CANCER VACCINE-INDUCED TUMOR IMMUNITY AND AUTOIMMUNITY

INVESTIGATING MECHANISMS OF CANCER VACCINE-INDUCED TUMOR IMMUNITY AND AUTOIMMUNITY

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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
of the Degree
Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2011) McMaster University
(Medical Sciences) Hamilton, Ontario

TITLE:	Investigating Mechanisms of Cancer Vaccine-Induced Tumor Immunity and Autoimmunity
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NUMBER OF PAGES:	xx, 255

— ABSTRACT —

INTRODUCTION: Pre-clinical and clinical data strongly support the feasibility of employing immunotherapy as a strategy to treat cancer.

METHODS: Using the B16F10 murine melanoma model, we have been investigating mechanisms of T cell-mediated antitumor immunity following immunization with dopachrome tautomerase (DCT), a melanoma-associated antigen.

RESULTS: In **Chapter 2**, we uncovered an interesting dichotomy whereby DCT-specific $CD4^+$ T cell-mediated tumor protection and autoimmunity are dependent on IL-4/STAT-6 and IFN-γ/STAT-4, respectively. Our data also revealed that this phenomenon is extrinsic of $CD4^+$ T cell polarization.

To gain further insight into the targets recognized by $CD4^+$ T cells, we conducted in **Chapter 3** extensive $CD4^+$ T cell epitope mapping experiments using overlapping peptide libraries. Interestingly, while we were able to identify "helper" epitopes within DCT that were required for maximal $CD8^+$ T cell expansion, we were unable to identify "effector" epitopes responsible for tumor rejection. Further examination of the requirements for the generation of $CD4^+$ T cell effector epitopes showed that post-translational modifications of the protein were involved.

In **Chapter 4**, we investigated the modest efficacy afforded by DCT immunization in the context of established B16F10 melanomas. Using intratumoral transcriptional analysis, we demonstrated that the vaccine rapidly promoted an IFN-γ-dependent immunosuppressive state inside the tumor. Concurrent treatment with the immunomodulatory antibodies anti-4-1BB and anti-PD-1 effectively counteracted this tumor immunosuppression, resulting in complete regression of tumors and long-term survival in 70% of the mice.

CONCLUSIONS: The research described in this thesis sheds new light into the mechanisms by which vaccine-mediated $CD4^+$ T cell responses participate to tumor rejection and autoimmunity. Moreover, our findings indicate that cancer vaccine-induced tumor immunosuppression significantly limits tumor regression, emphasizing the requirement of combinatorial approaches for successful cancer immunotherapy. Overall, our research offers new insight for future vaccine development.

— ACKNOWLEDGEMENTS —

As I reflect on my journey through grad school, I fully realize that it would not have been possible without the help of so many. I would like to thank all of you who have, in one way or another, contributed to my achievements:

First and foremost, I would like to express my deepest thanks and love to my parents Marie and Michel. Mom, Dad, I will forever be grateful to you for having instilled in me the values and merits of hard work, perseverance and education. I would not have made it this far without your unconditional love and support; thank you for continuously helping me pursue my goals and dreams. To my sisters Nathalie and Julie, thank you for your endless encouragement over the last years. To my late brother Mike, thank you for watching over me from where you are now and also reminding me every day to put things in life in perspective.

During my time at Mac, I was very fortunate to be co-supervised by Dr. Yonghong Wan and Dr. Jonathan Bramson. I sincerely want to thank both of you for your mentorship and guidance throughout the years. Thank you for believing in me and always pushing me as I slowly matured as a scientist. Your immense knowledge of science and true passion for research have been a constant source of motivation and inspiration; I could not have hoped for better role models as I began my career in your labs. Thank you. I would also like to thank Dr. Ali Ashkar who, committee meeting after committee meeting, provided useful insight into my various research projects.

None of the work presented in this thesis would have been possible without the assistance of very talented lab techs. Special thanks to Carole Evelegh, Chuyan Ying and Robin Parsons as well as Jane Ann Smith, Xueya Feng and Carol Lavery; great research and relationships arise from collaborative work and you've all been essential assets of the Bramson team.

To the past and present members of the Wan lab, especially Dr. Jeanette Boudreau, Sheng Zhang, Lea Luketic, Dr. Jonathan Pol, Liang Zhang, Dr. Khalil Karimi, Dr. Byram Bridle and Jordan Delanghe, as well as to the past and present members of the Bramson lab, especially Mike Ventresca, Bob McGray, Korosh Kianizad, Dr. Dana Nyholt, Laura Marshall, Dr. Natalie Grinshtein, Dr. Jen Bassett, Heather VanSeggelen, Dr. Stephanie Swift, Sanduni Liyanage, Dr. John Finn, Oula Akawi, Dr. Galina Denisova, Dr. Jamie Miller, Alina Lelic, Kristi Jenkins, Lesley Latham, Joni Hammill and Florentina Teoderascu, thank you all for your scientific help, but more importantly for the countless chats, laughs, sushi outings and coffee breaks we have shared over the last 6 years. Amazing friendships have developed between some of you and myself and I really hope these will last a lifetime. My experience in grad school would not have been same without all of you. Thank you.

To my dearest Quebecer girlfriends, Caroline Henry and Stéphanie Poirier, a big and sincere thank you from the bottom of my heart. This friendship that we have shared since elementary school is truly unique; your listening ears, moral support and encouragement have always helped me get through life's adventures.

I hope that we will continue to find the time to get together despite the distance that separates us.

I would like to acknowledge the Canadian Institutes of Health Research (CIHR) and the Terry Fox Foundation for funding the research presented in this thesis. I would like to particularly thank CIHR for awarding me scholarships during both my masters and my PhD training.

Last, but certainly not least, Szymon- I could never thank you enough for your infinite support and immeasurable love. I cannot imagine what the last 5 years in grad school would have been without you standing by my side each step of the way; you've always taken the time to listen to me go on and on about $CD4^+$ T cells and chimeric mice and also always found ways to brighten-up my bad lab days… Thank you for being there for me no matter what, for you patience and for your understanding. I love you. I would also like to thank your parents Aleksandra and Tomasz, as well as you brother Andrzej and his wife Gosia, for making me feel a part of your loving family since we first met. Thank you.

> *"The adventure of life is to learn. The purpose of life is to grow. The nature of life is to change. The challenge of life is to overcome. The essence of life is to care. The opportunity of life is to serve. The secret of life is to dare. The spice of life is to befriend. The beauty of life is to give. The joy of life is to love."*
>
> ☙ William Arthur Ward, American author and poet

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APPENDIX 1

— LIST OF ABBREVIATIONS AND SYMBOLS —

α	alpha
β	beta
Δ	delta
γ	gamma
μ	micro
4E-BP1	4E-binding protein 1
7-AAD	7-amino-actinomycin D
Ab (s)	antibody (ies)
Ad	adenovirus
AIDS	acquired immune deficiency syndrome
Aire	autoimmune regulator
APC (s)	antigen-presenting cell (s) or allophycocyanin (fluorochrome)
ATG	autophagy-related gene
BFA	brefeldin A
BM	bone marrow
CIITA	class II transactivator
CAR	chimeric antigen receptor
CCR#	C-C chemokine receptor
CD#	cluster of differenciation
cDNA	complementary DNA
CLIP	class II-associated invariant chain peptide
CMV	cytomegalovirus
CRE	cre recombinase
CSF	colony stimulating factor
CTL (s)	cytotoxic T lymphocyte (s)
CTLA-4	cytotoxic T lymphocyte antigen-4
CXCL#	chemokine (CXC) ligand
Cy	cychrome
DC (s)	dendritic cell (s)

DCT	dopachrome tautomerase
DHICA	dihydroxyindole carboxylic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNFB	2,4-dinitrobenzenesulfonic acid
DOPA	dihydroxyphenylalanine
ECL	enhanced chemiluminescence
EGFP	enhanced green fluorescent protein
ELISPOT	enzyme-linked immunosorbent spot assay
ER	endoplasmic reticulum
$EndoH_f$	endonuclease
FACS	fluorescence-activated cell sorting
FasL	Fas ligand
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
Foxp3	forkhead box P3
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA-3	GATA sequence binding transcription factor 3
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
GITR	glucocorticoid-induced TNFR-related protein
GM-CSF	granulocyte/monocyte-colony stimulating factor
gp75	glycoprotein 75 kDa
gp90	glycoprotein 90 kDa
gp100	glycoprotein 100 kDa
hDCT	human DCT
hgp100	human gp100
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
ICS	intracellular cytokine staining
i.d.	intradermal
IFN (s)	interferon (s)
Ii	invariant chain

IL	interleukin
i.m.	intramuscular
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
iTreg (s)	induced regulatory T cell (s)
kb	kilobase
kDa	kilodalton
LAG-3	lymphocyte activation gene-3
LC3	light chain 3
LCMV	lymphocytic choriomeningitis virus
LFA	lymphocyte function-associated antigen 1
mAb (s)	monoclonal antibody (ies)
M-CSF	monocyte colony stimulating factor
mDCT	murine DCT
MDSCs	myeloid-derived suppressor cells
MEM-F11	minimal essential medium F11
MFI	mean fluorescence intensity
mgp100	murine gp100
MHC	major histocompatibility complex
MHC I	major histocompatibility complex I
MHC II	major histocompatibility complex II
MOI	multiplicity of infection
mTOR	mammalian target of rapamycin
MTS	melanosomal transport signal
MyD88	myeloid differentiation primary response protein 88
nTreg (s)	natural regulatory T cell (s)
NK cell	natural killer cell
p70S6K	p70 ribosomal protein S6 kinase
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBL (s)	peripheral blood lymphocyte (s)
PBS	phoshate buffered saline
PCR	polymerase chain reaction

PD-1	programmed death 1
pDC (s)	plasmacytoid dendritic cell (s)
PD-L1	programmed death ligand 1
PD-L2	programmed death ligand 2
PE	phycoerythrin
Pfu	plaque-forming unit
PGE2	prostaglandin-E2
PMSF	phenylmethylsulfonyl fluoride
PNGaseF	N-glycosidase F
RAG2	recombination activating gene 2
rHuAd5	recombinant human adenovirus serotype 5
RIPA	acronym derived from the original application of this lysis buffer: the radioimmunoprecipitation assay
RNA	ribonucleic acid
RORγt	retinoic acid receptor related orphan receptor γt
RPMI	Roswell Park Memorial Institute
s.c.	subcutaneous
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SS	endoplasmic reticulum signal sequence
STAT	signal transducer and activator of transcription
SV40	simian virus 40
TAA (s)	tumor-associated antigen (s)
TAM (s)	tumor-associated macrophage (s)
TAP	transporter associated with antigen processing
Tapasin	TAP-associated protein
T-bet	T-box expressed in T cells
TCR	T cell receptor
T_H	T helper
T_{FH}	T follicular helper
TIL (s)	tumor-infiltrating lymphocyte (s)
TLR	toll-like receptor
TM	transmembrane domain

TNF	tumor necrosis factor
TNFR	TNF receptor
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TRAIL receptor
TRP-1	tyrosinase-related protein 1
TRP-2	tyrosinase-related protein 2
Treg (s)	regulatory T cell (s)
TSA (s)	tumor-specific antigen (s)
VEGF	vascular endothelial growth factor
VSV	vesicular stomatitis virus
WT	wild type

— CHAPTER 1 —

Introduction

1.0 Cancer

According to the World Health Organization, cancer accounts for 13% of all deaths worldwide, a figure that is expected to rise significantly over the next decades [1]. Successful treatment of cancer will ultimately rely on an in-depth understanding and appreciation of its origins and promoting factors. In this first section, following an overview of cancer, I will discuss briefly conventional cancer treatments and the concept of cancer immunotherapy.

1.1 Cancer is a heterogeneous disease

Cancer can be inherited, however, it is most often caused by extrinsic environmental factors such as pollutants, infections, lifestyle, dietary choices and tobacco use [2]. Epidemiological and histological studies have strongly suggested that tumor progression occurs over decades, following the sequential development of normal cells into hyperplastic, dysplastic, neoplastic and, finally, metastatic cells [2]. Cancer is believed to develop from a stepwise accumulation of genetic alterations which result in the inactivation of genes that suppress tumor development (tumor suppressor genes), deregulation of epigenetic events and activation of genes that promote tumor development (oncogenes) [2]. Such genetic and epigenetic aberrations impose over time significant stress on cancer cells, further promoting the accumulation of mutations [3]. Sequencing of human cancer genomes of numerous etiologies has revealed substantial genomic instability; indeed, thousands of mutations may accumulate in a given malignancy, with

considerably diverse mutation rates and patterns observed among individual cancers [4]. While most of the mutations are not thought to be tumorigenic, the whole genome analysis did find a significant number of potential "driver" mutations which could be involved in tumor formation [4]. Such findings have important implications for the treatment of cancer as they clearly illustrate the high degree of heterogeneity not only among cancer types, but also among cancer cells within a given tumor. Collectively, these (epi)genetic aberrations endow cancer cells with 8 characteristic hallmarks [5] that promote tumor formation: 1) sustained proliferative ability, 2) inhibition of growth suppression, 3) resistance to cell death, 4) replicative immortality, 5) pro-angiogenic ability, 6) invasiveness and metastatic potential, 7) reprogrammed energy metabolism and 8) evasion of immune destruction. It is now acknowledged in the field that fighting tumors, especially as they spread and become metastatic, will necessitate a multifaceted anticancer strategy.

1.2 Conventional cancer treatments

Classical cancer treatment modalities include surgery, chemotherapy and radiotherapy. Surgical resection remains the treatment of choice for solid tumors and is often accompanied by chemotherapy or localized/regional radiation therapy in order to prevent recurrence [6]. The majority of chemotherapeutic drugs act by impairing mitosis of rapidly dividing cells, a characteristic feature of most cancer cells [7]. Similarly, ionizing radiation causes DNA damage in the target cells,

consequently promoting loss of proliferation ability and apoptosis [8]. Given the non-specific cytotoxic nature of chemotherapy and radiation therapy, substantial concomitant side effects are frequently observed, notably in the bone marrow and mucosal tissues where rapidly dividing cells contribute to tissue homeostasis [6]. Further, tumors often develop resistance to chemotherapy [9] and radiation [10], which limits the effectiveness of conventional anticancer therapies against more advanced disease. Clearly, additional complementary therapies are required.

1.3 Cancer immunotherapy

The idea that host defenses can recognize and fight cancer cells has existed for hundreds of years and stemmed from the observation that spontaneous tumor regression would often coincide with acute infections [11]. The first active cancer immunotherapy, known as Coley's toxin, was tested by William Coley in the late 1800s and consisted of heat-killed *Streptococcus pyogenes* and *Serratia marcescens*. It was used to treat many cancers including sarcomas, carcinomas, melanomas, lymphomas and myelomas, with some success [12]. Significant progress has since been made towards the development of cancer immunotherapeutic strategies aimed at harnessing the immune system to target cancer malignancies. Indeed, increased overall survival has been reported in recent phase III clinical trials of cancer immunotherapy [13-16], illustrating the potential of these novel approaches in the clinic.

2.0 Cancer and the immune system

While tumor cell genetics and biology have been an important focus of cancer research over the last decades, our continually expanding knowledge of tumor and vaccine immunology has fuelled much interest in the promising application of cancer immunotherapies. In this section, I will review the basic aspects of immune responses.

2.1 Overview of innate and adaptive immunity

The human immune system encompasses an intricate network of cells, tissues and organs that protect us against a multitude of insults such as microbial infections, autoimmunity and cancer. Central to the immune system's complexity is its ability to distinguish "self" from "non-self" and "safe" from "dangerous". Numerous protective mechanisms are employed to tailor immune responses while concurrently avoiding damage to healthy surrounding tissues.

The first line of defense, known as innate immunity, consists of physical barriers (e.g. skin, mucosal membranes), biochemical barriers (e.g. enzymes, secretions) as well as immunological barriers (e.g. phagocytic cells, dendritic cells (DCs) natural killer (NK) cells, cytokines/chemokines, complement cascade) [17]. Innate immune cells share germline-encoded surface and cytoplasmic receptors that allow them to recognize pathogen-associated molecular patterns (PAMPs) found on a variety of microbes [18]. Consequently, innate immune cells can rapidly respond to infection.

