+ DC were isolated by MACS cell sorting and stained with different antibodies. The expression of CD80, CD86 and CD40 was measured by FACScan analysis. DC isolated from spleens of tumor-free animals were used as a control. The results from a representative experiment are shown. Experiment was repeated 4 times with similar data.

Taken together, these results demonstrate that DC isolated from the spleen of MC38-bearing mice had even more profound immunosuppression compared to cultured DC generated

from bone marrow precursors obtained from the same animals. Thus, tumor inhibits maturation of DC both *in vitro* and *in vivo*.

2.4 CONCLUSIONS

Interaction between tumor cells and cells of the immune system appear to be critical for tumor growth and progression. Tumor-derived factors dramatically alter the functional activity of immunocompetent cells, in particular DC. In this chapter we hypothesized that tumor alters DC maturation, activity and function. In fact, we had demonstrated that tumor cells decrease antitumor potential of DC *in vivo*. Cells generated from tumor-bearing animals do not suppress tumor growth in contrast to DC generated from tumor-free mice. In addition, we have shown that tumor induces a decreased functional activity of DC. The hypothesis was confirmed by our data showing suppressed IL-12 production, down-regulated expression of CD80 and CD86 co-stimulatory molecules on the surface of DC generated from tumor-bearing animals, and inhibited APC ability of DC both *in vitro and in vivo*. The most important finding was that DC generated from bone marrow precursors obtained from tumor-bearing mice have significantly lower expression of CD40 molecules compared to DC generated from control tumor-free animals. Furthermore, DC isolated from spleens of tumor-bearers also had a decreased expression of CD40. These data suggest that tumor induces inhibition of CD40 expression both *in vitro* and *in vivo*. Due to the fact that CD40 is an important maturation molecule for DC, our results suggest that tumor-derived factors suppress DC maturation and function, proposing a new target for testing a novel gene immunotherapy in cancer.

These results suggest that DC in the tumor microenvironment could be beneficial for generating antitumor immune responses. Our data also suggest that the tumor may actively decrease the number of tumor-infiltrating DC by inducing their premature death, inhibiting their immigration into the tumor site and homing within the tumor or by the general suppression of DC generation. Therefore, it is possible that defects in DC function in cancer could be responsible for a loss of tumor immunosurveillance.

Summary of conclusions:

1. DC generated form hematopoietic precursors obtained from MC38 or TS/A bearing mice display:

- Down-regulated expression of CD80 and CD86
- Decreased ability to induce T cell proliferation
- Decreased antitumor immunity
- Low IL-12 production
- Down-regulated expression of CD40 mRNA and protein

2. DC isolated from MC38 bearing mice display:

- Down-regulated expression of CD80 and CD86
- Low IL-12 production
- Down-regulated expression of CD40

3.0 CD40L IN ANTITUMOR IMMUNITY: ROLE OF DC

3.1 INTRODUCTION

The interaction between tumor cells and DC is critical for the regulation of an antitumor response. Based on their unique ability to stimulate T cells, DC have been tested as inducers of antitumor immunity in both preclinical models and clinical trials. Administration of DC pulsed with tumor antigens or tumor lysates was able to stimulate specific antitumor responses (132). Moreover, genetic modification of DC to express tumor antigens also resulted in effective immunization following inoculation into mice (133). Alternatively, the injection of non-pulsed DC directly into the tumor mass where DC can acquire tumor antigen followed by migration to lymph nodes to initiate tumor immunity has also been shown to be an effective strategy (103). The advantage of this approach is that it circumvents the needs for tumor-associated antigens.

There is considerable interest in applying DC to cancer immunotherapy. Promising results have been reported recently in clinical trials with purified or *ex vivo* generated DC pulsed with tumor-associated peptide or protein antigens (134-136). However, the absence of characterized TAA for many cancers forced the development of alternative DC-based vaccines in cancer. The recent experiments in animal tumor models and pilot studies in patients with melanoma and breast carcinoma demonstrated a striking antitumor potential of intratumoral administration of DC without addition of TAA (104, 112, 113, 137). Although direct evidence is

limited, several lines of evidence suggest that intratumoral DC play an important role in antitumor immune responses. For instance, increased numbers of tumor-infiltrating DC are associated with a better outcome in cancer patients with a variety of tumors (47, 138), and induction of DC migration into the tumor site by GM-CSF or chemokines resulted in development of antitumor immunity in both animals (139, 140) and humans (141). However, recent studies indicate that the generation, maturation, function, and survival of DC are all markedly inhibited in the tumor microenvironment (36, 43, 113, 124) explaining the reported limitations in the success of DC-based therapies and justifying the improvements of DC-based vaccines in cancer. Although it has been shown in murine tumor models that intratumoral administration of DC producing IL-12, CD40 or Bcl-x_L and thus protected from tumor-induced apoptosis, results in the generation of efficient antitumor immune responses, the mechanisms of these effects as well as other potential molecular targets in DC are unknown.

Intratumoral administration of DC may have several advantages: (i) it requires no isolation of tumor cells or tumor antigen(s), (ii) it also ensures the most suitable contact between DC and tumor cells leading to the generation of tumor-specific $CD8^+$ and $CD4^+$ T cells, (iii) the use of intratumoral injection of DC should result in a high-level of antigen processing and expression, (iv) finally, intratumoral DC may present previously unidentified epitops of the tumor antigen in association with different MHC molecules, whereas peptide vaccines are restricted by the HLA haplotype of the patient.

It has been demonstrated that the regulation of CD40/CD40L interaction is beneficial for the treatment of various diseases, including infections, cancer, autoimmune disorders, and side effects of transplantation. This suggests that different therapeutic strategies should be designed to control this pathway. For instance, transplantation and autoimmunity clinics will appreciate the

use of anti-CD40 or anti-CD40L antibodies or chimeric soluble proteins that can bind and neutralize CD40 or CD40L. Other approaches designed to stimulate antitumor immune responses may include development of compounds to trigger CD40 on APC in order to up-regulate co-stimulatory activity and cytokine secretion (142).

It has been suggested that soluble recombinant CD40L (sCD40L) may be used for an appropriate activation of CD40 on APC in tumor-bearing mice. For instance, Hirano et al. demonstrated that treatment of tumor-bearing SCID mice with sCD40L resulted in significant prolongation of animal survival (143). Stimulation of CD40 by its ligand directly inhibited growth of human breast carcinoma both *in vitro* and *in vivo*. Similarly, administration of plasmid expressing CD40 ligand resulted in initiation of effective immune responses *in vivo* (144). These results demonstrate that recombinant CD40L can be used *in vivo* to inhibit tumor growth. Alternative strategies to develop effective antitumor therapies by modulating CD40/CD40L interaction include the local delivery of CD40L overexpressing DC.

Based on all described studies, we <u>hypothesized</u> that overexpression of the CD40L at the tumor site will induce strong antitumor immunity. We proposed that CD40L would also be beneficial for stimulation and activation of tumor-infiltrating DC when administered locally to the growing tumors.

Here we have examined the antitumor effects of CD40L following three different routes of administration in two different murine tumor therapy models. We show that a systemic injection of recombinant mCD40L had no antitumor potential. In contrast, we demonstrate that direct injection of adenoviral vector encoding CD40L (Ad-CD40L) into established tumors resulted in a significant inhibition of the tumor growth in both murine colon MC38 and breast TS/A adenocarcinoma models. In addition, DC transduced with Ad-CD40L and administered to

the tumor site induced a strong specific antitumor response. Intratumoral injection of DC/CD40L resulted in a more effective antitumor response than injection of DC engineered to overexpress IL-12. Furthermore, CD40L rescued DC from tumor-induced DC dysfunction. Taken together, our data suggest that intratumoral delivery of CD40L by adenovirus-mediated gene transfer or by genetically engineered DC is a highly effective approach to generate antitumor immunity *in vivo*.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Female 6-8 week-old C57BL/6 (H-2^b) and Balb/c (H-2^d) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in groups of five in a 12:12 hour light:dark cycle with standard mice chow and water. All animals were acclimatized at least 2 weeks prior to experimentations. Animals were handled in accordance with IACUC protocols.

3.2.2 Tumor cell lines

MC38 colon, TS/A breast and 4-T.1 breast adenocarcinoma cells were maintained in RPMI 1640 medium (GIBCO BRL-Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (GIBCO BRL-Life Technologies).

3.2.3 Preparation of adenoviral vectors

Ad-CD40L was constructed, propagated, and titered according to the standard protocol previously described (145). Briefly, the murine CD40L cDNA (Immunex Corporation, Seattle, WA), was amplified by PCR using a set of primers to create a SalI site at the 5' end and a NotI

site at the 3' end. Then plasmids were digested by SalI and NotI restriction enzymes to release the CD40L cDNA, which was subcloned into SalI-NotI site of the adenovirus shuttle plasmid (pAdlox) to generate pAdlox/mCD40L. In this vector, the inserted cDNA sequence is expressed under the transcriptional control of the cytomegalovirus promoter. The plasmid was linearized with SfiI and co-transfected with the Ad-ψ5-derived, ΔE1, E3 deleted adenoviral backbone, into 293 cells by calcium phosphate precipitation. The recombinant Ad-CD40L vector was isolated from a single plaque, expanded in CRE8 cells, and purified by double cesium chloride gradient ultracentrifugation. The purified virus was extensively dialyzed against 10 mM Tris/1 mM $MgCl_2$ sterile viral buffer at 4°C, stored in aliquots at –80°C, and titered on CRE8 cells for plaque forming units (pfu). The generation of Ad-IL-12, Ad-LacZ, Ad-ψ5 and Ad-EGFP (enhanced green fluorescent protein) have been previously described (146).

3.2.4 DC generation

DC were generated from hematopoietic progenitors isolated from mouse bone marrow. Femur and tibia marrow cells from C57BL/6 or Balb/c mice were passed through a nylon cell strainer to remove pieces of bones and debris and depleted of erythrocytes by incubating with lysing buffer for 2 min. Then cell suspensions were incubated with anti-mouse B220, CD4 and CD8 antibodies for 1 h at 4ºC. This was followed by incubation with rabbit complement for 30 min at 37ºC to deplete B and T lymphocytes. The cells were then plated in 6-well plates ($0.2x10^6$ cells/ml 4 ml/plate) in a complete medium (RPMI 1640, 10% heat-inactivated FBS, 2mM L-glutamine, 10mM Hepes, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate) overnight. Then the non-adherent cells were collected and re-suspended in complete medium with addition of 1000U/ml mGM-CSF and mIL-4 (ENDOGEN, Woburn, MA). At Day 3 and 5, an additional dose of cytokines was added to the cell cultures. For adenoviral infection, DC were collected on

day 5 and washed in serum-free medium. Virus was added directly to the pellet (10^9 pfu/10^6 DC) and the cells incubated for 1 h at 37°C before plating in a complete medium.

3.2.5 Flow cytometry

For phenotypic analysis of bone marrow-derived DC (Day 7), FITC- or PE-conjugated monoclonal antibodies recognizing murine CD40L were used. After incubation with antibodies for 30 min at 4°C, cells were washed with PBS and analyzed on a FACStar using Cellquest FACS analysis software (Becton Dickinson, San Diego, CA). As a control to determine the percentage of transduced DC, cells were infected with Ad-eGFP (enhanced Green Fluorescent Protein) 48 hours before FACS analysis.

3.2.6 Tumor therapy models

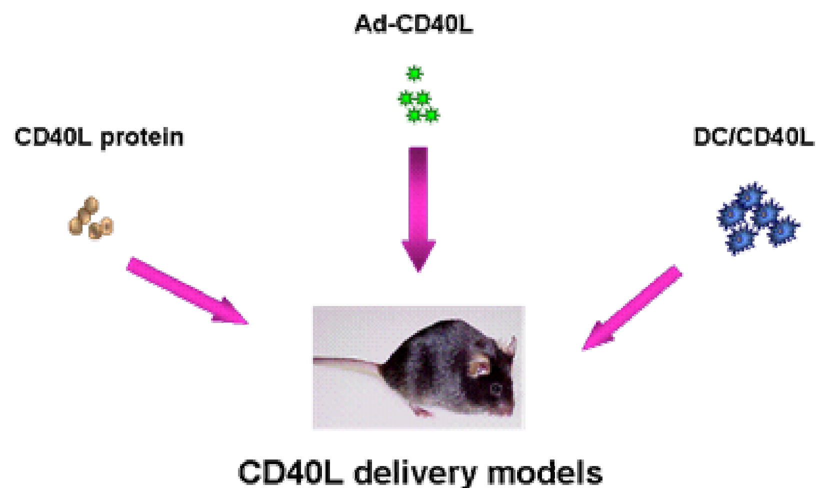


Figure 3 Three approaches to deliver CD40L into the tumor site. 1) Systemic administration of mCD40L protein; 2) Intratumoral administration of Ad-CD40L vector; 3) Intratumoral administration of DC transduced with Ad-CD40L.

7.5 X 10^4 MC38 cells or 2.0 X 10^5 TS/A or 4-T.1 cells were injected subcutaneously in the right flank of mice. On Day 7 when the tumor size reached 25-30 mm^2, intratumoral injection of: a) soluble CD40L trimeric protein (a gift from Immunex Corp); or b) 10^9 pfu of either control adenoviral vectors Ad-ψ5 or Ad-LacZ, or Ad-CD40L; or c) DC (10^6 cells) non-transduced or transduced with Ad-CD40L was performed in a 100 μl volume. Injection of HBSS served as an additional control. The size of each tumor was measured 2 times weekly with calipers and recorded as tumor area (mm^2). For some studies, where indicated, mice were injected with tumor cells in both flanks.

3.2.7 Data Analysis

Statistical analysis of experimental data was performed with a software package SigmaStat (STSS). For all analysis, the level of significance was set at a probability of 0.05 with results that had a p value less then the 0.05 considered significant. Student t-test was used for comparison between two groups after evaluation for normality. Two-way repeated measurements ANOVA was used to compare tumor growth for multiple groups of mice. All experiments were conducted with 5-7 mice per group receiving identical treatments. All experiments were repeated at least twice.

3.3 RESULTS

3.3.1 CD40L protein

It has been reported that CD40L protein might exhibit antitumor properties in a murine tumor model (143). To test its activity in the murine model with MC38 colon adenocarcinoma, tumor cells ($5x10^5$/100μl) were injected s.c. into C57BL/6 mice and animals were treated with soluble CD40L trimeric protein starting at Day 7 (100ng/day x 10). Treatment with FLT3 ligand (100 ng/day x 10) has been used as a positive control. Analysis of tumor growth demonstrated that sCD40L-based therapy did not result in a significant inhibition of tumor growth (**Figure 3.1**).

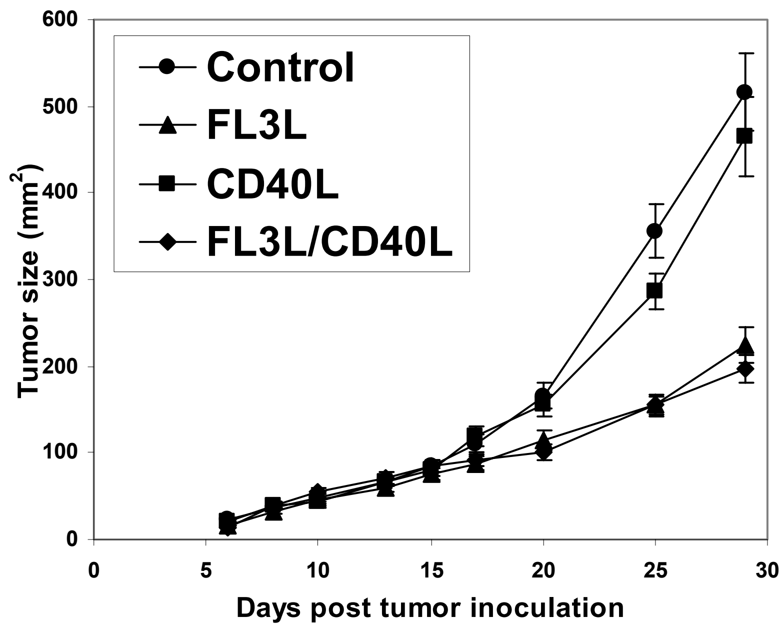


Figure 3. 1 Systemic administration of soluble CD40L protein has no significant effect on tumor growth in MC38 tumor model. MC38 tumor cells were inoculated s.c. into C57BL/6 mice at Day 0. Animals were injected with sCD40L protein at Day 7 for 10 days.

FLT3 ligand was used as a positive control. (N=5 mice/group). This experiment was repeated twice with similar results. Average of all experiments represents mean ± SE.

This experiment was repeated twice with similar results. Thus, these data suggest that soluble CD40L protein has no significant antitumor activity in the murine MC38 colon adenocarcinoma model. These results also justify the development of alternative genetic immunization approaches for tumor treatment.

3.3.2 Preparation of Ad-CD40L

It has been repeatedly demonstrated that DC can be efficiently transduced using an adenoviral vector-based technique. For the purpose of these studies, we have created and characterized several adenoviral vectors. Ad-CD40L was constructed, propagated, and titered according to the standard protocol previously described in the Materials and Methods section. Briefly, murine CD40L cDNA was amplified by PCR, digested by SalI and NotI and CD40L cDNA was subcloned into SalI-NotI site of the adenovirus shuttle plasmid (pAdlox). Next pAdlox/mCD40L plasmid was digested with SfiI and co-transfected with Ad-ψ5- derived, E1- and E3-deleted adenoviral backbone, into 293 cells by calcium phosphate precipitation (**Figure3.2 A**).

Recombinant adenoviruses were isolated from a single plaque, expanded in CRE8 cells, and purified by double cesium chloride gradient ultra-centrifugation (**Figure 3.2 B**). Purified virus was extensively dialyzed against 10 mM Tris/1 mM $MgCl_2$ sterile viral buffer at 4°C, and stored in aliquots at –80°C.

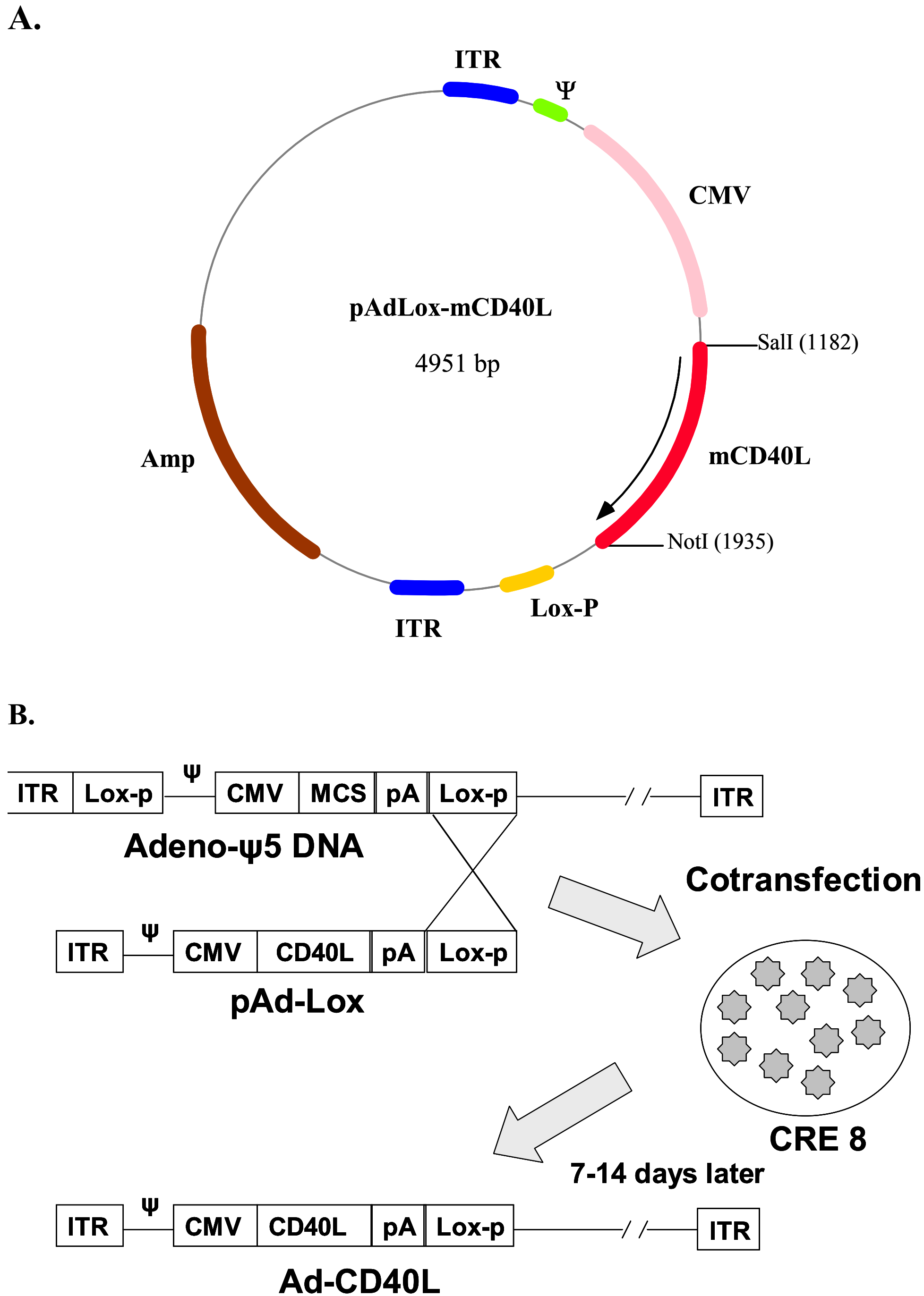


Figure 3.2 Preparation of Ad-CD40L vector. A. Ad-CD40L construct. **B.** Schematic representation of a construction of recombinant Ad-CD40L. Briefly, the murine CD40L cDNA was amplified by PCR and subcloned into SalI-NotI site of the adenovirus shuttle plasmid (pAdlox). pAdlox/mCD40L was linearized with SfiI and co-transfected with the Ad-ψ5-derived, ΔE1, ΔE3 deleted adenoviral

backbone, into 293 cells by calcium phosphate precipitation. The recombinant Ad-CD40L vector was isolated from a single plaque, expended in CRE8 cells, and purified over double cesium chloride gradient ultracentrifugation. The purified virus was extensively dialyzed against 10 mM Tris/1 mM $MgCl_2$ sterile viral buffer at 4°C, stored in aliquots at –80°C.

3.3.3 Examination of efficacy of Ad-CD40L transduction

To evaluate the efficacy of transduction and expression of CD40L, MC38 tumor cells were transduced with CD40L-encoding adenoviral vector (Ad-CD40L). As a negative control, tumor cells were either treated with saline or transduced with Ad-LacZ. Control and Ad-CD40L-transduced MC38 tumor cells were stained with anti-mCD40L antibody. **Figure 3.3** demonstrates that control MC38 tumor cells infected with Ad-ψ5 have no detectable surface expression of CD40L. However, cells infected with Ad-CD40L at an MOI of 100 expressed a high level of CD40L with more than 65% of cells positive for surface CD40L. This experiment was repeated five times with the similar results: 55-80 % of transduced cells were positive for CD40L expression. Thus, these data suggest that transduction of murine cells with the created adenovirus encoding CD40L is a highly effective way to infect cells resulting in a high level of protein expression.

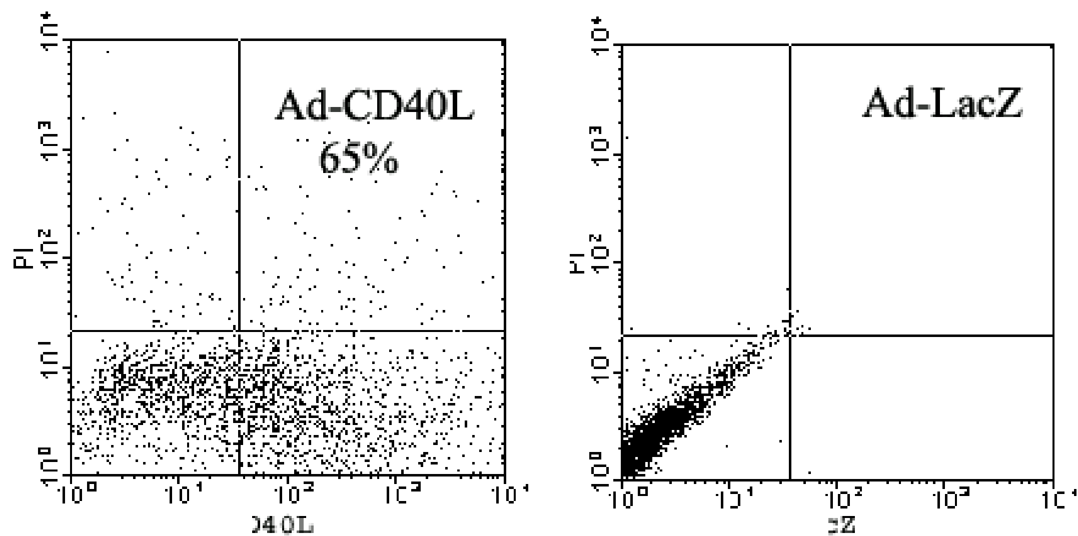
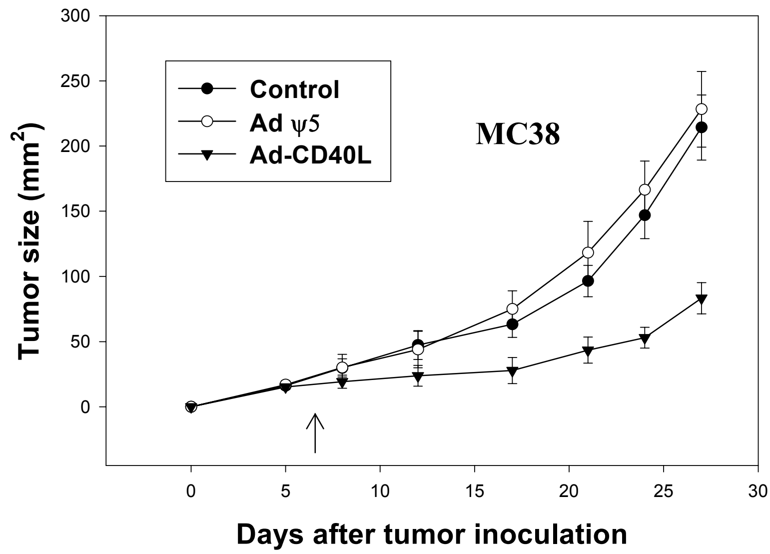


Figure 3.3 Evaluation of Ad-CD40L transduction efficiency. MC38 tumor cells were transduced with Ad-CD40L. As a negative control, tumor cells were transduced with Ad-LacZ. 24 h later cells were collected and stained with antibody against mCD40L. Expression of mCD40L was detected by FACS analysis. The experiment was repeated 4 times with similar results. Representative data are shown.

3.3.4 Intratumoral injection of Ad-CD40L inhibits tumor growth *in vivo*

To determine whether intratumoral delivery of CD40L gene was able to confer an antitumor effect *in vivo*, a murine MC38 adenocarcinoma tumor model was utilized. C57BL/6 mice were inoculated subcutaneously with wild type MC38 tumor cells. On Day 7, when the tumor size was 25 - 30 mm^2, 1 x 10^9 pfu of either Ad-CD40L or the control virus Ad-ψ5 were injected directly into the tumor. As shown in **Figure 3.4 A**, non-treated and Ad-ψ5 treated mice had similar rates of tumor growth with average tumor sizes of 214 ± 29 mm^2 and 228 ± 25 mm^2, respectively, Day 27. In contrast, a single intratumoral injection of Ad-CD40L significantly inhibited tumor growth (83 ± 13 mm^2, $p<0.001$). These data demonstrate that a single intratumoral administration of adenovirus encoding mCD40L results in a marked suppression of tumor growth.

A.



B.

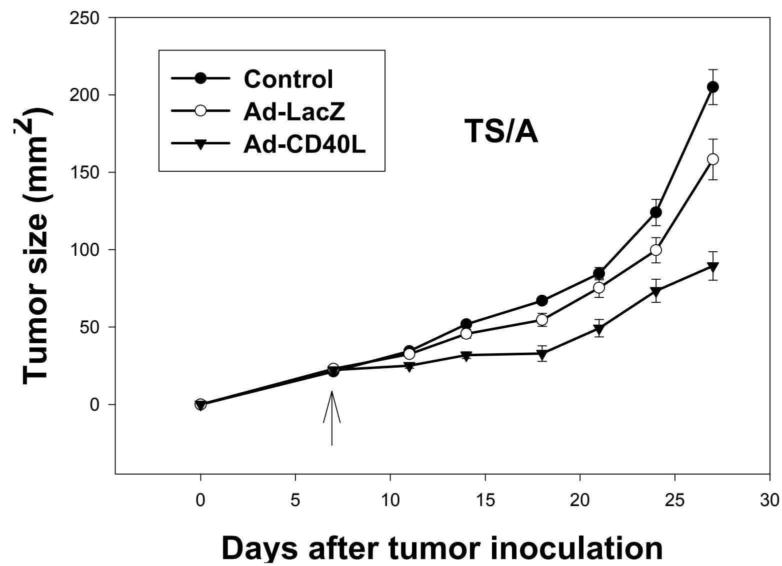


Figure 3.4 Intratumoral administration of Ad-CD40L induces a strong antitumor effect. A. Single injection of saline, Ad-ψ5 or Ad-CD40L (10^9 pfu/mouse) into the tumor site 7 days after MC38

tumor cells inoculation. Tumor size was measured twice a week. **B.** TS/A tumor-bearing mice were injected with either saline or 10^9 pfu of Ad-LacZ or Ad-CD40L on Day 7. Tumors were measured twice a week. (N=5 mice/group). Experiment was repeated twice with similar results. Data represent mean ± SE.