Adaptive immune responses are elicited concurrently to innate immune responses. Unlike innate immunity however, adaptive immunity relies on the prior proliferation of a small number of cells whose receptors are highly specific for target pathogens; temporally, adaptive immunity manifests itself after innate immune responses [17]. Immune cells of the adaptive system, namely B and T lymphocytes, each express unique receptors: B-cell receptors (BCRs) and T-cell receptors (TCRs). Somatic rearrangements of germline gene elements underline the exquisite specificity of individual BCRs and TCRs [19], permitting B and T cells to recognize virtually any pathogens. After their development in the bone marrow and thymus, naïve B and T cells traffic to secondary lymphoid organs, including the spleen and lymph nodes. Located at the host-pathogen interface, antigen-presenting cells (APCs) play a key role in the induction of adaptive immune responses. They do so by sampling antigens in their environment, processing them and subsequently presenting them to B and T cells in secondary lymphoid organs [20]. Adaptive immunity can be distinguished as humoral (present in the serum and bodily fluids) and cellular immunity. Humoral immunity is mediated by B cell-secreted antibodies (Abs) which, by means of neutralization, opsonization or complement activation, target antigens confined to extracellular spaces and mucosal membranes [21,22]. Contrary to humoral immunity, the role of cellular immunity is to identify and eliminate abnormal cells or cells that have been invaded by intracellular pathogens. This type of adaptive immunity requires the action of $CD4^+$ and $CD8^+$ T cell subsets [23]. T cell immunity relies on the

ability of APCs to present antigens in the form of peptides displayed onto major histocompatibility complex (MHC) molecules, which are termed "peptide-MHC complexes". TCR recognition of peptide-MHC complexes subsequently triggers the activation of T cells, which can then recognize non-APCs harboring the same peptide-MHC complexes. In addition to offering a broad repertoire of antigen specificity, an important hallmark of adaptive immunity pertains to its ability to generate "immunological memory". Following encounter with a pathogen, B and T cells first become activated and expand greatly in number to clear the infection; as this initial response eventually wanes, a small fraction of these cells becomes long-lived and maintains pathogen specificity. These memory cells can then rapidly mount robust immune responses upon re-encounter of the same pathogen [24,25].

2.2 $CD8^+$ T cell immunity

$CD8^+$ T cells, also called "cytotoxic T lymphocytes (CTLs)", play a fundamental role during adaptive responses as they actively take part in the clearance of intracellular pathogens and transformed cells [23]. Differentiation of naïve $CD8^+$ T cells into effector cells first requires antigenic recognition in an inflammatory setting. $CD8^+$ T cell proliferation, survival and effector functions depend on cellular signaling mediated by cytokines like as IL-12 [26] and type I IFNs [27] as well as ligation of the immune stimulatory receptor 4-1BB [28]. Two models have been proposed to explain the proliferation and differentiation of $CD8^+$ T cells. The

progressive differentiation model suggests that repeated random encounters with antigen, from which the various levels of antigenic signal accumulated dictate long-term T cell fate, are required throughout the expansion phase [29]. The alternative model, T cell programming, stipulates that one antigenic encounter is sufficient to instruct naïve $CD8^+$ T cell precursors to proliferate and that the context during which it happens (e.g. ligation of immune stimulatory receptors, cytokine milieu) determines long-term T cell fate [30]. The latter model appears to be the most probable one; it is supported by several studies demonstrating that only 24 hours of antigen stimulation is adequate to impart $CD8^+$ T cells with the ability to expand, develop effector functions and differentiate into memory cells [31-34]. Activated $CD8^+$ T cells kill their targets primarily by means of contact-mediated cytotoxicity. Following establishment of a secretory immunological synapse between the $CD8^+$ T cell and its target cell [35], contact-mediated cytotoxicity proceeds mainly via the migration and release of pre-formed lytic granules that contain death-inducing molecules such as perforin and granzymes [36,37]. Perforin functions by creating pores in the target cell membrane [38] allowing granzymes to enter the cell and activate non-overlapping caspase-mediated apoptotic pathways [39]. A secondary contact-dependent effector mechanism that also results in apoptosis is mediated via ligation of FasL by CTLs with its death receptor Fas, expressed on target cells [36,37]. Lastly, the effector cytokines secreted by CTLs, IFN-γ and TNF-α, can kill target cells through multiple indirect effects. These include increased MHC expression (renders infected cells more visible),

activation of innate cells such as macrophages and NK cells, inhibition of viral transcription and/or replication as well as death receptor (TNFR) mediated apoptosis [40,41].

2.3 $CD8^+$ T cells in cancer immunity

Numerous pre-clinical and clinical studies conducted during the 1980s initially demonstrated that autologous $CD8^+$ tumor infiltrating lymphocytes (TILs) expanded *ex-vivo* in the presence of IL-2 could result in some degree of cancer regression following adoptive transfer therapy [42]. Moreover, multiple studies in murine models have highlighted an important role for antitumor CTL responses in tumor rejection [43-45]. Several reports have shown that the perforin/granzyme pathway is the predominant effector pathway used by tumor-specific CTLs [46-48]. FasL-mediated apoptosis has also been observed as a tumor rejection mechanism in perforin-deficient mice [49]. Additionally, *in-vitro* work suggests that IFN-γ may be important for sensitizing tumor cells to death receptor-mediated apoptosis, an effect that results from increased expression of caspases [50] and Fas [51] in tumor cells upon IFN-γ treatment. These latter findings indicate that the direct killing ability of CTLs is likely facilitated by the collaborative action of IFN-γ.

2.4 $CD4^+$ T cell immunity

$CD4^+$ T cells play numerous important roles in adaptive immunity; this is illustrated by the existence of various subsets, which are characterized by distinct

differentiation pathways, cytokine profiles and functions. Two $CD4^+$ T cell subsets, T_H1 and T_H2, were originally identified in a seminal paper by Mosmann *et al* in 1986 [52]. Since then, other subsets have been characterized including regulatory T cells (Tregs) [53,54], T follicular helper cells (T_{FH}) [55], T_H9 [56,57] and T_H17 [58,59]. Figure 1 summarizes the diverse differentiation pathways and subset attributes. Whether these various $CD4^+$ T cell subsets should be viewed as independent lineages or "plastic/uncommitted" cells is matter of current debate. Although a description of the mechanisms possibly involved in such plasticity falls outside of the scope of this introduction, a summary of advances in the field is provided in recent review articles [60,61]. In light of the work presented in this thesis, I will only describe briefly the T_H1, T_H2, T_H17 and Treg $CD4^+$ T cell subsets.

T_H1 $CD4^+$ T cells Upon TCR triggering in an inflammatory setting, naïve $CD4^+$ T cells differentiate into T_H1 cells in the presence of IL-12 [62], IL-18 [63,64], type I IFNs [65] or IFN-γ [66], which are produced by DCs, monocytes, macrophages and NK cells in response to infections [67,68]. Differentiation is initiated by signaling through signal transducer and activator of transcription (STAT)-4 (IL-12, type I IFNs) or STAT-1 (IFN-γ). Activation of STAT-1 results in the induction of the transcription factor T-bet [66,69,70], which induces the expression of IFN-γ and upregulation of IL-12 receptor β2, forming a positive autoregulatory

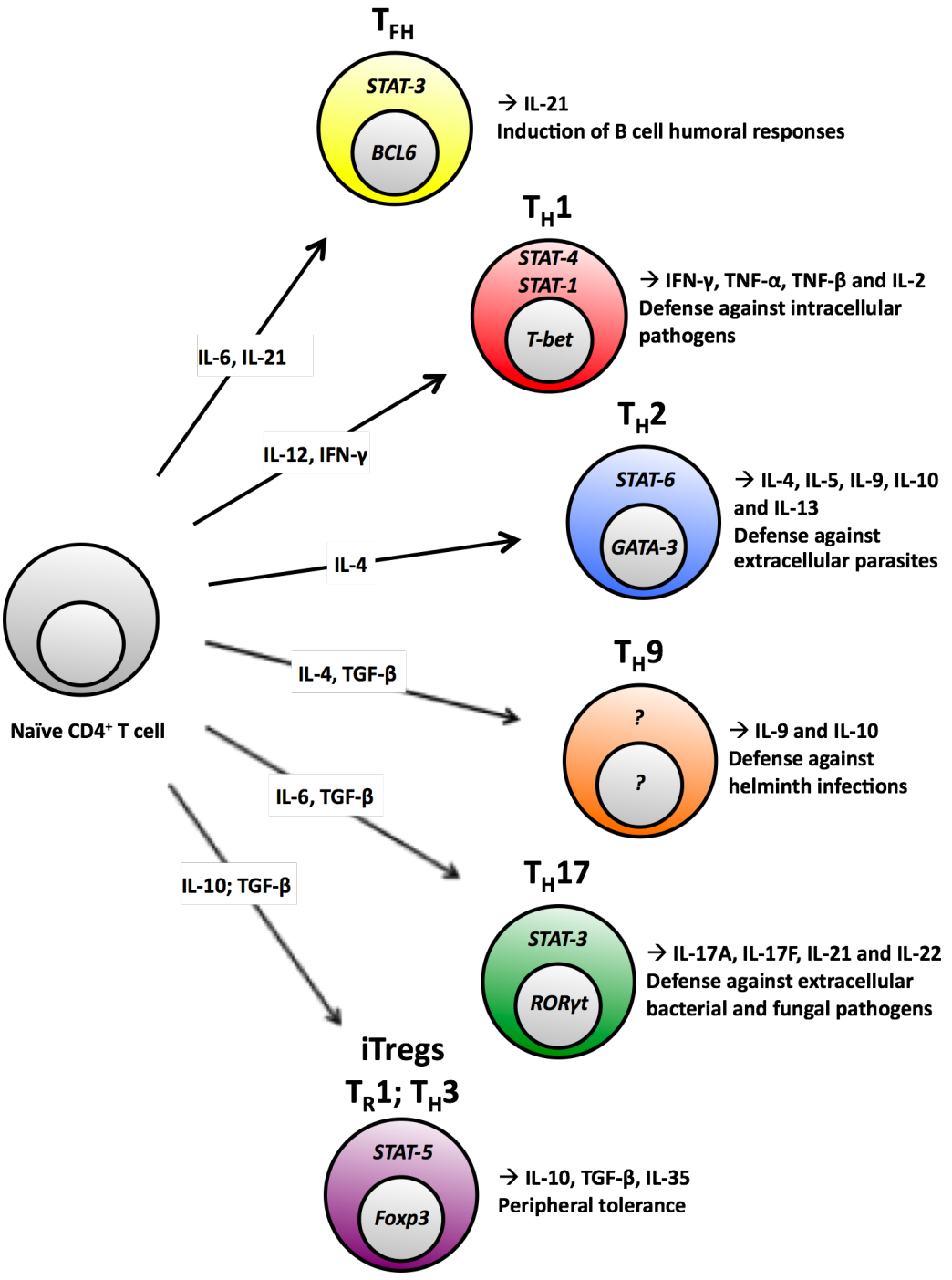

Figure 1. Differentiation of CD4$^+$ T cell subsets. The type of antigen and surrounding cytokine milieu dictate the differentiation of naïve CD4$^+$ T cells into effector CD4$^+$ T cells. Intracellular signalization and expression of subset-specific transcription factors further specify lineage commitment. (Figure adapted from O'Shea and Paul [60] and Zhu [71]).

loop that further amplifies T_H1 differentiation [72,73]. T_H1 cells are characterized by their production of proinflammatory cytokines such as IFN-γ, TNF-α and TNF-β as well as the T cell growth factor IL-2, all of which are important stimulators of innate and T cell responses [74]. T_H1 responses and IFN-γ are especially required for protection against a variety of intracellular bacteria [75-77], fungi [78] and viruses [79-81]; protective mechanisms include activation of macrophages and increased cytotoxic activity of $CD8^+$ T cells and NK cells [82]. Overt T_H1 immune responses have been implicated in several unwanted inflammatory reactions against self, including inflammatory bowel disease [83], graft-vs-host disease [84], type 1 diabetes [85] and rheumatoid arthritis [86].

T_H2 $CD4^+$ T cells IL-4 is considered to be the main cytokine driving the differentiation of naïve $CD4^+$ T cells into T_H2 cells [87,88]. It remains unclear what cells provide the initial source of IL-4 [89]. Signaling via the IL-4 receptor leads to the activation of STAT-6, an important signal transducer of T_H2 differentiation [90,91]. Activated STAT-6 translocates into the nucleus where it induces the expression of the transcription factor GATA-3 [92], referred to as the master regulator of T_H2 differentiation. STAT-6 also induces the expression of IL-4Rα [93] and c-maf [94], which reinforce T_H2 commitment via autocrine action of IL-4. Recent reports have also shown STAT-6-independent pathways of GATA-3 induction and T_H2 differentiation, which require either Notch [95] or WNT [96] signaling. GATA-3 functions by binding to the IL-4 locus where it induces the

production of IL-4, IL-5 and IL-13 [92]. The cytokines produced by T_H2 cells include IL-4, IL-5, IL-9, IL-10 and IL-13; they are mainly involved in B cell Ig class-switching to IgG1 and IgE as well as eosinophil recruitment [74]. As such, T_H2 responses are particularly important for the clearance of extracellular pathogens, including helminthes [97] and nematodes [98]; protection relies on humoral immunity and activation of eosinophils [82]. Unlike T_H1 responses, uncontrolled T_H2 responses have predominantly been associated with allergies [99] and atopic asthma [100].

T_H17 CD4$^+$ T cells T_H17 differentiation is initiated upon TCR stimulation and requires the combined action of TGF-β [101] and IL-6 [102]. While IL-23 was also thought to be an instigator of T_H17 differentiation [103], it has since been attributed a role in subsequent expansion of T_H17 cells given that expression of its receptor on CD4$^+$ T cells is induced by TGF-β [101]. IL-6 signaling is mediated by STAT-3 [104] and results in the expression of the transcription factor RORγt, which ensures T_H17 lineage commitment [105]. The cytokine profile of T_H17 CD4$^+$ T cells is primarily characterized by secretion of IL-17A (IL-17) and IL-17F [106] as well as IL-21 [107] and IL-22 [108]. Besides IL-21, which acts in an autocrine manner to sustain T_H17 differentiation [107], these cytokines target various cell types including endothelial and epithelial cells, fibroblasts, monocytes and macrophages [109], leading to the induction of antimicrobial proteins, inflammation (via IL-6 and CXCL8), neutrophil recruitment and stimulation of granulopoiesis (via G-CSF

and GM-CSF) [109]. T_H17 responses have been implicated in protection against numerous extracellular bacterial and fungal pathogens at mucosal surfaces [110]. Lastly, T_H17 cells are also regarded as key culprits of autoimmune pathologies. Indeed, using mouse models of multiple sclerosis [111], rheumatoid arthritis [112] and inflammatory bowel disease [113], it was demonstrated that uncontrolled T_H17 responses play a pivotal role in the induction and propagation of autoimmune reactions, a role which was previously attributed to T_H1 cells. These findings have now been extended to numerous human autoimmune diseases [114].

$CD4^+$ T regulatory cells (Tregs) Unlike the previously discussed $CD4^+$ T cell subsets, Tregs actually oppose immune activation through a variety of mechanisms to suppress the function of both conventional T cells and APCs, including: 1) the release of suppressive cytokines [115], 2) cytolysis [116,117], 3) metabolic disruption [118-120] and 4) interference with APC functions [121,122]. Tregs fall into 2 categories: natural Tregs (nTregs) and induced Tregs (iTregs (T_R1 and T_H3)) [123]. The surface phenotypic markers common to both nTregs and iTregs resemble those of an activated T cell: CD25, CTLA-4, GITR and $CD45RB^{low}$ [123]. Expression of the transcription factor Foxp3 further characterizes Tregs [124,125], with the exception of the Tr1 subset [126]. Natural Tregs develop in the thymus [127]. The importance of nTregs was clearly demonstrated during the mid-1990s in pre-clinical experiments where depletion of the $CD25^+$ cells resulted in lethal autoimmune diseases [53,54]. Contrary to nTregs, iTregs arise in the periphery from

conventional naïve $CD4^+$ T cells and include the T_R1 and T_H3 subsets, which are induced by IL-10 [126] and TGF-β [128,129], respectively.

2.5 $CD4^+$ T cells in cancer immunity

The role of antitumor $CD4^+$ T cell responses, aside from supporting optimal cancer-specific CTL expansion and effector functions [130-132], appears to be multifaceted. Indeed, using pre-clinical models, 2 groups have demonstrated a critical requirement for tumor-specifc $CD4^+$ T cells in rendering the tumor milieu amenable to recruitment of antitumor $CD8^+$ T cells [133,134]. Importantly, tumor-specific $CD4^+$ T cells can also actively take part in tumor rejection. For instance, early work investigating adoptive transfer therapy in a murine leukemia model showed that $CD4^+$ T cells conferred tumor immunity to tumor-bearing hosts [135,136]. Using *in-vivo* depletion of $CD4^+$ T cells or MHC I-negative tumor cells, an important role for $CD4^+$ T cell-mediated tumor protection was reported in 2 pre-clinical studies in which irradiated melanoma cells engineered to express granulocyte-macrophage colony stimulating factor (GM-CSF) were used as a vaccination platform [137,138]. These latter results illustrate the fact that $CD4^+$ T cells can mediate tumor protection independently of $CD8^+$ T cells. A recent report by Perez-Diez *et al* demonstrated that antitumor $CD4^+$ T cells were even more efficient than $CD8^+$ T cells, on a "per-T-cell" basis, at killing tumor cells [139]. Several mechanisms of $CD4^+$ T cell-mediated tumor destruction have been elucidated. For instance, these cells can directly kill tumor cells as initially

reported by Topalian *et al* in the case of a human melanoma expressing MHC II [140]. Although it is generally believed that tumor cells from solid tumors do not express MHC II, it appears that a proportion of cells within most tumor types do harbor MHC II at their surface [141]; however, the percentage of MHC II-positive tumor cells varies within the tumor and among individuals [142]. Alternatively, $CD4^+$ T cells can also mediate tumor protection independently of MHC II expression by tumor cells. It is thought that tumor antigens released at the tumor site are indirectly presented by APCs to tumor-specific $CD4^+$ T cells which then kill tumor cells via secretion of cytokines or recruitment of non-specific innate cells. Indeed, T_H1-derived IFN-γ has been shown to exert anti-angiogenic effects [143], mediate direct tumor cytotoxicity in the presence of TNF-α [144] or upregulate the expression of MHC molecules [145], therefore increasing the recognition and elimination of tumor cells. Furthermore, IFN-γ can activate macrophages from which the production of nitric oxide and super-oxide plays a role in tumor killing [146,147]. As for T_H2 cells, they can promote tumor destruction via IL-4-mediated recruitment and activation of eosinophils, which act as downstream effectors by releasing cytotoxic granules [146,148,149]. As is the case for IFN-γ, IL-4 can also inhibit tumor growth through its anti-angiogenic properties [150,151]. The role of T_H17 cells in mediating tumor clearance remains to be fully investigated. Indeed, while a number of studies have demonstrated a role for IL-17 in enhancing CTL effector function and DC maturation, others have reported that IL-17 harbors pro-angiogenic properties and therefore increases tumorigenicity [152].

The regulatory role of Tregs in cancer immunity is also well recognized. Tregs, originally termed "T suppressors", were first observed to suppress antitumor immunity in a pre-clinical study conducted in 1980 [153]. The importance of their suppressive impact on cancer immunity was clearly illustrated in pre-clinical tumor studies in which Tregs were depleted using anti-CD25 antibodies [154,155]. The relative contribution of nTregs versus iTregs to suppression of antitumor immunity has yet to be defined [156], but it does appear that the mechanisms employed by the 2 cell types differ. Indeed, T_H3 and T_R1 iTreg subsets are thought to primarily promote contact-independent suppression of conventional T cells and APCs via their abundant secretion of IL-10 and TGF-β while nTregs are believed to mediate their suppressive activity via contact-dependent mechanisms [156]. Overall, Tregs are regarded as an important immune inhibitory cell population in antitumor immunity.

2.6 Tumor antigens

Antitumor T cells target and destroy tumor tissue due to their ability to recognize tumor antigens. Consequently, cancer immunotherapeutic strategies, such as cancer-specific vaccination, rely on the identification of tumor antigens [157]. Following the discovery of the first tumor antigen in 1991, MAGE-1 [158], significant technological advances such as *in-silico* epitope prediction, chromatography/mass spectrometry and gene expression profiling have facilitated the identification and epitope characterization of over 100 antigens recognized by

antitumor T cells [159]. Tumor antigens, as illustrated in Table 1 below, are broadly classified into tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs) [157,160-162].

Table 1. Classification of tumor antigens

Antigen class	Antigen subclass	Description	Examples*
Tumor-specific antigens (TSAs)	Viral	Derived from oncogenic viruses	EBNA-1, HPV-16 E6/E7
	Unique	Point-mutation / aberrant splicing	CDK4, K-ras, p53
Tumor-associated antigens (TAAs)	Cancer testis	Transcriptionally activated genes otherwise silent in adult tissues	BAGE, GAGE, MAGE, NY-ESO-1
	Differentiation	Shared between tumors and the tissues they are originate from	DCT, gp100, tyrosinase, TRP-1, CEA, PSA
	Overexpressed	Overexpressed compared to normal tissues, found in different tumors	cyclin D1, MUC1, p53, survivin, telomerase

* See review articles [157,160-162] for more comprehensive listings of tumor antigens.

TSAs are sub-divided among the following 2 types: 1) antigens derived from tumor-causing viruses and 2) unique antigens that arise from genetic mutations or aberrant RNA splicing within the tumor cell. However, there are few "common" TSAs. Since most TSAs will be unique to individual tumors, deploying TSAs as the basis for cancer immunotherapy would require sequencing of individual tumors, which remains a daunting task. Most tumor antigens identified to date and targeted in the clinic are TAAs belonging to one of the following categories: 1) cancer testis antigens, which are the product of reactivated genes that are normally silent in adult tissues, except testis, 2) differentiation antigens, which are

shared between normal and cancerous tissues and 3) overexpressed antigens, which are ubiquitously expressed, but to a higher extent in tumors.

2.7 Antigen presenting cells (APCs)

Three cell types are viewed as "professional" APCs based on their constitutive expression of MHC II: macrophages, B cells and dendritic cells (DCs) [163]. DCs, however, given their prime location within the T cell-rich areas of the LNs [164], are considered as the most efficient APCs. DCs originate from bone marrow progenitors and constitute a heterogeneous population which includes many subsets that can be distinguished based on their tissue distribution, phenotype, ability to process and present antigens as well as cytokine profile [165]. They are broadly classified into plasmacytoid and myeloid DCs, with the latter ones including langerhans cells and interstitial DCs found in the skin and tissues, respectively [165]. The "immature" state of DCs, which prevails in most tissues, is largely defined by poor antigen presentation ability and lack of costimulatory signals for T cell activation. In contrast to immature DCs, "mature" DCs are characterized by an increased ability to present antigens and upregulation of costimulatory molecules required for T cell activation. Immature DCs, consistent with their role as sentinels, are continuously sampling antigens in their environment via macropinocytosis (ingestion of large quantities of fluids) [166] and receptor-mediated phagocytosis [167] for presentation to B and T cells in secondary lymphoid organs. DC migration relies on the expression of different chemokine

receptors. For instance, DCs are recruited to inflamed tissues in response to chemokines such as CCL2 (via CCR2) as well as CCL3, CCL4 and CCL5 (via CCR1, CCR3 and CCR5) [168,169]. DC maturation requires proinflammatory cytokines (ex. IL-1 and TNF-α) and recognition of PAMPs or danger signals (tissue injury) [170-172]; it is characterized by: 1) enhanced antigen presentation via upregulation of MHC I and II [173,174], 2) expression of the lymphoid tissue homing receptor CCR7 [175], 3) expression of costimulatory molecules (CD80/CD86) and 4) cytokine production (TNF-α, IL-6, and IL-12) [176].

In the steady state, i.e. in the absence of infection/inflammation and cytokines, acquisition of antigen by immature DCs and recognition by T cells induces T cell anergy (functional inactivation), deletion (removal by apoptosis) or, alternatively, promotes a "tolerance" state [177]. Such DCs are referred to as "tolerogenic DCs". Pre-clinical and clinical studies conducted during the late 1990s showed that DCs found in cancer lesions and draining lymph nodes of tumor-bearing animals and cancer patients displayed a decreased ability to present antigens [178,179]. It is now recognized that the immunosuppressive environment found within tumors significantly impairs the ability of DCs to elicit T cell-mediated antitumor immunity due to maintenance of an immature DC state [180]. "Tumor-conditioned" DCs therefore also represent a form of tolerogenic DCs.

2.8 Antigen processing and presentation

Antigens are presented to $CD8^+$ and $CD4^+$ T cells in the context of MHC I and MHC II, respectively (FIG 2). MHC I is expressed on all nucleated cells while MHC II is constitutively expressed by professional APCs [181].

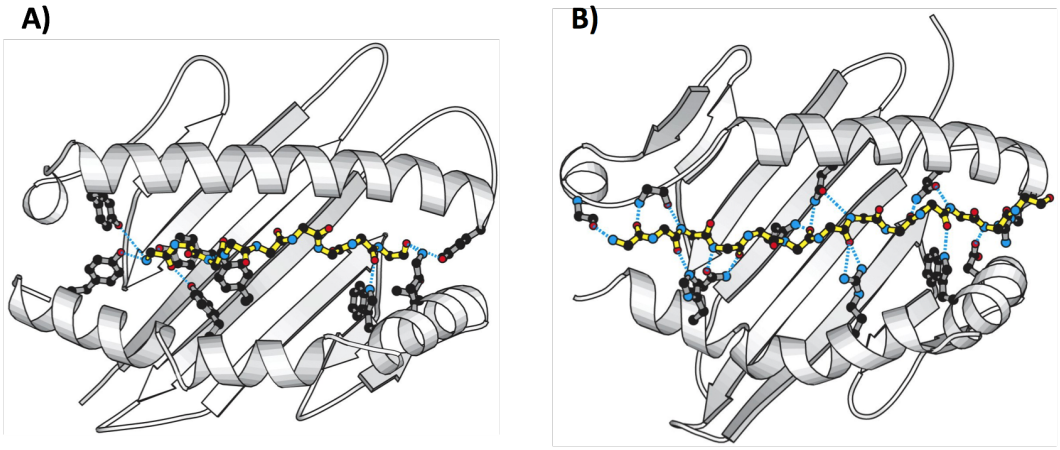

Figure 2. Structure of peptide-MHC complexes. Schematic representation (top view) of peptides bound to **(A)** MHC I and **(B)** MHC II binding grooves. (Source: Kenneth *et al* [182]).

MHC I presentation MHC I molecules are composed of 2 polypeptide chains, the α chain and the β_2-microglobulin, which are assembled in the endoplasmic reticulum (ER) [181]. The MHC I groove accommodates peptides that are generally 8-10 amino acids in length [183]. Peptides loaded onto MHC I are typically derived from proteins that are degraded by the proteasome, which generates peptides with varied lengths that are optimally trimmed either by cytoplasmic proteases or by ER aminopeptidases [184]. Intracellular proteins

targeted for degradation by the proteasome are first labelled through ubiquitylation [185]. Peptides are transported from the cytoplasm into the ER using the transporter associated with antigen processing (TAP) complex [186] prior to MHC I loading via a peptide-loading complex made of calreticulin, ERp57 and tapasin [187]. Peptide binding results in stabilization of the MHC molecule, which is then routed to the cell surface via the endosomal pathway (Fig. 3A). Extracellular antigens can also be loaded onto MHC I by means of "cross-presentation" (FIG. 3B-D) [182], a process that is suspected to be important for presentation of tumor antigens [188].

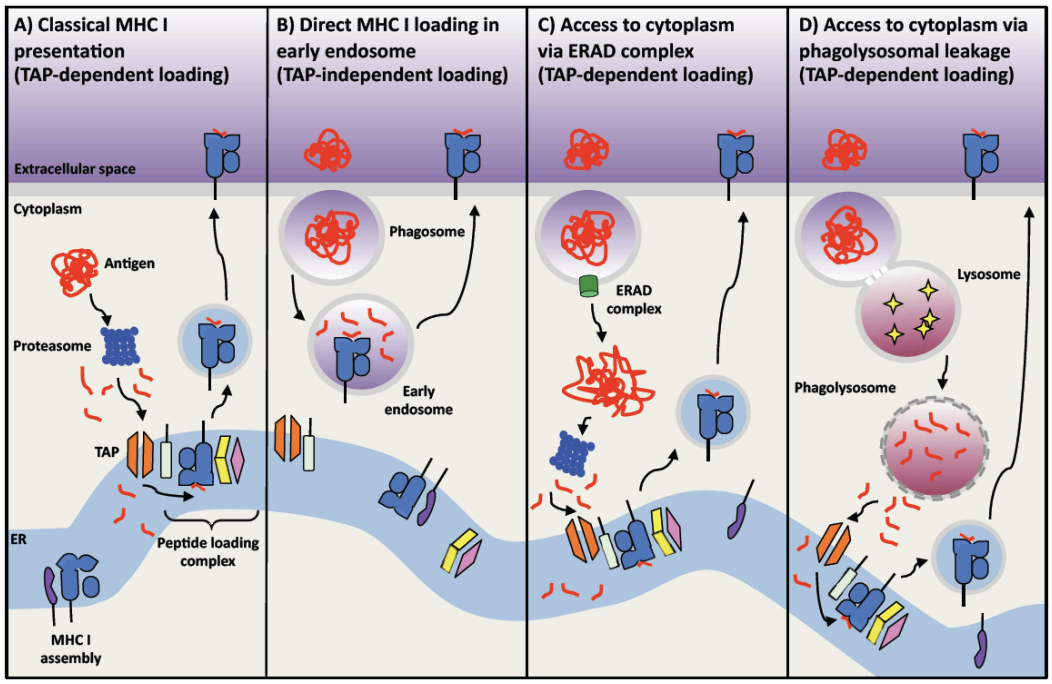

Figure 3. MHC I presentation. **(A)** Classical MHC I presentation pathway. **(B-D)** Putative cross-presentation pathways. **(B)** Peptides derived from internalized antigens may be loaded directly onto MHC I molecules, which have been found to colocalize with MHC II molecules in early endosomes. **(C)** Extracellular antigens may access the cytoplasm using the ER-associated degradation (ERAD) machinery, which has also been found in phagosomes. **(D)** Antigens may "leak" into the cytoplasm through increased permeability or rupture of phagolysosomal membranes. (Figure adapted from Murphy *et al* [182] and Rock and Shen [188]).