To evaluate whether the therapeutic effect of direct, intratumoral injection of Ad-CD40L was tumor specific, a Balb/c syngeneic breast tumor cell line TS/A was used in the next set of experiments. The TS/A cells were inoculated on Day 0 and palpable tumors were injected with Ad-CD40L on Day 7. As shown in **Figure 3.4 B**, growth of TS/A tumor was significantly inhibited by the treatment with Ad-CD40L (78 ± 9 mm^2) compared to either the untreated control group (201 ± 13 mm^2) or to the group treated with control Ad-LacZ (152 ± 17 mm^2) ($p<0.001$). These results demonstrate that the antitumor effect induced by Ad-CD40L is not MC38 tumor or C57BL/6 strain specific. However, the antitumor effect of Ad-CD40L treatment was less effective in the TS/A model than in the MC38 model.

3.3.5 Transduction of DC with Ad-CD40L *in vitro*

We have demonstrated in Chapter 1 that tumor cells secrete soluble factors that are able to reduce expression of CD40 on DC and consequently, block DC maturation. Moreover, our results suggest that DC obtained from tumor bearing mice express lower levels of co-stimulatory molecules resulting in reduced ability to stimulate T cell activities. Since it has been recently shown that CD40 ligation on DC results in increased expression of CD40 and enhanced DC function, it is possible that overexpression of CD40L on DC may be beneficial for function of CD40-deficient tumor-derived DC by an autocrine/paracrine up-regulation of their maturation in the tumor microenvironment. To test the efficacy of DC transduction, murine bone marrow-derived DC were transduced with Ad-CD40L on Day 5 and analyzed by flow cytometry on Day 7 (**Figure 3.5**).

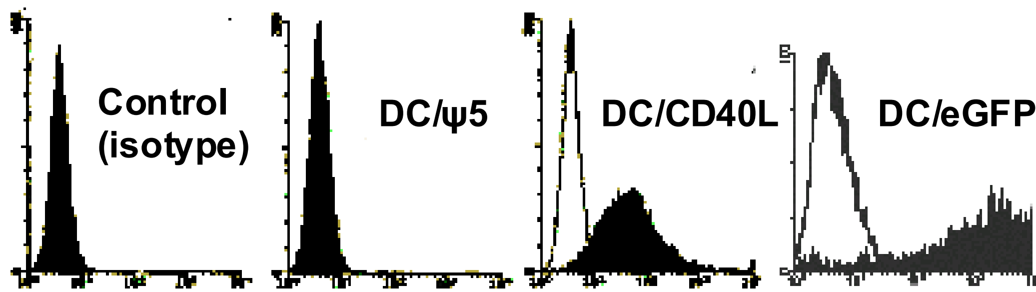


Figure 3.5 Evaluation of efficacy of DC transduced with Ad-CD40L. DC precursors were generated from tumor-free animals and transduced with Ad-CD40L or Ad-ψ5 on Day 5. Transduction of murine DC with adenoviral vector encoding mCD40L was measured by FACScan analysis. Ad-eGFPwas used as a positive control. The results of representative experiment are shown. Similar data was observed in a set of 5 independent experiments.

Transduction of bone marrow-derived DC with Ad-CD40L resulted in expression of CD40L in greater than 80% of DC. Similarly, greater than 95% of the DC were eGFP positive following infection with Ad-eGFP (**Figure 3.5**). These data demonstrated that DC can be efficiently transduced with Ad-CD40L.

2.3.6 Transduction of DC with Ad-CD40L increases their antitumor activity *in vivo*

Our *in vitro* results suggested that overexpression of CD40L on DC may be beneficial for the induction of antitumor immunity since it should induce DC maturation and function (see Chapter 3). To examine the antitumor efficacy of CD40L overexpressing DC, bone marrow-derived DC were infected with Ad-CD40L and injected directly into Day 7 MC38 tumors. As controls, tumors were treated with either DC transduced with Ad-LacZ vector or with untreated non-transduced DC. As expected, mice in both control groups developed rapidly growing tumors (untreated DC: 401 ± 21 mm^2; Ad-LacZ: 302 ± 18 mm^2). In contrast, mice immunized with DC

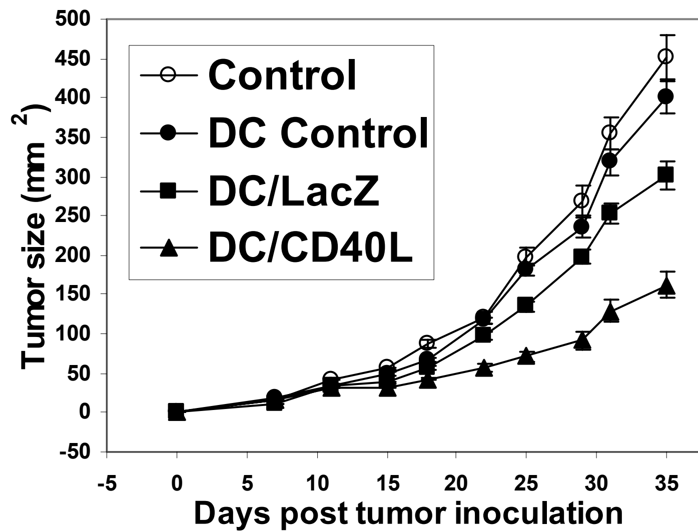
transduced with Ad-CD40L, showed a significant inhibition (162 ± 17 mm^2) of tumor growth ($p<0.001$) (**Figure 3.6 A**). These data demonstrate that a single intratumoral administration of DC/CD40L induces a strong antitumor response *in vivo*.

To evaluate the antitumor effect of DC/CD40L in a different tumor model and another mouse strain, Balb/c mice were inoculated with TS/A breast carcinoma cells and DC/CD40L were administered intratumoraly. Since we have previously demonstrated that Ad-IL-12 infected DC display a strong antitumor activity in a local therapy model (123, 147) treatment of TS/A tumor with Ad-IL-12-infected DC served as an additional positive control. As shown in **Figure 3.6 B**, animals in both control groups, and mice treated with control DC or DC infected with Ad-LacZ developed rapidly growing tumors (398 ± 23 mm^2 and 312 ± 28 mm^2, respectively) by Day 32. Animals in the DC/IL-12 group showed a significant suppression (103 ± 15 mm^2) of tumor growth ($p<0.001$). However, mice treated with DC/CD40L displayed a complete tumor rejection with 83% of mice being tumor-free on Day 23. These experiments were repeated twice with similar results. Our data clearly demonstrate that DC genetically modified by Ad-CD40L infection induce a significant antitumor effect in the TS/A tumor model following intratumoral injection. Moreover, the results suggest that DC/CD40L treatment is more effective than DC/IL-12 in conferring a sustained antitumor response.

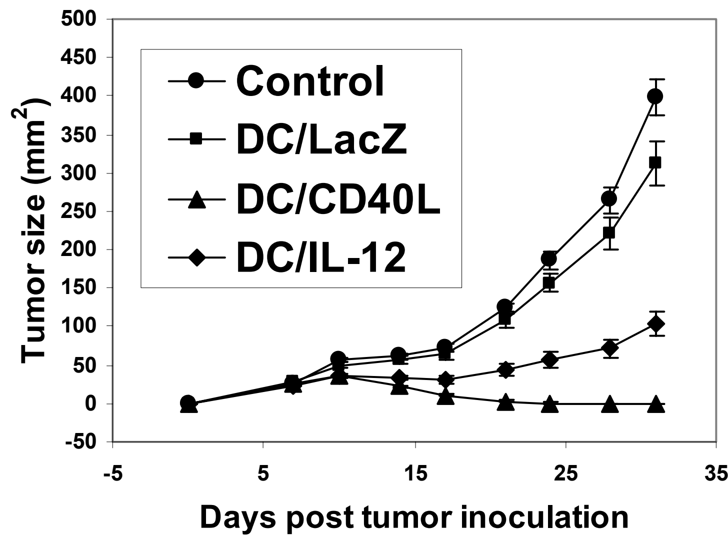
To determine whether tumor rejection induced by DC/CD40L is accompanied by the induction of a specific immune memory, DC/CD40L-treated tumor-free mice were re-challenged with either TS/A tumor cells or a non-related Balb/c syngeneic control 4-T.1 breast adenocarcinoma cells on Day 35. Additional controls included administration of both tumor cell lines into naive syngeneic mice. No tumor growth was observed following TS/A tumor re-challenge in mice previously treated with DC/CD40L (**Figure 3.6 C**). All other groups of mice

developed tumors at the expected growth rate. These results demonstrate that Balb/c mice treated with intratumoral DC/CD40L developed a specific, long-term systemic antitumor immune response and immune memory.

A.



B.



C.

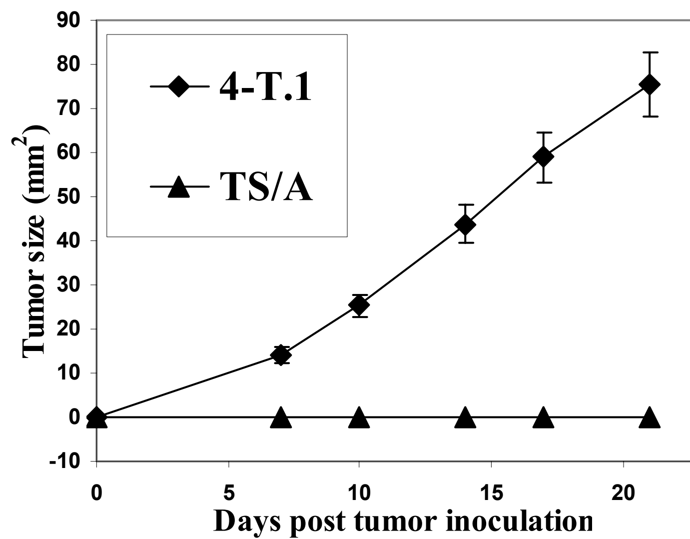


Figure 3.6 Antitumor effect of DC transduced with Ad-CD40L in two tumor models. A. DC were transduced with Ad-CD40L (100 M.O.I.) and injected into 7 day-old MC38 tumor. As controls, DC treated with saline or transduced with Ad-ψ5 were used. Tumor growth was measured twice a week and plotted as a function of time. **B.** DC were transduced with Ad-CD40L and injected into the tumor site of TS/A tumor on Day 7. Control groups were treated with non-modified DC or DC transduced with Ad-LacZ. DC/IL-12-treated group was used as a positive control. **C.** Generation of immune memory by DC/CD40L therapy. Tumor-free mice, rejected TS/A tumors after DC/CD40L treatment, were re-challenged with either TS/A or 4-T.1 cells. Tumor growth was measured twice a week. (N=5 mice/group). Representative results of 3 independent experiments are shown as mean ± SE.

3.3.7 CD40L rescues DC from tumor-induced suppression of antitumor activity

To evaluate the antitumor effect of DC generated from tumor-bearing mice and overexpressing CD40L, DC were transduced with Ad-CD40L on Day 5. As controls, non-transduced DC and DC infected with Ad-LacZ were used. On Day 7 DC were inoculated into the tumor site of TS/A-bearing mice. **Figure 3.7** demonstrates that DC generated from tumor-bearing animals had a strong suppression of antitumor effect in both control groups compare to DC generated from

tumor-free animals. For example, saline control group display size of 434.5 ± 14.4 mm^2 tumors. DC generated from tumor-free mice induced a stronger antitumor effect compare to DC generated from TS/A-bearers (326.6 ± 5.6 mm^2 in Cont/Cont groups vs 404.4 ± 11.1 mm^2 in Cont/Tu group). Similarly, DC from LacZ/Cont group (302.4 ± 8.75 mm^2) had smaller tumor size compare to DC from LacZ/Tu group (357.0 ± 11 7 mm^2).

In addition, **Figure 3.7** shows that DC in CD40L/control group induced a strong antitumor response (6.4 ± 1.5 mm^2, $p<0.001$). However, DC generated from tumor-bearing mice and infected with Ad-CD40L also dramatically suppressed tumor growth (168.6 ± 8.1 mm^2, $p<0.05$) compared to control groups. This elicited antitumor effect was not as strong as in DC generated from control mice and transduced with Ad-CD40L but it was significant ($p<0.05$). These data demonstrate that CD40L rescues DC from tumor-mediated suppression of DC activities.

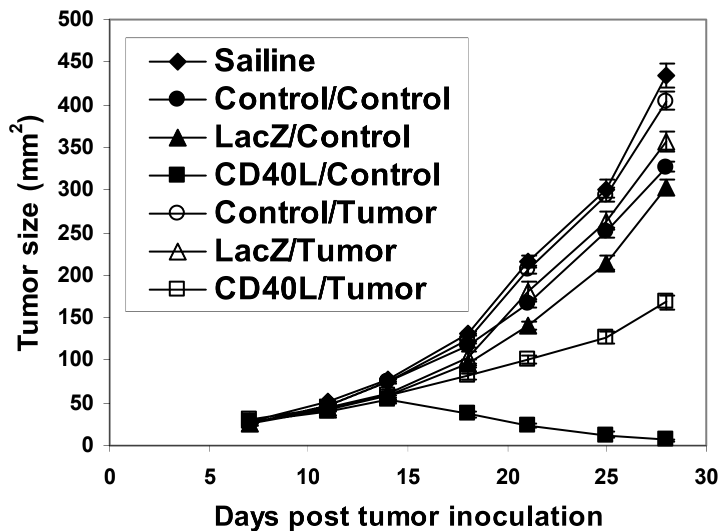


Figure 3.7 Transduction with CD40L rescues DC from tumor-induced suppression. DC precursors generated from control and tumor-bearing mice, were transduced with Ad-CD40L on Day 5 and injected into the tumor site of TS/A-bearing mice. DC/LacZ and

uninfected DC were used as negative controls. Tumor size was measured twice a week. (N=5 animals/group). Experiment was repeated twice with similar results. Average data represent mean ± SE.

3.4 CONCLUSIONS

In this set of experiments we have demonstrated that overexpression of CD40L at the tumor site by injecting adenovirus or transduced DC generated strong antitumor immunity. Interestingly, direct injection of recombinant mCD40L protein did not induce a significant antitumor effect. In contrast, a single injection of Ad-CD40L directly to the tumors produced strong antitumor immunity in MC38 and TS/A tumor models. Similarly, introduction of DC genetically modified to overexpress CD40L at the tumor site induced a significant antitumor effect. Furthermore, CD40L transduced DC caused a complete rejection of TS/A tumors with generation of specific immune memory. In addition, CD40L was able to restore antitumor activity in DC generated from tumor-bearing mice. Taken together, our results demonstrate that CD40L overexpression on DC induces a significant antitumor immunity in mice and genetically modified DC expressing high levels of CD40L could be considered as a novel gene immunotherapy to treat cancer. However, the means of action of CD40L on DC is not clear. Therefore, next, we determined the primary mechanisms responsible for the generation of antitumor immunity in mice by DC/CD40L-based immunotherapy (next Chapter).

Summary of conclusions:

- Systemic administration of CD40L protein does not inhibit MC38 tumor growth
- Administration of Ad-CD40L at the tumor site induces significant inhibition of MC38 and TS/A tumor growth
- Intratumoral administration of DC transduced with Ad-CD40L elicits a strong antitumor effect in MC38 tumor model
- Intratumoral injection of DC modified to overexpress CD40L induces significant antitumor immunity with the generation of specific long-lasting immune memory
- CD40L up-regulates the antitumor activity of DC generated from tumor-bearing mice

4.0 MECHANISM OF ANTITUMOR ACTIVITY OF LOCAL DELIVERY OF CD40L *IN VIVO*

4.1 INTRODUCTION

CD154 (CD40 ligand, CD40L), an important co-stimulatory molecule primarily expressed on activated CD4+ helper T cells (142), is a 33-36 kD type II integral membrane glycoprotein from the TNF superfamily. The receptor for CD154 is CD40, which is expressed on a variety of cell types. In hematopoietic cells, CD40 is present on CD34+ hematopoietic progenitors, B cell progenitors, mature B lymphocytes, plasma cells, monocytes, DC, eosinophils, basophils, and subpopulations of T lymphocytes. CD40 is also expressed on non-hematopoietic cells including endothelial cells, fibroblasts, and epithelial cells (142). The interaction between CD40 and CD40L is critical for the generation of cell-mediated immune responses (76, 80, 128), including antitumor immunity. For example, mice vaccinated with a CD40L expressing plasmid did not develop metastatic tumors following challenge with a lethal dose of tumor cells (144). In addition, it was observed that treatment with anti-CD40L monoclonal antibody inhibited the generation of protective immune responses after the administration of three potent tumor vaccines: irradiated MCA 105 tumor cells, MCA 105 tumor cells admixed with *Corynebacterium parvum* adjuvant, and irradiated B16 melanoma cells transduced with the gene

encoding GM-CSF (88). Further confirmation of the role of CD40/CD40L interactions in tumor immunity was provided by the overt tumor susceptibility of mice deficient for the CD40 receptor (88).

Professional APC, in particular DC, play a central role in the induction of T cell and T-dependent immune responses. Ligation of CD40 expressed by DC provides the signals required by the APC for initiation of adaptive immune responses. In particular, CD40 provides an important signal for the maturation and function of DC both *in vitro* and *in vivo*. Furthermore, CD40 ligation has been also shown to inhibit both spontaneous (148) and tumor-induced apoptosis of DC (113), and protects B cells from apoptotic death by up-regulating the apoptosis inhibitory protein Bcl-x_L (149). CD4+ T cells stimulate DC through a CD40-CD40L interaction resulting in DC that can effectively stimulate CD8+ T cell responses. Gene transfer of CD40L to DC appears to stimulate a CD8+ response in the absence of CD4+ T cells (150).

After capture of tumor antigen(s) DC undergo maturation and migrate to regional lymph nodes where the presentation of antigenic peptides to T lymphocytes takes place. Thus their correct functioning as APC involves localization in tissues and trafficking via the lymph to lymphoid organs. It is believed that migration of DC to the draining lymph nodes is an important component of initiation of the adaptive immune response. However, nothing is known about the mechanisms responsible for homing and trafficking of DC after CD40 ligation. We have demonstrated in Chapter 2 that CD40L-transduced DC display a strong antitumor potential when injected into the tumor tissue. However, it is unclear whether administration of CD40L-transduced DC into tumor-bearing host alters DC homing within the tumor tissue or DC trafficking to the regional lymph nodes. The regulation of CTL activity and survival by DC

within the tumor tissues, as well as presentation of tumor antigens to T cells by DC within the lymph nodes are required for the immune response.

In Chapter 2 we demonstrated that CD40L-based immunotherapy induces strong antitumor immunity when administered into the tumor site. Here, we hypothesized that CD40L, in addition to stimulating DC to overexpress co-stimulatory molecules and produce high levels of IL-12, would increase DC survival and trafficking in/out of tumor tissue, and augment the ability of DC to present tumor antigen to T cells and induce CTL activity. In addition, we proposed that CD40L will be able to rescue DC from tumor-induced DC dysfunction. The rationale for the proposed study was based on data showing that CD40 ligation plays an important role in DC generation, activation and function.

The main goal of these set of experiments was to investigate the primary mechanisms responsible for the strong antitumor immunity of DC overexpressing CD40L. We performed a series of experiments to verify the important role of CD40L on DC maturation, activities, survival, autostimulation and trafficking. We have demonstrated that transduction of DC with CD40L increases expression of CD40, CD80, CD86 and class I molecules and production of IL-12. Furthermore, transduction of DC generated from tumor bearers rescues DC from tumor-mediated suppression of expression of co-stimulatory molecules. Additionally, increased production of IL-12 by CD40L-transduced DC was not inhibited by tumor cells, suggesting additional pathways of the antitumor activity of DC/CD40L. Moreover, we have demonstrated that CD40L increases DC survival in growth-factor depleted cultures, induces CTL activity, antigen presentation to T cells, and trafficking from tumor tissue to the lymphoid organs. Also, we showed that DC/CD40L-based therapy increased accumulation of CD11c+ DC at the tumor

site. Taken together, we have demonstrated that stimulation of major DC functions and activities could explain the antitumor effect of CD40L-based immunotherapy.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Female 6-8 week-old C57BL/6 (H-2^b) and Balb/c (H-2^d) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in groups of five in a 12:12 hour light:dark cycle with standard mice chow and water. All animals were acclimatized at least 2 weeks prior to experimentations. Animals were handled in accordance with IACUC protocols.

4.2.2 Tumor cell lines

MC38 colon and TS/A breast adenocarcinoma cells were maintained in RPMI 1640 medium (GIBCO BRL-Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (GIBCO BRL-Life Technologies).

A single-cell suspension of tumor cells (0.2 ml) was injected s.c. into the right flank. Injection of HBSS served as a negative control. C57BL/6 mice received s.c. injection of 5 X 10^5 MC38 cells. Balb/c mice received s.c. injection of 2 X 10^5 TS/A cells.

4.2.3 DC generation

DC were generated from hematopoietic progenitors isolated from mouse bone marrow using control tumor-free or tumor-bearing mice. Femur and tibia marrow cells from C57BL/6 or Balb/c mice were passed through a nylon cell strainer to remove pieces of bones and debris and depleted

of erythrocytes by incubating with lysing buffer for 2 min. Then cell suspensions were incubated with anti-mouse B220, CD4 and CD8 antibodies for 1 h at 4ºC. This was followed by incubation with rabbit complement for 30 min at 37ºC to deplete B and T lymphocytes. The cells were then plated in 6-well plates ($0.2x10^6$ cells/ml 4 ml/plate) in a complete medium (RPMI 1640, 10% heat-inactivated FBS, 2mM L-glutamine, 10mM Hepes, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate) overnight. Then the non-adherent cells were collected and re-suspended in complete medium with addition of 1000U/ml mGM-CSF and mIL-4 (ENDOGEN, Woburn, MA). On Days 3 and 5, an additional dose of cytokines was added to the cell cultures. At Day 7, DC were collected and used for the intratumoral injections (10^6 DC/mouse) or other assays.

To examine morphological characteristics of DC, DC were collected and fixed on microscope slides using a Cytospin centrifuge (Shandon Lipshaw, Pittsburgh, PA). After drying for 5-10 min, cells were fixed and stained with a LeucoStat Stain Kit (Fisher Scientific, Pittsburgh, PA), and morphology of cells was observed using a low power microscope.

4.2.4 DC survival

For DC survival studies, transduced DC were collected at Day 7 and re-plated (at concentration 10^6 cells/well) in complete DC medium without addition of IL-4 and GM-CSF. Live and dead cells were counted every day for 3 weeks using Trypan blue exclusion method. Cells were collected and re-suspended in 1 ml of medium. 30 µl of cell suspension and 30 µl of Trypan blue solution were mixed well and a 10 µl aliquot was applied to each side of a hematocytometer. Cells were counted under 10 X magnification. Percent Viable = (number of live cells / total cell count) X 100. Data waere plotted as a percentage of viable DC over time.

4.2.5 Direct effect of DC on tumor cells

DC were plated in 96-well plates at different concentrations in triplicate. Then TS/A cells were added to each well at 5 X 10^4 cells/well concentration. Cells were pulsed with 1 µCi/well of ^{3}H-thymidine and harvested using a Skatron (Skatron, Lier, Norway) cell harvester at either Day 7, 8 or 9. Proliferation of tumor cells was determined by measuring uptake of ^{3}H-thymidine on a MicroBeta TRILUX liquid scintillation counter (WALLAC, Gaithersburg, MD). Tests were performed in triplicate and results were expressed as the mean counts per minute (cpm) ± standard deviation (SD).

4.2.6 Flow cytometry

For phenotypic analysis of bone marrow-derived DC (Day 7), FITC- or PE-conjugated monoclonal antibodies recognizing murine CD11c, CD80, CD86, MHC class I and class II, and CD40 molecules were used (Pharmingen, San Diego, CA). After incubation with antibodies for 30 min at 4°C, cells were washed with PBS and analyzed on a FACStar using Cellquest FACS analysis software (Becton Dickinson, San Diego, CA).

4.2.7 IL-12 production

IL-12 production by DC was determined by p70 IL-12 ELISA. A standard curve was generated using recombinant murine IL-12 (R & D Systems, Minneapolis, MN). To induce IL-12 expression, DC cultures were treated with inactivated *Staphylococcus aureus* (0.1% v/v of essentially non-viable cell suspension), (Sigma, St Louis, MO) 20 µl/well per 1x10^6 DC cells for 24 hr.

4.2.8 MLR Assay

Functional activity of DC was determined in one-way allogeneic MLR using T lymphocytes. Allogeneic T cells were obtained from spleen cell suspensions by passage through the nylon

wool columns after the lysing of red blood cells. Cultured DC generated from control or tumor-bearing mice were added to T cells (3 X 10^5 cells/well) at different ratios (10^2-10^6 cells per well) in final volume of 200 µl per well. Cells were mixed in 96-well round-bottom plates and incubated for 3 days. Cell cultures were pulsed with 1 µCi of ^{3}H-thymidine/well (DuPont-NEN, Boston, MA) for 16-18 hr and harvested using a Skatron (Skatron, Lier, Norway) cell harvester. Proliferation of T cells was determined by measuring uptake of ^{3}H-thymidine on a MicroBeta TRILUX liquid scintillation counter (WALLAC, Gaithersburg, MD). Tests were performed in triplicates and results were expressed as the mean counts per minute (cpm) ± standard deviation (SD).

4.2.9 Presentation of TAA by DC *in vitro*

Similarly, a syngeneic MLR assay was used to determine functional activities of DC pulsed with tumor antigens. For these studies, TS/A cells were frozen and thawed 3 times to obtain tumor antigens as tumor cells lysates. Then DC were incubated with tumor lysates for 24 hr at different ratios (1:1, 1:10, 1:100 DC : Tumor cells number, before they were destroyed) and washed. To separate live and dead cells, cell suspensions were loaded onto NycoPrep solution (1:1 mixed 1.068 and 1.077 g/ml, Nycomed Pharma AS, Oslo, Norway) and centrifuged for 20 min at 400 x g. The cells from the interface exhibited a viability of > 98% (trypan blue-negative or 'live' DC), and the cells from the bottom exhibited a viability of < 2 % (trypan blue-positive or 'dead' DC). Next, syngeneic T cells were isolated from spleen of Balb/c mice and co-incubated with pulsed DC for 3 days. Proliferation of T cells was measured as described in the MLR assay. Non-pulsed DC were used as a control.

4.2.10 Cytotoxic T lymphocyte assay.

To evaluate the levels of CTL activity, splenocytes were pooled from control mice, mice treated with control DC, or DC transduced with Ad-LacZ or Ad-CD40L, 7 days post intratumoral injection of DC. The spleen cells were re-stimulated *in vitro* with irradiated (6000 rad) TS/A cells at a responder to stimulator ratio of 10:1 in culture medium supplemented with 10 IU/ml IL-2. Cytolytic activity was assayed after 6 days of incubation. Target cells (TS/A and irrelevant control YAC-1 cells) were labeled with 100 μCi/$1x10^6$ cells of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) for 1 h and plated in round-bottom 96-well plates at $1x10^4$ cells/well after washing. Effector cells were added at various Effector : Target ratios in triplicate. The total reaction volume was kept constant at 200 μl/well. After cells were incubated for 4 h at 37 ºC/ 5% CO_2, the ^{51}Cr release was measured in a gamma-counter. The amount of ^{51}Cr spontaneously released was obtained by incubating target cells in medium alone. The maximum amount of ^{51}Cr incorporated was determined by adding 10% SDS. The percentage lysis was calculated as follows: % lysis = {(experimental release – spontaneous release)/(maximum release – spontaneous release)} x 100.

4.2.11 Immunohistochemistry

Tumor tissue sections were embedded in OCT compound (Miles, Elkhart, IN), snap-frozen on dry ice, and stored at -80ºC. Six μm cryostat sections wee air-dried and fixed in ice-cold acetone. Then slides were washed in PBS and incubated with CD11c antibodies (N418; Serotec) at 1:800 dilution for 1 hr at room temperature. Biotinylated mouse anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:500 dilution was used as a secondary antibody for 45 min at room temperature. After developing with peroxidase chromogen kit (3-amino-9-ethylcarbazol, Biomega, Foster City, CA) for 8 min, counterstaining was performed

with hematoxylin. Two investigators analyzed the slides independently to determine the number of positive cells per area. At least 10 different areas were analyzed. Negative controls included staining with irrelevant isotype antibodies. DC infiltration per high power field was graded 0 through 5 as follows: 0, absence of DC; 1, a small quantity of DC (2-5% stained cells); 2, a medium infiltration (5-20%); 3, medium to high amount of DC (20-50%); 4, a high number of DC present (widely distributed cells throughout the whole slide).

4.2.12 DC trafficking studies

DC were generated, cultured and transduced with Ad-EGFP, Ad-LacZ or Ad-CD40L on Day 5. Non-transduced DC were used as a negative control. On Day 7 DC were collected, stained with Cy3 (DiI cell-labeling solution, Molecular Probes, Eugene, OR; 5 µl/1 X 10^6 DC/1 ml) for 30 min at 37ºC, washed 3 times in warm medium and injected (10^6 cells/mouse) into the tumor site of TS/A-bearing mice. Two hr, 24 hr or 48 hr later, tumor, spleen and lymph nodes were removed from one mouse in each group, placed on ice and analyzed by 2-photon microscopy methods. Fresh tissues were imaged for the presence of Cy3-positive cells using a multiphoton laser scanning confocal microscope system comprising a titanium-sapphire ultrafast tunable laser system (Coherent Mira Model 900-F), Olympus Fluoview confocal scanning electronics, an Olympus IX70 inverted system microscope, and custom built input-power attenuation and external photomultiplier detection systems. Single-plane image acquisition utilized two-photon excitation at 850nm with Olympus water-immersion objectives (20X UApo 0.7NA, 60X UplanApo, 1.2NA). Emission filters comprised a HQ535/50m filter (green emission), a 565dclp dichroic mirror and a HQ610/75m filter (red emission).

4.2.13 Data Analysis

Statistical analysis of experimental data was performed with a Sigma Stat software package STSS. For all analysis, the level of significance was set at a probability of 0.05 with results that had a p value less then the 0.05 considered significant. Student t-test was used for comparison between two groups after evaluation for normality. Two-way repeated measurements ANOVA was used to compare tumor growth for multiple groups of mice. All experiments were conducted with 5-7 mice per group receiving identical treatments. All experiments were repeated at least twice.

4.3 RESULTS

4.3.1 Transduction of DC with CD40L induces significant IL-12 production

The interaction between CD40/CD40L is an essential trigger for IL-12 production by DC, important for induction of Th1 responses *in vivo* ((80) and regulation of Th1/Th2 balance (83, 84). To evaluate whether CD40L-modified DC induce IL-12 production, DC from C57BL/6 mice were treated with saline or infected with Ad-LacZ or Ad-CD40L. DC infected with Ad-IL-12 were used as a positive control. Twenty four hours post-transduction, both control and transduced DC cultures were treated with heat-inactivated *Staphylococcus aureus* (*S. aureus),* a potent inductor of IL-12 expression in cultured DC (151). The results revealed only a slight difference in *S.aureus*-induced IL-12 production between control DC and DC transduced with Ad-LacZ. However, DC infected with Ad-CD40L produced a significantly higher level of IL-12 (5500 ± 578 pg/ml/10^5 cells/24 hr) ($p<0.01$) that was similar to the level of IL-12 production by

Ad-IL-12-infected DC (5400 ± 649 pg/ml ml/10^5 cells/24 hr) (**Figure 4.1**). The level of IL-12 production from the Ad-CD40L and Ad-IL-12 infected DC could be further simulated with treatment by the *S.aureus*. Similar results were observed in DC generated from Balb/c mice (data not shown). Thus, CD40L-transduction induces production of high levels of IL-12 by DC.

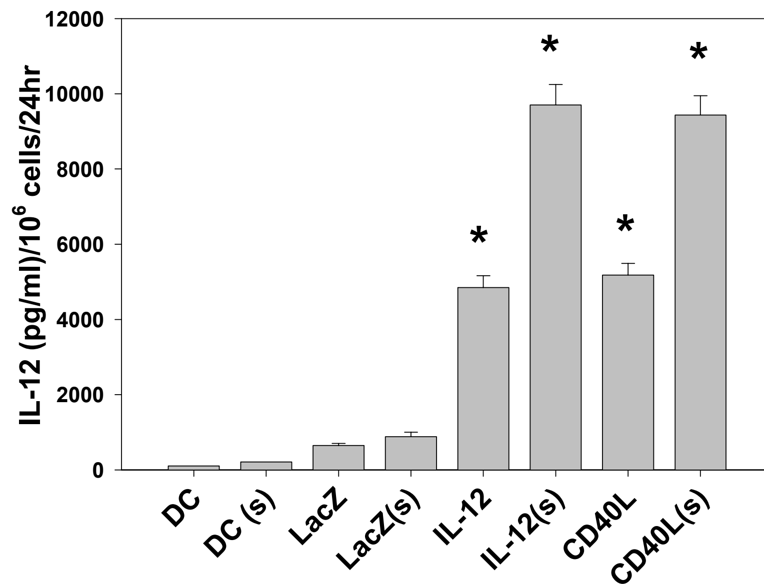


Figure 4. 1 CD40L transduction stimulates IL-12 production. DC were generated from C57BL/6 mice and transduced with Ad-CD40L or Ad-LacZ vectors. Ad-IL-12 was used as a positive control. Half of DC from each group were stimulated with *S. aureus* on Day 6. 24 hr later, cell-free supernatants were collected and the concentration of IL-12 was measured by p70 IL-12 ELISA. The mean ± SD of 3 independent experiments are shown. Groups: DC, control untreated DC; DC (s), control DC stimulated with *S. aureus*; LacZ, DC transduced with Ad-LacZ, LacZ (s), DC/LacZ stimulated with *S. aureus*; IL-12, DC transduced with Ad-IL-12; IL-12 (s), DC/IL-12 stimulated with *S. aureus*; CD40L, DC transduced with Ad-CD40L, CD40L (s), DC/CD40L stimulated with *S. aureus*. (*) $p<0.001$.

4.3.2 CD40L transduction protects DC from tumor-induced suppression of IL-12 production

To investigate whether CD40L could protect DC from tumor-induced dysfunction, we co-incubated DC (1 X 10^6 cells/well) with TS/A tumor cells (2 X 10^6 cells/well) for 24 or 48 hr. TS/A cells were added in the 6-well-plate inserts, to prevent a direct interaction between DC and tumor cells.

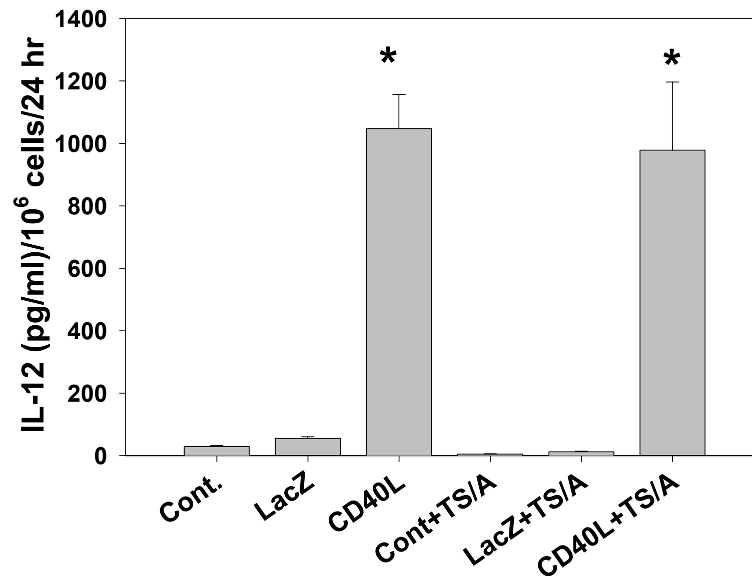


Figure 4. 2 CD40L transduction rescues DC from tumor-suppressed IL-12 secretion. Transduced DC were co-incubated with TS/A tumor cells for 48 hr, added in inserts with 0.4 µm pore size. Cell-free supernatants were collected and IL-12 production was measured by p70 IL-12 ELISA. Results of a representative of 3 independent experiments are demonstrated as mean ± SD. Groups: Cont, non-transduced DC; LacZ, DC transduced with Ad-LacZ; CD40L, DC transduced with Ad-CD40L; Cont+TS/A, non-transduced DC incubated with TS/A cells; LacZ+TS/A, DC/LacZ incubated with TS/A cells; CD40L+TS/A, DC/CD40L incubated with TS/A cells.

Cell-free supernatants were collected and IL-12 production was measured by ELISA. Our data demonstrated that tumor cells did not have a significant influence on DC modified with CD40L (**Figure 4.2**). Therefore, our result suggested that CD40L protects DC from tumor-mediated suppression of IL-12 secretion by DC.

4.3.3 CD40L-transduced DC express high levels of co-stimulatory molecules

To examine the effect of CD40L gene transfer to DC, the morphology and phenotype of the transduced cells were analyzed. DC derived from control bone marrow cultures exhibited the veiled dendrite morphology typical for DC and displayed a characteristic set of DC surface markers (**Table 2**). The genetically modified control DC expressed similar levels of MHC class I and II molecules, co-stimulatory molecules CD80, CD86, ICAM-1 adhesion molecules, and integrin CD11c. Infection of DC with Ad-CD40L at a 100 MOI resulted in a marked increase in the levels of expression of CD80, CD86, CD40 and MHC class I molecules. For example, 45.6% of CD40L-transfected DC expressed CD86 molecules in comparison with 24.6% and 26.9% in non-transduced and Ad-ψ5 cells, respectively ($p<0.05$). Similar results were observed for CD80 and CD40 expression. Interestingly, expression of MHC class I molecules on DC was also increased upon CD40L transduction confirming that overexpression of CD40L on DC stimulates their maturation by an autocrine and/or paracrine manner. For instance, control and Ad-ψ5-treated DC expressed 25.8% and 34.0% I^{ab}, respectively. In contrast, DC/CD40L up-regulated I^{ab} levels to 48.7% ($p<0.05$). These data suggested that CD40L transduction increases the levels of co-stimulatory molecules expressed on DC which might be directly related to up-regulation of DC ability to activate T cells.

Table 2 Transduction of DC with Ad-CD40L stimulates expression of CD40, CD80, CD86 and class I molecules.

	CD11c	**CD80**	**CD86**	**I ^{ab}**	**CD40**
Control	75.7%	32.5%	24.6%	25.8%	21.8%
Ad-ψ5	73.9%	40.2%	26.9%	34.0%	28.4%
CD40L	75.4%	56.7%*	55.6%**	48.7%*	49.2%**

DC were generated from C57BL/6 mice and transduced with CD40L on Day 5. Uninfected DC or DC transduced with Ad- ψ5 were used as controls. Expression of co-stimulatory molecules was measured by FACScan analysis on Day 7. Average of 4 independent experiments is shown. (*p<0.05, **p<0.01)

Next, we tested whether CD40L is able to restore suppressed expression of CD40, CD80 and CD86 on DC generated from tumor-bearing mice. Generated DC were cultured in complete medium and transduced with CD40L on Day 5. Untreated and LacZ-transduced DC were used as controls. Cells from tumor-free mice served as an additional control. Interestingly, DC generated from tumor-bearing mice also displayed increased levels of co-stimulatory molecules after transduction with Ad-CD40L compared to controls. **Figure 4.3** demonstrates that similar to DC generated from C57BL/6 mice, the phenotype of DC generated from Balb/c animals was significantly influenced by CD40L. For instance, the level of CD86 was incresed from 31% in untreated control and 48% in LacZ-transduced groups to 70% in the DC/CD40L group (p<0.01). Similar effects were observed for the levels of CD80, I^{ad} and CD40. Levels of CD40 were increased almost two fold: control groups expressed 33% of CD40, LacZ – 48% and CD40L amplified levels up to 74% (p<0.05). An analogous pattern was observed in DC cultures generated from TS/A-bearing mice. Even though general levels of co-stimulatory molecules

were low in both control groups on DC generated from tumor bearers compared to DC generated from tumor-free animals, CD40L was able to elevate them to the values similar to control DC/CD40L group.

Figure 4.3 shows that in tumor-bearers CD40L increased CD86 from 20% in Cont/Tu and 28% in LacZ/Tu groups to 61% ($p<0.01$). Levels of CD40 were raised from 22% in Cont/Tu and 26% in LacZ/Tu groups to 68% in CD40L/Tu group by transduction with Ad-CD40L. These results suggest that CD40L transduction stimulates maturation of DC and is one of the mechanisms involved in antitumor immunity induced by DC modified to express CD40L *in vivo*.

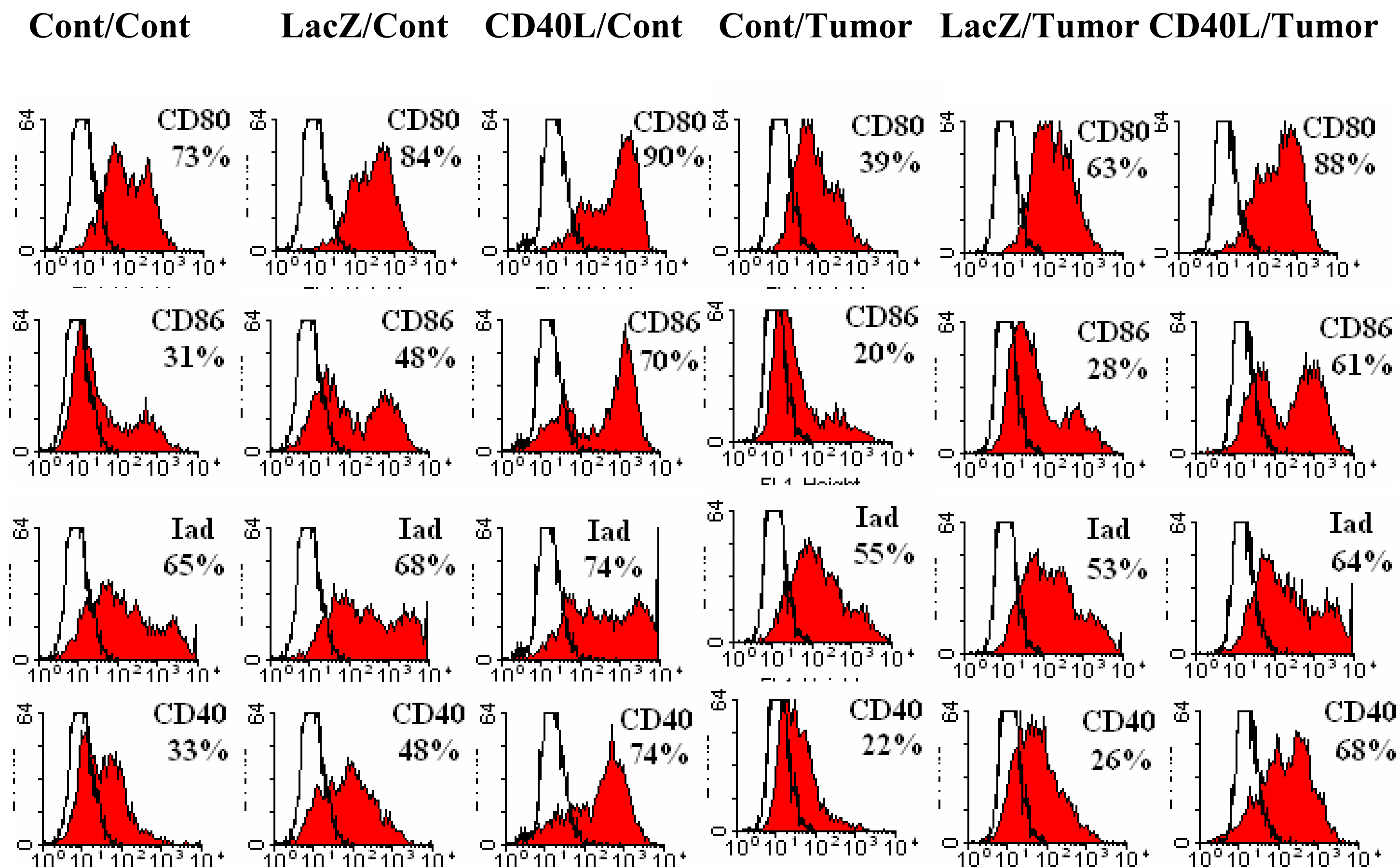


Figure 4. 3 Transduction with Ad-CD40L up-regulates expression of co-stimulatory molecules on DC and rescues DC from tumor-mediated down-regulation of CD80, CD86 and CD40 expression. DC were

generated from control and TS/A-bearing mice. Cells were transduced and stained with antibodies for different co-stimulatory molecules, expression of which was measured by FACScan analysis. Data from representative of 3 independent experiments is shown. Groups: Cont/Cont, non-transduced DC from tumor-free animals; LacZ/Cont, DC transduced with Ad-LacZ from tumor-free animals; CD40L/Cont, DC transduced with Ad-CD40L from tumor-free animals; Cont/Tumor, non-transduced DC from tumor-bearing mice; LacZ/Tumor, DC transduced with Ad-LacZ from tumor-bearing mice; CD40L/Tumor, DC transduced with Ad-CD40L from tumor-bearing mice.

4.3.4 CD40L induces clustering of transduced DC

Based on our previously described data, we hypothesized that overexpression of CD40L on transduced DC caused activation of DC by an autocrine/paracrine manner. To test this hypothesis, we transduced bone marrow-derived DC with Ad-CD40L vector and evaluated cluster formation in control and treated cultures. Non-treated DC and DC transduced with Ad-ψ5 vector served as negative controls. As shown in **Figure 4.4**, CD40L-expressing DC formed multiple spontaneous clusters, the numbers of which were significantly higher than in control groups. In contrast, DC from control groups did not organize themselves in such structures. Therefore, it is likely that CD40L-transduced DC activate each other in the tight clusters by multiple CD40 ligations resulting in accelerated maturation of cells as evidenced by increased expression of co-stimulatory molecules CD80 (B7-1), CD86 (B7-2), MHC molecules, and increased production of IL-12. Furthermore, these results demonstrate that transduction of DC with Ad-CD40L induces clustering of these cells and support our hypothesis that CD40L-transfected DC might be activated in an autocrine or paracrine manner.

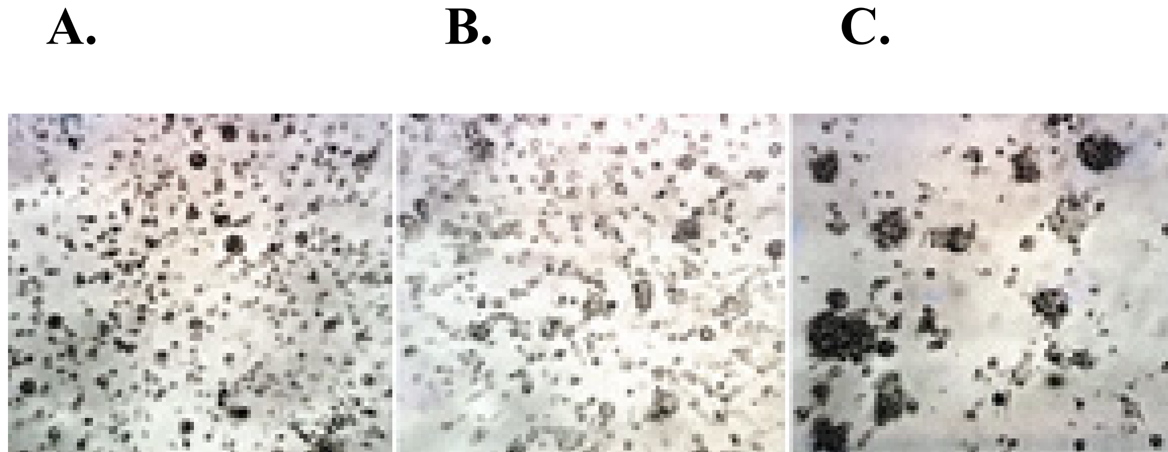


Figure 4. 4 Cluster formation by CD40L-transduced DC. Bone marrow-derived DC transduced with Ad-CD40L displayed an intensive spontaneous cluster formation in cultures (**C**). Control non-transduced DC (**A**) and transduced DC (**B**) were not organized in cluster structures. The results from a representative experiment are shown. Similar data were obtained in five separate experiments.

4.3.5 DC/CD40L do not have a direct antitumor effect *in vitro*

To rule out the possibility that DC/CD40L might have a direct inhibitory effect on tumor cells, TS/A tumor cells ($5x10^5$ cells/well) were incubated in the presence of either unmodified DC or DC transduced with Ad-CD40L at different ratios (1:10 and 1:50 Effector : Target ratio) (**Figure 4.5**). The proliferation rate of tumor cells was analyzed using ^{3}H-thymidine incorporation. The results of these experiments showed no significant effect of all tested DC groups on tumor cell growth *in vitro* ($p>0.3$). Thus the significant antitumor effects observed following DC/CD40L treatment *in vivo* appear not to be mediated through a direct effect of DC on tumor cells.

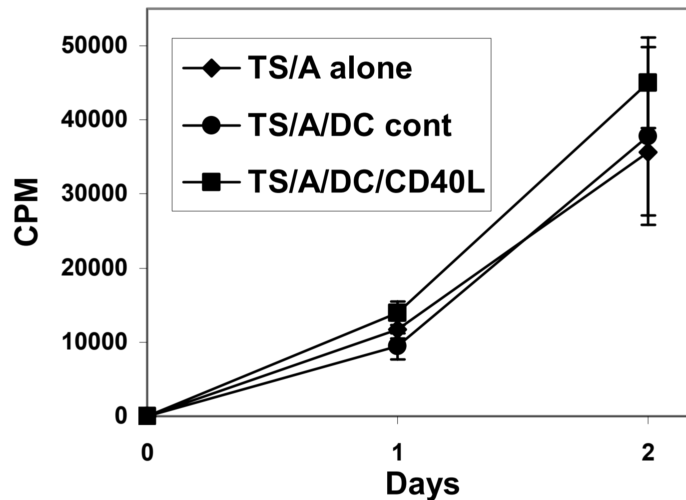


Figure 4. 5 DC transduced with Ad-CD40L do not have a direct effect on TS/A tumor growth *in vitro*. DC were transduced with Ad-CD40L on Day 5. Untreated DC were used as a control. On Day 7 cells were collected and co-incubated with TS/A cells at different concentrations. The proliferation of the tumor cells was analyzed using ^{3}H-thymidine incorporation 0, 24 and 48 hr after incubation. TS/A cells proliferation was plotted as a function of time (days). Means from 3 experiments ± SD are shown.

4.3.6 CD40L increases survival of DC in growth factor-depleted cultures

To examine CD40L effect on DC survival, we generated DC cultures from control mice, transduced DC on Day 5 with Ad-LacZ or Ad-CD40L, collected cells on Day 7, and re-plated cells in complete medium without GM-CSF and IL-4. Cells were counted every day for 3 weeks. **Figure 4.6** demonstrates that DC/CD40L survived much longer in cytokine-free medium compared to controls. For example, on Day 8 only 10% of DC were alive in Control and DC/LacZ groups. In contrast, 65% of DC overexpressing CD40L were still viable ($p<0.001$). These data suggest that CD40L increases survival of DC in growth factor-depleted medium.

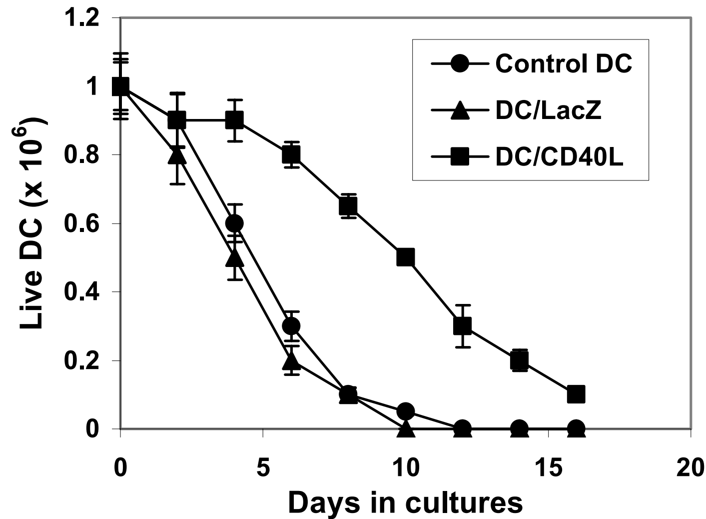


Figure 4. 6 Transduction with CD40L prolongs survival of DC in growth factor-depleted cultures. DC transduced with CD40L or control LacZ vectors were collected on Day 7 and re-plated in complete medium without IL-4 and GM-CSF. Cells were collected (1 well/group) and counted every day using the Trypan blue exclusion method. Y-axis: number of live DC in cultures. The means ± SD from two independent experiments are shown.

4.3.7 Transduction of DC with CD40L significantly augments their ability to present TAA to T cells

To determine the effect of CD40L on DC function, the ability of DC to present tumor antigen to T cells was evaluated in syngeneic models. Cultured DC were transduced with Ad-CD40L on Day 5 and incubated with TS/A tumor lysate on Day 6 for 24 hr. DC were passed through Ficoll gradients to get rid of dead cells. On Day 7, T cells were isolated from syngeneic mice and incubated with pulsed DC at different Effector : Target ratios. DC overexpressing CD40L were able to stimulate T cell proliferation significantly compared to control DC (**Figure 4.7**). In addition, the DC/CD40L group pulsed with TS/A antigen stimulated T cells even more extensively than non-pulsed DC/CD40L. For instance, at 1:5 ratio: Control, 3108 ± 46.8 cpm;

LacZ, 1128 ± 20 cpm, CD40L – 13478 ± 342 cpm; Cont + Ag, 2583 ± 280 cpm; LacZ + Ag, 4976 ± 418; and CD40L + Ag, 19623 ± 13 cpm ($p<0.001$). These data demonstrate that pulsing of DC transduced with CD40L with specific tumor antigen induces significant antigen presentation to T cells.

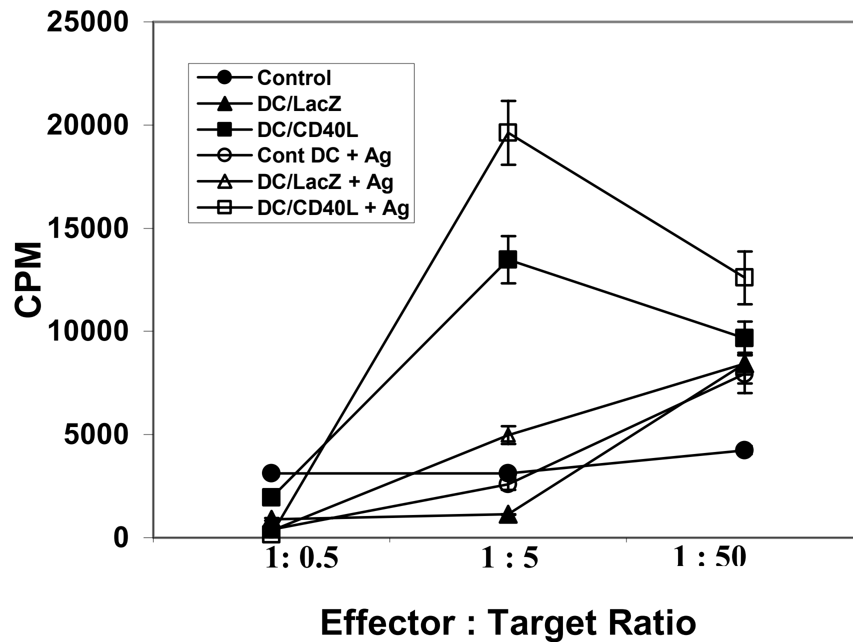


Figure 4. 7 CD40L-transduction significantly amplifies the ability of DC to present tumor antigen to T cells. Syngeneic MLR. DC generated from Balb/c mice and cultured for 5 days were transduced with Ad-CD40L. Untreated and LacZ-transduced DC were used as controls. On Day 6, DC were pulsed with TS/A tumor antigen. Twenty four hours later DC were collected and co-incubated with T cells, isolated from spleens of Balb/c mice at different Effector : Target ratios in triplicate. T cell proliferation was measured by ^{3}H-thymidine incorporation. The experiment was repeated 2 times with similar results and means ± SD from a representative experiment are shown.

4.3.8 Transduction of DC with CD40L induces DC ability to efficiently stimulate T cell proliferation

To examine whether CD40L was able to rescue DC from tumor-induced suppression, the ability of DC to stimulate T cell proliferation in an allogeneic MLR was measured. DC from control and TS/A tumor-bearing mice were transduced with Ad-LacZ or Ad-CD40L and incubated with T cells, isolated from allogeneic mice. Our data show that tumor inhibits the ability of DC to stimulate T cell proliferation in non-transduced and LacZ groups (at 1:3 ratio: Cont/Cont, 75016.8 ± 7191.8 cpm vs Cont/Tu, 53317.5 ± 3509.4 cpm; LacZ/Cont, 184268.8 ± 14880.8 cpm vs LacZ/Tu, 142848.6 ± 13336.2 cpm, $p<0.01$) (**Figure 4.8**).