MHC II presentation MHC II α/β heterodimers are first assembled in the ER along with the invariant chain (Ii), whose cytoplasmic tail contains an endosomal pathway targeting motif [189]. Once located in late endosomal/lysosomal vesicles (named MHC II compartments), Ii is degraded by lysosomal proteases and a small fragment, the class II-associated invariant chain peptide (CLIP), remains bound to the MHC II peptide groove [190]. Phagocytosis and macropinocytosis shuttle exogenous antigenic material into vesicles termed phagosomes, which eventually fuse with lysosomes [191] to create an organelle known as a phagolysosome. Upon fusion, the phagolysosomes acidify, allowing for maximal activity of various hydrolases, proteases and peptidases required for antigen degradation [192]. MHC II loading is next performed by the chaperone HLA-DM (H2-M in mice), which catalyzes the exhange of CLIP for antigenic peptides [193]. Evidence also supports an alternative antigen processing model, called MHC-guided processing, which suggests that large antigenic fragments are first loaded onto MHC molecules prior to trimming by peptidases [194]. The MHC II peptide groove is less constrained than that of MHC I and allows for binding of longer peptides, generally between 12 and 25 amino acid long, of which 9 are located within the groove [195]. Loaded MHC II molecules are next transported to the surface for presentation to $CD4^+$ T cells (Fig. 4A). A number of studies have shown that MHC II loading can result from autophagy [196-198], a process by which cells, during periods of stress, engulf cytoplasmic content (proteins, macromolecules, organelles and microorganisms) for degradation in lysosomes

(FIG. 4C-D) [199]. Further investigation is required to understand the contribution of autophagy to MHC II presentation. Lastly, it appears that intracellular proteins trafficking along the endosomal pathway may also cross over to MHC II compartments; this MHC II endogenous loading has for instance been described for melanocyte proteins such as TRP-1 [200] and gp100 [201].

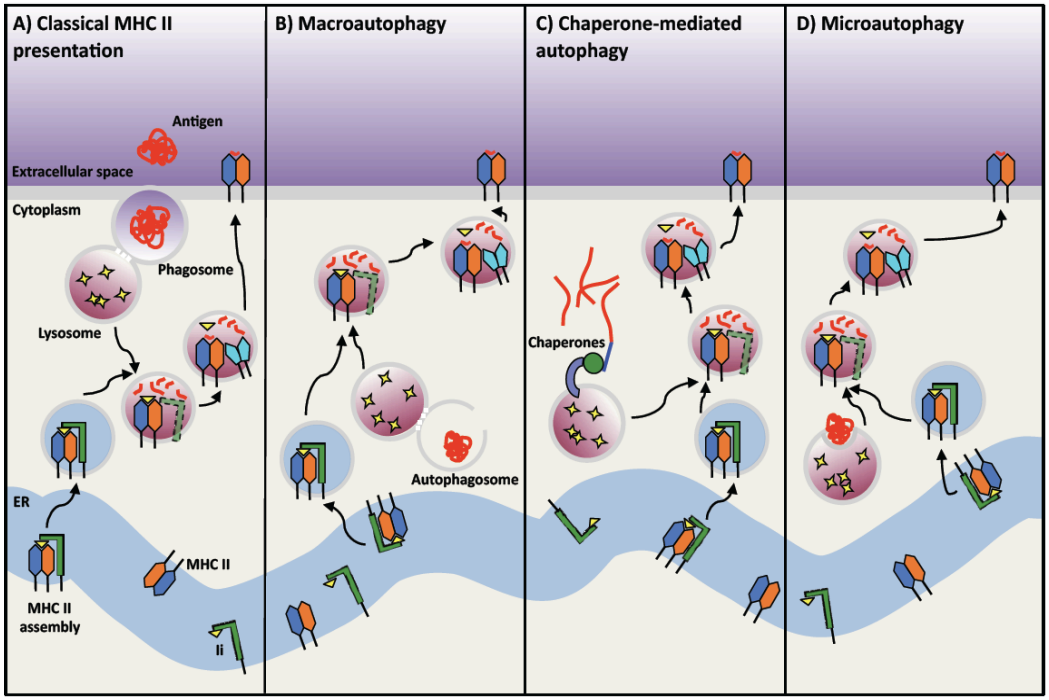

Figure 4. MHC II presentation. (A) Classical MHC II presentation pathway. **(B-D)** Autophagy pathways. **(B)** Macroautophagy involves the formation of an autophagosomal vesicle and subsequent fusion with lysosomes. **(C)** Chaperone-mediated autophagy depends on the recognition of a lysosomal degradation target sequence by chaperones. **(D)** Microautophagy results from cytoplasm budding into lysosomes. (Figure adapted from Murphy *et al* [182] and Mizushima [202]).

2.9 Dendritic cell (DC)-induced T cell activation

T cell activation takes place in T cell-rich areas of secondary lymphoid tissues. DCs and T cells first interact in a non-specific manner until TCRs specifically

associate with peptide-MHC complexes. DC-mediated T cell activation then occurs following the formation of an immunological synapse that is stabilized sequentially by 3 types of interactions: 1) peptide-MHC complexes with TCRs, 2) MHC I or II with CD8 or CD4 co-receptors and 3) ICAM-1 with LFA-1 (adhesion molecules) [203]. A 3-signal model is used to describe DC-induced T cell activation and differentiation. Signal 1 relays information about the antigen and is delivered upon binding of peptide-MHC complexes with TCRs. Signal 2 conveys information regarding the inflammatory nature of the microenvironment in which the antigen was captured and is mediated by ligation of "costimulatory" molecules on the DC, such as CD80 and CD86, to corresponding receptors on the T cells, in this case CD28. This latter signal induces IL-2 production and expression of its receptor IL-2R, therefore promoting expansion and survival of responding T cells [204]. While signals 1 and 2 are sufficient to induce proliferation of naïve T cells on their own, signal 3 is instrumental in directing the differentiation of T cells, especially $CD4^+$ T cells, towards optimal effector phenotypes and is dictated by the cytokine milieu and cytokines produced by interacting DCs [205]. Full activation of $CD8^+$ T cells is generally thought to depend on the ability of $CD4^+$ T cells to provide "help", in the form of T cell growth factors (cytokines), hence the name of "T helper (T_H)" cells. Three independent studies have demonstrated a role for "DC licensing" in activating $CD8^+$ T cell responses [206-208]. According to this model, expression of CD40L on activated $CD4^+$ T cells, via its interaction with CD40 expressed on DCs, subsequently licenses them to activate $CD8^+$ T

cells through secretion of IL-12 and IL-15 [206-208]. These experiments were notably carried out using non-infectious agents [206-208]. Studies with infectious agents, such as *Listeria monocytogenes* or lymphocytic choriomeningitis virus (LCMV), have however demonstrated that primary $CD8^+$ T cell responses can be generated independently of $CD4^+$ T cells [209]; this implies that DC licensing can be bypassed, most likely through direct activation of DCs by microbial products [18]. Nonetheless, activation of $CD8^+$ T cells in the absence of $CD4^+$ T cells significantly impairs the generation of $CD8^+$ T cell memory responses [210-212], an effect that may be due in part to insufficient IL-2 production during the primary response [213].

2.10 Regulation of T cell homeostasis

T cell homeostasis describes the fine control of magnitude, effector functions and duration of T cell responses and depends on the interpretation of diverse stimuli, including costimulatory and inhibitory receptor ligation. Following TCR triggering, a series of positive stimulatory reinforcing signals are required to achieve optimal activation. Costimulatory receptors of the CD28 and TNFR families appear to provide the greatest stimulatory signal [204,214]. While CD28 is constitutively expressed on T cells, the TNFR family members are inducible following T cell activation [204,214]. Among others, the TNFR family members 4-1BB and OX40 play key roles in T cell proliferation [28,215-217] and memory formation [218-220]. Their ligands 4-1BBL and OX40L, are expressed on activated

professional APCs and activated T cells, respectively [214]. The CD28 family also encodes a collection of receptors that function as negative regulators of T cell function, termed immune checkpoints. Notably, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death 1 (PD-1) both harbor important suppressive functions in T cell homeostasis [221]. CTLA-4 associates with the costimulatory molecules CD80 and CD86 (with higher affinity comparatively to CD28) while PD-1 is the receptor for programmed death ligand 1 (PD-L1) and PD-L2 [221]. Ligation of either inhibitory receptor results in impaired TCR signaling and therefore inhibits T cell cytokine production, proliferation and survival [221]. Homeostatic regulation of T cell responses is crucial to ensure not only the generation of optimal immune responses to fight infections, but also the prevention of immune-mediated pathologies [222].

Immunomodulation of antitumor T cell immunity Loss of T cell effector functions, a phenomenon referred to as T cell exhaustion, has been associated with chronic antigen stimulation, such as in the case of cancer [223]. Strategies using immunomodulatory antibodies (Abs) to improve antitumor immunity have highlighted the importance of T cell regulatory pathways in the context of cancer immunity [224]. Agonist Abs act by triggering costimulatory molecules expressed on activated effector T cells [214]. Using animal models, enhanced antitumor immunity has been reported with agonist Abs targeting 4-1BB [225,226] and OX40 [227,228]. While anti-OX40 remains to be tested in the clinic, anti-4-1BB agonist

Abs offered evidence of enhancing antitumor activity and were well tolerated in phase I studies [229,230]. Antagonist Abs, which aim to block inhibitory signals involved in downmodulation of T cell responses, are also being examined. Pre-clinical work has clearly shown the potential of PD-1 antagonist Abs in increasing antitumor responses [231,232]. Although many anti-PD-1 phase I trials are still active, clinical benefit as well as one complete remission have already been reported thus far [233,234]. Most of the progress seen with immunomodulatory Abs has been obtained with anti-CTLA-4 therapy. Indeed, results from a stage III/IV melanoma phase III trial published last year revealed an overall survival benefit associated with anti-CTLA-4 treatment [14]; the US Food and Drug Administration (FDA) has recently approved its use in the clinic. Importantly, many pre-clinical cancer vaccination studies have demonstrated a synergistic effect following the combination of different T cell immunomodulatory Abs over their use as single agents [232,235-237]. These latter findings strongly suggest that targeting multiple T cell regulatory pathways can highly benefit cancer immunotherapeutic approaches.

2.11 Tolerance mechanisms

Given the vast possibilities of TCR rearrangement, high affinity TCRs often arise which can recognize self-derived peptides. If T cells bearing such TCRs were allowed to circulate and become activated, this could result in significant autoimmune pathology. The immune system has therefore co-evolved safeguards

to maintain tolerance to self-antigens, which include central and peripheral tolerance mechanisms. While immune tolerance is an important homeostatic control to prevent unwanted autoimmune syndromes, it is also a major barrier to cancer immunotherapy. Understanding and overcoming these mechanisms of immune tolerance is key to the development of effective T cell-based immunotherapies.

Central tolerance Central tolerance takes place in the thymus. T cell precursors, termed thymocytes, are selected in the thymus based on their relative ability to interact with self-MHC. "Positive selection" is the first step in the development of a broad, but limited, T cell repertoire whereby only T cells that can recognize self-MHC are selected [238,239]. Thymocytes whose TCRs cannot recognize host MHC molecules die "by neglect" via apoptosis. When positive selection yields T cells whose TCRs recognize MHC molecules presenting self-peptides with high affinity, these T cells are removed from the repertoire by means of "negative selection" (apoptosis) or, alternatively, are converted to nTregs [240]. Elimination of T cells specific for antigens which are not found in the thymus is critically important and depends on the ability of medullary thymic epithelial cells to expose T cells to the antigenic diversity found in peripheral tissues. This is accomplished by the autoimmune regulator (Aire) protein, which allows the promiscuous ectopic expression and presentation of tissue-restricted antigens [241].

Peripheral tolerance The process of negative selection in the thymus does not entirely eliminate potentially autoreactive T cells before they enter the periphery. Peripheral tolerance mechanisms are therefore in place to prevent the deleterious activation of self-reactive T cells and include: suppression by Tregs (discussed in section 2.4), induction of T cell anergy (functional inactivation) and deletion [242].

Anergy is characterized by a state of functional inactivation resulting from the recognition of self-antigen without costimulation [243]. The properties most often ascribed to anergic T cells include decreased proliferation potential and production of IL-2 as well as lower responsiveness to IL-2 [244]. T cells can become anergic in 2 different scenarios: presentation of self-antigen by immature/tolerogenic DCs [177] or by non-APCs, which inherently lack costimulation [245]. An intrinsic form of T cell anergy [246] is supported by the T cell expression of the inhibitory receptors CTLA-4 and PD-1, which are key to the homeostatic regulation of T cell responses [221]. It has been suggested, based on the expression pattern of these ligands, that CTLA-4 and PD-1 control autoreactive T cells in lymphoid structures and peripheral tissues, receptively [246]. Maintenance of an immature state in the absence of infection/inflammation is central to the "tolerogenic" role of DCs in peripheral tolerance. Contrary to the ingestion of necrotic cellular debris, the routine uptake of apoptotic cellular debris by DCs is insufficient to trigger their maturation [170]. This can be explained by the fact that apoptotic cell membranes contain 2 ligands, Gas6 and Protein S, which promotes the association of their immune inhibiting TAM receptors (TAM: Tyro, Axl and

MerTK) with type I interferon receptors in DCs, resulting in the prevention of TLR and cytokine signaling [247]. The central role of these DC inhibiting receptors in self-tolerance is exemplified by the fact that mice lacking TAM receptors develop systemic autoimmunity as a result of DC hyperactivation and massive lymphoproliferation [248].

When faced with repeated exposure to self-antigen in absence of costimulation, autoreactive T cells may be "deleted", in other words eliminated through activation-induced cell death (AICD), a form of apoptosis dependent on IL-2/IL-2R and Fas/FasL signaling [249]. Interestingly, although this type of deletional tolerance is mainly thought to depend on presentation by immature tolerogenic DCs, it has also recently been linked to Aire-dependent [250] and independent [251] promiscuous ectopic expression of tissue-restricted antigens in lymph nodes.

3.0 Cancer immunotherapy

Cancer immunotherapeutic strategies have emerged as a promising complementary approach to treat cancers as they exploit the ability of the patient's own immune system to seek, identify and destroy malignant cells. In this section, I will summarize the origins of the cancer immunotherapy era, provide an overview of the various strategies and discuss the risk for autoimmune pathologies.

3.1 Cancer immunosurveillance and immunoediting

The idea that the immune system plays a protective role against tumor development is gaining increased acceptance among the scientific and medical communities. The concept of "cancer immunosurveillance" was first proposed in 1957 by Macfarlane Burnet who hypothesized [252]:

> "It is by no means inconceivable that small accumulations of tumor cells may develop and, because of their possession of new antigenic potentialities, provoke an effective immunological reaction with regression of the tumor and no clinical hint of its existence."

This theory received much attention initially, but was eventually abandoned based on the observation that athymic nude mice, which are immune-deficient, showed an incidence of carcinogen-induced tumor formation that was comparable to wild type mice [253]. Subsequent studies, however, revealed that these athymic nude mice, although immunologically impaired, do retain some functional T cells [254]. The cancer immunosurveillance hypothesis re-surfaced during the 1990s with reports showing that IFN-γ [255,256] and perforin [257], key effector molecules of the adaptive system, were both involved in preventing tumor formation. The hypothesis was formally proven in 2001 by Schreiber's group who demonstrated that the incidence of carcinogen-induced tumor formation was higher in RAG2-deficient mice, which completely lack B and T lymphocytes, compared to wild type mice [258]. The immunosurveillance hypothesis has evolved into the "cancer immunoediting" hypothesis [259], which describes a dynamic process of tumor development that encompasses 3 phases, or "3 Es": elimination, equilibrium and

escape [259]. The elimination phase corresponds to the original theory of immunosurveillance. It is evidenced clinically by the fact that immunosuppressed individuals like AIDS and transplant patients have a higher incidence of tumor formation [260,261]. These data argue that a dysfunctional immune system leads to an increased cancer risk and strongly suggest that the immune system is constantly eliminating tumor cells before they become noticeable. The equilibrium phase, as its name suggests, corresponds to a phase during which the immune system recognizes tumor cells and controls their growth without fully eliminating them. The presence of an equilibrium phase in humans is supported by the observation that malignancies have been "transmitted" via organ transplantation [262]. An interesting case report by MacKie [263] describes 2 patients who developed metastatic melanoma within 2 years of both receiving an allogeneic kidney transplant from a person who had had a primary melanoma lesion resected 16 years prior to the organ donation, demonstrating that the metastases had been dormant, or "in equilibrium", within the donor kidneys since the time of primary resection. Lastly, immune escape begins when the immunological pressure exerted by the immune system during the equilibrium phase results in tumor cell variants that can no longer be recognized or controlled; such tumor deposits then become clinically apparent.