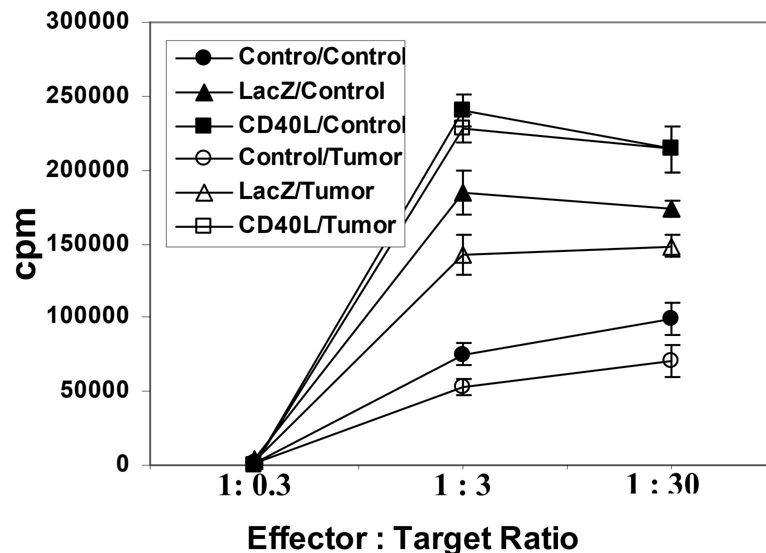


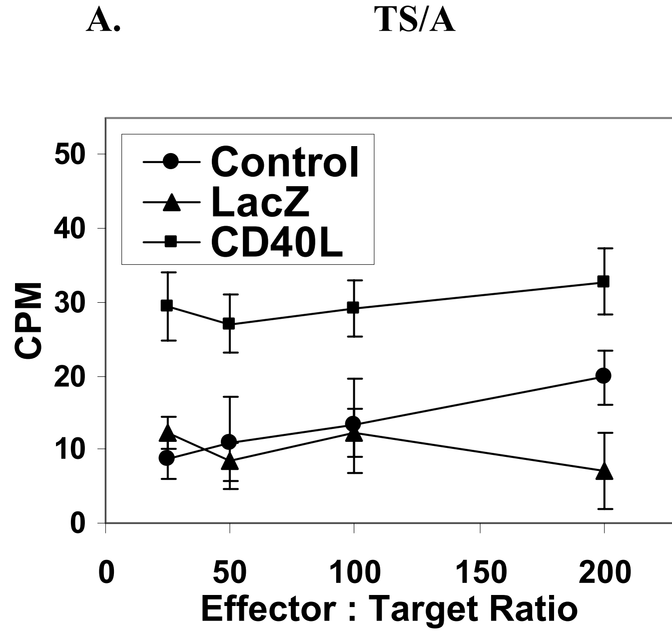
Figure 4. 8 Transduction with Ad-CD40L induces DC ability to stimulate T cell proliferation. DC generated from control and TS/A-bearing mice were transduced with Ad-CD40L or Ad-LacZ. Spleens from C57BL/6 mice were collected and T cells were isolated. T cells and DC were co-incubated at different ratios and pulsed with ^{3}H-thymidine 3 days later. Incorporation of ^{3}H-thymidine was calculated to measure T cell proliferation. Experiment was repeated twice. Means ± SD from a representative experiment are shown.

However, both CD40L treated groups demonstrated similar levels of T cell stimulation (CD40L/Cont: 239753.7 ± 10961.1 cpm and CD40L/Tu: 228113.7 ± 9580.5 cpm, $p<0.001$). Therefore, our data demonstrate that CD40L induces a significant stimulation of T cell proliferation and rescues DC from tumor-mediated suppression of DC activities.

4.3.9 Treatment of tumor-bearing mice with Ad-CD40L elicits CTL response

It has been previously demonstrated that CD8+ T cells play a key role in tumor regression after administration of an Ad-CD40L vector (152). To determine whether treatment with DC transduced with CD40L stimulates a CTL response against tumor cells, tumor-bearing Balb/c mice were intratumorally treated with control non-transduced DC or DC transduced with either Ad-LacZ or Ad-CD40L. Splenocytes were harvested 6 days later and analyzed for specific CTL activity against TS/A or irrelevant Yac-1 cells. The results shown on **Figure 4.9** demonstrate that there was no significant CTL activity against TS/A cells in animals treated with control or LacZ-transduced DC. In contrast, Ad-CD40L transduction significantly enhanced specific cytolytic activity. These CTL, however, did not exhibit cytotoxicity against Yac-1 cells. Taken together, these results demonstrate that the vaccination of mice with DC transduced with Ad-CD40L can induce a long-lasting immune response with activation of CTL directed against the nonimmunogenic cell line TS/A.

A. TS/A



B. Yac-1

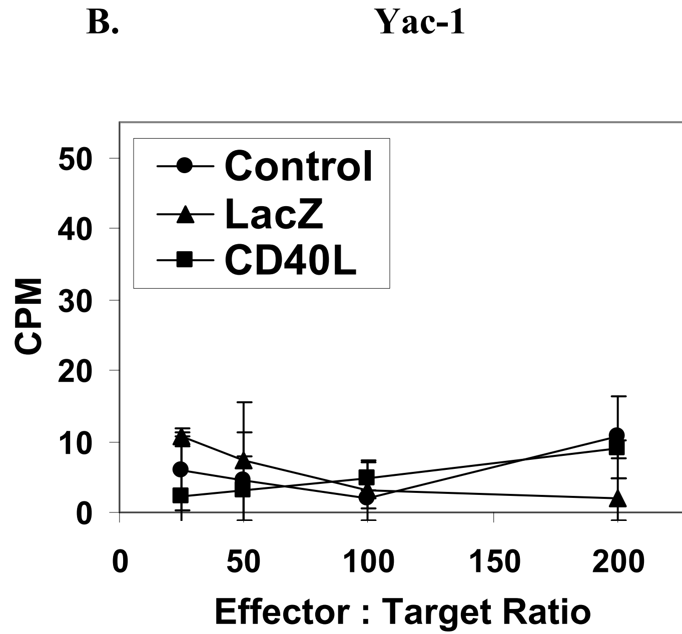


Figure 4. 9 Intratumoral DC/CD40L-based therapy induces cytotoxic T lymphocyte (CTL) response. Balb/c mice were treated with control, or LacZ- or CD40L-transduced DC, injected into the TS/A tumor site. Splenocytes were harvested 6 days later and analyzed for

specific CTL activity against TS/A cells in a standard ^{51}Cr-release assay. Irrelevant Yac-1 cells were used as a negative control. Mean ± SD from a representative of 2 experiments are shown.

4.3.10 Increased accumulation of intratumoral CD11c+ DC in TS/A tumors following the DC-based immunotherapy

The number and function of DC in cancer patients are likely to play an important role in the regulation of tumor growth. Clinical studies have demonstrated that higher number of tumor-infiltrating DC is associated with better prognosis. To evaluate tumor infiltration by DC, tumors were collected 2 hr, 24 hr and 48 hr after DC injection from Control, DC/LacZ and DC/CD40L groups. Then, tumor sections were stained with anti-CD11c (N418) antibody to measure DC presence in the tumors. **Table 3** and **Figure 4.10** show that a high level of staining was already present in DC/CD40L group 24 hr after immunotherapy (2.9 ± 0.74).

Table 3 Summary of immunohistochemical analysis of DC/CD40L-based anticancer immunotherapy.

	Control DC	DC/LacZ	DC/CD40L	Negative cont.
24 hr	0.60 ± 0.62	0.80 ± 0.42	2.90 ± 0.74	0.00
48 hr	1.7 ± 0.95	2.3 ± 0.67	3.80 ± 0.42	0.00

Tumor tissue sections were embedded in OCT compound, snap-frozen on dry ice, and 6 μm cryostat sections fixed, washed and incubated with CD11c antibodies (N418; Serotec) at 1:800 dilution (1 hr rm temp), biotinylated mouse anti-rat IgG at 1:500 dilution (secondary antibodies, 45 min rm temp). After developing with peroxidase chromogen kit for 8 min, counterstaining was performed with hematoxylin. Ten different areas were analyzed. Negative control included staining with irrelevant isotype

antibodies. DC infiltration per high power field was graded 0 through 5 as follows: 0, absence of DC; 1, a small quantity of DC (2-5% stained cells); 2, a medium infiltration (5-20%); 3, medium to high amount of DC (20-50%); 4, a high number of DC present (widely distributed cells throughout the whole slide).

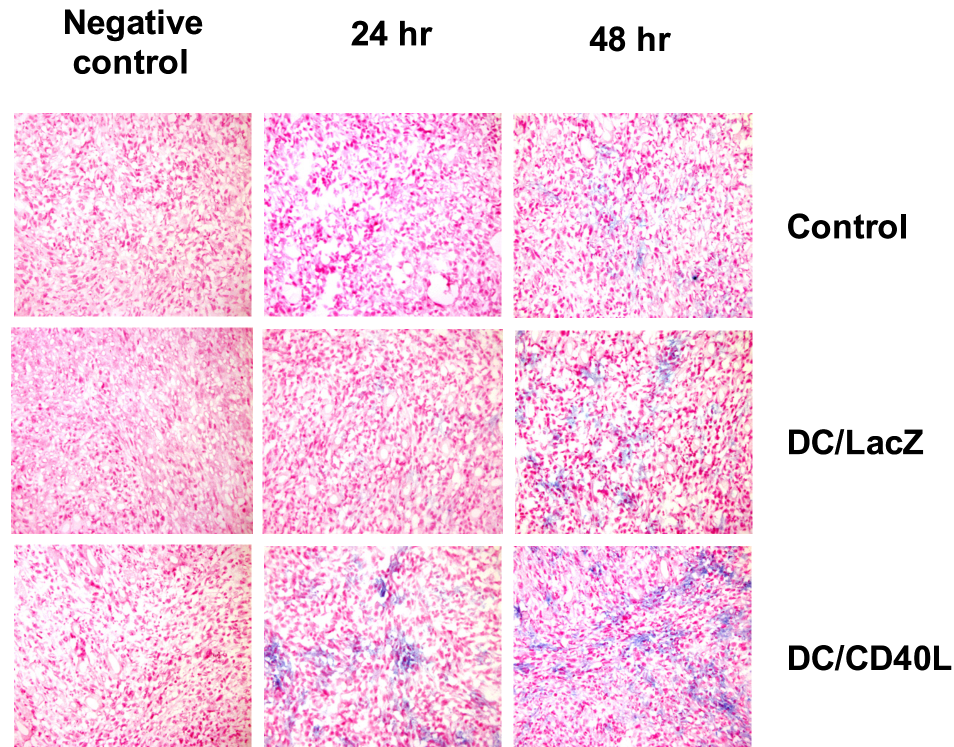


Figure 4. 10 DC/CD40L-based therapy stimulates accumulation of CD11c+ DC at the tumor site. Tumor-bearing mice were treated with DC transduced with Ad-CD40L or Ad-LacZ. Tumors were isolated 2, 24 and 48 hr post inoculation. Fresh-frozen sections were stained with anti-CD11c antibodies and analyzed for the presence of CD11c+ cells by immunohistochemistry. Blue staining represents CD11c+ DC. The experiment was repeated 2 times. Representative results are shown. Therapy groups: Control – non-transduced DC, DC/LacZ – DC transduced with Ad-LacZ, DC/CD40L – DC transduced with Ad-CD40L.

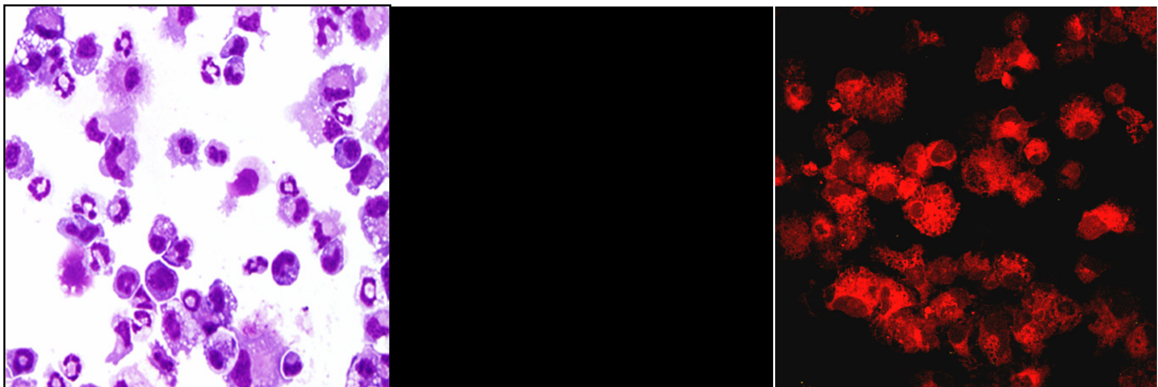
Control and LacZ-treated groups did not have a measurable amount of DC staining at this time point (DC/LacZ: (0.8 ± 0.42), Control: (0.6 ± 0.62)). After 48 hr post DC injection, DC were detected at high levels in Control (1.7 ± 0.95) and LacZ-treated (2.3 ± 0.67) groups. However, in DC/CD40L group, infiltrating DC were present in significantly higher (3.8 ± 0.42) amounts compared to the 24 hr time point. These data demonstrate that CD40L increases tumor-infiltrating DC after DC-based therapy.

4.3.11 Developing methods to evaluate trafficking of DC *in vivo*

To examine DC trafficking patterns in vivo, we first developed a method for finding injected DC in the mouse. On Day 7, DC were collected and stained with Cy3. Morphological characteristics of DC were measured by LeucoStat Stain Kit 30 min later. Control DC stained with Cy3 display a typical DC morphology (**Figure 3.11 A**) and the staining did not influence DC appearance.

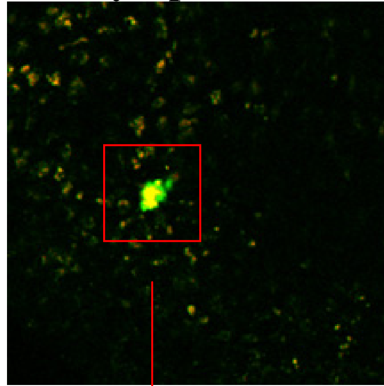
DiI/Cy3 is an active labeling compound that produces conjugates that are brighter, more photostable and emit less background than most standard fluorescent labels (absorption at 549 nm, emission at 565 nm). In addition, due to it's highly lypophilic nature, DiI/Cy3 has an ability to uniformly label cell in aqueous culture medium.

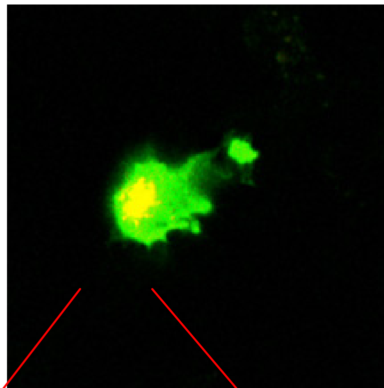
A.



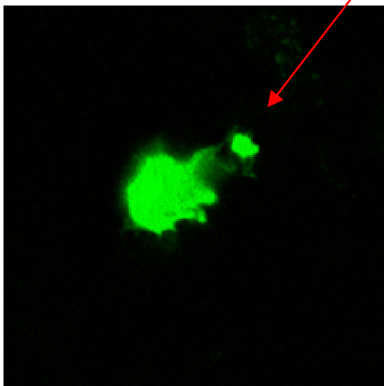
B.

Lymph Node





EGFP only



Cy 3 only

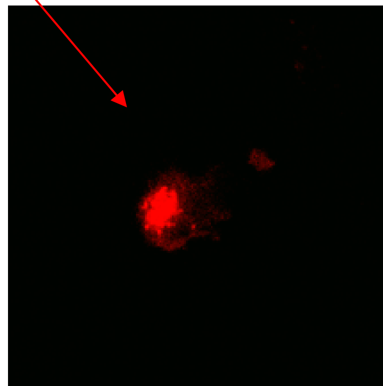


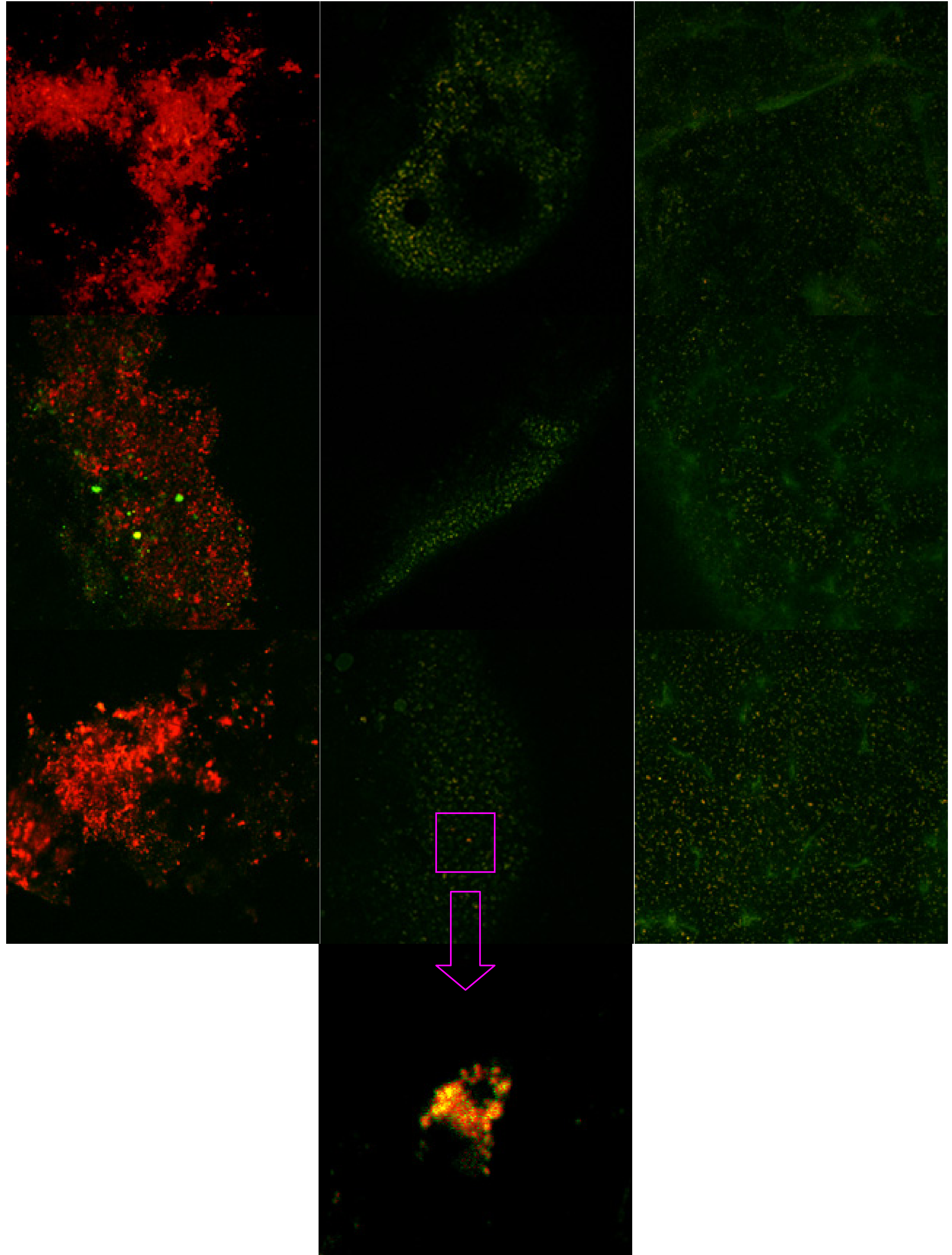
Figure 4. 11 Development of method to evaluate trafficking of DC injected into the tumor site. A. Cy3 staining does not influence DC morphology. DC were stained with: a) LeucoStat Staining to study DC morphology; or b) Cy3 staining. **B.** DC injected into the tumor site travel to the lymph nodes. DC transduced with Ad-EGFP were stained with Cy3 for 30 min and injected into the tumor site of TS/A-bearing mice. 24 hr later, mice were sacrificed and lymph nodes were isolated. Fresh tissues were imaged using a multiphoton laser scanning confocal microscope system. Data typical of those obtained in a series of 3 independent experiments are shown.

Next, we examined DC trafficking patterns after intratumoral inoculation. To control that Cy3 stained cells were injected, Ad-eGFP was used. This double color-analysis was needed to co-localize red and green DC. Cultured DC were transduced with Ad-eGFP, stained with Cy3 (24 hr later) and injected into the tumor site. 2 hr, 24 hr and 48 hr after treatment, tumors and lymph nodes were collected from animals. Fresh tissues were imaged using a multiphoton laser scanning confocal microscope system. Green cells were found in lymph nodes 24 and 48 hr post DC injection (**Figure 4.11 B**). These cells also display a typical DC shape. This Figure also shows that DC found in lymphatic organ have both green and red staining. These data suggested that our method of studying DC trafficking could be used for analyzing DC migration from tumor tissues after injection.

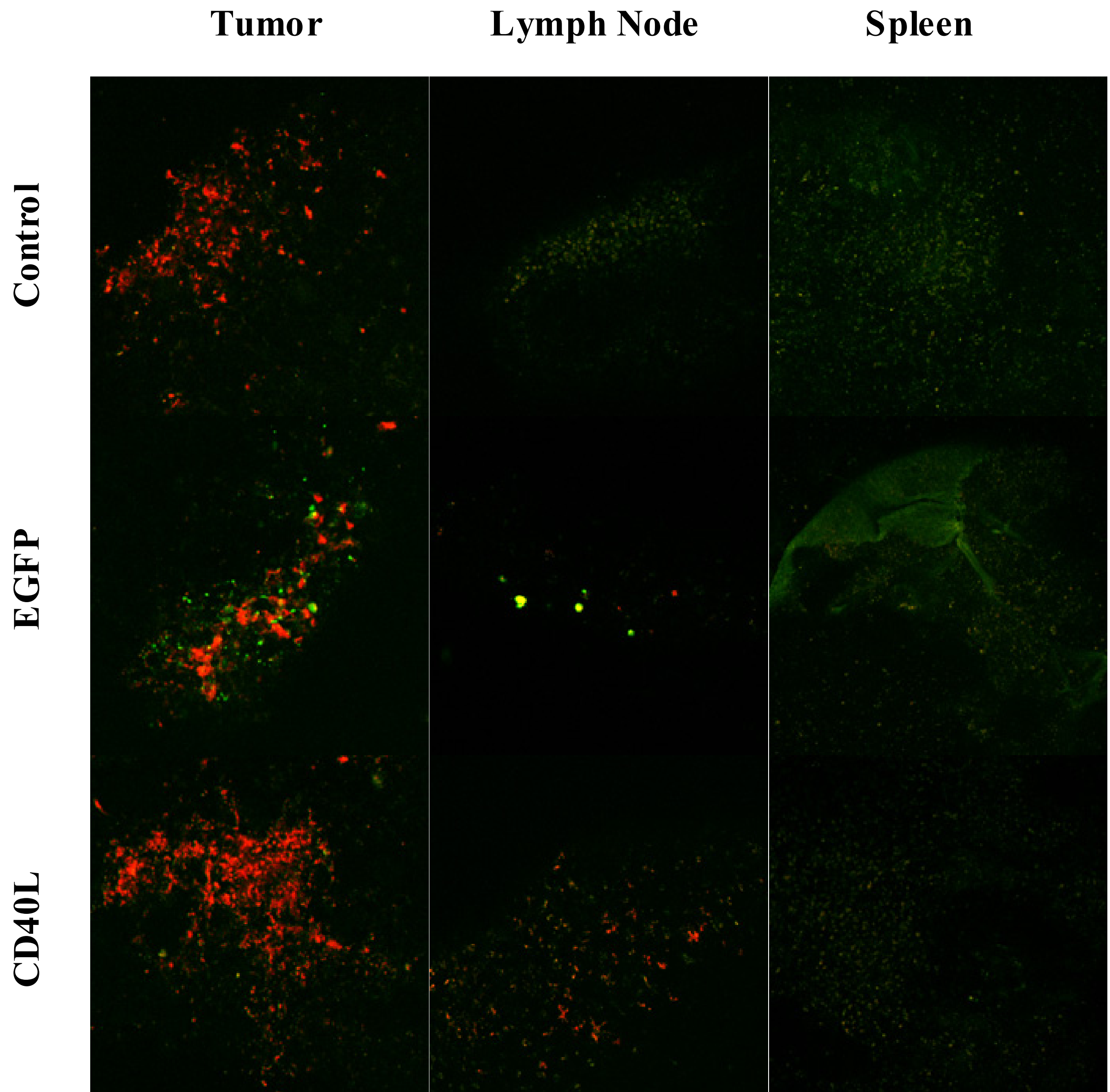
4.3.12 CD40L stimulates DC trafficking to the lymphatic system in DC-based immunotherapy

To examine whether CD40L transduction modulates DC trafficking and homing *in vivo*, we generated DC, transduced them with Ad-CD40L or control Ad-eGFP vectors, stained DC with Cy3, and injected them into the tumor site in Balb/c mice. Tumors, lymph nodes and spleens were collected 2, 24 and 48 hr after treatment from one mouse from each group. Confocal microscopy of fresh tissues demonstrated that DC from CD40L-treated group migrate from tumors faster than untreated DC or DC/EGFP (**Figure 4.12**). For instance, DC were already

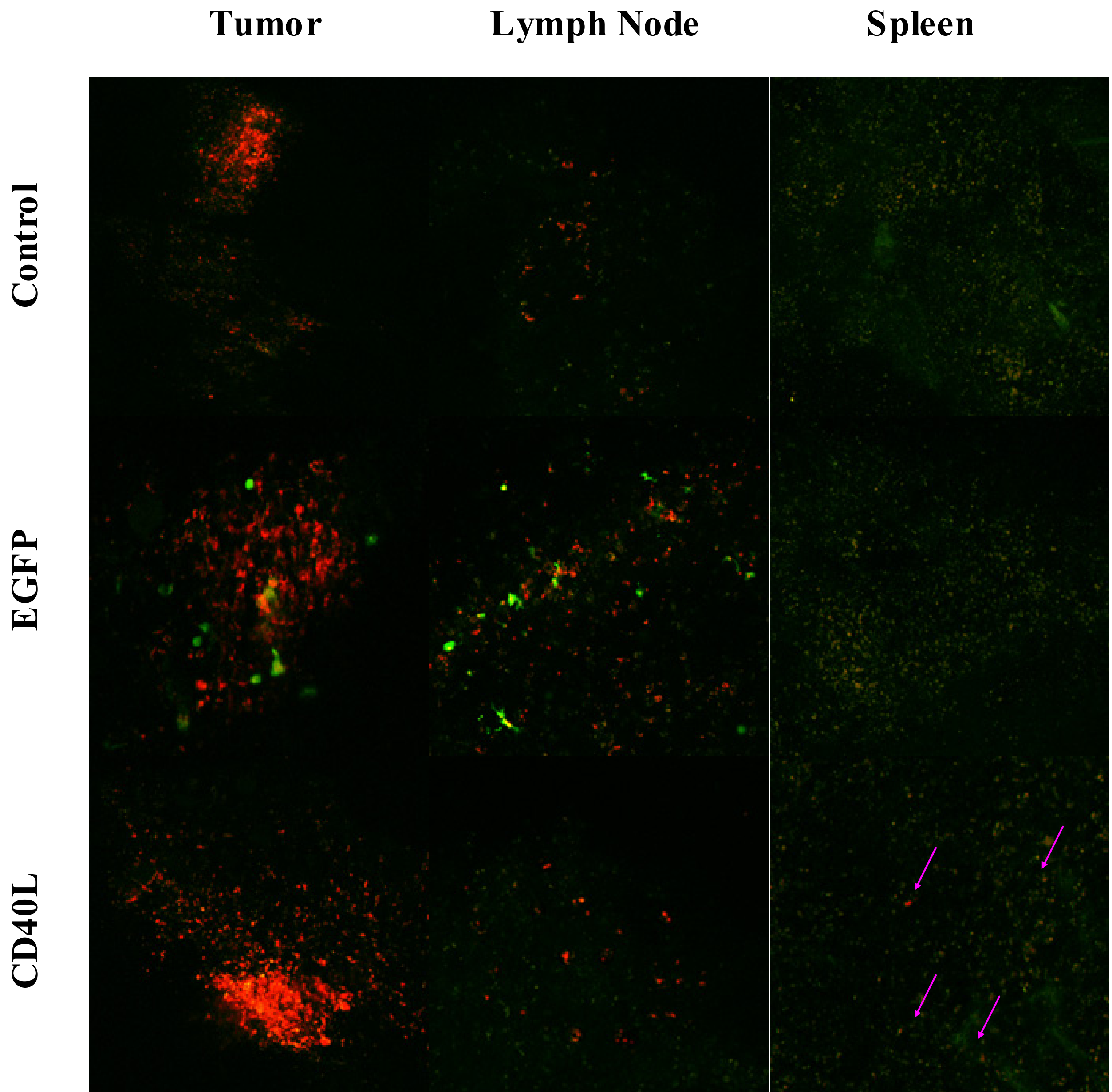
detectable in lymph nodes 2 hr post injection only in DC/CD40L group. In addition, in these mice 24 hr later there were many red cells present in lymph nodes and some were found in spleen 48 hr later suggesting that CD40L stimulates DC migration from tumor tissue. In contrast, DC were observed in lymph nodes only 24 hr after treatment in both control or EGFP-treated mice. Also, there were no DC present in spleens of these animals at 48 hr. These results suggest that transduction of DC with Ad-CD40L induces a fast migration of injected DC from tumor site to lymphoid tissues which could explain the effective generation of antitumor immunity by DC/CD40L.


Tumor
Lymph Node
Spleen
Control
EGFP
CD40L

B.



C.



D.

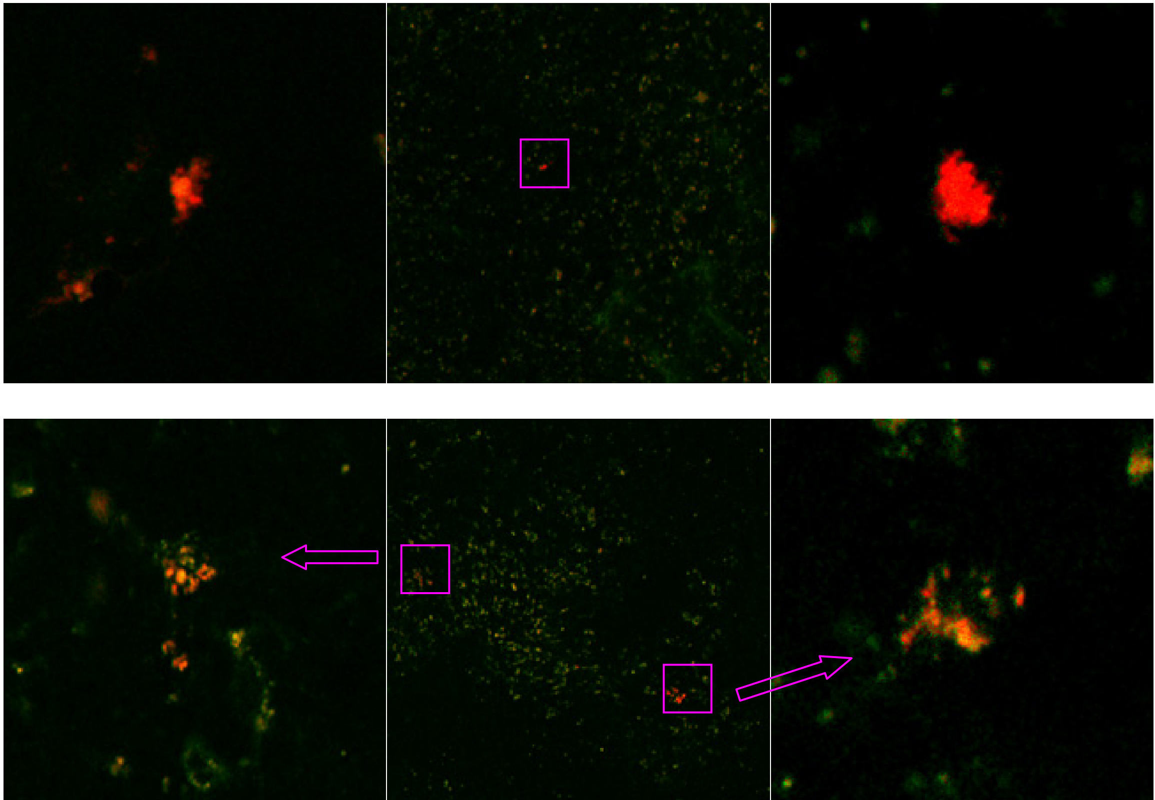


Figure 4. 12 CD40L-transduction stimulates trafficking of DC injected into the tumor site to lymphoid organs. DC transduced with Ad-CD40L and stained with Cy3 were injected into the TS/A tumors growing for 7 days in Balb/c mice. Non-transduced DC and Ad-EGFP-transduced DC were used as controls. Tumors, spleens and lymph nodes were isolated 2, 24 and 48 hr after DC injection. Fresh tissues were imaged using a multiphoton laser scanning confocal microscope system. Tissues isolated: **A.** 2 hr; **B.** 24 hr; and **C.** 48 hr after DC injection. **D.** DC were detectable in the spleens of mice treated with DC/CD40L 48 hr post treatment. (N=3 animals/group). Representative results of 2 independent studies are shown.

4.4 CONCLUSIONS

Taken together, our data demonstrate several mechanisms of how CD40L-modified DC generate strong antitumor immunity *in vivo*. For example, it is likely that CD40L-transfected DC activate each other in tight clusters by multiple CD40 ligation, resulting in accelerated maturation of cells as evidenced by an increase in expression of the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2), MHC molecules, and increased production of IL-12. In addition, CD40L protects DC from tumor-mediated dysfunction by inducing significant secretion of IL-12 and high expression of co-stimulatory molecules; DC generated from tumor-bearing mice had expression levels comparable to those seen in DC generated from tumor-free animals. CD40L rescues DC function previously suppressed by tumor cells. We have also demonstrated that CD40L increases DC survival in cultures, induces DC to stimulate and activate T cells, in addition to boosting tumor antigen presentation to T cells. Furthermore, CD40L increases DC accumulation at the tumor site and stimulates trafficking of injected DC to the lymph organs for effective antigen presentation.

Summary of conclusions:

CD40L transduction of DC:

- Significantly induces IL-12 production by DC
- Rescues DC from tumor-mediated suppression of IL-12 secretion
- Considerably increases expression of CD40, CD80, CD86 and class I molecules on DC
- Rescues DC from tumor-induced down-regulation of CD40, CD80 and CD86 expression
- Induces clustering of DC, and, thus, autocrine/paracrine activation
- Increases survival of DC in cultures
- Drastically up-regulates ability of DC to present TAA to T cells
- Significantly stimulates proliferation of T cells
- Activates CTL response
- Stimulates infiltration of tumor-associated DC
- Induces fast migration of injected DC to secondary lymphoid tissues

5.0 COMPARATIVE ANALYSIS OF CD40L WITH OTHER TNF FAMILY LIGANDS: RANKL AND 4-1BBL

5.1 INTRODUCTION

The ligands of the Tumor Necrosis Factor Ligand superfamily are cytokines which control the interaction between many different cell types. For example, they mediate dialogue between T and B cells, T cells and DC, T cells and monocytes, and T cells themselves. These molecules play a critical role in the regulation of proliferation, activation, differentiation, and maturation of lymphoid, myeloid and other hematopoietic cells, as well as various non-hematopoietic cell types including tumor cells. Different members of TNF family of proteins have been demonstrated to have a significant antitumor potential in a number of tumor models. The most described in generating antitumor immunity are CD40L and 4-1BBL.

For instance, the interaction between CD40 and its ligand is an important element in the development of antitumor immunity. Significant inhibition of CD40+ human breast cancer cell line proliferation was described after therapy with soluble recombinant human CD40L (143). Similarly, retroviral transduction of MC38 colon adenocarcinoma cells with the CD40L gene resulted in inhibition of tumor growth (118). In addition, intratumoral administration of Ad-CD40L was tested in three different murine tumor models. In the melanoma and colon cancer murine models, injection of a recombinant adenovirus vector expressing CD40L resulted in

sustained tumor regression and tumor-free status in >60% of animals (60). Intratumoral administration of Ad-CD40L also significantly suppressed the growth of established immunogenic Lewis lung carcinoma tumors, but to a lesser extent.

4-1BBL also plays an important role in initiating antitumor immune responses. This protein is expressed on activated T cells and delivers a co-stimulatory signal for T cell activation and growth. Multiple studies have demonstrated that increased 4-1BB-mediated signaling in the tumor microenvironment leads to the induction of antitumor immunity. For example, Melero et al. have shown that treated with anti-4-1BB monoclonal antibodies can eliminate established large tumors in mice, including the poorly immunogenic Ag104A sarcoma and the highly tumorigenic P815 mastocytoma (92). Also, adenovirus-mediated gene therapy delivering a combination of IL-12 and 4-1BBL in mice with hepatic metastases induced by colon cancer cells lead to long-term survival (153). These results were confirmed by others who have demonstrated an antitumor effect of Ad-4-1BBL (154).

DC are required for stimulation and expansion of naïve T cells and induction of specific antitumor immunity (97). However, it has been demonstrated that tumor cells release factors which cause dysfunction of DC. In addition, tumors induced premature apoptotic death of DC (18). These data suggest a new mechanism of tumor-induced immunosuppression and explain why many immunotherapeutic approaches met only limited success.

CD40L, RANKL and 4-1BBL were shown to play an important role in DC function. For example, CD40 ligation on DC promotes their differentiation and maturation into effective inducers of cell-mediated immunity (78). In Chapter 3 we demonstrated that CD40L-transduction induces DC maturation, trafficking to lymphoid organs, enhances DC survival, T cell activation, stimulates secretion of IL-12 and increases CTL activity. Furthermore, CD40

ligation enhances the efficiency of DC to present antigen to T cells by up-regulating the expression of CD80, CD86 and adhesion molecules CD54/ICAM, CD58/LFA-3 on DC (3). In Chapter 1 we demonstrated that transduction of DC with CD40L results in significant augmentation of their antitumor potential.

RANKL is known as DC-restricted survival factor that mediates T cell and DC communication and may provide a tool to selectively enhance of DC activity (61, 62). RANKL was reported to induce the expression of proinflammatory cytokines, like IL-6 and IL-1, and T cell growth and differentiation factors IL-12 and IL-15 in addition to enhancing DC survival (63, 155). Furthermore, it has been shown that treatment of antigen-pulsed DC with RANKL before immunization enhanced their adjuvant capacity and elicits improved T cell priming in vivo, such that both primary and memory T cell immune responses are enhanced (62). By enumerating migratory DC in the draining lymph nodes and by studying their function in stimulating T cell responses in an increase in the number of *ex vivo* antigen-pulsed DC that are found in the T cell areas of lymph nodes.

In contrast, the role of 4-1BBL in DC vaccination in cancer has not been yet investigated. It has been shown, however, that 4-1BB on DC could play an important role in activation of these cells through DC/DC and DC/tumor interactions by inducing maturation of DC, up regulating co-stimulatory molecules, and inducing IL-12 and IL-6 secretion by DC (64, 65). Also, the antitumor effect of RANKL was not studied yet. Furthermore, the effects of transduced DC with 4-1BBL and RANKL were not evaluated either. In this study, we have evaluated whether expression of CD40L, RANKL, and 4-1BBL at the site of the tumor and DC elicits an immune response to established tumors in mice.

We have tested adenoviral vectors expressing CD40L, RANKL and 4-1BBL on two different tumor models (colon MC38 and breast TS/A adenocarcinomas) and two mouse strains. We hypothesized that overexpression of RANKL and 4-1BBl at the tumor site might induce strong antitumor immunity. In addition, we proposed that transduction of DC with these vectors would stimulate DC function. The rational for this hypothesis was the results from Chapters 1 and 2 demonstrating that CD40L transduction induces DC to generate a strong antitumor immunity by up-regulating their function.

We tested our hypothesis by measuring RANKL and 4-1BBL antitumor effects and comparing it to CD40L-induced antitumor immunity. We have demonstrated that all tested TNF ligands have a significant antitumor effect on both MC38 and TS/A tumor models, whether they are administered as adenoviral vectors or transduced DC. In addition, transduction of DC with RANKL and 4-1BBL, similar to CD40L, significantly prolongs survival of DC in culture. However, only DC transduced with Ad-CD40L secrete elevated levels of IL-12 and express high levels of surface markers. Our data suggest that since CD40L, RANKL and 4-1BBL-based therapy induces DC survival in cultures, transduction of DC might significantly improve the efficacy of DC-based immunotherapies for cancer.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Female 6-8 week-old C57BL/6 (H-2^b) and Balb/c (H-2^d) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in groups of five in a 12:12 hour

light:dark cycle with standard mice chow and water. All animals were acclimatized at least 2 weeks prior to experimentations. Animals were handled in accordance with IACUC protocols.

5.2.2 Tumor cell lines

MC38 colon and TS/A breast adenocarcinoma cells were maintained in RPMI 1640 medium (GIBCO BRL-Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (GIBCO BRL-Life Technologies).

5.2.3 Tumor therapy model

7.5 $X10^4$ MC38 cells or 2.0 X 10^5 TS/A cells were injected s.c. into the right flank of mice. On Day 7, when the tumor size reached 25-30 mm^2, intratumoral injection of 10^9 pfu of either control adenoviral vector Ad-LacZ or Ad-CD40L or 10^6 DC non-transduced or transduced with Ad-CD40L was performed in 100 µl volume. Administration of HBSS served as an additional control. The tumor size was measured twice a week with calipers and recorded as tumor area (mm^2).

To determine the direct effect of transduced DC, TS/A tumor cells ($5x10^5$ cells/well) were incubated in the presence of either unmodified DC or DC transduced with Ad-CD40L, Ad-RANKL or Ad-4-1BBL at different ratios (1:10 and 1:50 effector:target ratio). The growth rate of the tumor cells was analyzed using ^{3}H-thymidine incorporation. Proliferation of tumor cells in cultures was assessed by incorporation of ^{3}H-thymidine after 4-hour pulsing period with 1mCi/well.

5.2.4 Preparation of adenoviral vectors

Ad-CD40L, Ad-RANKL and 4-1BBL were constructed, propagated, and titered according to the standard protocol previously described (Chapter 2). The purified virus was extensively dialyzed

against 10 mM Tris/1 mM $MgCl_2$ sterile viral buffer at 4°C, stored in aliquots at –80°C, and titered on CRE8 cells for plaque forming units (pfu).

5.2.5 DC generation

DC were prepared as previously described in Chapter 1. Briefly, DC were generated from hematopoietic progenitors isolated from mouse bone marrow of C57BL/6 or Balb/c mice. Then cell suspensions were depleted from red blood cells, B and T lymphocytes and macrophages. The cells were then plated in 6-well plates ($0.2x10^6$ cells/ml 4 ml/plate) in complete medium with addition of 1000U/ml mGM-CSF and mIL-4 (ENDOGEN, Woburn, MA). At Day 3 and 5, an additional dose of cytokines was added to the cell cultures. For adenoviral infection, DC were collected on Day 5 and washed in serum-free medium. Virus was added directly to the pellet (10^9 pfu/10^6 DC) and the cells incubated for 1 h at 37°C before plating in complete medium.

5.2.6 Flow cytometry

For phenotypic analysis of DC (Day 7), FITC- or PE-conjugated monoclonal antibodies recognizing murine CD40L molecule were used. After incubation with antibodies for 30 min at 4°C, cells were washed with PBS and analyzed on a FACStar using Cellquest FACS analysis software (Becton Dickinson, San Diego, CA). As a control to determine the percentage of transduced DC, cells were infected with Ad-EGFP 48 hours before FACS analysis.

5.2.7 IL-12 production

IL-12 production by DC was determined by p70 IL-12 ELISA. A standard curve was generated using recombinant murine IL-12 (R & D Systems). As a positive control, DC cultures were treated with inactivated *Staphylococcus aureus* (0.1% v/v of essentially non-viable cell suspension, Sigma, St Louis, MO) 20 µl/well per $1x10^6$ DC cells.

5.2.8 Data Analysis

Statistical analysis of experimental data was performed with a software package STSS. For all analysis, the level of significance was set at a probability of 0.05 with results that had a p value less then the 0.05 considered significant. Student t-test was used for comparison between two groups after evaluation for normality. Two-way repeated measurements ANOVA was used to compare tumor growth for multiple groups of mice. All experiments were conducted with 5-7 mice per group receiving identical treatments. All experiments were repeated at least twice.

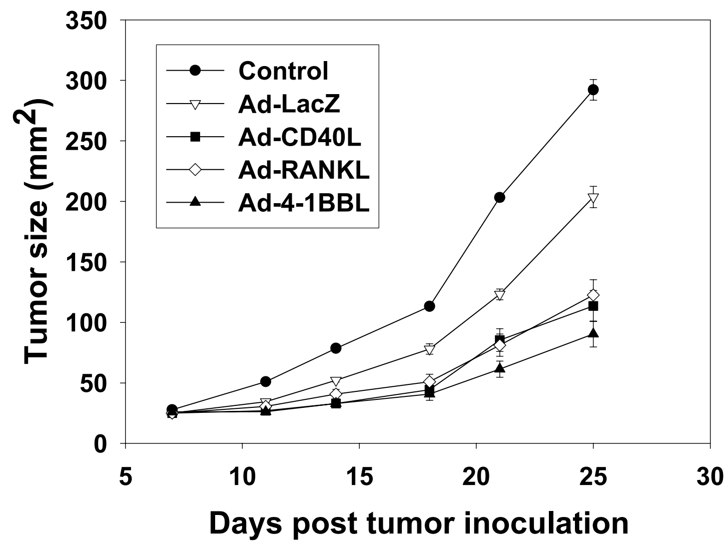
5.3 RESULTS

5.3.1 Intratumoral injection of Ad-CD40L, Ad-RANKL and Ad-4-1BBL inhibits tumor growth *in vivo*

To examine the anti-tumor efficacy of local gene transfer of CD40L, RANKL and 4-1BBL, a ΔE1 and E3 adenoviral vector expressing murine CD40L, RANKL and 4-1BBL, respectively, were constructed as described in the Materials and Methods. To determine whether intratumoral delivery of these genes was able to confer an antitumor effect *in vivo*, a murine colon MC38 adenocarcinoma tumor model was utilized in these series of experiments. Syngeneic C57BL/6 mice were inoculated s. c. with wild type MC38 tumor cells. On Day 7, when the tumor size was 25 - 30 mm^2, 10^9 pfu of either Ad-CD40L, Ad-RANKL, Ad-4-1BBL, or the control virus Ad-LacZ were injected directly into the tumor mass. As shown in **Figure 5.1 A**, non-treated and Ad-LacZ treated mice had fast rates of tumor growth with average tumor sizes of 292 ±8.5 mm^2 and 203 ± 8.9 mm^2 respectively at Day 25. In contrast, a single intratumoral injection of Ad-CD40L

significantly inhibited tumor growth (90 ± 11 mm^2, $p<0.001$). Similarly, Ad-RANKL and Ad-4-1BBL significantly decreased tumor growth (123 ± 13 mm^2 and 113 ± 12 mm^2, respectively, $p<0.001$). These data demonstrate that a single intratumoral administration of adenovirus encoding mCD40L, mRANKL and m4-1BBL results in a marked suppression of tumor growth and that treatment with Ad-4-1BBL induces the strongest antitumor effect out of all 3 tested TNF ligands. These results suggest that intratumoral treatment with CD40L, RANKL and 4-1BBL induced strong antitumor immunity in mice.

A.



B.

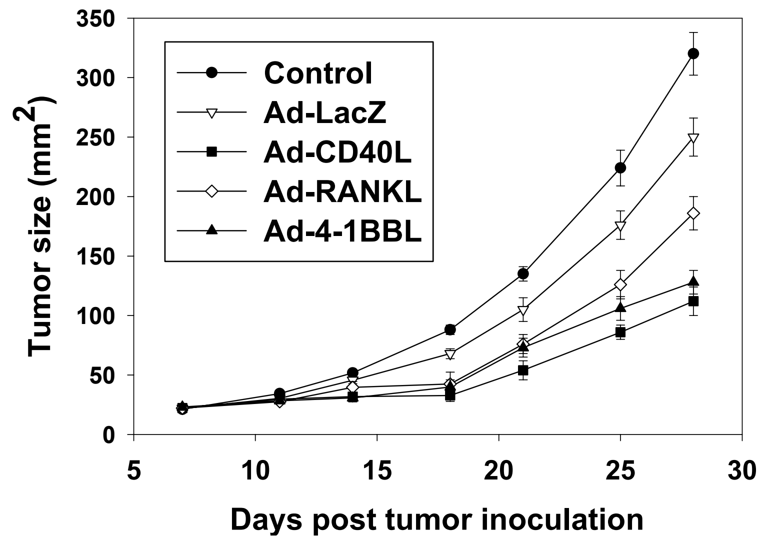


Figure 5. 1 Antitumor effect of intratumoral administration of adenoviral vectors encoding the CD40L, RANKL and 4-1BBL genes. A. MC38 tumor-bearing mice were inoculated with 10^9 pfu of Ad-ψ5, Ad-4-1BBL, Ad-RANKL or Ad-CD40L on Day 7, tumor size was measured twice a week. **B.** TS/A tumor-bearing mice were treated with Ad-4-1BBL, Ad-RANKL or Ad-CD40L vectors. Saline and Ad-LacZ were used as negative controls. Tumor size was measured twice a week. (N=5 mice/group). Experiment was repeated twice and representative results are shown (mean ± SE).

To evaluate whether the therapeutic effect of direct, intratumoral injection of adenoviral vectors was tumor specific, a Balb/c syngeneic breast tumor cell line TS/A was used in the next set of experiments. The TS/A cells were inoculated subcutaneously on Day 0. On Day 7, when tumor sizes reached 25-30 mm^2, Ad-CD40L, Ad-RANKL or Ad-4-1BBL were injected directly into tumors (N=5). Control groups were treated with saline or Ad-LacZ. As shown in **Figure 5.1 B**, the growth of the TS/A tumor was significantly inhibited by treatment with Ad-CD40L (106 ± 10 mm^2, $p<0.001$) and Ad-4-1BBL (86 ± 6 mm^2, $p<0.001$) compared to either the untreated control group (224 ± 15 mm^2) or to the group treated with control LacZ adenoviral vector (176 ±

12 mm^2) ($p<0.005$). Ad-RANKL also induced a significant inhibition of tumor growth (126 ± 12 mm^2), however it was less dramatic than in CD40L- and 4-1BBL-treated groups. These results demonstrate that the antitumor effect induced by tested adenoviral vectors is not MC38 tumor or C57BL/6 strain specific. However, the antitumor effect of Ad-4-1BBL treatment was slightly more effective in the TS/A model than the MC38 model.

5.3.2 Transduction of DC with Ad-CD40L, Ad-RANKL and Ad-4-1BBL

We have recently demonstrated that CD40 ligation on DC results in increased expression of CD80, CD86, CD40, enhanced DC function, and a strong antitumor effect (18, 156). Similarly, it has been shown that both RANKL and 4-1BBL increase the levels of co-stimulatory molecules on the DC surface and induce secretion of IL-12, IL-6 and IL-15 by these cells (61, 65). In addition, it has been demonstrated that RANKL plays a critical role in DC survival (68, 157). Therefore, it is possible that overexpression of RANKL and 4-1BBL on DC may be beneficial for function of DC by an autocrine/paracrine up-regulation of their maturation in the tumor microenvironment.

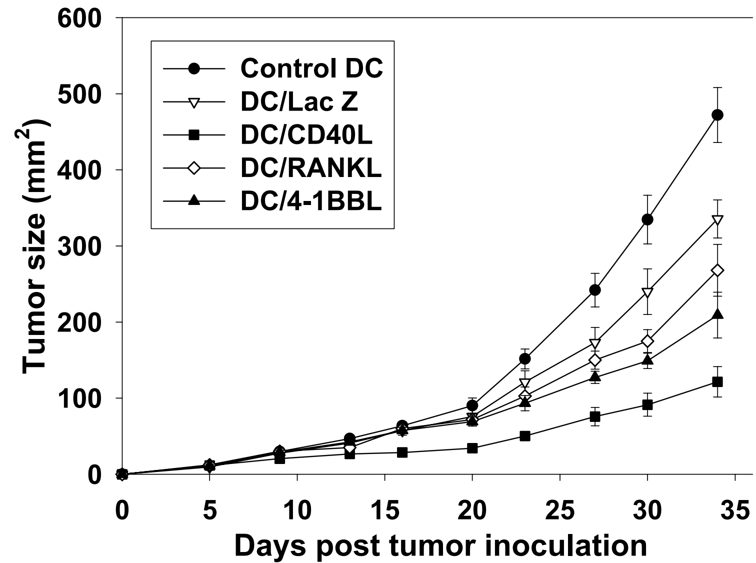
To examine the antitumor efficacy of CD40L-, RANKL- or 4-1BBL-overexpressing DC, bone marrow-derived DC were infected with either Ad-CD40L, Ad-RANKL or Ad-4-1BBL and injected directly into Day 7 MC38 tumors. As controls, tumors were treated with either DC transduced with Ad-LacZ vector or with untreated non-transduced DC. As expected, mice in both control groups developed rapidly growing tumors (on Day 30 untreated DC: 334 ± 32 mm^2; Ad-LacZ: 240 ± 30 mm^2). In contrast, mice immunized with DC transduced with Ad-RANKL and Ad-4-1BBL, showed significant inhibition of tumor growth (175 ± 15 mm^2 and 149 ± 10 mm^2, respectively, $p<0.001$) (**Figure 5.2 A**). Treatment with DC overexpressing CD40L showed more dramatic suppression of tumor growth. As shown on **Figure 5.2 A,** the tumor size was only

91 ± 15 mm^2 in this group ($p<0.001$). These results demonstrate that a single intratumoral administration of DC which were modified to overexpress tested TNF ligands induces a strong antitumor response *in vivo*.

To evaluate the antitumor effect of DC transduced with the TNF family ligands in a different tumor model and another strain of mouse, Balb/c mice were inoculated with TS/A breast carcinoma cells and DC/CD40L, DC/RANKL or DC/4-1BBL were administered intratumoraly. As shown in **Figure 5.2 B**, mice treated with control DC or DC infected with Ad-LacZ developed rapidly growing tumors (316 ± 13 mm^2 and 295 ± 14 mm^2, respectively) by Day 31. Animals in DC/RANKL and DC/4-1BBL groups showed a significant suppression of tumor growth (105 ± 24 mm^2 and 71 ± 16 mm^2, respectively) ($p<0.001$). However, mice treated with DC/CD40L displayed complete tumor rejection with 4 out of 5 mice being tumor-free on Day 21. These experiments were repeated twice with similar results.

Our data demonstrate that DC genetically modified to express CD40L, RANKL and 4-1BBL exhibit a significant antitumor effect in the TS/A tumor model following intratumoral injection, with complete tumor rejection in the case of DC/CD40L treatment.

A. MC38



B. TS/A

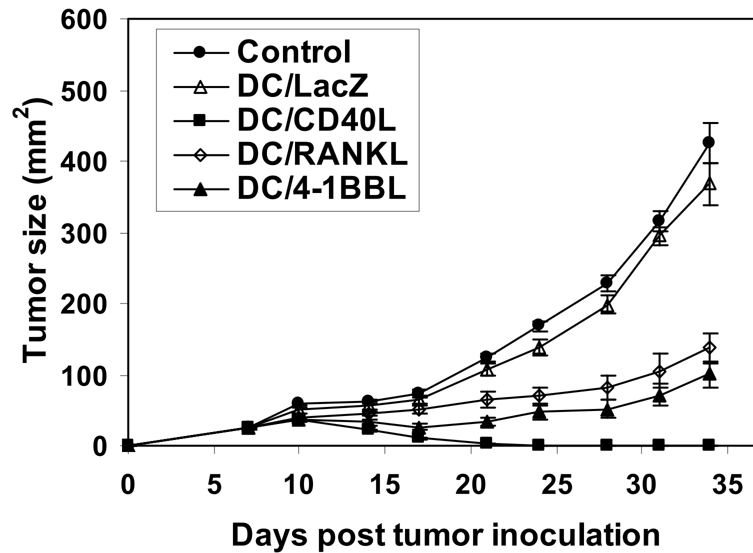


Figure 5. 2 Effect of DC transduced with Ad-CD40L, Ad-RANKL or Ad-4-1BBL on tumor growth *in vivo*. A. DC were transduced with Ad-CD40L, Ad-RANKL or AD-4-1BBL (100 MOI) and injected into MC38 tumor-bearing mice at the site of the tumor. As controls, DC treated with saline or transduced with Ad-LacZ were used. **B.** DC were transduced with Ad-CD40L, Ad-RANKL or Ad-4-1BBL and administered at the site of the TS/A tumors. Control groups were

treated with non-modified DC or DC transduced with Ad-LacZ. Tumor size was measured twice a week. (N=5 mice/group). Means ± SE from representative of 2 independent experiments are shown.

5.3.3 Ad-CD40L, Ad-RANKL and Ad-4-1BBL-infected DC do not have a direct effect on tumor growth *in vitro*

We have demonstrated above that DC genetically modified to express CD40L, RANKL or 4-1BBL induce a significant antitumor effect in mice. This effect could be due to direct antitumor properties of these DC. To rule out the possibility that modified DC have a direct inhibitory effect on tumor cell growth, we co-incubated modified DC with TS/A tumor cells 24 hours after transduction with TNF ligands.

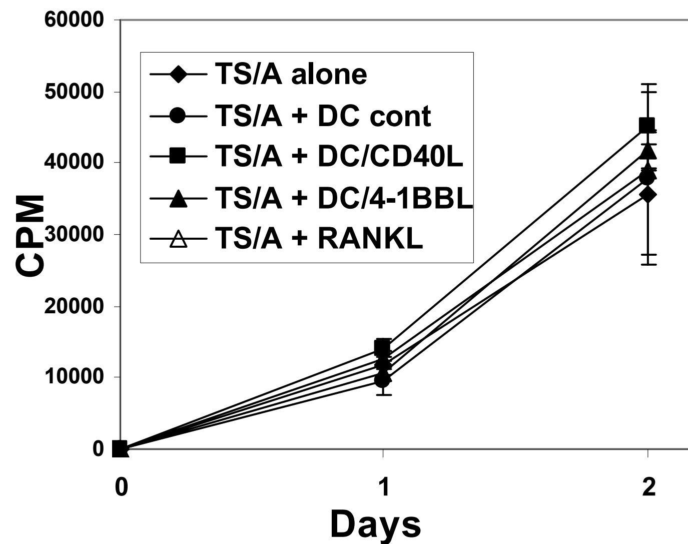


Figure 5. 3 Lack of direct effect of DC transduced with Ad-CD40L, Ad-RANKL or Ad-4-1BBL on TS/A tumor growth *in vitro*. Tumor cells were incubated with non-transduced DC or DC transduced with Ad-CD40L, Ad-RANKL or Ad-4-1BBL for 0, 1, and 2 days *in vitro*. Proliferation of tumor cells in cultures was assessed by incorporation of ^{3}H-thymidine after 4-hour pulsing period with 1mCi/well. Mean ± SD of 3 experiments is shown.

Out data demonstrated that TS/A tumor growth was not influenced by unmodified or modified DC. As shown in **Figure 5.3**, all groups had similar growth curves. Thus, the significant antitumor effects observed following the DC/CD40L, DC/RANKL and DC/4-1BBL treatments *in vivo* appear not to be mediated through a direct effect of DC on tumor cells.