3.2 Cancer immunotherapeutic strategies

The main cancer immunotherapeutic strategies involve treatment with recombinant cytokines, monoclonal antibodies, cancer vaccines, and adoptive T cell transfer therapies. Since my thesis work is focused on T cell-based immunotherapies, in the interest of space, I will not be discussing the applications of cytokines and tumor-targeted monoclonal antibodies; interested readers are directed to the following review articles [264,265]. Rather, I will focus on cancer vaccines and adoptive T cell therapies.

Cancer vaccines Cancer vaccines can either be administered prophylactically or therapeutically. Prophylactic cancer vaccines are used to prevent cancer formation, as in the case of vaccines against human papillomavirus, which causes cervical cancer [266]. The antigens targeted by such vaccines are typically viral antigens and immunity depends on the generation of neutralizing antibodies [266]. Therapeutic cancer vaccines are used to treat an existing cancer [267]. As previously mentioned, most tumor antigens targeted by therapeutic cancer vaccines are self non-mutated tumor-associated antigens (TAAs). Consequently, the available TAA-specific T cells often display low affinity TCRs due to central tolerance [268] and limited functionality due to peripheral tolerance [242]. Thus, to be effective, cancer vaccines must be able to overcome the mechanisms of immune tolerance.

Two main strategies employed to circumvent immune tolerance and induce/enhance antitumor responses are the use of heteroclitic epitope peptides and "xenoimmunization" [269]. Heteroclitic epitope peptides, also referred to as "altered self-peptides", are peptides whose amino acid sequence has been modified based on the knowledge of MHC and TCR contact residues. Such modifications can either increase the binding affinity of the peptide for MHC, rendering peptide-MHC complexes more stable [270] or, alternatively, increase the affinity between peptide-MHC complexes and TCRs [271]. As for the xenoimmunization strategy, i.e. the use of homologous tumor antigens from different species, it is built on the concept that the xenoantigen is different enough from the native self-protein to overcome tolerance, but similar enough to provide cross-reactivity. Several groups, including ours, have shown that immunizing mice with human melanoma-associated antigens, but not the murine equivalents, could successfully induce antitumor immunity in pre-clinical melanoma models [272-277]. In a number of circumstances, the xenoantigen has been shown to contain heteroclitic $CD8^+$ T cell epitopes that lead to increased $CD8^+$ T cell immunity [45,270,278]. Our group has also demonstrated an alternate mechanism where a heteroclitic $CD4^+$ T cell epitope in the xenoantigen augments $CD8^+$ T cell immunity through the provision of helper signals [279]. Overall, these results provided proof-of-principle that appropriately designed vaccination strategies can overcome tolerance mechanisms and generate T cell-mediated antitumor immunity.

The richest source of tumor antigens is tumor cells themselves. The use of irradiated autologous tumor cells was considered early on as a cancer vaccination platform. To simplify the manufacturing process, allogeneic tumor cell lines were developed from cancers of various etiologies and chemically or genetically modified to increase their immunogenicity. The greatest antitumor effect of such approach was initially demonstrated in a pre-clinical study making use of irradiated tumor cells expressing GM-CSF [137], which acts to recruit DCs and promote T cell responses. Although pre-clinical results were encouraging, GM-CSF-expressing tumor cell vaccines have only resulted in modest antitumor immunity in the clinic [280].

Pre-clinical data from the early 1990s showed that intramuscular injection of naked DNA was a straightforward method for generating immune responses [281]. Plasmid DNA vaccines therefore represented an attractive form of cancer immunotherapy due to their low cost of production and high safety profile. Again, despite initial promise from animal studies, clinical evidence has clearly shown that DNA vaccines are of rather low immunogenicity [282]. Several DNA vaccine-enhancing strategies aimed at evaluating routes of administration, concurrent adjuvant therapy and prime-boost immunization have been the focus of recent clinical studies [283].

The use of recombinant viruses encoding TAAs for cancer immunotherapy has several advantages. For instance, they are highly immunogenic as they provide an inflammation stimulus, or “danger signal”,

required for initiating an immune response. This is exemplified by the fact that such infectious agents can directly elicit primary $CD8^+$ T cell responses as previously mentioned (section 2.9) [209]. Moreover, viral vectors are easily expanded *in-vitro*, making them suitable for large-scale production necessary for their implementation in the clinic [284]. Importantly, the inherent adjuvant property of recombinant viruses was shown to readily overcome tolerance to poorly immunogenic TAAs in early pre-clinical studies [285,286]. Phase I clinical studies have been conducted with several recombinant viral vectors including adenovirus, avipox virus, vaccinia virus and herpes virus; while the degree of antitumor responses was highly variable between cancer types and vector platforms used, significant disease stabilization was reported in numerous patients [284]. Interestingly, a prostate cancer phase II clinical trial has recently reported improved overall survival after initial priming with vaccinia virus vaccine and subsequent boosting injections with a fowlpox vaccine, both encoding 2 prostate-specific transgenes as well as 3 synergistic costimulatory molecules ("TRICOM": CD80, ICAM-1 and LFA-3) [15]. This latter finding underscores the fact that the effectiveness of recombinant virus-based vaccines can be improved.

Another promising area of cancer immunotherapy makes use of *ex-vivo* expanded, TAA- or TSA-loaded DCs known as "DC-based vaccines". Pioneering work from Inaba *et al* demonstrated that murine DCs could be expanded to large numbers *in-vitro* from bone marrow precursors [287]. Subsequently, mouse studies provided proof-of-principle that *ex-vivo* loaded DCs (peptide-pulsed) could

generate protective tumor immunity [44,288,289]. Following the establishment of methods to expand DCs *ex-vivo* from human blood $CD34^+$ precursor cells and monocytes [290,291], DC-based vaccines were tested in the clinic. Typically, DCs are expanded *ex-vivo* from precursor cells, subsequently loaded with TAAs and matured with a cytokine cocktail prior to reinfusion [292]. The feasibility and efficacy of this approach were demonstrated in a phase III prostate cancer clinical trial where extended survival was observed [13]; these findings led to the FDA approval of the first cell-based cancer immunotherapy in 2010.

Adoptive T cell transfer therapies Adoptive T cell transfer therapy represents an alternative cancer immunotherapeutic approach which has progressed considerably since the 1980s [293]. The greatest success of adoptive T cell therapy has been seen in melanoma where tumor infiltrating lymphocytes (TILs) were expanded *ex-vivo* using IL-2 and anti-CD3 and infused in high numbers, along with $CD34^+$ hematopoietic stem cells, into patients pretreated with 12Gy total body irradiation [294]. Indeed, objective clinical responses were observed in 72% of the patients, with a complete response rate of 40% [294]. Retrospective analysis of the *ex-vivo* expanded TILs showed a correlation between the proliferation potential (CD27 expression and telomere length) and clinical response, demonstrating the importance of using "young" TILs for adoptive cell transfer therapy [294,295]. The potential use in adoptive T cell transfer therapy of genetically modified T cells containing either "transgenic TCRs" or

"chimeric antigen receptors (CARs)" has also received considerable attention. Both of these techniques have the substantial advantage of making use of readily available, non-specific autologous T cells found in the periphery (likely less subjected to tumor-mediated suppression as in the case of TILs) as starting material. The first approach consists of inserting a construct encoding a TCR of known tumor antigen specificity into T cells by means of *ex-vivo* transduction [296]. A study published in 2009 reported objective cancer regression in 30% of the melanoma patients treated with transgenic TCR T cells, which was however accompanied by significant, albeit manageable, signs of autoimmunity [297]. As for the second approach, CARs, put simply, are genetic constructs composed of an antigen-specific antibody fragment linked to TCR signaling components [298]. *Ex-vivo* transduction of T cells with CARs prior to expansion and reinfusion allows for re-targeting of T cells towards tumor antigens in a MHC-unrestricted manner. One obvious limitation of this approach pertains to the fact that the tumor antigen has to be expressed at the cell surface. Several clinical trials are underway to evaluate whether sufficient *in-vivo* expansion, persistence as well as functionality of CAR-transduced T cells can be obtained to mediate tumor clearance [298]. While considerable knowledge has been gained regarding adoptive T cell transfer therapy, many factors currently limit its successful implementation in the clinic. For instance, *ex-vivo* culture of tumor biopsies sometimes fails to grow and expand tumor-specific TILs for a yet unknown reason [299]. While this is not a problem when using genetically modified T cells, many concerns still remain.

Indeed, lack of persistence and expansion have limited the clinical efficacy of CAR-expressing T cells for instance [300]. Importantly, significant toxicities, including lethality, have also been reported following adoptive transfer of CAR-expressing T cells [300]. Various strategies are currently under investigation to improve the survival, function and safety profile of transferred T cells [301]. However, whether patient-customized adoptive cell transfer therapy constitutes a commercially viable cancer immunotherapeutic approach remains questionable given the associated labor cost and technical issues pertaining to scalability and manufacturing [301].

3.3 Risk of autoimmunity

Overcoming tolerance against TAAs and generating strong antitumor T cell immunity are not without any pathological consequences as T cells can also target cells expressing the same antigens in normal tissues [302]. In the melanoma setting, development of autoimmunity has correlated with better antitumor responses, prompting researchers to suggest that "inducing autoimmune disease to treat cancer" [303] is an integral approach to cancer immunotherapy. While tissues and organs such as skin melanocytes, thyroid, breasts, ovaries, testis and prostate are considered to be non-essential, autoimmunity against vital organs should be avoided if at all possible. As cancer immunotherapeutic strategies become more and more potent however, the severity of autoimmune pathology is likely to increase. Indeed, a recent review article by Amos *et al* highlights the fact that

adoptive T cell transfer therapy using genetically modified T cells is associated with more significant autoimmune adverse events, including lethality in some cases [304]. We, and others, have shown in pre-clinical models that antitumor immunity can be achieved without triggering autoimmunity [276,305-307], suggesting that autoimmunity may not be an unavoidable consequence of overcoming tolerance. A better understanding of the mechanisms leading to autoimmune pathology is required in order to allow the design of more specific cancer immunotherapies.

4.0 Immune escape and immunosuppression

Evasion from immune destruction (termed "immune escape") has been described as an emerging hallmark of cancer [5] and is now regarded as the main underlying reason behind the lack of efficacy of many cancer immunotherapies in the clinic. In this section, I will review the different mechanisms used by tumor cells to escape immune attack.

4.1 Defects in antigen presentation

One of the main stratagems employed by tumors to circumvent antitumor T cell immunity is to "hide" by altering their antigen presentation ability. Downregulation or loss of MHC I expression is a common mechanism used by tumors of diverse etiologies to escape $CD8^+$ T cell attack [308] and strongly

correlates with disease progression [309]. The various molecular mechanisms responsible for MHC I downregulation/loss have been well characterized [310]. Briefly, total MHC I loss can occur following mutation of the β2-microglobulin chain [311,312] while partial MHC I loss can be attributed to loss of heterozygosity of chromosome 6, which results in deletion of one allele of each of the MHC I haplotypes [312]. Coordinated downregulation of MHC I expression and various components of the antigen presentation machinery by tumor cells has been reported in both murine [313] and human [314] tumors. Analysis of a panel of human melanoma cell lines has revealed defects in antigen presentation in 67% of the cell lines tested; downregulation of MHC I and the antigen presentation machinery was most commonly observed and also found to be reversible with IFN-γ treatment [315]. While these latter findings suggest that *in-vivo* MHC I expression on tumor cells should be restored upon exposure to killer cell-derived IFN-γ, presence or absence of tumor cells with loss of MHC I expression likely dictates the ultimate response to $CD8^+$ T cell-dependent cancer immunotherapies [309].

4.2 Resistance to apoptosis

Another method employed by tumor cells to evade immune attack by $CD8^+$ T cells is resistance to apoptosis. For instance, tumor cells can avoid cell death via the expression of anti-apoptotic molecules such as survivin [316] or, alternatively, by inhibiting the perforin-granzyme pathway [317]. The death ligands, FasL and TRAIL, have been shown to play a role in cancer immunosurveillance [318] and, as

such, a commonly reported anti-apoptotic mechanism is downregulation or loss of expression by tumor cells of the death receptors Fas [319] and TRAIL-R [320]. Tumor cells can also resist apoptosis by cleavage of Fas (and presumably TRAIL-R) through the effects of matrix metalloproteinase-7 [321] or even by expressing soluble Fas, which acts as a competitive antagonist of Fas [322]. Lastly, expression of decoy receptors has also been shown to help tumor cells neutralize the effect of FasL [323].

4.3 Soluble immunosuppressive factors produced by tumors

Tumor cells produce a variety of soluble immunosuppressive factors, of which TGF-β, IL-10, prostaglandin-E2 (PGE2) and vascular endothelial growth factor (VEGF) have been the most well characterized to date [324]. TGF-β and IL-10 are secreted in large amounts by malignant cells as well as Tregs, tumor-infiltrating DCs and tumor-associated macrophages (TAMs) [325-327]. Both cytokines can suppress T cell and APC functions via numerous mechanisms including: inhibition of IL-2/IFN-γ production and CTL effector functions [328] as well as impairment of DC differentiation from monocytes and DC maturation [327,328]. Moreover, TGF-β and IL-10 promote the conversion of conventional $CD4^+$ T cells into TGF-β-secreting T_H3 iTregs [128,129] and IL-10-producing T_R1 iTregs [126], further suppressing antitumor immune responses. Production of PGE2, a downstream effect of overexpression of the inducible isoform of the enzyme cyclooxygenase 2 by tumor cells, has not only been linked to angiogenesis and invasiveness [329], but also negative modulation of immune responses. Among

others, PGE2 can suppress DC activity in secondary lymphoid organs as well as T cell proliferation and effector cytokine production [330]. Additionally, *in-vitro* PGE2 treatment has been shown to induce Foxp3 expression and stimulate suppressive functions in both Tregs and conventional $CD4^+$ T cells [331]. Lastly, VEGF, produced at high levels by tumor cells [332], plays a pivotal role in cancer-associated angiogenesis [333]. VEGF can also indirectly interfere with T cell immunity by recruiting immunosuppressive cells within the tumor microenvironment and by impairing DC activation [332]. Cumulatively, these soluble immunosuppressive factors potently inhibit antitumor immune responses.

4.4 Inhibitory cell populations

Assorted chemokines produced by tumor and stromal cells are responsible for attracting various immune cell populations within the tumor microenvironment [334]. Together, the combined actions of tumor, stromal and immune cells result in tissue remodeling, angiogenesis and ultimately, tumor progression [335]. Immune inhibitory cells, including tolerogenic DCs (described in sections 2.7 and 2.11), Tregs (described in sections 2.4 and 2.5), tumor-associated macrophage [336] and myeloid-derived suppressor cells [337], are regarded as important mediators of tumor escape.

Tumor-associated macrophages (TAMs) Cancer cells, via the secretion of chemokines such as CCL2, CCL5 and VEGF among others, recruit circulating

monocytes inside the tumor microenvironment and stimulate their differentiation to tumor-associated macrophages (TAMs) [334,338,339]. TAMs make up a major component of the leukocytic infiltrate and are highly plastic immune cells. Indeed, TAMs exist mainly in 2 main polarization states, the "classically" activated M1 state and the "alternatively" activated M2 state [336,339,340]; M2-like states, which result from interactions with Tregs or B cells, have also been described [341,342]. Similarly to T_H1-T_H2 $CD4^+$ T cell differentiation, IFN-γ contributes to M1 polarization while IL-4 and IL-13 drive M2 responses [336]. Of the two subsets, M2 cells are the ones prevailing in malignancies and promote immune suppression mainly via the following mechanisms: decreased IL-12 production and increased IL-10 and TGF-β production [343], impaired tumoricidal functions [343] as well as secretion of CCL22, which recruits Tregs [344]. Additionally, as a consequence of L-arginine catabolism, arginase-1 expression by M2 TAMs [343] further suppresses T cell responses via TCR signaling inhibition and cell cycle arrest [345,346]. Lastly, in contrast to M1 cells, M2 cells also express many tissue remodeling and pro-angiogenic factors that support tumor growth [336]. Collectively, these immunosuppressive M2 macrophage characteristics have been linked to tumor escape and metastasis [338].

Myeloid-derived suppressor cells (MDSCs) Under normal conditions, immature myeloid progenitor cells generated in the bone marrow migrate to the periphery where they differentiate into granulocytes, macrophages and DCs.

However, under pathological circumstances, such as in the presence of cancer, differentiation of immature myeloid cells is impaired and instead results in the generation of incompletely matured monocytic or granulocytic cells, which lack surface markers associated with monocytes, macrophages and DCs, and display immune suppressive features. Collectively, these cells are known as myeloid-derived suppressor cells (MDSCs) [347]. The expansion of MDSCs is regulated by many factors produced either by tumor or stromal cells (e.g. prostaglandins, M-CSF, GM-CSF, VEGF) while their activation is mediated by factors produced mainly by activated T cells, but also tumor cells (e.g. IFN-γ, IL-4, IL-13, TGF-β) [337]. The numerous immunoregulatory facets of MDSCs have been described in detail by Gabrilovich and Nagaraj in a recent review article [337]. Among others, one of the first suppressive activities attributed to MDSCs is depletion of L-arginine mediated by the arginase-1 enzyme, which is upregulated in MDSCs [348]. L-arginine deficiency results in TCR signaling inhibition and cell cycle arrest [345,346]. MDSCs also express inducible nitric oxide synthase (iNOS), which catabolizes L-arginine to produce nitric oxide, consequently mediating downregulation of MHC II molecules [349], T cell apoptosis [350] and impairment of TCR recognition [351]. Lastly, there is also evidence showing that MDSCs may promote the development of Tregs, however contradictory findings warrant further investigations [337].

5.0 Cancer vaccination model used in this thesis

We have used the murine B16F10 melanoma as a tumor model because it shares many TAAs with human melanomas. Our prototype vaccine vector is a replication-deficient recombinant adenovirus (Ad); previous screening by our lab of Ad vectors expressing a number of melanoma TAAs showed that human dopachrome tautomerase (hDCT) provides the most robust protective immunity [276,277] (unpublished data). Results presented in chapters 2 and 3 were generated using AdhDCT vectors while results included in chapter 4 made use of AdhDCT, but also Adhgp100.

5.1 Melanoma

Melanoma is a very aggressive and deadly type of skin cancer that arises from transformed melanocytes for which the incidence rate has been steadily increasing over the past decades [352]. Standard treatments include resection, which may be followed by beam radiation or cytokine adjuvant therapy depending on the malignant stage; patients who progress to stage IV typically have a median survival of less than one year [352]. Melanoma has been considered a good target for the development of immunotherapies based on the discovery that tumors are often infiltrated with high frequencies of TILs recognizing TAAs including gp100, MelanA/Mart-1, tyrosinase, tyrosinase-related protein (TRP)-1 and DCT [353]. Murine B16F10 melanoma is a poorly immunogenic tumor cell line that first originated from a subcutaneous tumor in a C57BL/6 mouse from which cell lines

with an enhanced metastatic potential were subsequently selected to mimic more closely the phenotype of human melanoma cells [354,355]. As previously mentioned, B16F10 cells express several murine counterparts of the TAAs identified in human melanomas, therefore providing researchers with a relevant pre-clinical model.

5.2 Model tumor-associated antigens (TAAs)

DCT Also known as tyrosinase-related protein (TRP)-2, DCT is a highly immunogenic TAA that we have been employing to characterize the biological pathways responsible for tumor protection following immunization. Located in melanosomes, which are special organelles responsible for melanin synthesis in melanocytes, DCT is an enzyme responsible for converting the pigment intermediate DOPAchrome to DHICA [356]. It has an expected molecular weight of 59 kd, but is expressed as 2 isoforms of 69 kDa and ~80 kDa, which result from post-translational glycosylation taking place in the ER as well as in the golgi apparatus [357]. DCT was first identified in 1996 as a human TAA recognized by TILs from melanoma patients [358]. Shortly after, another group also found DCT to be recognized by TILs derived from B16F10 tumors [359]. Human DCT (hDCT) shares 83% identity with murine DCT (mDCT), making this protein an excellent candidate for xenoimmunization [274]. Indeed, we and others have shown that immunization of wild type mice with hDCT results in robust antitumor immunity that protects against a high-dose B16F10 cell challenge [274,276,279,360]. As

demonstrated by Bowne *et al* [274] and our group [276], tumor rejection following DCT immunization requires both $CD4^+$ and $CD8^+$ T cells, but not NK cells or B cells. $DCT_{180\text{-}188}$ (SVYDFFVWL) has been identified as the immunodominant $CD8^+$ T cell epitope from DCT [361] and is fully conserved in mDCT.

gp100 gp100, expressed both by normal melanocytes and melanoma cells, is a heavily glycosylated protein involved in the early steps of melanosome biogenesis [362]. Human gp100 (hgp100) was cloned in 1993 and identified as one of the diagnostic human melanoma markers recognized by the NKI-beteb, HMB-45 and HMB-50 monoclonal antibodies [363]. The murine gp100 (mgp100) homologue was subsequently cloned from a B16 melanoma cDNA library and sequence comparison revealed 77% identity with human gp100 [364]. As its name suggests, the molecular weight of gp100 is 100 kDa [365]. Shortly after its identification as a TAA, hgp100 was shown to be recognized by melanoma TILs [366,367], which were involved in tumor regression following adoptive transfer therapy [367]. In the pre-clinical setting, xenoimmunization using either plasmid DNA constructs [275,278] or viral vectors [45,364] expressing hgp100 was able to break tolerance and elicit some degree of protection against B16F10 challenge in a $CD8^+$ T cell-dependent manner. The protection afforded by xenoimmunization was due to the presence of a heteroclitic immunodominant CD8 epitope, $hgp100_{25\text{-}33}$ (KVPRNQDWL), which displayed higher avidity for MHC I than the murine counterpart $mgp100_{25\text{-}33}$ (EGSRNQDWL) [45,278]. Overall however,

protection elicited by hgp100 xenoimmunization is rather weak and, as shown by our group, is due to inefficient presentation of $mgp100_{25-33}$ by B16F10 cells [277].

5.3 Recombinant adenovirus platform

The vaccine prototypes that we have been investigating in the B16F10 model are composed of recombinant human adenovirus type 5 vectors that express either hDCT or hgp100 as xenoantigens. The replication-defective rAd platform that we employ is a first generation vector, which lacks the E1 and E3 regions of its genome. The E1 genes code for proteins that play a key role in virus replication while the E3 genes code for proteins that subvert the host defense mechanisms [368]. The first generation rAd vectors are propagated *in-vitro* in cell lines stably transfected with E1 proteins, providing in *trans* the necessary elements for replication. First-generation rAd vectors can accommodate up to 8 Kb of transgene DNA into their genome and can be easily expanded *in-vitro* to high titers [369]. They can infect a wide range of dividing and non-dividing cell types [369].

6.0 Scope of thesis research

The overall goal of my PhD thesis research was to further our understanding of the mechanisms behind DCT-specific T cell-mediated antitumor immunity and autoimmunity. The research objectives set forth during my PhD studies were

based on previous and concurrent findings generated by our lab, which are briefly described below.

6.1 Work by our group relevant to thesis research

Earlier investigations by our lab have shown that immunization with AdhDCT elicits important tumor protection which requires both $CD4^+$ and $CD8^+$ T cells [276]. Interestingly, this study also revealed that the development of autoimmunity is linked to inflammation within the skin, demonstrating that autoimmunity can possibly be avoided following vaccination against self-antigens [276]. We have also reported that hDCT immunization results in frequencies of $DCT_{180\text{-}188}$-specific $CD8^+$ T cells that are 5-10 fold higher than immunization with mDCT [279]; extensive epitope mapping experiments demonstrated that this is due to a heteroclitic $CD4^+$ T cell helper epitope in hDCT, $hDCT_{89\text{-}101}$ (KFFHRTCKCTGNF) [279]. We were unable to identify any CD4 epitopes responsible for eliciting effector $CD4^+$ T cell responses observed in hDCT-mediated tumor protection [279]. Concurrently, other studies conducted in our lab using the same vaccination platform in a therapeutic challenge setting showed minimal benefit against established tumors [370,371]; analysis of DCT-specific TILs showed decreased cytokine production and cytotoxic functions compared to DCT-specific cells found in the periphery [370].

6.2 Research objectives

The work described in the result chapters of my thesis was framed around the following 3 research objectives:

Objective 1 Examine the relative roles of effector $CD4^+$ T cell subsets in mediating tumor protection and autoimmune depigmentation following immunization with AdhDCT (**Chapter 2**). Results pertaining to this objective have been published in the following manuscript:

> Zhang, S., Bernard, D., Khan, W.I., Kaplan, M.H., Bramson, J.L. & Wan, Y. $CD4^+$ T-cell-mediated anti-tumor immunity can be uncoupled from autoimmunity via the STAT4/STAT6 signaling axis. *Eur J Immunol* (2009) [372].

Objective 2 Investigate the importance of antigen processing with regard to antitumor $CD4^+$ T cell responses elicited following AdhDCT immunization (**Chapter 3**). Results pertaining to this objective have been published in the following manuscript:

> Bernard, D., Ventresca, M.S., Marshall, L.A., Evelegh, C., Wan, Y. & Bramson, J.L. Processing of tumor antigen differentially impacts the development of helper and effector CD4+ T-cell responses. *Mol Ther* **18**, 1224-1232 (2010) [373].

Objective 3 Evaluate the therapeutic effectiveness of AdhDCT immunization combined with monoclonal antibodies targeting inhibitory and costimulatory T cell receptors as well as the mechanisms resulting in tumor progression or

clearance (**Chapter 4**). Results pertaining to this objective have been summarized in the following manuscript:

> A. J. Robert McGray, Dannie Bernard, Florentina Teoderascu, Ryan Kelly, Mayank Jha, Caitlin Gregory, Jennifer D. Bassett, Yonghong Wan and Jonathan L. Bramson. Early IFN-γ production by vaccine-induced T cells provokes tumor adaptation and PD-1 ligand-mediated suppression of local T cell activity (2011).

— CHAPTER 2 —

$CD4^+$ T cell-mediated anti-tumor immunity can be uncoupled from autoimmunity via the STAT4/STAT6 signaling axis

$CD4^+$ T cell-mediated anti-tumor immunity can be uncoupled from autoimmunity via the STAT4/STAT6 signaling axis

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Published in the *European Journal of Immunology*
Vol. 39(5), pp. 1252-1259, May 2009

Preface: *The research presented in this manuscript is the result of a collaboration between myself and Sheng Zhang, a former Master student in Dr. Wan's laboratory. My participation to this experimental work spanned from 2006 to 2008. I designed and optimized the IL-4 and IL-17 ELISPOT assays used in this study as well as performed the ex-vivo T cell analyses. Sheng Zhang conducted the in-vivo work. Briefly, I contributed to figure 1 and generated the data presented in figure 4. I also helped Dr. Wan prepare the manuscript and address the reviewers' comments. Dr. Waliul I. Khan and Dr. Mark H. Kaplan (collaborators) provided the STAT6 and STAT4 knockout mice, respectively. This work was supervised by Dr. Wan who, along with Dr. Bramson, provided experimental guidance and contributed to the interpretation of the results.*

Title: $CD4^+$ T cell-mediated anti-tumor immunity can be uncoupled from autoimmunity via the STAT4/STAT6 signaling axis

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Running Title: Separation of anti-tumor immunity and autoimmunity

Keywords: $CD4^+$ T cells, vaccination, cytokines, anti-tumor immunity, autoimmunity

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ABSTRACT

Previous reports have suggested that autoimmune sequelae may be an unavoidable consequence of successful immunization against tumor-associated antigens which are typically non-mutated self-antigens. Using a melanoma model, we demonstrated that $CD4^+$ T cell-mediated anti-tumor immunity and autoimmunity could be separated by modulating the STAT4/STAT6 signaling axis. Our results have revealed an unexpected dichotomy in the effector phase following cancer vaccination where anti-tumor immunity is mediated via a STAT6 and IL-4-dependent pathway whereas autoimmune pathology is mediated via STAT4 through a mechanism that relies partially on IFN-γ. Our results offer a possibility to elicit specific anti-tumor responses without triggering unwanted tissue autoimmune diseases.

INTRODUCTION

Many cancer vaccines are directed at antigens that are not only expressed within the tumor but are also present on normal healthy tissues. Although it has been suggested that breaking self-tolerance through successful application of cancer vaccines will inevitably result in autoimmune sequelae, the true relationship between anti-tumor and autoimmune pathology remains uncertain [1-3]. Using a

murine melanoma model, we and others have demonstrated that the development of effective anti-tumor immunity also results in autoimmune depigmentation (manifested as vitiligo) and this appears to be true for both T cell- and antibody-mediated mechanisms [1,4]. Concomitant development of anti-tumor immunity and autoimmune pathology appears to occur more readily when there is trauma/inflammation within the skin or the vaccination method is intensified [1]. These results suggest that vaccine-associated autoimmune destruction may not be avoidable, especially when there is a concurrent inflammatory event in healthy tissues that express antigens carried by the vaccine.

The important role of $CD4^+$ T cells as helpers to fully engage B cell and $CD8^+$ T cell differentiation has been well established but their function as effectors in anti-tumor immunity has been insufficiently studied. Increasing evidence indicates that $CD4^+$ T cells are able to mediate tumor destruction without direct interaction with tumor cells and that $CD4^+$ T cells may provide even greater anti-tumor effect than $CD8^+$ T cells [5-8]. Furthermore, reports from various groups, including ours, have demonstrated the ability of $CD4^+$ T cells to reject CTL-resistant, MHC class II-negative tumors [7,9,10]. Therefore, understanding the potential for $CD4^+$ T cells as anti-tumor effectors is of broad value to both MHC class II-positive and -negative tumors. With regard to MHC class II-negative tumors, it is believed that APCs infiltrating the tumor bed process tumor antigen and present it to $CD4^+$ T cells. In turn, the tumor antigen-stimulated $CD4^+$ T cells can produce IFN-γ (Th1) or IL-4 (Th2); both of these cytokines display anti-

angiogenic properties and are able to recruit other tumoricidal cells such as NK, macrophages or eosinophils into the tumor [5,10-12]. These indirect destructive mechanisms may prove advantageous over the direct effector functions mediated by $CD8^+$ T cells as the indirect mechanisms should not be affected by MHC expression levels on the tumor cell and consequently may deter the development of tumor escape variants due to antigen loss. Little is known, however, about the risk of autoimmune pathology caused by $CD4^+$ effector T cells and the mechanisms regulating their functions against cancerous and normal tissues. Some insight can be gained from a recent publication that found the anti-tumor potency of $CD4^+$ T cells was directly related to their capacity to produce depigmentation in the skin [13], reinforcing the concept that these two processes are linked.

We have previously reported that immunization with a recombinant adenovirus (Ad) expressing human dopachrome tautomerase (DCT; also as known as tyrosinase-related protein-2) could induce $CD4^+$ T cell-mediated tumor protection and autoimmune vitiligo, independent of $CD8^+$ T cells [1]. In the present study, we demonstrate that anti-tumor effector functions in this model are IL-4 and STAT6-dependent, whereas autoimmunity requires IFN-γ and STAT4 signaling. Our results offer a possibility to elicit specific anti-tumor responses without triggering unwanted tissue autoimmune diseases.