5.3.4 Induction of IL-12 production by transduced DC

Activation of DC induces secretion of different cytokines which could play an important role in the generation of antitumor immunity. For example, the interaction between CD40/CD40L is an essential trigger for IL-12 production by DC. In addition, it has been demonstrated that RANKL and 4-1BBL also stimulate DC to secrete IL-12. To evaluate the level of IL-12 production by the different populations of DC, cells were generated form bone-marrow of both C57BL/6 and Balb/c mice. DC were genetically modified with Ad-LacZ, Ad-CD40L, Ad-RANKL or Ad-4-1BBL or untreated. Cell-free medium was collected 24 hr post transduction and IL-12 levels were measured by IL-12 ELISA. The results revealed only a slight difference in *S.aureus*-induced IL-12 production between control DC and DC transduced with Ad-LacZ, Ad-RANKL and Ad-4-1BBL. However, DC infected with Ad-CD40L produced a significantly higher level of IL-12 independent of the mice strain. DC/CD40L generated from C57BL/6 mice secreted 5500 ± 578 pg/ml and generated from Balb/c mice secreted 7750 ± 622 pg/ml (**Figure 5.4**, $p<0.0001$). These data demonstrate that out of the tested TNF ligands, only transduction with Ad-CD40L stimulated DC to produce significant levels of IL-12.

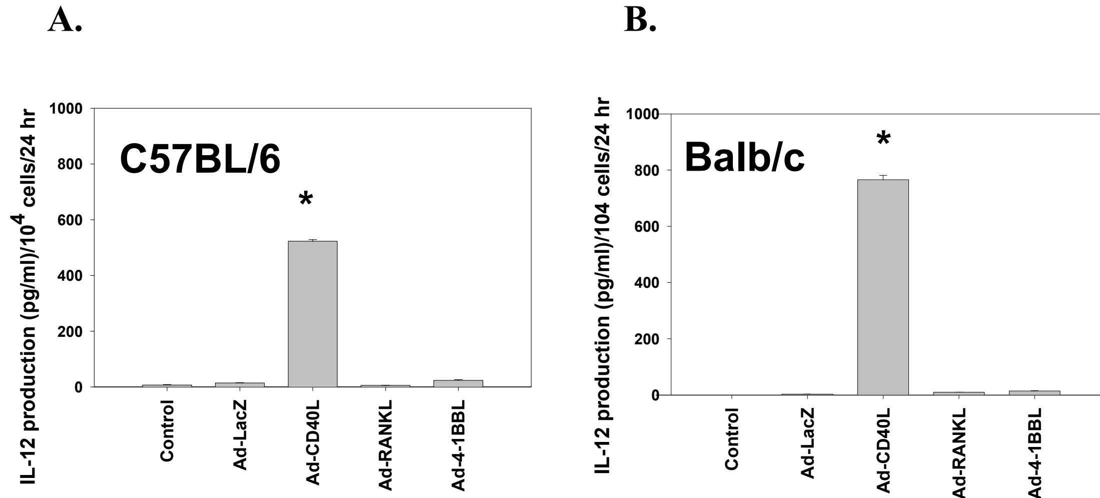
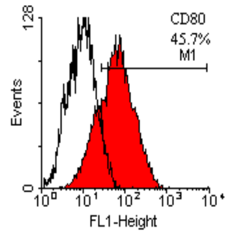


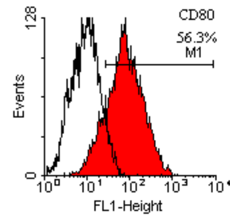
Figure 5. 4 Transduction of DC with different TNF family member genes modulates IL-12 production. Murine bone marrow-derived DC were transduced with either Ad-LacZ, Ad-RANKL, Ad-4-1BBL or Ad-CD40L as described above. 24 hours later, cell-free supernatants were collected for IL-12 determination. IL-12 production was measured by a p70 (IL-12) ELISA. Mean ± SD of a representative experiment are shown. The experiment was repeated 2 times. **A.** DC generated from C57BL/6 mice. **B.** DC generated from Balb/c mice.

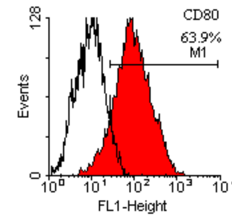
5.3.5 Evaluation of the effect of DC transduced with Ad-CD40L, Ad-RANKL and Ad-4-1BBL on expression of co-stimulatory molecules

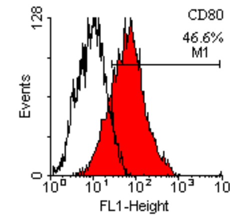
To examine the effect of CD40L, RANKL or 4-1BBL gene transfer to DC, DC morphology and phenotype were analyzed. DC derived from control bone marrow cultures exhibited the veiled dendrite morphology typical for DC and displayed a characteristic set of DC surface markers (**Figure 5.5, Table 4**). The genetically modified control DC and DC/RANKL and DC/4-1BBL expressed similar levels of the MHC class I and II molecules, the co-stimulatory molecules CD80, CD86, ICAM-1 adhesion molecules, and integrin CD11c. Infection of DC with Ad-CD40L at a 100 MOI resulted in a marked increase in the expression of CD80, CD86 and CD40 molecules.

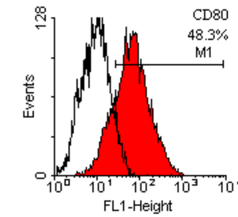
Control | **Ad-LacZ** | **Ad-CD40L** | **Ad-RANKL** | **Ad-4-1BBL**

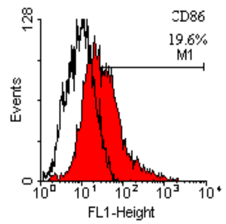


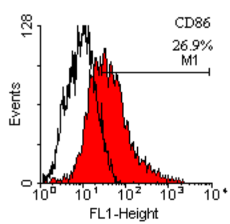


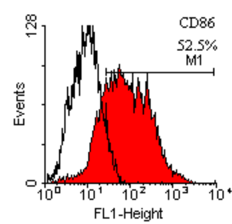


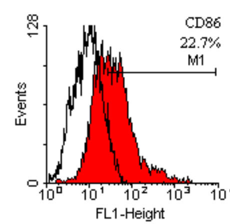


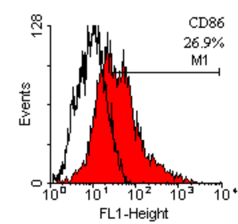


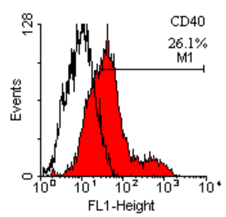


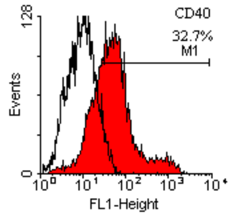


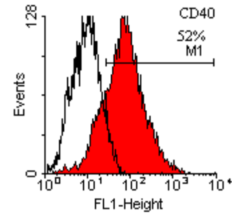


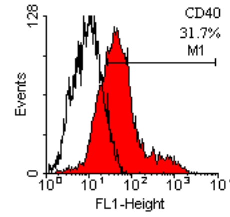












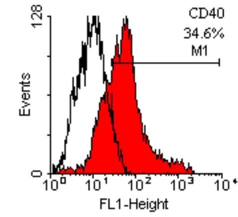


Figure 5. 5 Phenotyping of DC transduced with Ad-CD40L, Ad-RANKL and Ad-4-1BBL. Bone marrow-derived DC were transduced with the Ad-CD40L, Ad-RANKL or Ad-4-1BBL genes and analyzed by Flow cytometry. Data from a representative of 3 independent experiments are shown.

For example, 53% ($p<0.01$) of CD40L-transfected DC expressed CD86 molecules in comparison with 20% and 27% in non-transduced and Ad-LacZ cells, respectively. Similar results were observed for CD80 and CD40 expression. Interestingly, expression of MHC class II molecules on DC was also similar in all groups. In Chapter 2 we demonstrated that transduction of DC with Ad-CD40L increases their antitumor activity *in vivo*. Our *in vitro* results suggested that overexpression of CD40L on DC may be beneficial for the induction of antitumor immunity since it should up-regulate DC maturation and function. However, our data demonstrate that overexpression of RANKL and 4-1BBL on DC has no effect on the expression of co-stimulatory molecules and IL-12 secretion. Thus, the mechanism of antitumor effect of DC transduced with these ligands is different from DC/CD40L.

Table 4 The effect of the TNF family ligands on the expression of co-stimulatory molecules on transduced DC.

	Control	Ad-LacZ	Ad-CD40L	Ad-RANKL	Ad-4-1BBL
CD11c	76 ± 2%	74 ± 3%	78 ± 3%	79 ± 3%	76 ± 3%
CD80	46 ± 3%	53 ± 2%	68 ± 3%*	49 ± 3%	50 ± 4%
CD86	20 ± 2%	25 ± 3%	53 ± 2%*	28 ± 3%	29 ± 2%
I^{ad}	80 ± 3%	78 ± 4%	87 ± 3%	82 ± 4%	78 ± 2%
CD40	26 ± 1%	30 ± 2%	59 ± 2%*	34 ± 2%	38 ± 3%

DC were transduced with Ad-CD40L, Ad-RANKL or Ad-4-1BBL on Day 5. Non-transduced DC or DC transduced with Ad-LacZ were used as controls. On Day 7 DC were collected and stained with antibodies against

different co-stimulatory molecules, the expression of which was detected by FACScan analysis. The experiment was repeated 3 times. Mean ± SD of experiment are shown (* p<0.001).

5.3.6 CD40L, RANKL and 4-1BBL increase survival of DC in growth factor-depleted cultures

CD40 ligation has been shown to inhibit both spontaneous (148), and tumor-induced apoptosis of DC (123). Similarly, it has been shown that RANKL increases DC survival by inhibiting apoptosis of DC (68). To evaluate the role of TNF ligands on DC survival, we incubated $1x10^6$ cells/well of transduced DC in the medium without addition of mGM-CSF and mIL-4. Cells were counted every day for 3 weeks. DC transduced with CD40L, RANKL and 4-1BBL formed multiple spontaneous clusters (data not shown). DC which were untreated or transduced with Ad-LacZ did not appear to be clustered.

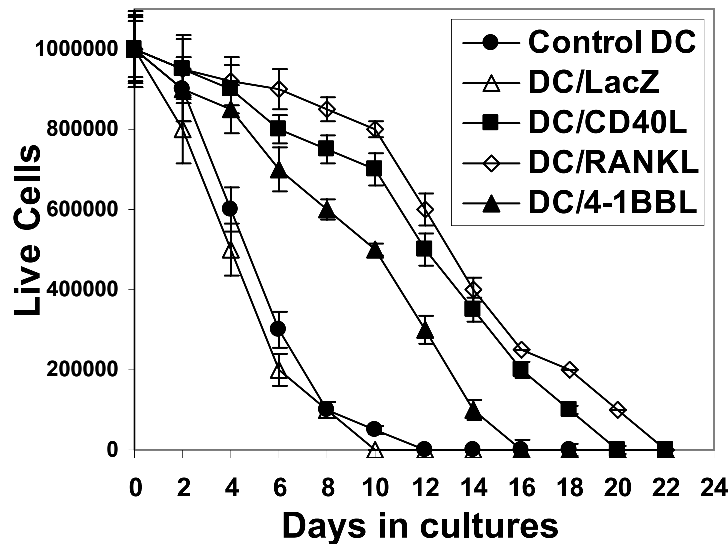


Figure 5. 6 The TNF ligands prolong DC survival in cultures. Control DC and DC transduced with Ad-LacZ, Ad-CD40L, Ad-RANKL or Ad-4-1BBL were grown in medium without GM-CSF and IL-4.

Cells were counted using Trypan blue exclusion method every other day. Experiment was repeated 3 times. Mean ± SD are shown.

Figure 5.6 demonstrates that DC modified with either one of tested TNF ligands survived in growth factor-depleted cultures longer than control or DC/LacZ. For example, on Day 10 none of the control and less then 10% of DC transduced with Ad-LacZ were present in cultures. However, 50% of DC/4-1BBL, 75% of DC/CD40L and 85% of DC/RANKL were still viable. These data suggest that DC genetically modified to overexpress CD40L, RANKL or 4-1BBL survive longer in cytokine-free cultures. Taken together, these data suggest that a significant prolongation of DC survival in cultures is the mechanism of induction of antitumor immunity shared by all three tested ligands.

5.4 CONCLUSIONS

Our data demonstrate that all tested TNF ligands induce a strong antitumor immunity when injected directly into the tumor site in the form of adenoviral vectors or genetically modified DC (**Table 5**). However, out of all adenoviral vectors, Ad-4-1BBL had the strongest effect on MC38 tumor growth and Ad-CD40L and Ad-4-1BBL had the strongest inhibition of TS/A tumor. In contrast, DC transduced with Ad-CD40L has the most significant antitumor effect in both MC38 and TS/A tumor models compare to DC/RANKL or DC/4-1BBL. In addition, we showed that out of three tested TNF ligands, only CD40L induced IL-12 production and up-regulation of co-stimulatory molecules in transduced DC. RANKL and 4-1BBL did not exhibit effects on IL-12 secretion and co-stimulatory molecules expression on transduced DC. However, all tested ligands induced survival of DC in growth factor-depleted cultures. The longest survival was

observed in the DC/RANKL group. This could be one of the mechanisms playing an important role in generating antitumor immunity by DC transduced with tested TNF ligands. Taken together our data demonstrate that CD40L, RANKL and 4-1BBL play in important role in DC-generated antitumor effect and could be used as genetic immune vaccines for cancer treatment.

Table 5 Summary of antitumor effect of the TNF family ligands.

Delivery approaches	Antitumor effect MC38	Antitumor effect TS/A
sCD40L	-	
Ad-CD40L	++	+
DC/CD40L	+++	+++++
Ad-RANKL	++	+
DC/RANKL	++	+++
Ad-4-1BBL	++	+
DC/4-1BBL	++	+++

Summary of conclusions:

1. CD40L:

- Ad-CD40L induces a significant antitumor effect on MC38 tumor model
- Ad-CD40L dramatically suppresses TS/A tumor growth
- Transduced DC considerably inhibit MC38 tumor growth
- Transduced DC generate a strong antitumor immunity with complete rejection of TS/A tumors
- Transduction stimulates DC to produce high levels of IL-12
- Transduction up-regulates expression of co-stimulatory molecules
- Transduction significantly prolongs survival of DC in cultures

2. RANKL:

- Ad-RANKL stimulates a strong antitumor effect on MC38 tumor model
- Ad-RANKL significantly decreases TS/A tumor growth
- Transduced DC considerably inhibit MC38 tumor growth
- Transduced DC induce a dramatic antitumor effect on TS/A tumor
- Transduction significantly induces survival of DC in culture

3. 4-1BBL:

- Ad-4-1BBL induces a strong antitumor effect on MC38 tumor
- Ad-4-1BBL dramatically suppress TS/A tumor growth
- Transduced DC significantly inhibit MC38 tumor growth
- Transduced DC stimulate a strong antitumor effect on TS/A tumor
- Transduction significantly induces survival of DC in culture

6.0 DISCUSSION

The absence of characterized tumor-associated antigens for many cancers forced the development of alternative DC-based therapies. The recent pilot study in patients with melanoma and breast carcinoma demonstrated a potent antitumor potential of intratumoral administration of DC without addition of tumor antigens (104). Combination of intratumoral injection of DC with cytokines might further increase the efficacy of the therapy. For example, an intratumoral injection of syngeneic DC in combination with the low dose of adenoviral vector encoding TNF-α elicited marked tumor suppression without toxicity and tumor-specific immune responses in four tumor models (137). Using an adenoviral vector expressing CD40L and the number of DC that alone had no effect on tumor growth, Kikuchi et al. have reported that the growth of CT26 colon adenocarcinoma and B16 melanoma murine s.c. tumors is significantly suppressed by direct administration of DC into established tumors that had been pretreated with Ad-CD40L two days previously (158). We have shown here that intratumoral administration of DC genetically modified to express IL-12 or CD40L significantly inhibits tumor growth or causes complete tumor rejection in murine prostate, colon or breast carcinoma models (147, 156). These data demonstrate that the combination of intratumoral administration of DC together with cytokines could evoke tumor suppression without systemic toxicity, providing a new paradigm for the use of cytokines and DC as antitumor therapy.

Different members of TNF family of proteins have demonstrated a significant antitumor potential in a number of preclinical murine and human tumor models. However, the mechanisms of their action as well as the sensitivity of malignant cells were different. We have investigated the antitumor properties of adenoviral vectors encoding CD40L, RANKL, and 4-1BBL, as well as genetically engineered DC overexpressing these ligands, using murine tumor cells both *in vitro* and *in vivo* (**Table 6**).

Table 6. Comparison analysis of properties of the tested TNF family ligands.

	Survival	Up-regulation of CD40 CD80 CD86	IL-12 production	Antitumor effect of adenoviral vectors	Antitumor effect of transduced DC
CD40L	++++	+++ ++ +++	++++++++	+++	+++++++++
RANKL	++++	+ + +	-	+++	++++
4-1BBL	+++	+ + +	-	+++	++++

Here we hypothesized that genetic modification of tumor cells to express TNF ligands will trigger TNF receptors on local APC to present tumor antigen to T cells *in vivo*. Our results suggest that administration of Ad-4-1BBL, Ad-RANKL or Ad-CD40L on Day 7 after tumor inoculation resulted in significant inhibition of MC38 and TS/A tumor growth, whether they were administered as adenoviral vectors or transduced DC. However, none of the tested ligands had a direct antitumor effect *in vitro*. The effect of CD40L-based therapy is in agreement with our and other previously described results which showed that Ad-CD40L-treatment of these tumor lines is very effective (60, 156). Similarly, the antitumor effect of Ad-4-1BBL supports

recently shown data (154). However, antitumor property of Ad-RANKL and DC transduced with Ad-RANKL or Ad-4-1BBL were never studied. In addition, the induction of DC survival in cultures by 4-1BBL transduction is also a novel finding.

We have shown that overexpression of CD40L on DC stimulated production of IL-12 by these cells. In contrast, we observed that RANKL and 4-1BBL did not induce IL-12 production by DC. These data are not in agreement with several investigators (61, 65, 153) who showed that these ligands stimulate DC to secrete IL-12. However, these researchers used soluble proteins or stably-expressing cell lines to activate DC. Maybe adenoviral vectors and transduced DC should be activated in different ways. Also, DC/RANKL and DC/4-1BBL, in contrast to DC/CD40L, did not show a significant increase in DC maturation. Co-stimulatory molecules on DC surface were comparable to the control and LacZ-treated groups, not elevated like in CD40L-transduced DC. These results suggest that RANKL and 4-1BBL used different mechanisms in generating antitumor effect than CD40L.

Our data demonstrate that therapy with adenoviral vectors encoding CD40L, RANKL or 4-1BBL and DC transduced with these vectors elicits a significant antitumor effect in two different tumor models. Furthermore, all tested TNF ligands increase DC survival in growth factor-depleted cultures. CD40L, RANKL and 4-1BBL enhance DC survival dramatically compared to control groups. It is possible that prolongation of DC survival could be one mechanism that is common to all three molecules in inducing antitumor immunity in mice. Therefore, CD40L, RANKL and 4-1BBL-based immunotherapies could be an effective approach for inducing antitumor immunity following a local intratumoral administration.

Taken together, our data suggest that the treatment of tumor-bearing mice with an intratumoral administration of adenovirus encoding the mCD40L, mRANKL or m4-1BBL genes or DC genetically modified to express these genes caused a significant inhibition of tumor growth *in vivo*. However, DC/CD40L displayed the highest antitumor effect which results in a tremendous decrease of MC38 tumor and a complete rejection of TS/A tumors. We concluded that Ad-CD40L transduction of DC or tumor cells at the tumor site is the most effective approach to induce specific long lasting antitumor immunity, as compared to Ad-RANKL and Ad-4-1BBL. In addition, CD40L transduction of DC induced a significant production of IL-12 and up-regulation of co-stimulatory molecules. Therefore, our data suggested that among all tested TNF ligands, Ad-CD40L transduction of DC at the tumor site is the most effective approach to induce specific long lasting antitumor immunity.

The effectiveness of CD40L-based treatments was not surprising because CD40 ligation plays a critical role in DC life cycle. It is well established that CD40-CD40L interaction plays an important role in the generation of antitumor immunity and the regulation of DC generation, maturation, survival and function (70, 128). In fact, it has been demonstrated that CD40L enhances DC efficiency to present antigen to T cells (66, 159), up-regulates the expression of co-stimulatory molecules CD80, CD86, CD40, MHC class I/II, adhesion molecules ICAM, and LFA-3 on DC (3), heightens secretion of proinflammatory cytokines IL-12, IL-6, IL-8, TNF-α (66) by DC, and enhances the DC survival (118, 156). Activation by CD40L in the presence of proinflammatory cytokines or INF-γ was necessary for the "cross-priming" function of DC, by which ingested tumor apoptotic body-derived antigens were presented to T cells in the context of HLA class I, leading to the activation of tumor-specific cytotoxic T effector cells (160-162). Therefore, in the following research we concentrated our attention on studying CD40L effects on

DC. We hypothesized that CD40 stimulation may enhance the effectiveness of the host's antitumor immune response afferently by improving antigen presentation, and efferently by enhancing cytotoxic activity and cytokine secretion of DC. Therefore, we focused on evaluating the role of CD40L in generating antitumor immunity in mice.

Based on the know properties of CD40L in different stages of the DC life cycle, including generation, maturation, trafficking, and antigen presentation we have chosen a systemic administration of soluble CD40L protein to generate antitumor immunity. In addition, it is known that addition of protein to the tumor site will have only a short duration of effect due to high rate of degradation and might influence only tumor cells locally instead of activating DC in the body. On the other side it has been shown that systemic administration of sCD40L may induce significant antitumor effects in other tumor models. However, the effect of sCD40L has not been evaluated in murine MC38 colon adenocarcinoma. Thus, we have chosen to use a systemic administration of CD40L protein to evaluate its potential effect on tumor. However, our data suggest that soluble CD40L protein has no significant antitumor activity in the murine MC38 model. These results also justify the development of alternative genetic immunization approaches for tumor treatment.

The ability of CD40L gene transfer to stimulate antitumor immunity *in vivo* has been demonstrated previously by several groups. Stable expression of CD40L on MC38 colon carcinoma cells and MCA 205 fibrosarcomas resulted in inhibition of tumor growth following inoculation of the genetically modified tumor cells (117, 118). Similarly, it has been demonstrated that the *in vivo* genetic modification of tumor cells to express CD40L will trigger CD40 on local antigen-presenting cells to present tumor antigen to T cells, thus eliciting antitumor immunity to suppress growth of the tumor (60).

We have examined the antitumor effects of two different methods of intratumoral delivery of the CD40L gene, either directly by adenoviral injection or indirectly by injection of genetically modified DC. Our results demonstrate that the treatment of both MC38 and TS/A tumor-bearing mice with intratumoral administration of adenovirus encoding the mCD40L gene caused a significant inhibition of tumor growth. Similarly, it has been shown that Ad-CD40L treatment of established AC29 tumor induces a significant antitumor effect (163) and intratumoral injection of one of two synchronous tumors resulted in regression of both. However, Yanagi et al. demonstrated that Ad-CD40L treatment of hepatocellular carcinoma induces only weak antitumor immunity (164). These data are not in agreement with our results since we have shown that Ad-CD40L considerably suppresses tumor growth. Furthermore, we have demonstrated that the use of Ad-CD40L infected DC resulted in a significant anti-tumor response in MC38 tumor model, caused a complete rejection of TS/A tumors in 80% of mice, and induced a specific systemic immunity. These data are similar to Kikuchi's, who showed that DC genetically modified to express CD40L elicit strong antitumor effects in the B16 melanoma tumor model (165). In addition, it has been demonstrated that simultaneous administration of Ad-CD40L and naïve DC induces tumor regression (158).

In the next set of experiments we focused on identification of primary mechanisms responsible for the significant antitumor immunity induced by DC genetically modified to express CD40L. To test this, we examined different functional activities of transduced DC.

First, we have shown that DC modified to overexpress CD40L produce high amounts of IL-12, which is one of the most important cytokines secreted by DC. It plays a critical role in regulation of Th1/Th2 balance and in generation of antitumor immunity by DC. In addition, the expression of CD40, CD80, CD86 and class II was elevated by CD40L transduction of DC,

which is in agreement with multiple studies (3, 66). This was an important observation, because co-stimulatory molecules play a major role in T cell activation by DC.

Next, we demonstrated that the adhesiveness of DC was altered by CD40L expression, with the modified cells becoming organized into clusters. Moreover, DC transduced with Ad-CD40L survived significantly longer in cultures compared to control DC. Therefore, it is likely that CD40L-transfected DC activate each other in tight clusters by multiple CD40-CD40L interactions, resulting in accelerated maturation of cells reflected by the increased expression of co-stimulatory molecules CD80, CD86, and MHC molecules, as well as increased production of IL-12.

Next, we conducted studies to determine if overexpression of CD40L on DC increases their potential to present tumor antigen to T cells, which is the most important function of DC. We tested this hypothesis in syngeneic MLR assay. We demonstrated that CD40L-transduced DC display significantly higher levels of tumor antigens presentation to T cells *in vitro* compared to control DC. These data were confirmed in subsequent studies which showed that CD40L-transduced DC induce higher levels of cytotoxic T cell activation *in vivo* when compared to control cells. These results are in agreement with others which demonstrated that transduction of DC with Ad-CD40L stimulates cytotoxic T lymphocytes (166, 167). Therefore, these data serve as additional support for our main findings demonstrating that DC modified with CD40L gene elicit a strong antitumor effect *in vivo* (Chapter 2).

In addition, immunohistochemical staining of tumor tissues obtained form control and treated mice demonstrated an increased accumulation of T effectors within the tumor and an increased accumulation of tumor-infiltrating CD11c+ DC in animals treated with CD40L-transduced DC. Our data show that an elevated accumulation of DC at the tumor site in

DC/CD40L-treated group was observed as early as 24 hr post injection and was still increased 1 week later. This finding is in good agreement with numerous clinical data demonstrating that higher number of DC within the tumor was associated with the better prognosis and lower metastasis in cancer patients (47, 96, 126, 127).

Furthermore, trafficking studies have demonstrated that transduction with CD40L induces migration of injected DC from tumors to lymphoid tissues. DC transduced with CD40L were found in lymph nodes 2 hr and in spleens 48 hr post treatment. In contrast, in control groups DC were present in lymph nodes only 24 hr post treatment and were not found in spleens of these animals. These findings were important because we showed that not only CD40L induces high infiltration of tumors with DC but, also, stimulates the migration of activated DC to the lymph nodes to present tumor antigen to T cells and generate antitumor effect. This could be one of the most important mechanisms of CD40L-mediated antitumor immunity by modified DC.

Therefore, analyzing all described mechanisms, we speculate that immunotherapy with genetically modified DC overexpressing CD40L induces many important DC functions which play a major role in generation of a strong specific antitumor immunity by modified DC.

Taken together (**Figure 6.1**), we suggest that intratumoral administration of DC genetically modified to overexpress CD40L significantly induces maturation, survival, IL-12 production, and infiltration of DC at the tumor site. In addition, CD40L transduction stimulates DC migration from tumor site and trafficking to lymph nodes and other lymphoid organs. In the lymph nodes, DC transduced with Ad-CD40L display an elevated ability to present TAA and stimulate T cell proliferation. Taken together, CD40L significantly up-regulates antitumor

immune response. Furthermore, DC modified to express the CD40L gene considerably increase CTL response, which inhibits tumor growth and causes tumor rejection.

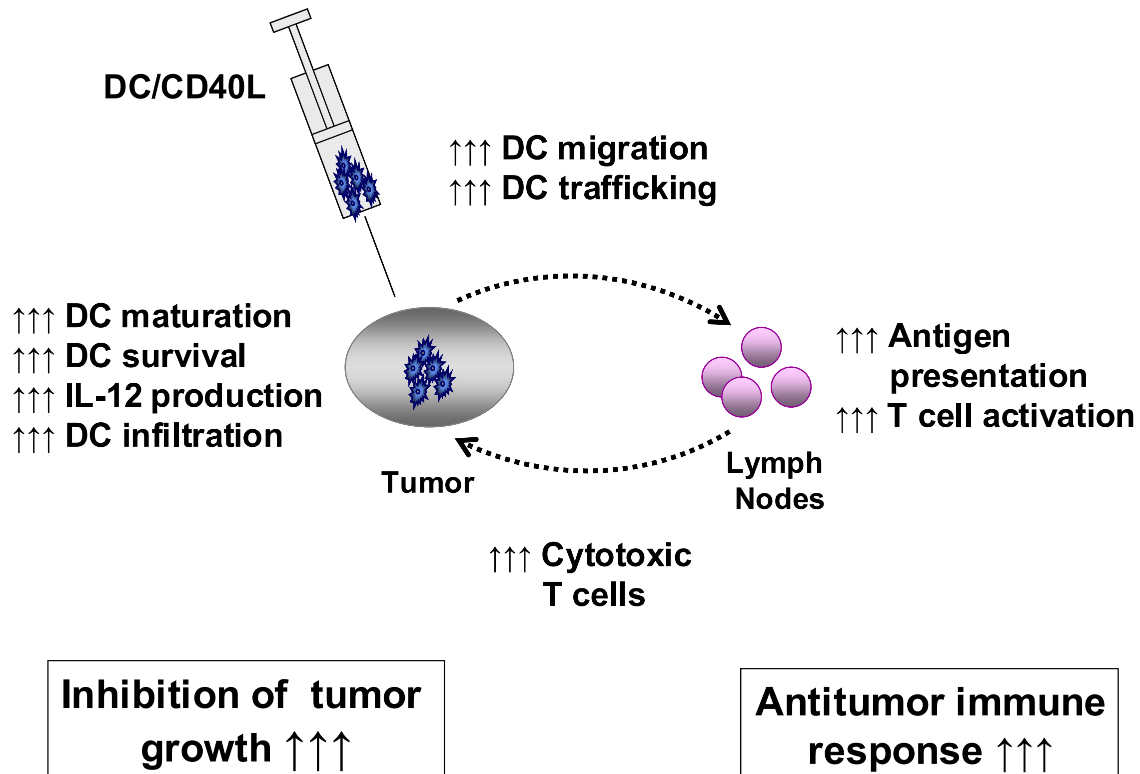


Figure 6. 1 Summary of the antitumor effect generated by DC/CD40L-based immunotherapy.