RESULTS

Conventional CD4$^+$ T cells, but not CD4$^+$ NKT cells, are required to mediate anti-tumor immunity and autoimmunity

We have previously reported that immunization with AdhDCT could protect C57BL/6 mice against a lethal B16F10 melanoma challenge and autoimmune vitiligo [1]. Interestingly, tumor protection and vitiligo induction were abrogated only by in vivo depletion of both CD4$^+$ and CD8$^+$ T cells at the effector phase, while depletion of either subset alone did not affect the outcome of the two events, suggesting that CD4$^+$ T cells are efficient in mediating tumor and normal tissue destruction in the absence of CD8$^+$ T cells. However, since NKT cells also express CD4, it is possible that we had misinterpreted the outcome of the anti-CD4 treatment. Therefore, we evaluated the activity of AdhDCT immunization in CD8-depleted, CD1-deficient mice (CD1$^{-/-}$), which lack iNKT cells and CD8$^+$ T cells. Under these conditions, the CD1$^{-/-}$ mice both resisted tumor challenge and developed vitiligo demonstrating that iNKT cells were not involved in either anti-tumor immunity or autoimmune vitiligo (Table 1). Importantly, since both tumor protection and vitiligo development are independent of iNKT cells but could be abrogated by simultaneous treatment with anti-CD8 and anti-CD4, these results support an effector role for vaccine-induced CD4$^+$ T cells (Table 1).

CD4-mediated effector functions do not require antibodies

It has previously been reported that immunization with tyrosinase-related protein-1 (TRP-1) can elicit $CD4^+$ T cell-dependent immune responses against both melanoma and melanocytes through induction of antigen-specific antibodies [3,4]. Although studies by our group and others have demonstrated that protection following immunization with DCT is mediated primarily by T cells [1,4], it is nevertheless possible that under extreme conditions, such as $CD8^+$ T cell depletion, a role for antibodies as effectors may emerge. To confirm that the CD4-dependent, CD8-independent immune protection was due to $CD4^+$ T cell effector function and not simply due to their role as helpers for B cell differentiation, we tested CD8-depleted, B cell-deficient ($B^{-/-}$) mice for induction of anti-tumor immunity and autoimmune vitiligo. As summarized in Table 1, both tumor protection and vitiligo occurred in $B^{-/-}$ mice in the absence of $CD8^+$ T cells demonstrating the vaccine-induced antibodies were not playing a significant role as effectors in this model. Simultaneous depletion with CD8 and CD4 antibodies in $B^{-/-}$ mice fully abrogated both protective immunity and the development of vitiligo further confirming the role of $CD4^+$ T cells as effectors in both of these processes.

CD4-mediated anti-tumor immunity and autoimmunity are differentially regulated by IL-4 and IFN-γ

The results described above confirmed a role for AdhDCT-induced, DCT-specific $CD4^+$ T cells as effector cells that provide anti-tumor immunity and autoimmune vitiligo. Since characterization of the effector $CD4^+$ T cell response requires concomitant depletion of $CD8^+$ T cells, we opted to use CD8α-deficient mice ($CD8^{-/-}$) to simplify our further investigation of DCT-specific $CD4^+$ T cell immunity. $CD8^{-/-}$ mice immunized with AdhDCT were fully protected against B16F10 challenge and this protection was completely abrogated by the depletion of $CD4^+$ T cells one day prior to tumor challenge (Fig. 1A), confirming dependence upon $CD4^+$ T cells. To confirm antigen-specificity, $CD8^{-/-}$ mice were immunized with AdhDCT and challenged with B78H.1, a variant of B16 melanoma that does not express the DCT antigen. All mice succumbed to tumor challenge confirming that the AdhDCT-mediated anti-tumor protection in this model was antigen-specific (not shown). Furthermore, AdhDCT-immunized $CD8^{-/-}$ mice all developed vitiligo in a CD4-dependent manner demonstrating that both $CD4^+$ T cell-mediated anti-tumor immunity and autoimmunity could be generated in this model (Fig. 1A). Finally, neither protection nor vitiligo development was affected by the treatment with anti-NK1.1 antibody, confirming that neither NK nor any NKT populations were required for either outcome (Fig. 1A).

Characterization of the $CD4^+$ T cell response in $CD8^{-/-}$ mice revealed the presence of T cells that could produce IFN-γ and IL-4 but not IL-17A (Fig. 1B), suggesting that AdhDCT gives rise to a mixed response of Th1 and Th2 cells without Th17 cells. Both Th1 and Th2 $CD4^+$ T cells can mediate tumor and/or tissue destruction through cytokine production. In this regard, IFN-γ and IL-4 have been reported to cause damage to the tumor microenvironment by suppressing angiogenesis and/or acting as chemoattractants for tumoricidal cells such as NK, macrophages or eosinophils [6,11,12]. To determine whether these cytokines play any role in anti-tumor and autoimmune responses in our model, $CD8^{-/-}$ mice were immunized with AdhDCT and depleted of either IL-4 or IFN-γ during the effector phase. Surprisingly, tumor protection was completely abrogated by neutralizing IL-4 but not IFN-γ (Fig. 1C). By contrast, vitiligo was diminished by blocking IFN-γ while IL-4 depletion had no obvious impact (Fig. 1C).

Anti-tumor and autoimmune effects can be blocked through STAT6 and STAT4 deletion, respectively

Although the divergence described in the previous paragraph appears to suggest that anti-tumor and autoimmune effects may require differential polarization of $CD4^+$ T cells, both IL-4 and IFN-γ can be produced by Th0 cells and non-T cell populations [14-16]. We thus set out to evaluate vaccination outcomes in Stat4-deficient ($Stat4^{-/-}$) and Stat6-deficient ($Stat6^{-/-}$) mice. STAT4 is specifically

phosphorylated in response to IL-12, and IL-12-dependent Th1 development and IFN-γ production are largely impaired in Stat4-deficient mice while Th2 functions in these mice are intact [17]. In contrast, STAT6 is activated in response to IL-4 and STAT6-deficient T cells cannot differentiate into IL-4-producing Th2 cells but they can develop into IFN-γ producing Th1 cells [18].

As shown in Figure 2A, immunization of $Stat6^{-/-}$ mice with AdhDCT resulted in both protection from tumor challenge and development of vitiligo in the absence of $CD4^+$ T cells, suggesting that $CD8^+$ T cell-mediated anti-tumor immunity and autoimmunity were fully intact in this mouse strain. Interestingly, when $Stat6^{-/-}$ mice were immunized with AdhDCT and depleted of $CD8^+$ T cells, tumor protection was lost but vitiligo was not affected indicating that anti-tumor immunity was dependent upon STAT6 signalling.

Strikingly, we observed the opposite outcome for $CD4^+$ T cell-mediated responses in $Stat4^{-/-}$ mice. Similar to the $Stat6^{-/-}$ model, immunization of $Stat4^{-/-}$ mice with AdhDCT resulted in both tumor protection and vitiligo development demonstrating that the $CD8^+$ T cell-mediated immune function was intact in this mouse strain. However, following depletion of $CD8^+$ T cells, AdhDCT-immunized $Stat4^{-/-}$ mice were still able to resist tumor challenge but they no longer developed vitiligo (Fig. 2B). Compared to the result in Figure 1C where IFN-γ-depletion only attenuated vitiligo, STAT4 deletion appears to be more effective than elimination of a particular cytokine to prevent autoimmune pathology. These results indicate that anti-tumor immunity and autoimmune

pathology mediated by $CD4^+$ T cells can be separated along the STAT4/STAT6 signaling axis.

STAT4/STAT6 signaling does not influence $CD4^+$ T cell differentiation following AdhDCT immunization

In light of the involvement of STAT4 signaling for Th1 and Th17 development, we repeated these experiments in $p40^{-/-}$ mice which lack both the IL-12 and IL-23 cytokines that play an important role in the elaboration of Th1 and Th17 functions [19,20]. Immunization of $p40^{-/-}$ mice with AdhDCT resulted in both protection from tumor growth and vitiligo development suggesting that the effect of the STAT4 deletion may not be at the level of $CD4^+$ T cell differentiation (Fig. 3).

To directly evaluate whether the absence of either STAT4 or STAT6 resulted in differential programming of DCT-specific $CD4^+$ T cells to produce cytokines, splenocytes from wild type, $Stat4^{-/-}$ and $Stat6^{-/-}$ mice immunized with AdhDCT were restimulated with a pool $CD4^+$ T cell peptide epitopes from hDCT. Total numbers of DCT-specific $CD4^+$ T cells were enumerated by the CD154 mobilization assay, which detects antigen-specific $CD4^+$ T cells independently of their cytokine secretion profile [21]. Specific cytokine-producing $CD4^+$ T cells were determined by intracellular staining (IFN-γ and IL-2) as well as ELISPOT (IL-4). Strikingly, deletion of either STAT4 or STAT6 had no impact upon the numbers of DCT-specific $CD4^+$ T cells (Fig. 4A) or the frequencies of IFN-γ-, IL-2- and IL-4-secreting cells (Fig. 4B and C) relative to matched wild type control mice. In

light of these data, we infer that the $CD4^+$ T cell population elicited by AdhDCT is likely Th0 or a mix of Th1 and Th2 responses.

DISCUSSION

To our knowledge, this is the first report that demonstrates a reciprocal role of STAT4 and STAT6 signaling pathways in the development of autoimmune pathology and anti-tumor immunity mediated by effector $CD4^+$ T cells. Although antibody-mediated blockade of IL-4 and IFN-γ produced effects similar to the STAT6 and STAT4 deficiency, respectively, there was no evidence of altered Th polarization in the STAT-deficient mice arguing against the possibility that these reciprocal effects were reflective of a mixed population of DCT-specific Th1 and Th2 cells. Rather, our data suggest that the STAT proteins may play an important role in the signaling pathway that is extrinsic to $CD4^+$ T cell differentiation.

The absence of autoimmune vitiligo in $Stat4^{-/-}$ mice, even when the skin was deliberately damaged with a highly inflammatory agent, is a striking observation. It is consistent with reports from murine models of autoimmunity, such as experimental autoimmune encephalomyelitis and diabetes, where disease pathology was associated with signaling via STAT4, while STAT6 signaling pathway was associated with resistance to disease pathology [22,23]. This paradigm was previously interpreted as a reflection of the opposing functions of Th1

(STAT4-dependent) and Th2 (STAT6-dependent) effector cells, where pathogenic Th1 cells were believed to be counter-regulated by Th2 cells [22]. However, the role of Th1 cells in autoimmune pathology has been challenged by the recent discovery of Th17 cells and the associated evidence revealing that Th17 are responsible for some autoimmune pathologies that had been previously ascribed to Th1 cells [24]. Although STAT4 signaling influences the development of both Th1 and Th17 responses [25], our data do not support a role for either population in the development of autoimmune pathology in our model. The Stat4$^{-/-}$ mice showed no evidence of skewing in CD4^{+} T cell populations relative to WT or Stat6$^{-/-}$ mice. Vitiligo developed in mice lacking p40, a subunit for both IL-12 and IL-23 that are critical for Th1 and Th17 differentiation [19], arguing against a role for polarized CD4^{+} T cells in our vitiligo model. The likelihood of Th17 cells contributing to vitiligo in our model is further refuted by our inability to measure any IL-17-producing DCT-specific CD4^{+} T cells. Although neutralizing IFN-γ largely diminished autoimmune vitiligo, it could be detected in all immunized animals including the Stat4$^{-/-}$ mice, suggesting that other signaling pathways exist to bypass STAT4-dependent production of IFN-γ in this model [26-28]. We believe that our data are consistent with a role of STAT4 signaling downstream of the effector CD4^{+} T cells as seen in a diabetes adoptive transfer model where diabetogenic CD4^{+} T cells failed to produce disease in Stat4$^{-/-}$ hosts [29]. If our hypothesis is proven to be correct, then targeting of STAT4 with specific inhibitors may provide a useful tool for suppressing autoimmune sequelae

following cancer immunotherapy using $CD4^+$ T cell-dependent strategies.

Another striking observation is that tumor rejection was mediated by IL-4 and dependent on the STAT6 signaling pathway. More importantly, this anti-tumor mechanism did not evoke fulminant autoimmune pathology. The utility of Th2 cells or IL-4 as anti-tumor effectors has been reported by others and in some cases, Th2 cells were more effective than Th1 cells in the clearance of CTL-resistant tumors [10]. Furthermore, a human clinical trial of Canvaxin, a cell-based vaccine, found that the magnitude of vaccine-induced Th2 immunity correlated with overall survival, and anti-tumor Th2 cells can be identified in cancer patients supporting the possibility of generating such populations in humans [30,31]. As discussed above, we did not observe any influence of STAT6 signaling on the differentiation of DCT-specific $CD4^+$ T cells, suggesting that STAT6 is also required downstream of the effector $CD4^+$ T cells. Consistent with our observation, Mattes et al demonstrated that tumor regression mediated by transferred Th2 effector cells was dependent on host expression of STAT6 [10]. Furthermore, there is ample evidence that STAT6 plays a critical role in the late phase of allergic asthma, in addition to its requirement in Th2 differentiation [32,33]. In light of the data presented herein, it is reasonable to investigate strategies for the development of cancer vaccines where the inoculum is designed to bias toward a Th2/IL-4 response rather than typical strategies that aim for Th1/IFN-γ production.

In summary, our results clearly indicate that CD4-mediated autoimmune vitiligo and tumor immunity produced by immunization with AdhDCT are differentially regulated by the STAT4/STAT6 signaling axis. This appears to be a unique feature of $CD4^+$ T cell-mediated processes because the DCT-specific $CD8^+$ T cells in both $Stat4^{-/-}$ and $Stat6^{-/-}$ mice were capable of producing both anti-tumor immunity and autoimmune vitiligo. Although our AdhDCT vaccine did not result in Th1/Th2 differentiation, designing vaccines that can deviate antigen-specific Th1/Th2 balance should further increase anti-tumor immunity and offer a novel strategy to control autoimmune sequelae without the need to target individual cytokines.

MATERIALS AND METHODS

Animals

C57BL/6 (H-2^b) female mice were obtained from Charles River Laboratories (Wilmington, MA). Different gene knockout mice on the C57BL/6 background, including CD8α-deficient ($CD8^{-/-}$; Jackson Laboratories, Bar Harbor, ME), IL-12-deficient (IL-12 $p40^{-/-}$; provided by Jeanne Magram, Hoffmann-La Roche, NJ), Igμ-deficient ($B^{-/-}$; Jackson Laboratories), CD1d-deficient ($CD1^{-/-}$; provided by Albert Bendelac, University of Chicago), Stat6-deficient ($Stat6^{-/-}$; provided by Klaus Matthaei, Australian National University) [18], and Stat4-deficient mice

(Stat4$^{-/-}$) [17] were bred in our specific pathogen-free animal facility. All animal experiments were approved by the McMaster Animal Research Ethics Board.

Cell culture and viral vectors

B16F10, a melanoma cell line derived from C57BL/6 mice and B78H.1, a variant of B16 melanoma that does not express the DCT antigen (obtained from Hyam I. Levitsky, Johns Hopkins University) were cultured in complete MEM-F11 media containing 10% FBS. AdhDCT is an E1/E3-deleted adenovirus that expresses the full-length human DCT gene and AdBHG is an E1/E3-deleted virus that expresses no transgene [1].

Immunization and tumor challenge

Mice were immunized with 1 x 10^8 pfu of AdhDCT by intramuscular injection. Negative control mice received PBS or AdBHG. Fourteen days later, mice were challenged subcutaneously in the right hind flank with 1 x 10^5 B16F10 cells. Tumor size was monitored daily and measured twice a week for 60 days.

Vitiligo induction

Our previous studies showed that manifest autoimmune pathology following intramuscular vaccination with AdhDCT could be intensified by delivery of an inflammatory agent to the skin [1]. To intensify the autoimmune pathology used as a read-out for these studies, mice were immunized with AdhDCT and, fourteen

days later, their skins were "painted" with 120 µl of 2,4-dinitrobenzenesulfonic acid (DNFB, 0.2% diluted in acetone/olive oil at a 4:1 ratio) (Sigma-Aldrich, St. Louis, MO). Vitiligo development was monitored weekly for 8 weeks after the DNFB challenge.