Therefore, our data demonstrate that intratumoral administration of DC transduced with Ad-CD40L is a very effective immunotherapy for cancer. We have demonstrated that transduction of DC with Ad-CD40L induces a strong activation, stimulation and maturation of DC. In addition, DC/CD40L display a prolonged survival, up-regulated TAA presentation, significant T cell activation potential, increased tumor infiltration and induced trafficking from

the tumor site to the lymphatic tissues. Taken together, all these dramatically amplified DC functions produce a strong antitumor immunity with generation of specific immune memory.

All the previously described experiments were based on examining the functional activity of DC generated from tumor-free animals. However, the main purpose of DC-based immunotherapy is generation or isolation of DC from cancer patient, activating these cells by different methods, and injecting these modified DC back into patient's blood, skin, lymph nodes or tumors. Therefore, interactions between tumor cells and cells of the immune system might be critical for tumor growth and progression. In fact, tumor-derived factors were shown to dramatically alter the function and survival of immunocompetent cells in the local tumor microenvironment as well as systemically (4, 47, 48). Plentiful data clearly demonstrate the importance of developing therapies directed to protect DC and their precursors from tumor-induced down-regulation of cell differentiation, function and survival. Thus, better understanding of the mechanisms involved in the tumor-mediated inhibition of DC generation, maturation, and function may led to the development of novel strategies for the prevention of immunosuppression in cancer patients and the development of novel DC-based immunotherapeutic strategies.

It is well known that DC play a crucial role in the generation of antitumor immunity. We verified that tumors suppress the efficiency of DC-based therapies in cancer using two tumor models. We noticed that untreated DC have antitumor potential when injected into the tumor site. However, when we used DC generated from tumor-bearing mice, our data demonstrate that these DC did not induce an antitumor effect. The growth curve of tumors treated with DC generated from tumor-bearing mice was similar to growth of untreated tumors. These data suggested that tumor inhibits the antitumor potential of DC.

In the next set of experiments, we examined the functional activity responsible for the decreased antitumor potential of DC in cancer. First, we demonstrated that DC generated from colon or breast adenocarcinoma-bearing mice show a marked inhibition of maturation when compared to DC generated from tumor-free animals. Our data have shown that DC generated form tumor-bearers produce a decreased amount of IL-12 and expressed down-regulated levels of CD80 and CD86. These results are in agreement with studies demonstrating that tumor suppresses the expression of co-stimulatory molecules on DC (18, 44, 45). It is well known that if antigen-presenting cells do not express CD80 or CD86, they will fail to provide an appropriate second signal for T cells. In fact, we have demonstrated that DC generated from tumor bearers displayed a decreased ability to stimulate T cell proliferation compared to control DC generated from tumor-free mice. A similar toleragenic effect of melanoma-associated DC was also reported: tumor-derived DC from progressing metastases might actively induce tolerance in the immune system against tumor tissue. This process might ultimately lead to acceptance of metastatic tumor growth with a “silenced” immune system incapable of rejecting the tumor (39).

Furthermore, DC as well as bone marrow precursors from tumor-bearing animals expressed low levels of CD40 protein and CD40 mRNA. This was of a special interest to us, since, as has been mentioned, CD40 ligation plays a critical role in the DC life cycle. Thus, we have suggested that CD40 ligation on tumor-derived DC might induce lower levels of IL-12 production due to inhibition of CD40 expression. In addition, down-regulated expression of CD40 on DC in cancer could explain why CD40L transduced DC had the strongest antitumor effect compared to two other tested treatments (DC/RANKL and DC/4-1BBL). Furthermore, down-regulation of CD40 expression on DC generated from tumor-bearing mice was accompanied by inhibition of expression of co-stimulatory molecules CD80 and CD86, and

suppression of the ability of DC to stimulate proliferation of T cells. In addition, more profound inhibition of DC maturation was observed *in vivo* in mice bearing s.c.- or i.v.-injected MC38 tumor cells. Endogenous CD11c+ DC isolated from the spleen of tumor-bearers expressed significantly lower levels of CD40, CD80 and CD86 molecules compared to control DC. These data showed that tumor inhibited DC both *in vitro* and *in vivo*. Thus, it could be concluded that abrogation of DC maturation is a common feature of tumor cell DC interaction both *in vitro* and *in vivo*.

Taken together, our data suggest that tumor-induced DC dysfunction could be generated by down-regulation of CD40 expression on DC, and, thus, suppressing CD40-mediated signaling. Therefore, protection of DC from tumor-induced DC dysfunction could be an important mechanism in generating strong antitumor immunity. For instance, recovery of decreased CD40 signaling (18) may potentially increase the efficacy of anticancer immunotherapy. Thus, we hypothesized that in order to overcome deficient CD40 signaling in DC in cancer, the genetic modification of DC with the CD40L gene might induce enhanced antitumor responses by protecting DC from tumor-mediated suppression.

In the next set of experiments we examined whether CD40L-transduction of DC might overcome tumor-induced suppression of antitumor potential of DC. We generated DC from tumor-bearing and tumor-free mice, transduced them with Ad-CD40L and injected into the tumor site. As controls, non-transduced DC and DC transduced with Ad-LacZ were used. Our results have shown that transduction of DC generated from tumor bearing mice with CD40L rescues DC from tumor-induced suppression. DC generated from TS/A-bearing mice display a significant antitumor effect compared to controls.

Thus, better understanding of the mechanisms involved in the tumor-mediated inhibition of DC generation, maturation, and function may led to the development of novel strategies for the prevention of immunosuppression in cancer patients and the development of novel DC-based immunotherapeutic strategies. Therefore, in the next set of experiments we evaluated the effect of CD40L-transduction on DC and whether infection of DC with Ad-CD40L would protect/rescue DC from tumor-induced dysfunction.

Next, we tested the hypothesis that autocrine/paracrine CD40 ligation on genetically modified DC might restore suppressed IL-12 production by DC in cancer. DC were generated from control and TS/A-bearing mice and measured IL-12 production. Our data showed that DC/CD40L group secretes similar levels of IL-12 whether they were from control or tumor-bearing animals. This suggests that transduction of DC with the CD40L gene protects them from tumor-induced inhibition of IL-12 production and this phenomenon is involved in increased antitumor potential of CD40L-transduced DC in the tumor microenvironment.

Furthermore, we have shown that CD40L transduction of DC generated from TS/A-bearing mice rescues tumor-suppressed expression of co-stimulatory molecules. We have demonstrated that DC from tumor bearers, which were transduced with Ad-CD40L, display a significant increase in CD80, CD86 and CD40 expression compared to controls. Furthermore, CD40L-transduction restores the expression of these co-stimulatory molecules to similar levels, expressed by DC/CD40L group from tumor-free animals. Thus, our data suggest that transduction of DC generated from tumor-bearing mice rescues DC from tumor-mediated suppression of the expression of co-stimulatory molecules, which are known to play a crucial role in stimulation of T cells and induction of antitumor responses.

In addition, we have tested whether CD40L transduction protects the ability of DC to induce proliferation of T cells in the allogeneic MLR assay. Our data demonstrate that DC transduced with Ad-CD40L display similar levels of T cell stimulation whether they were generated form the control or tumor-bearing mice. These results suggest that the transduction with Ad-CD40L induces the ability of DC to stimulate T cell proliferation and could explain the significant antitumor effect generated by DC/CD40L-based immunotherapy.

Taken together, our results demonstrate that DC generated from tumor-bearing mice display a decreased antitumor potential. We have shown that the CD40L transduction of DC not only stimulates induction of a strong antitumor immunity of control DC, but also up-regulates the potential of DC generated from tumor-bearing mice to generate strong antitumor immunity. This is a novel mechanism of antitumor effect of transduced DC. Therefore, our results demonstrate that CD40L-based immunotherapy induces strong antitumor immunity and protects DC from tumor-mediated suppression of their antitumor potential (**Figure 6.2)**. In addition, these data suggest that CD40L might increase the resistance of DC to tumor-induced inhibition of DC maturation and function.

The significance of our research is that the primary mechanisms responsible for the antitumor immunity generated by genetically modified DC have not been yet studied. In our research, we focused on evaluating different DC functional activities induced by CD40L transduction which might play a critical role in generating a significant antitumor effect. Moreover, we developed a novel method to study trafficking of DC injected into the tumor site. The migration pattern of DC has not been evaluated yet.

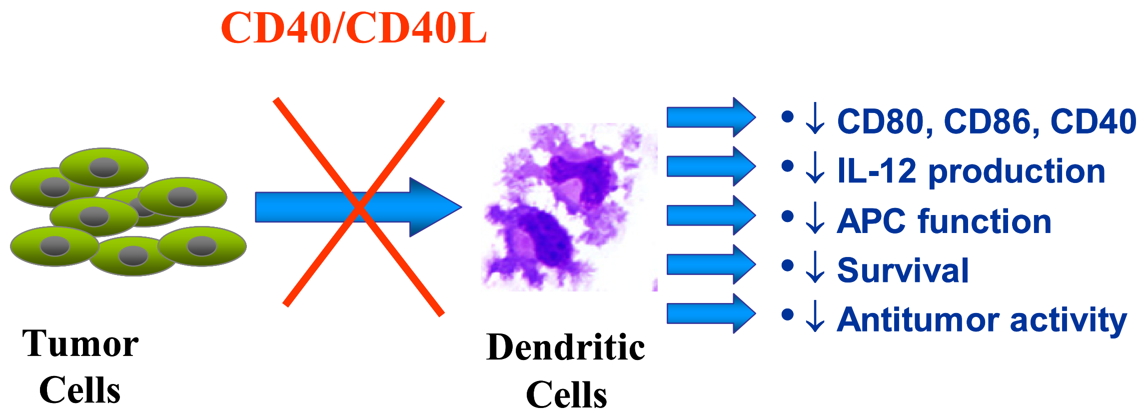


Figure 6. 2 CD40L-based immunotherapy stimulates strong antitumor immunity and protects DC from tumor-mediated suppression of their functional activities.

It has been demonstrated that tumor-secreted factors induce DC dysfunction. However, we used a novel approach to protect DC function in cancer. Our data suggest that the immune gene therapy with CD40L-transduced DC protects DC from tumor-mediated suppression.

In addition, the role of RANKL and 4-1BBL in DC immunobiology has not been defined. We have shown that immunotherapies with both adenoviral vectors and transduced DC expressing RANKL or 4-1BBL induce strong antitumor immunity in both tested tumor models. Furthermore, DC modified with these two genes display prolonged survival in cultures.

Overall, our data provide supporting evidence for the concept of antitumor immunotherapy based on the administration of Ad-CD40L or DC transduced with adenoviral vector encoding CD40L. Immunotherapy with modified DC playing a key role in the induction of specific antitumor immune responses seems to be one of the promising alternative approaches in the treatment of cancer. Genetically modified DC overexpressing CD40L protein may serve as a basis for all DC-based clinical protocols, including the development of therapies for patients with cancer, HIV and infectious diseases.

FUTURE DIRECTIONS

All together our data demonstrate that DC genetically modified to overexpress CD40L could be used as a therapeutic approach in cancer. We have shown that the transduction of DC with CD40L up-regulates many important DC functions and protects DC from tumor-induced suppression. However, there are still a lot of questions that require further investigation.

The infiltration of DC into tumors has been associated with significantly prolonged patient survival and reduced incidence of metastasis in patients with different types of cancer (102). However, several studies have demonstrated that tumor induces apoptotic death of DC (123). Therefore, investigation of the mechanisms involved in tumor-induced apoptosis of DC will allow for the definition of effective means to protect DC from premature death and thus improve tumor-specific immune responses.

Tumors secrete multiple soluble factors which have a significant effect on DC development and function. Thus, the interaction between soluble factors and DC will require further study to reveal the intricate processes leading up to the tumor-induced inhibition of DC. It is important to examine different intracellular mechanisms induced/suppressed in DC by tumor-associated molecules.

We have demonstrated that tumor-mediated inhibition of the functional maturation of DC might be mediated by down-regulated expression of CD40 on DC, and, thus, deficient CD40-mediated signaling. Further understanding of the cellular and intracellular mechanisms

responsible for inhibition of CD40 expression on DC will allow us to protect DC formation and restore the suppressed antitumor potential of these cells in cancer.

We have demonstrated that CD40L transduction of DC significantly increases the antitumor potential of DC generated from tumor-bearing animals. However, this therapy did not completely rescue DC from tumor-mediated suppression. Thus, further investigations of the mechanisms involved in the inhibition of antitumor potential of DC could lead to the discovery of novel therapeutic agents that will be directed to decrease tumorogenicity and increase immunogenicity of DC.

APPENDIX

Our research was presented in the following publications, book chapters and lectures:

PUBLICATIONS:

1 Pirtskhalaishvili G., Shurin G.V., **Yurkovetsky Z.R.**, Esche C., Gambotto A., Robbins P., Shurin M.R. Transduction of dendritic cells with Bcl-x_L increases their resistance to prostate cancer-induced apoptosis and antitumor effect in mice. *J. Immunol*. **165**:1956-1964, 2000.

2 Tourkova I.L., **Yurkovetsky, Z.R**., Shurin M.R., Shurin G.V. Mechanisms of T cell activation by dendritic cells in the MLR assay. *Immunol. Let*., **78**:75-82, 2001.

3 Shurin M.R., **Yurkovetsky Z.R.,** Barksdale E. Jr., Shurin G.V. Inhibition of CD40 expression during dendropoiesis by tumor: Role of GM3 and IL-10. International Journal of Cancer. 101(1):61-8, 2002 Sep 1.

4 Tourkova I.L., **Yurkovetsky Z.R.**, Gambotto A., Shurin M.R., Shurin G.V. Increased Function and Survival of IL-15-transfected Human Dendritic Cells are Mediated by Up-regulation of IL-15Rα and Bcl-2. Journal of Leukocyte Biology. 72(5):1037-45, 2002 Nov.

5 Satoh Y., Esche C., Gambotto A., Shurin G.V., **Yurkovetsky Z.R**., Robbins P.D., Watkins S.C., Todo S., Lotze M.T., Herberman R.B., Shurin M.R. Local Administration of IL-12-transfected Dendritic Cells Induces Antitumor Immune Responses to Colon Adenocarcinoma in the Liver in Mice. *J. Exp. Therap. Oncol*.

6 Yamabe K., Peron J.M., Esche C., **Yurkovetsky Z.R.,** Watkins S., Lotze M.T., **Shurin M.R.** Lymphoid and myeloid dendritic cells: Functional differences between in vivo and in vitro generated cells. Submitted.

7 **Yurkovetsky Z.R.**, Shurin G.V., Gambotto A., Kim S.H., Shurin M.R., Robbins P.D. Intratumoral administration of adenovectors encoding the CD40L gene or dendritic cells transduced with CD40L vector induces antitumor immunity in mice. Cancer gene therapy. Submitted.

8 **Yurkovetsky Z.R.**, Shurin G.V., Shurin M.R., Robbins P.D. Comparative analysis of antitumor activity of CD40L, RANKL, and 4-1BBL in vivo following intratumoral administration of viral vectors or transduced dendritic cells. In preparation.

9 **Yurkovetsky Z.R.,** Shurin G.V., Shurin M.R., Robbins P.D. Transduction with avenoviral vector encoding CD40L protects dendritic cells from tumor-induced dysfunction. In preparation.

BOOK CHAPTERS AND REVIEWS:

1. Shurin G.V., **Yurkovetsky Z.R**., Shurin M.R. Tumor-induced dendritic cell dysfunction. In: *Mechanisms of Tumor Escape*. A.Ochoa (Ed.). Harwood Academic Publishers. 2002.

2. **Yurkovetsky Z.R.**, Shurin G.V., Robbins P.D., Shurin M.R. The TNF-Superfamily of Proteins for Cancer Therapy. *Recent Research Development in Cancer*, Vol.2, 2001.

3. **Yurkovetsky Z.R**., Tourkova I.L., Balkir L., Perez L., Shurin G.V., Chatta G.S., Shurin M.R. Malfunction of the dendritic cell system in cancer. In press.

ABSTRACTS:

1. Esche C., Shurin G., **Yurkovetsky Z.R.,** Barksdale E., Lotze M.T., Shurin M.R. The maturation level of dendritic cells (DC) modifies the sensitivity to melanoma-induced apoptosis. *J. Invest. Dermatol*. **113**:475, 1999.

2. Pirtskhalaishvili G., Gambotto A., Esche C., **Yurkovetsky Z.R**., Lotze M.T., Shurin M.R. IL-12 and Bcl-xl gene transfection of murine dendritic cells protects them from prostate cancer-induced apoptosis and improves their antitumor activity. *J. Urol*. **163** (Suppl): 105, 2000.

3. Tourkova I., Shurin G.V., Gambotto A., **Yurkovetsky Z.R.,** Shurin M.R. Transfection of dendritic cells with IL-15 gene protects them from prostate cancer induced apoptosis. Keystone Meeting “Dendritic cells’. Taos, NM, 2001.

4. Shurin G.V., **Yurkovetsky Z.R.,** Shurin M.R. Suppressed maturation of dendritic cells in cancer is mediated by down-regulation of CD40 expression. Keystone Meeting “Dendritic cells’. Taos, NM, 2001.

5. **Yurkovetsky Z.R.**, Gambotto A., Kim S.H., Shurin M.R., Robbins P .D. Intratumoral administration of Ad-CD40L or DC/CD40L elicited effective antitumor immunity in mice. 4th annual ASGT meeting, 2001.

6. **Yurkovetsky Z.R.,** Gambotto A., Kim S.H., Shurin M.R., Robbins P .D. Intratumoral administration of Ad-CD40L or DC/CD40L elicited effective antitumor immunity in mice. 13th annual Virology Symposium, 2002.

7. **Yurkovetsky Z.R.,** Robbins P.D. Antitumor effect of adenoviral vectors expressing CD40L, RANKL or 4-lBBL. ASGT 5th annual meeting, 2002.

8. Kin S.H., **Yurkovetsky Z.R.,** Robbins P .D. Combined immunotherapy of a murine mammary tumor using genetically modified dendritic cells. ASGT 5th annual meeting, 2002.

9. **Yurkovetsky Z.R.,** Robbins P.D. Antitumor effect of adenoviral vectors expressing CD40L, RANKL or 4-lBBL. MGB retreat, 2002.

10. **Yurkovetsky Z.R.,** Shurin M.R., Robbins P.D. Intratumoral administration of adeno CD40L or dendritic cells overexpressing CD40L elicited effective antitumor immunity in mice. Era of Hope, DOD breast cancer research program meeting, 2002.

INVITED SEMINARS, LECTURESHIPS, AND PRESENTATIONS

1. Intratumoral administration of adeno CD40L or dendritic cells overexpressing CD40L elicited effective antitumor immunity in mice fl. Era of Hope, DOD breast cancer research program meeting, 2002.

BIBLIOGRAPHY

1. Ioannides, C. G., and T. L. Whiteside. 1993. T cell recognition of human tumors: implications for molecular immunotherapy of cancer. *Clin Immunol Immunopathol 66:91*.

2. Janeway, C. A., Jr., and K. Bottomly. 1994. Signals and signs for lymphocyte responses. *Cell 76:275*.

3. Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med 184:747*.

4. Kavanaugh, D. Y., and D. P. Carbone. 1996. Immunologic dysfunction in cancer. *Hematol Oncol Clin North Am 10:927*.

5. Wojtowicz-Praga, S. 1997. Reversal of tumor-induced immunosuppression: a new approach to cancer therapy. *J Immunother 20:165*.

6. Guillou, P. J., P. C. Sedman, and C. W. Ramsden. 1989. Inhibition of lymphokine-activated killer cell generation by cultured tumor cell lines in vitro. *Cancer Immunol Immunother 28:43*.

7. Lapointe, H., H. Lampe, and D. Banerjee. 1992. Head and neck squamous cell carcinoma cell line-induced suppression of in vitro lymphocyte proliferative responses. *Otolaryngol Head Neck Surg 106:149*.

8. Whitehead, J. S., and Y. S. Kim. 1980. An inhibitor of lymphocyte proliferation produced by a human colonic adenocarcinoma cell line in culture. *Cancer Res 40:29*.

9. Tartter, P. I., B. Steinberg, D. M. Barron, and G. Martinelli. 1989. Transfusion history, T cell subsets and natural killer cytotoxicity in patients with colorectal cancer. *Vox Sang 56:80*.

10. Herberman, R. B. 2002. Cancer immunotherapy with natural killer cells. *Semin Oncol 29:27*.

11. Mickel, R. A., D. J. Kessler, J. M. Taylor, and A. Lichtenstein. 1988. Natural killer cell cytotoxicity in the peripheral blood, cervical lymph nodes, and tumor of head and neck cancer patients. *Cancer Res 48:5017*.

12. Guillou, P. J., C. W. Ramsden, S. S. Somers, and P. C. Sedman. 1989. Suppression of the generation of lymphokine-activated killer (LAK) cells by serum-free supernatants of in vitro maintained tumour cell lines. *Br J Cancer 59:515*.

13. Berd, D., and M. J. Mastrangelo. 1987. Effect of low dose cyclophosphamide on the immune system of cancer patients: reduction of T-suppressor function without depletion of the CD8+ subset. *Cancer Res 47:3317*.

14. Jaffe, M. L., H. Arai, and G. J. Nabel. 1996. Mechanisms of tumor-induced immunosuppression: evidence for contact-dependent T cell suppression by monocytes. *Mol Med 2:692*.

15. Hirte, H., and D. A. Clark. 1991. Generation of lymphokine-activated killer cells in human ovarian carcinoma ascitic fluid: identification of transforming growth factor-beta as a suppressive factor. *Cancer Immunol Immunother 32:296*.

16. Letessier, E. M., M. Sacchi, J. T. Johnson, R. B. Herberman, and T. L. Whiteside. 1990. The absence of lymphoid suppressor cells in tumor-involved lymph nodes of patients with head and neck cancer. *Cell Immunol 130:446*.

17. Shurin, G. V., M. R. Shurin, S. Bykovskaia, J. Shogan, M. T. Lotze, and E. M. Barksdale, Jr. 2001. Neuroblastoma-derived gangliosides inhibit dendritic cell generation and function. *Cancer Res 61:363*.

18. Shurin, M. R., Z. R. Yurkovetsky, I. L. Tourkova, L. Balkir, and G. V. Shurin. 2002. Inhibition of CD40 expression and CD40-mediated dendritic cell function by tumor-derived IL-10. *Int J Cancer 101:61*.

19. Wikstrom, P., J. Damber, and A. Bergh. 2001. Role of transforming growth factor-beta1 in prostate cancer. *Microsc Res Tech 52:411*.

20. Ivanovic, V. V., N. Todorovic-Rakovic, M. Demajo, Z. Neskovic-Konstantinovic, V. Subota, O. Ivanisevic-Milovanovic, and D. Nikolic-Vukosavljevic. 2002. Elevated plasma levels of transforming growth factor-beta(1) (TGF-beta(1)) in patients with advanced breast cancer: association with disease progression. *Eur J Cancer 39:454*.

21. Park, C., W. S. Kim, Y. Choi, H. Kim, and K. Park. 2002. Effects of transforming growth factor beta (TGF-beta) receptor on lung carcinogenesis. *Lung Cancer 38:143*.

22. Idobe, Y., Y. Murawaki, Y. Kitamura, and H. Kawasaki. 2003. Expression of transforming growth factor-beta 1 in hepatocellular carcinoma in comparison with the non-tumor tissue. *Hepatogastroenterology 50:54*.

23. Mouri, H., K. Sakaguchi, T. Sawayama, T. Senoh, T. Ohta, M. Nishimura, A. Fujiwara, M. Terao, Y. Shiratori, and T. Tsuji. 2002. Suppressive effects of transforming growth factor-beta1 produced by hepatocellular carcinoma cell lines on interferon-gamma production by peripheral blood mononuclear cells. *Acta Med Okayama 56:309*.

24. Haase, C., T. N. Jorgensen, and B. K. Michelsen. 2002. Both exogenous and endogenous interleukin-10 affects the maturation of bone-marrow-derived dendritic cells in vitro and strongly influences T-cell priming in vivo. *Immunology 107:489*.

25. McKallip, R., R. Li, and S. Ladisch. 1999. Tumor gangliosides inhibit the tumor-specific immune response. *J Immunol 163:3718*.

26. Naama, H. A., V. E. Mack, G. P. Smyth, P. P. Stapleton, and J. M. Daly. 2001. Macrophage effector mechanisms in melanoma in an experimental study. *Arch Surg 136:804*.

27. Eisengart, C. A., J. R. Mestre, H. A. Naama, P. J. Mackrell, D. E. Rivadeneira, E. M. Murphy, P. P. Stapleton, and J. M. Daly. 2000. Prostaglandins regulate melanoma-induced cytokine production in macrophages. *Cell Immunol 204:143*.

28. Proescholdt, M. A., M. J. Merrill, B. Ikejiri, S. Walbridge, A. Akbasak, S. Jacobson, and E. H. Oldfield. 2001. Site-specific immune response to implanted gliomas. *J Neurosurg 95:1012*.

29. Yotnda, P., P. Mintz, K. Grigoriadou, F. Lemonnier, E. Vilmer, and P. Langlade-Demoyen. 1999. Analysis of T-cell defects in the specific immune response against acute lymphoblastic leukemia cells. *Exp Hematol 27:1375*.

30. Bagot, M., L. Boumsell, and A. Bensussan. 1999. [Contribution of immunotherapy to the treatment of T-cell lymphoma of the skin]. *Presse Med 28:1911*.

31. Echchakir, H., M. Bagot, G. Dorothee, D. Martinvalet, S. Le Gouvello, L. Boumsell, S. Chouaib, A. Bensussan, and F. Mami-Chouaib. 2000. Cutaneous T cell lymphoma reactive CD4+ cytotoxic T lymphocyte clones display a Th1 cytokine profile and use a fas-independent pathway for specific tumor cell lysis. *J Invest Dermatol 115:74*.

32. Sheu, B. C., R. H. Lin, H. C. Lien, H. N. Ho, S. M. Hsu, and S. C. Huang. 2001. Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer. *J Immunol 167:2972*.

33. Kobayashi, H., M. Wood, Y. Song, E. Appella, and E. Celis. 2000. Defining promiscuous MHC class II helper T-cell epitopes for the HER2/neu tumor antigen. *Cancer Res 60:5228*.

34. Esche, C., A. Lokshin, G. V. Shurin, B. R. Gastman, H. Rabinowich, S. C. Watkins, M. T. Lotze, and M. R. Shurin. 1999. Tumor's other immune targets: dendritic cells. *J Leukoc Biol 66:336*.

35. Shurin, G. V., M. T. Lotze, and E. M. Barksdale. 2000. Neuroblastoma inhibits dendritic cell differentiation and function. *Curr Surg 57:637*.

36. Shurin, G. V., M. Aalamian, G. Pirtskhalaishvili, S. Bykovskaia, E. Huland, H. Huland, and M. R. Shurin. 2001. Human prostate cancer blocks the generation of dendritic cells from CD34+ hematopoietic progenitors. *Eur Urol 39 Suppl 4:37*.

37. Gabrilovich, D. I., M. P. Velders, E. M. Sotomayor, and W. M. Kast. 2001. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. *J Immunol 166:5398*.

38. Garcia-Plata, D., M. Lessana-Leibowitch, A. Palangie, J. Gulliemette, D. Sedel, L. Mendez, and E. Mozos. 1995. Immunophenotype analysis of dendritic cells and lymphocytes associated with cutaneous malignant melanomas. *Invasion Metastasis 15:125*.

39. Enk, A. H., H. Jonuleit, J. Saloga, and J. Knop. 1997. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int J Cancer 73:309*.

40. Gabrilovich, D. I., J. Corak, I. F. Ciernik, D. Kavanaugh, and D. P. Carbone. 1997. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin Cancer Res 3:483*.

41. Nestle, F. O., G. Burg, J. Fah, T. Wrone-Smith, and B. J. Nickoloff. 1997. Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T cell co-stimulatory molecules and are impaired as antigen-presenting cells. *Am J Pathol 150:641*.

42. Gabrilovich, D. I., H. L. Chen, K. R. Girgis, H. T. Cunningham, G. M. Meny, S. Nadaf, D. Kavanaugh, and D. P. Carbone. 1996. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med 2:1096*.

43. Aalamian, M., G. Pirtskhalaishvili, A. Nunez, C. Esche, G. V. Shurin, E. Huland, H. Huland, and M. R. Shurin. 2001. Human prostate cancer regulates generation and maturation of monocyte-derived dendritic cells. *Prostate 46:68*.

44. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature 356:607*.

45. Reiser, H., G. J. Freeman, Z. Razi-Wolf, C. D. Gimmi, B. Benacerraf, and L. M. Nadler. 1992. Murine B7 antigen provides an efficient costimulatory signal for activation of murine T lymphocytes via the T-cell receptor/CD3 complex. *Proc Natl Acad Sci U S A 89:271*.

46. Chaux, P., N. Favre, B. Bonnotte, M. Moutet, M. Martin, and F. Martin. 1997. Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression. A role in the immune tolerance to antigenic tumors. *Adv Exp Med Biol 417:525*.

47. Shurin, G. V., Z. R. Yurkovetsky, and M. R. Shurin. 2003. Tumor-induced dendritic cell dysfunction. *The Tumor Immunology and Immunotherapy Series 2:112*.

48. Yurkovetsky, Z. R., I. L. Tourkova, L. Balkir, L. Perez, G. V. Shurin, G. S. Chatta, and M. R. Shurin. In press. Malfunction of the dendritic cell system in cancer.

49. Tas, M. P., P. J. Simons, F. J. Balm, and H. A. Drexhage. 1993. Depressed monocyte polarization and clustering of dendritic cells in patients with head and neck cancer: in vitro restoration of this immunosuppression by thymic hormones. *Cancer Immunol Immunother 36:108*.

50. Thurnher, M., C. Radmayr, R. Ramoner, S. Ebner, G. Bock, H. Klocker, N. Romani, and G. Bartsch. 1996. Human renal-cell carcinoma tissue contains dendritic cells. *Int J Cancer 68:1*.

51. Cosman, D. 1994. A family of ligands for the TNF receptor superfamily. *Stem Cells 12:440*.

52. Gravestein, L. A., and J. Borst. 1998. Tumor necrosis factor receptor family members in the immune system. *Semin Immunol 10:423*.

53. Zhou, T., J. D. Mountz, and R. P. Kimberly. 2002. Immunobiology of tumor necrosis factor receptor superfamily. *Immunol Res 26:323*.

54. Mitsiades, N., V. Poulaki, S. Tseleni-Balafouta, D. A. Koutras, and I. Stamenkovic. 2000. Thyroid carcinoma cells are resistant to FAS-mediated apoptosis but sensitive tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res 60:4122*.

55. Gruss, H. J., and S. K. Dower. 1995. Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. *Blood 85:3378*.

56. Herr, I., C. Posovszky, L. D. Di Marzio, M. G. Cifone, T. Boehler, and K. M. Debatin. 2000. Autoamplification of apoptosis following ligation of CD95-L, TRAIL and TNF-alpha. *Oncogene 19:4255*.

57. Ahmad, M., and Y. Shi. 2000. TRAIL-induced apoptosis of thyroid cancer cells: potential for therapeutic intervention. *Oncogene 19:3363*.

58. Kuninaka, S., T. Yano, H. Yokoyama, Y. Fukuyama, Y. Terazaki, T. Uehara, T. Kanematsu, H. Asoh, and Y. Ichinose. 2000. Direct influences of pro-inflammatory cytokines (IL-1beta, TNF-alpha, IL-6) on the proliferation and cell-surface antigen expression of cancer cells. *Cytokine 12:8*.

59. Lasek, W., A. Mackiewicz, A. Czajka, T. Switaj, J. Gol b, M. Wiznerowicz, G. Korczak-Kowalska, E. Z. Bakowiec-Iskra, K. Gryska, D. Izycki, and M. Jakobisiak. 2000. Antitumor effects of the combination therapy with TNF-alpha gene-modified tumor cells and interleukin 12 in a melanoma model in mice. *Cancer Gene Ther 7:1581*.

60. Kikuchi, T., and R. G. Crystal. 1999. Anti-tumor immunity induced by in vivo adenovirus vector-mediated expression of CD40 ligand in tumor cells. *Hum Gene Ther 10:1375*.

61. Josien, R., B. R. Wong, H. L. Li, R. M. Steinman, and Y. Choi. 1999. TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J Immunol 162:2562*.

62. Josien, R., H. L. Li, E. Ingulli, S. Sarma, B. R. Wong, M. Vologodskaia, R. M. Steinman, and Y. Choi. 2000. TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J Exp Med 191:495*.

63. Wong, B. R., R. Josien, and Y. Choi. 1999. TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. *J Leukoc Biol 65:715*.

64. Kim, Y. J., G. Li, and H. E. Broxmeyer. 2002. 4-1BB Ligand Stimulation Enhances Myeloid Dendritic Cell Maturation from Human Umbilical Cord Blood CD34(+) Progenitor Cells. *J Hematother Stem Cell Res 11:895*.

65. Futagawa, T., H. Akiba, T. Kodama, K. Takeda, Y. Hosoda, H. Yagita, and K. Okumura. 2002. Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells. *Int Immunol 14:275*.

66. Wurtzen, P. A., M. H. Nissen, and M. H. Claesson. 2001. Maturation of dendritic cells by recombinant human CD40L-trimer leads to a homogeneous cell population with enhanced surface marker expression and increased cytokine production. *Scand J Immunol 53:579*.

67. Ouaaz, F., J. Arron, Y. Zheng, Y. Choi, and A. A. Beg. 2002. Dendritic cell development and survival require distinct NF-kappaB subunits. *Immunity 16:257*.

68. Wong, B. R., R. Josien, S. Y. Lee, B. Sauter, H. L. Li, R. M. Steinman, and Y. Choi. 1997. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med 186:2075*.

69. Paulie, S., A. Rosen, B. Ehlin-Henriksson, S. Braesch-Andersen, E. Jakobson, H. Koho, and P. Perlmann. 1989. The human B lymphocyte and carcinoma antigen, CDw40, is a phosphoprotein involved in growth signal transduction. *J Immunol 142:590*.

70. van Kooten, C., and J. Banchereau. 2000. CD40-CD40 ligand. *J Leukoc Biol 67:2*.

71. Yellin, M. J., S. Winikoff, S. M. Fortune, D. Baum, M. K. Crow, S. Lederman, and L. Chess. 1995. Ligation of CD40 on fibroblasts induces CD54 (ICAM-1) and CD106 (VCAM-1) up-regulation and IL-6 production and proliferation. *J Leukoc Biol 58:209*.

72. Karmann, K., C. C. Hughes, J. Schechner, W. C. Fanslow, and J. S. Pober. 1995. CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc Natl Acad Sci U S A 92:4342*.

73. Gormand, F., F. Briere, S. Peyrol, M. Raccurt, I. Durand, S. Ait-Yahia, S. Lebecque, J. Banchereau, and Y. Pacheco. 1999. CD40 expression by human bronchial epithelial cells. *Scand J Immunol 49:355*.

74. Armitage, R. J., C. R. Maliszewski, M. R. Alderson, K. H. Grabstein, M. K. Spriggs, and W. C. Fanslow. 1993. CD40L: a multi-functional ligand. *Semin Immunol 5:401*.

75. Alderson, M. R., R. J. Armitage, T. W. Tough, L. Strockbine, W. C. Fanslow, and M. K. Spriggs. 1993. CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. *J Exp Med 178:669*.

76. Mackey, M. F., J. R. Gunn, C. Maliszewsky, H. Kikutani, R. J. Noelle, and R. J. Barth, Jr. 1998. Dendritic cells require maturation via CD40 to generate protective antitumor immunity. *J Immunol 161:2094*.

77. Peguet-Navarro, J., C. Dalbiez-Gauthier, F. M. Rattis, C. Van Kooten, J. Banchereau, and D. Schmitt. 1995. Functional expression of CD40 antigen on human epidermal Langerhans cells. *J Immunol 155:4241*.

78. Banchereau, J., B. Dubois, J. Fayette, N. Burdin, F. Briere, P. Miossec, M. C. Rissoan, C. van Kooten, and C. Caux. 1995. Functional CD40 antigen on B cells, dendritic cells and fibroblasts. *Adv Exp Med Biol 378:79*.

79. Imaizumi, K., T. Kawabe, S. Ichiyama, H. Kikutani, H. Yagita, K. Shimokata, and Y. Hasegawa. 1999. Enhancement of tumoricidal activity of alveolar macrophages via CD40-CD40 ligand interaction. *Am J Physiol 277:L49*.

80. van Kooten, C., and J. Banchereau. 1997. Functional role of CD40 and its ligand. *Int Arch Allergy Immunol 113:393*.

81. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med 179:1109*.

82. Cella, M., F. Sallusto, and A. Lanzavecchia. 1997. Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol 9:10*.

83. Straw, A. D., A. S. MacDonald, E. Y. Denkers, and E. J. Pearce. 2003. CD154 plays a central role in regulating dendritic cell activation during infections that induce Th1 or Th2 responses. *J Immunol 170:727*.

84. Shurin, M. R., L. Lu, P. Kalinski, A. M. Stewart-Akers, and M. T. Lotze. 1999. Th1/Th2 balance in cancer, transplantation and pregnancy. *Springer Semin Immunopathol 21:339*.

85. Steinbrink, K., M. Wolfl, H. Jonuleit, J. Knop, and A. H. Enk. 1997. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol 159:4772*.

86. Gao, J. X., J. Madrenas, W. Zeng, M. J. Cameron, Z. Zhang, J. J. Wang, R. Zhong, and D. Grant. 1999. CD40-deficient dendritic cells producing interleukin-10, but not interleukin-12, induce T-cell hyporesponsiveness in vitro and prevent acute allograft rejection. *Immunology 98:159*.

87. van Kooten, C., and J. Banchereau. 1997. Functions of CD40 on B cells, dendritic cells and other cells. *Curr Opin Immunol 9:330*.

88. Mackey, M. F., J. R. Gunn, P. P. Ting, H. Kikutani, G. Dranoff, R. J. Noelle, and R. J. Barth, Jr. 1997. Protective immunity induced by tumor vaccines requires interaction between CD40 and its ligand, CD154. *Cancer Res 57:2569*.

89. Salih, H. R., S. G. Kosowski, V. F. Haluska, G. C. Starling, D. T. Loo, F. Lee, A. A. Aruffo, P. A. Trail, and P. A. Kiener. 2000. Constitutive expression of functional 4-1BB (CD137) ligand on carcinoma cells. *J Immunol 165:2903*.

90. Xiang, J. 1999. Expression of co-stimulatory 4-1BB ligand induces significant tumor regression and protective immunity. *Cancer Biother Radiopharm 14:353*.

91. Melero, I., J. V. Johnston, W. W. Shufford, R. S. Mittler, and L. Chen. 1998. NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. *Cell Immunol 190:167*.

92. Melero, I., N. Bach, K. E. Hellstrom, A. Aruffo, R. S. Mittler, and L. Chen. 1998. Amplification of tumor immunity by gene transfer of the co-stimulatory 4-1BB ligand: synergy with the CD28 co-stimulatory pathway. *Eur J Immunol 28:1116*.

93. Hamblin, T. J. 1996. From dendritic cells to tumour vaccines. *Lancet 347:705*.

94. Boon, T., P. Coulie, M. Marchand, P. Weynants, T. Wolfel, and V. Brichard. 1994. Genes coding for tumor rejection antigens: perspectives for specific immunotherapy. *Important Adv Oncol:53*.

95. Rosenthal, F. M., K. S. Zier, and B. Gansbacher. 1994. Human tumor vaccines and genetic engineering of tumors with cytokine and histocompatibility genes to enhance immunogenicity. *Curr Opin Oncol 6:611*.

96. Becker, Y. 1992. Anticancer role of dendritic cells (DC) in human and experimental cancers--a review. *Anticancer Res 12:511*.

97. Shurin, M. R. 1996. Dendritic cells presenting tumor antigen. *Cancer Immunol Immunother 43:158*.

98. Mayordomo, J. I., T. Zorina, W. J. Storkus, L. Zitvogel, M. D. Garcia-Prats, A. B. DeLeo, and M. T. Lotze. 1997. Bone marrow-derived dendritic cells serve as potent adjuvants for peptide-based antitumor vaccines. *Stem Cells 15:94*.

99. Ribas, A., L. H. Butterfield, and J. S. Economou. 2000. Genetic immunotherapy for cancer. *Oncologist 5:87*.

100. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245*.

101. Girolomoni, G., and P. Ricciardi-Castagnoli. 1997. Dendritic cells hold promise for immunotherapy. *Immunol Today 18:102*.

102. Lotze, M. T., M. Shurin, I. Davis, A. Amoscato, and W. J. Storkus. 1997. Dendritic cell based therapy of cancer. *Adv Exp Med Biol 417:551*.

103. Lotze, M. T. 1997. Getting to the source: dendritic cells as therapeutic reagents for the treatment of patients with cancer. *Ann Surg 226:1*.

104. Triozzi, P. L., R. Khurram, W. A. Aldrich, M. J. Walker, J. A. Kim, and S. Jaynes. 2000. Intratumoral injection of dendritic cells derived in vitro in patients with metastatic cancer. *Cancer 89:2646*.

105. Rosenberg, S. A. 2001. Progress in human tumour immunology and immunotherapy. *Nature 411:380*.

106. Banchereau, J., B. Schuler-Thurner, A. K. Palucka, and G. Schuler. 2001. Dendritic cells as vectors for therapy. *Cell 106:271*.

107. Bodey, B., B. Bodey, Jr., S. E. Siegel, and H. E. Kaiser. 2000. Failure of cancer vaccines: the significant limitations of this approach to immunotherapy. *Anticancer Res 20:2665*.

108. Thompson, T. 2001. Cancer vaccines: advances give hope for future therapy. *J Natl Cancer Inst 93:1044*.

109. Nielsen, M. B., and F. M. Marincola. 2000. Melanoma vaccines: the paradox of T cell activation without clinical response. *Cancer Chemother Pharmacol 46 Suppl:S62*.

110. Marincola, F. M., E. M. Jaffee, D. J. Hicklin, and S. Ferrone. 2000. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol 74:181*.

111. Matzinger, P. 1998. An innate sense of danger. *Semin Immunol 10:399*.

112. Candido, K. A., K. Shimizu, J. C. McLaughlin, R. Kunkel, J. A. Fuller, B. G. Redman, E. K. Thomas, B. J. Nickoloff, and J. J. Mule. 2001. Local administration of dendritic cells inhibits established breast tumor growth: implications for apoptosis-inducing agents. *Cancer Res 61:228*.

113. Pirtskhalaishvili, G., G. V. Shurin, A. Gambotto, C. Esche, M. Wahl, Z. R. Yurkovetsky, P. D. Robbins, and M. R. Shurin. 2000. Transduction of dendritic cells with Bcl-xL

increases their resistance to prostate cancer-induced apoptosis and antitumor effect in mice. *J Immunol 165:1956*.

114. Matsui, S., J. D. Ahlers, A. O. Vortmeyer, M. Terabe, T. Tsukui, D. P. Carbone, L. A. Liotta, and J. A. Berzofsky. 1999. A model for CD8+ CTL tumor immunosurveillance and regulation of tumor escape by CD4 T cells through an effect on quality of CTL. *J Immunol 163:184*.

115. Ribas, A., L. H. Butterfield, B. Hu, V. B. Dissette, A. Y. Chen, A. Koh, S. N. Amarnani, J. A. Glaspy, W. H. McBride, and J. S. Economou. 2000. Generation of T-cell immunity to a murine melanoma using MART-1-engineered dendritic cells. *J Immunother 23:59*.

116. Dhodapkar, M. V., R. M. Steinman, J. Krasovsky, C. Munz, and N. Bhardwaj. 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med 193:233*.

117. Couderc, B., L. Zitvogel, V. Douin-Echinard, L. Djennane, H. Tahara, G. Favre, M. T. Lotze, and P. D. Robbins. 1998. Enhancement of antitumor immunity by expression of CD70 (CD27 ligand) or CD154 (CD40 ligand) costimulatory molecules in tumor cells. *Cancer Gene Ther 5:163*.

118. Esche, C., A. Gambotto, Y. Satoh, V. Gerein, P. D. Robbins, S. C. Watkins, M. T. Lotze, and M. R. Shurin. 1999. CD154 inhibits tumor-induced apoptosis in dendritic cells and tumor growth. *Eur J Immunol 29:2148*.

119. Radoja, S., and A. B. Frey. 2000. Cancer-induced defective cytotoxic T lymphocyte effector function: another mechanism how antigenic tumors escape immune-mediated killing. *Mol Med 6:465*.

120. Kiessling, R., K. Wasserman, S. Horiguchi, K. Kono, J. Sjoberg, P. Pisa, and M. Petersson. 1999. Tumor-induced immune dysfunction. *Cancer Immunol Immunother 48:353*.

121. Hoffmann, T. K., G. Dworacki, T. Tsukihiro, N. Meidenbauer, W. Gooding, J. T. Johnson, and T. L. Whiteside. 2002. Spontaneous apoptosis of circulating T lymphocytes in patients with head and neck cancer and its clinical importance. *Clin Cancer Res 8:2553*.

122. Shurin, G. V., V. Gerein, M. T. Lotze, and E. M. Barksdale, Jr. 1998. Apoptosis induced in T cells by human neuroblastoma cells: role of Fas ligand. *Nat Immun 16:263*.

123. Pirtskhalaishvili, G., G. V. Shurin, C. Esche, Q. Cai, R. R. Salup, S. N. Bykovskaia, M. T. Lotze, and M. R. Shurin. 2000. Cytokine-mediated protection of human dendritic cells from prostate cancer-induced apoptosis is regulated by the Bcl-2 family of proteins. *Br J Cancer 83:506*.

124. Katsenelson, N. S., G. V. Shurin, S. N. Bykovskaia, J. Shogan, and M. R. Shurin. 2001. Human small cell lung carcinoma and carcinoid tumor regulate dendritic cell maturation and function. *Mod Pathol 14:40*.

125. Tsuge, T., M. Yamakawa, and M. Tsukamoto. 2000. Infiltrating dendritic/Langerhans cells in primary breast cancer. *Breast Cancer Res Treat 59:141*.

126. Takahashi, A., K. Kono, J. Itakura, H. Amemiya, R. Feng Tang, H. Iizuka, H. Fujii, and Y. Matsumoto. 2002. Correlation of vascular endothelial growth factor-C expression with tumor-infiltrating dendritic cells in gastric cancer. *Oncology 62:121*.

127. Yin, X., M. Lu, L. Liang, Y. Lai, J. Huang, and Z. Li. 2002. [Infiltration of dendritic cells and lymphocytes in hepatocellular carcinoma tissue]. *Zhonghua Wai Ke Za Zhi 40:336*.

128. Mackey, M. F., R. J. Barth, Jr., and R. J. Noelle. 1998. The role of CD40/CD154 interactions in the priming, differentiation, and effector function of helper and cytotoxic T cells. *J Leukoc Biol 63:418*.

129. Myers, J. N., A. Mank-Seymour, L. Zitvogel, W. Storkus, M. Clarke, C. S. Johnson, H. Tahara, and M. T. Lotze. 1998. Interleukin-12 gene therapy prevents establishment of SCC VII squamous cell carcinomas, inhibits tumor growth, and elicits long-term antitumor immunity in syngeneic C3H mice. *Laryngoscope 108:261*.

130. Lotze, M. T., L. Zitvogel, R. Campbell, P. D. Robbins, E. Elder, C. Haluszczak, D. Martin, T. L. Whiteside, W. J. Storkus, and H. Tahara. 1996. Cytokine gene therapy of cancer using interleukin-12: murine and clinical trials. *Ann N Y Acad Sci 795:440*.

131. Zitvogel, L., B. Couderc, J. I. Mayordomo, P. D. Robbins, M. T. Lotze, and W. J. Storkus. 1996. IL-12-engineered dendritic cells serve as effective tumor vaccine adjuvants in vivo. *Ann N Y Acad Sci 795:284*.

132. Zitvogel, L., J. I. Mayordomo, T. Tjandrawan, A. B. DeLeo, M. R. Clarke, M. T. Lotze, and W. J. Storkus. 1996. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med 183:87*.

133. Wan, Y., J. Bramson, A. Pilon, Q. Zhu, and J. Gauldie. 2000. Genetically modified dendritic cells prime autoreactive T cells through a pathway independent of CD40L and interleukin 12: implications for cancer vaccines. *Cancer Res 60:3247*.

134. Hsu, F. J., C. Benike, F. Fagnoni, T. M. Liles, D. Czerwinski, B. Taidi, E. G. Engleman, and R. Levy. 1996. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med 2:52*.

135. Nestle, F. O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg, and D. Schadendorf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med 4:328*.

136. Murphy, G. P., B. A. Tjoa, S. J. Simmons, J. Jarisch, V. A. Bowes, H. Ragde, M. Rogers, A. Elgamal, G. M. Kenny, O. E. Cobb, R. C. Ireton, M. J. Troychak, M. L. Salgaller, and A. L. Boynton. 1999. Infusion of dendritic cells pulsed with HLA-A2-specific prostate-specific membrane antigen peptides: a phase II prostate cancer vaccine trial involving patients with hormone-refractory metastatic disease. *Prostate 38:73*.

137. Kianmanesh, A., N. R. Hackett, J. M. Lee, T. Kikuchi, R. J. Korst, and R. G. Crystal. 2001. Intratumoral administration of low doses of an adenovirus vector encoding tumor necrosis factor alpha together with naive dendritic cells elicits significant suppression of tumor growth without toxicity. *Hum Gene Ther 12:2035*.

138. Shurin, M. R., and D. I. Gabrilovich. 2001. Regulation of Dendritic Cell System by Tumor. *Cancer Res Therapy and Control 11:65*.

139. Mach, N., S. Gillessen, S. B. Wilson, C. Sheehan, M. Mihm, and G. Dranoff. 2000. Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res 60:3239*.

140. Sharma, S., M. Stolina, L. Zhu, Y. Lin, R. Batra, M. Huang, R. Strieter, and S. M. Dubinett. 2001. Secondary lymphoid organ chemokine reduces pulmonary tumor burden in spontaneous murine bronchoalveolar cell carcinoma. *Cancer Res 61:6406*.

141. Kusumoto, M., S. Umeda, A. Ikubo, Y. Aoki, O. Tawfik, R. Oben, S. Williamson, W. Jewell, and T. Suzuki. 2001. Phase 1 clinical trial of irradiated autologous melanoma cells adenovirally transduced with human GM-CSF gene. *Cancer Immunol Immunother 50:373*.

142. Grewal, I. S., and R. A. Flavell. 1998. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol 16:111*.

143. Hirano, A., D. L. Longo, D. D. Taub, D. K. Ferris, L. S. Young, A. G. Eliopoulos, A. Agathanggelou, N. Cullen, J. Macartney, W. C. Fanslow, and W. J. Murphy. 1999. Inhibition of human breast carcinoma growth by a soluble recombinant human CD40 ligand. *Blood 93:2999*.

144. Gurunathan, S., K. R. Irvine, C. Y. Wu, J. I. Cohen, E. Thomas, C. Prussin, N. P. Restifo, and R. A. Seder. 1998. CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J Immunol 161:4563*.

145. Hardy, S., M. Kitamura, T. Harris-Stansil, Y. Dai, and M. L. Phipps. 1997. Construction of adenovirus vectors through Cre-lox recombination. *J Virol 71:1842*.

146. Gambotto, A., T. Tuting, D. L. McVey, I. Kovesdi, H. Tahara, M. T. Lotze, and P. D. Robbins. 1999. Induction of antitumor immunity by direct intratumoral injection of a recombinant adenovirus vector expressing interleukin-12. *Cancer Gene Ther 6:45*.

147. Satoh, Y., C. Esche, A. Gambotto, G. V. Shurin, Z. R. Yurkovetsky, P. D. Robbins, S. C. Watkins, S. Todo, R. B. Herberman, M. T. Lotze, and M. R. Shurin. 2002. Local administration of IL-12-transfected dendritic cells induces antitumor immune responses to colon adenocarcinoma in the liver in mice. *J Exp Ther Oncol 2:337*.

148. Ludewig, B., D. Graf, H. R. Gelderblom, Y. Becker, R. A. Kroczek, and G. Pauli. 1995. Spontaneous apoptosis of dendritic cells is efficiently inhibited by TRAP (CD40-ligand) and TNF-alpha, but strongly enhanced by interleukin-10. *Eur J Immunol 25:1943*.

149. Fang, W., K. A. Nath, M. F. Mackey, R. J. Noelle, D. L. Mueller, and T. W. Behrens. 1997. CD40 inhibits B cell apoptosis by upregulating bcl-xL expression and blocking oxidant accumulation. *Am J Physiol 272:C950*.

150. Ribas, A., L. H. Butterfield, S. N. Amarnani, V. B. Dissette, D. Kim, W. S. Meng, G. A. Miranda, H. J. Wang, W. H. McBride, J. A. Glaspy, and J. S. Economou. 2001. CD40 cross-linking bypasses the absolute requirement for CD4 T cells during immunization with melanoma antigen gene-modified dendritic cells. *Cancer Res 61:8787*.

151. Tourkova, I. L., Z. R. Yurkovetsky, M. R. Shurin, and G. V. Shurin. 2001. Mechanisms of dendritic cell-induced T cell proliferation in the primary MLR assay. *Immunol Lett 78:75*.

152. Sun, Y., D. Peng, J. Lecanda, V. Schmitz, M. Barajas, C. Qian, and J. Prieto. 2000. In vivo gene transfer of CD40 ligand into colon cancer cells induces local production of cytokines and chemokines, tumor eradication and protective antitumor immunity. *Gene Ther 7:1467*.

153. Martinet, O., V. Ermekova, J. Q. Qiao, B. Sauter, J. Mandeli, L. Chen, and S. H. Chen. 2000. Immunomodulatory gene therapy with interleukin 12 and 4-1BB ligand: long- term remission of liver metastases in a mouse model. *J Natl Cancer Inst 92:931*.

154. Yoshida, H., Y. Katayose, M. Unno, M. Suzuki, H. Kodama, S. Takemura, R. Asano, H. Hayashi, K. Yamamoto, S. Matsuno, and T. Kudo. 2003. A novel adenovirus expressing human 4-1BB ligand enhances antitumor immunity. *Cancer Immunol Immunother 52:97*.

155. Cremer, I., M. C. Dieu-Nosjean, S. Marechal, C. Dezutter-Dambuyant, S. Goddard, D. Adams, N. Winter, C. Menetrier-Caux, C. Sautes-Fridman, W. H. Fridman, and C. G. Mueller. 2002. Long-lived immature dendritic cells mediated by TRANCE-RANK interaction. *Blood 100:3646*.

156. Yurkovetsky, Z. R., G. V. Shurin, A. Gambotto, S. H. Kim, M. R. Shurin, and P. D. Robbins. Submitted. Intratumorlal administration of Ad-CD40L or dendritic cells transduced with Ad-CD40L induces antitumor immunity.

157. Lum, L., B. R. Wong, R. Josien, J. D. Becherer, H. Erdjument-Bromage, J. Schlondorff, P. Tempst, Y. Choi, and C. P. Blobel. 1999. Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a

TNF family member involved in osteoclastogenesis and dendritic cell survival. *J Biol Chem 274:13613*.

158. Kikuchi, T., N. Miyazawa, M. A. Moore, and R. G. Crystal. 2000. Tumor regression induced by intratumor administration of adenovirus vector expressing CD40 ligand and naive dendritic cells. *Cancer Res 60:6391*.

159. Kikuchi, T., S. Worgall, R. Singh, M. A. Moore, and R. G. Crystal. 2000. Dendritic cells genetically modified to express CD40 ligand and pulsed with antigen can initiate antigen-specific humoral immunity independent of CD4+ T cells. *Nat Med 6:1154*.

160. French, R. R., H. T. Chan, A. L. Tutt, and M. J. Glennie. 1999. CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. *Nat Med 5:548*.

161. Diehl, L., A. T. den Boer, S. P. Schoenberger, E. I. van der Voort, T. N. Schumacher, C. J. Melief, R. Offringa, and R. E. Toes. 1999. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat Med 5:774*.

162. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478*.

163. Friedlander, P. L., C. L. Delaune, J. M. Abadie, M. Toups, J. LaCour, L. Marrero, Q. Zhong, and J. K. Kolls. 2003. Efficacy of CD40 Ligand Gene Therapy in Malignant Mesothelioma. *Am J Respir Cell Mol Biol*.

164. Yanagi, K., Y. Nagayama, K. Nakao, A. Saeki, K. Matsumoto, T. Ichikawa, H. Ishikawa, K. Hamasaki, N. Ishii, and K. Eguchi. 2003. Immuno-gene therapy with adenoviruses expressing fms-like tyrosine kinase 3 ligand and CD40 ligand for mouse hepatoma cells in vivo. *Int J Oncol 22:345*.

165. Kikuchi, T., M. A. Moore, and R. G. Crystal. 2000. Dendritic cells modified to express CD40 ligand elicit therapeutic immunity against preexisting murine tumors. *Blood 96:91*.

166. Terheyden, P., P. Straten, E. B. Brocker, E. Kampgen, and J. C. Becker. 2000. CD40-ligated dendritic cells effectively expand melanoma-specific CD8+ CTLs and CD4+ IFN-gamma-producing T cells from tumor-infiltrating lymphocytes. *J Immunol 164:6633*.

167. Loskog, A., T. H. Totterman, A. Bohle, and S. Brandau. 2002. In vitro activation of cancer patient-derived dendritic cells by tumor cells genetically modified to express CD154. *Cancer Gene Ther 9:846*.