Immunodepletion and neutralization studies

Immunodepletion studies were conducted using the following mAbs from American Type Culture Collection (ATCC, Manassas, VA): GK1.5 (anti-CD4), 2.43 (anti-CD8) and PK136 (anti-NK1.1). Purified mAbs (100 µg) were injected i.p. 2 days before tumor or DNFB challenge and then twice a week for 4 weeks. A rat IgG (Sigma, St. Louis, MO) was used as control. The efficiency of specific depletion of lymphocyte subsets was >98% by flow cytometry using different clones of antibodies (data not shown). To block IFN-γ or IL-4 activity in vivo, mice were injected i.p. with 200 µg of purified mAb R4-6A2 (anti-IFN-γ; ATCC) or 11B11 (anti-IL-4; ATCC) based on the following protocol. Antibody administration started 2 days before the tumor or DNFB challenge and then once every other day for two weeks. At that point, tumor growth was evident in control animals and the DNFB-caused lesion was completely healed.

Enzyme-linked immunospot (ELISPOT) assays

The ELISPOT assay was performed as described previously [34]. Spleens from AdhDCT-immunized mice were harvested 10 days post-immunization, which

corresponds to the peak of the $CD4^+$ T cell response. Briefly, splenocytes (5 x 10^5) were aliquoted into the appropriate wells and restimulated with DMSO or a peptide pool corresponding to the immunodominant ($hDCT_{88-102}$, RKFFHRTCKCTGNFA) and subdominant ($hDCT_{242-254}$, LPYWNFATGRNEC; $hDCT_{449-463}$, DQLGYSYAIDLPVSV) $CD4^+$ T cell epitopes from hDCT (10 μg/ml each) [34]. The number of cytokine producing cells was enumerated using an ImmunoSpot 3B analyzer (Cellular Technology Ltd.). Results are shown as mean +/- SEM from 10 mice pooled from 2 separate experiments.

Intracellular cytokine staining (ICS)

Intracellular cytokines were visualized using a protocol we described previously [34]. Briefly, aliquots of 2 x 10^6 splenocytes were plated in 96-well U-bottomed plates and restimulated with DMSO or with the peptide pool previously described for 1 hour at 37°C, 5% CO_2. Brefeldin A (BD Biosciences) was added to a final concentration of 5 μg/ml and the incubation was continued for another 4 hours. The cells were then stained with anti-CD4-PeCy5.5 (clone RM4-5), treated with Cytofix/Cytoperm (BD Biosciences) and stained with anti-IFN-γ-APC (clone XMG1.2), or anti-IL-2-PE (clone JES6-5H4). Alternatively, the cells were stained with anti-CD154-APC (clone MR1), which was added at the beginning of the restimulation, followed by the addition of 2 μM of monensin (BD Biosciences) instead of Brefeldin A. All antibodies were purchased from BD Biosciences with the exception of the anti-CD154-APC antibody, which was

obtained from eBioscences. Data from stained samples were acquired using either an LSRII or a FACSCanto (BD Biosciences) and analyzed using FlowJo (Treestar). Results are shown as mean +/- SEM from 10 mice pooled from 2 separate experiments.

Statistical analysis

A two-tailed, paired Student *t* test was used for the analysis.

ACKNOWLEDGMENTS

We would like to thank Drs. A. Bendelac and K. I. Matthaei for providing the breeding pairs of CD1 knockout and Stat6 knockout mice, respectively. We also thank Carole Evelegh and Chuyan Ying for preparing the viruses and antibodies used in these experiments. This work was supported by grants to Y.W. from the Canadian Institutes of Health Research [MOP-67066] and the Ontario Cancer Research Network. D.B. was supported by a studentship from the Canadian Institutes for Health Research.

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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FIGURE LEGENDS

Figure 1. Both tumor protection and vitiligo induction require $CD4^+$ T cells and depend on IL-4 and partially on IFN-γ, respectively. $CD8^{-/-}$ mice were immunized with either PBS, AdBHG or AdhDCT and then challenged 14 days post-immunization with 1 x 10^5 B16F10 cells via s.c. injection or 120 µl of DNFB painted on the shaved skin. (***A***) Depletion of $CD4^+$ T cells or $NK1.1^+$ cells was performed by Ab injection starting on day 12 and subsequently administered twice a week for 4 weeks. (***B***) DCT-specific $CD4^+$ T cell responses were measured via IFN-γ, IL-4 and IL-17 ELISPOT assays using a hDCT CD4 peptide pool to restimulate splenocytes derived from day 10 AdhDCT-immunized $CD8^{-/-}$ mice. (***C***) Depletion of IL-4 or IFN-γ was performed by Ab injection starting on day 12 and then administered every other day for two weeks. The results are summarized from two individual experiments with 5-6 mice for each group. The ELISPOT results are shown as mean +/- SEM from 10 mice pooled from 2 separate experiments. Photographs are representative of 15 mice in each group from 3 separate experiments.

Figure 2. CD4-mediated anti-tumor immunity and autoimmunity are differentially regulated by the STAT4/STAT6 signaling axis. $Stat6^{-/-}$ (***A***) or $Stat4^{-/-}$ (***B***) mice were immunized with either PBS, AdBHG or AdhDCT and challenged 14 days post-immunization with 1 x 10^5 B16F10 cells via s.c. injection

or 120 μl DNFB. Depletion of $CD4^+$ or $CD8^+$ T cells was performed by Ab injection starting on day 12 and then administered twice a week for 4 weeks. The results are summarized from two individual experiments with 5-6 mice for each group. Photographs are representative of 10-15 mice in each group.

Figure 3. CD4-mediated STAT4-dependent autoimmunity does not rely on Th1 or Th17 cells. IL-12 $p40^{-/-}$ mice were immunized with either PBS, AdBHG or AdhDCT and challenged 14 days post-immunization with 1 x 10^5 B16F10 cells via s.c. injection or 120 μl DNFB. Depletion of $CD4^+$ or $CD8^+$ T cells was performed by Ab injection starting on day 12 and then administered twice a week for 4 weeks. The results are summarized from two individual experiments with 5-6 mice for each group. Photographs are representative of 10 mice in each group.

Figure 4. DCT-specific $CD4^+$ T cells harbor an unpolarized Th0 phenotype. Splenocytes from day 10 AdhDCT-immunized WT, $Stat6^{-/-}$ and $Stat4^{-/-}$ mice were restimulated with a hDCT CD4 peptide pool and $CD4^+$ T cell responses were measured via ICS of CD154 (***A***) or IFN-γ/IL-2 (***B***) and IL-4 ELISPOT (***C***). Results are shown as mean +/- SEM from 10 mice pooled from 2 separate experiments. No results comparing WT vs $Stat6^{-/-}$ or WT vs $Stat4^{-/-}$ mice were found to be statistically significantly different.

Table 1. Induction of protective immunity and vitiligo following immunization with AdhDCT in $CD1^{-/-}$ and $B^{-/-}$ mice. $CD1^{-/-}$ or $B^{-/-}$ mice were immunized with 1 x 10^8 pfu AdhDCT for 14 days and then challenged s.c. with 1 x 10^5 ($CD1^{-/-}$ mice) or 5 x 10^5 ($B^{-/-}$ mice) B16F10 cells or painted on the shaved skin of the back with 120 µl DNFB. Antibodies were injected beginning two days before the tumor or DNFB challenge and then once every other day for two weeks. The onset of tumor formation and development of vitiligo were monitored for 60 days. Immunization with PBS or a control vector AdBHG had no impact on tumor protection and vitiligo induction (not shown).

	$CD1^{-/-}$		**$B^{-/-}$**	
Immunodepletion	**Protection**	**Vitiligo**	**Protection**	**Vitiligo**
None	12/12	10/10	15/15	15/15
CD4	5/5	5/5	10/10	10/10
CD8	8/8	10/10	10/10	10/10
CD4 + CD8	0/7	0/9	0/10	0/10

FIGURE 1-

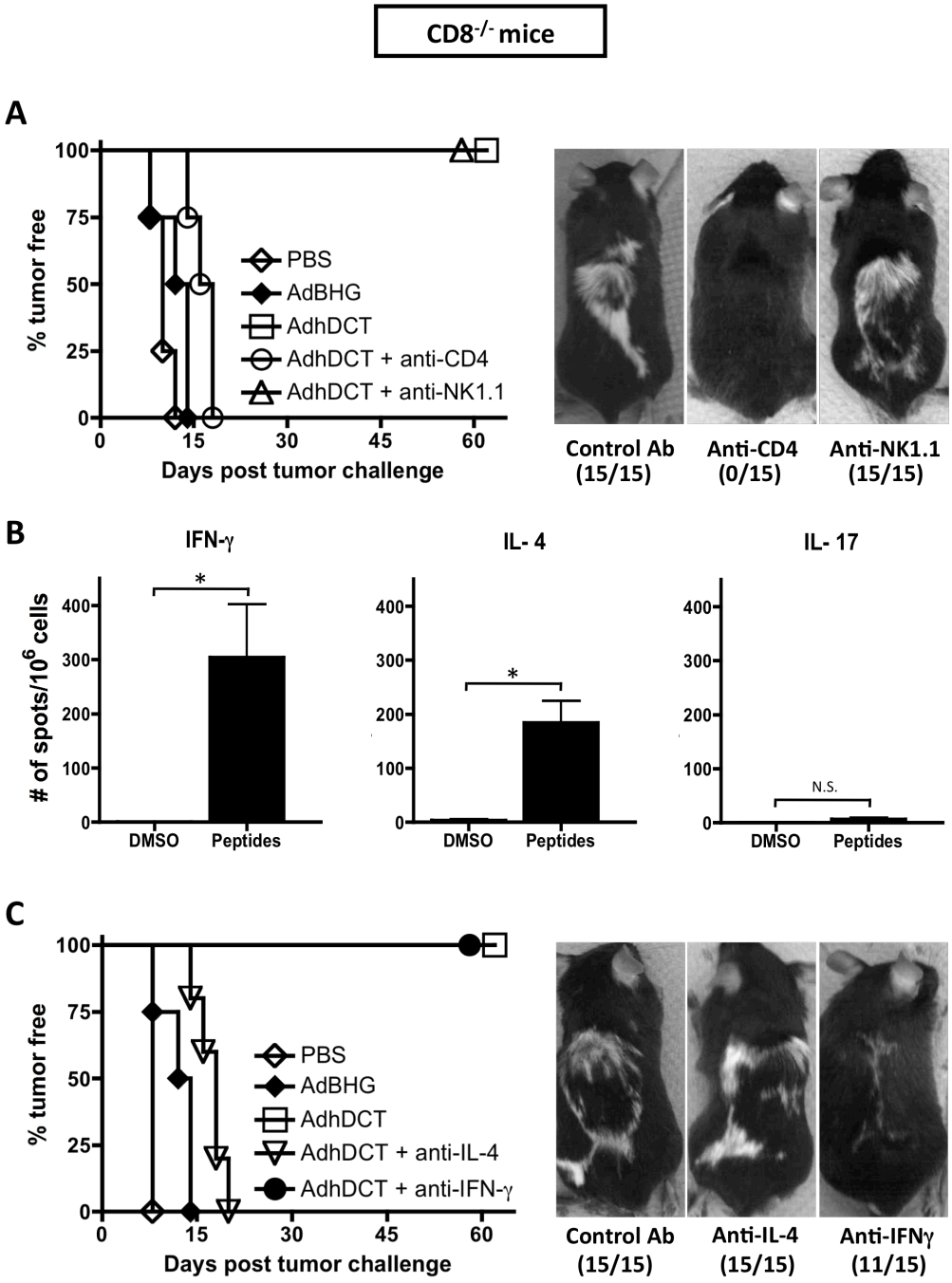

CD8-/- mice
A
100
75
50
25
0
% tumor free
0
15
30
45
60
Days post tumor challenge
PBS
AdBHG
AdhDCT
AdhDCT + anti-CD4
AdhDCT + anti-NK1.1
Control Ab (15/15)
Anti-CD4 (0/15)
Anti-NK1.1 (15/15)
B
IFN-γ
IL- 4
IL- 17
# of spots/10^6 cells
400
300
200
100
0
*
*
N.S.
DMSO
Peptides
C
100
75
50
25
0
% tumor free
0
15
30
45
60
Days post tumor challenge
PBS
AdBHG
AdhDCT
AdhDCT + anti-IL-4
AdhDCT + anti-IFN-γ
Control Ab (15/15)
Anti-IL-4 (15/15)
Anti-IFNγ (11/15)

FIGURE 2-

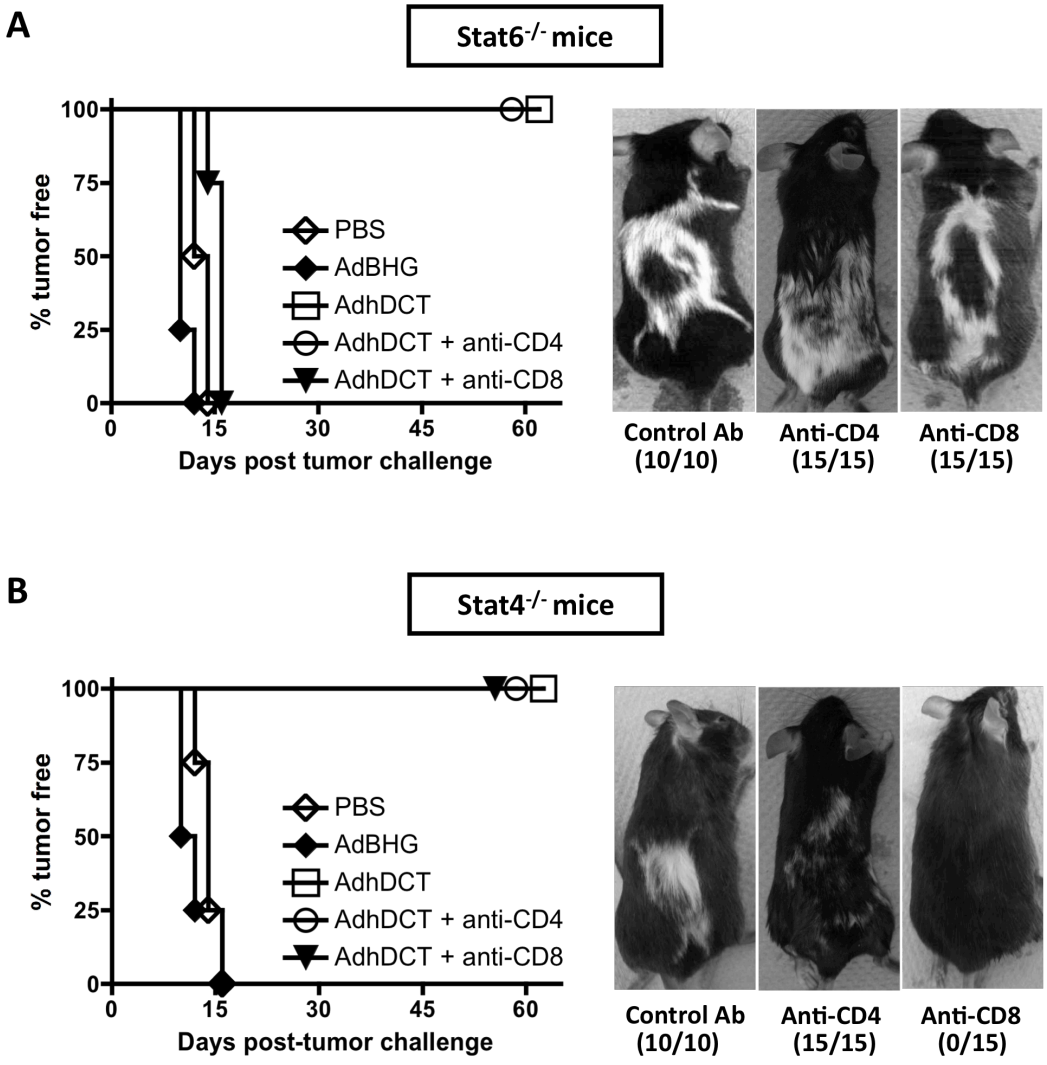

A
Stat6-/- mice
100
75
50
25
0
% tumor free
0
15
30
45
60
Days post tumor challenge
PBS
AdBHG
AdhDCT
AdhDCT + anti-CD4
AdhDCT + anti-CD8
Control Ab (10/10)
Anti-CD4 (15/15)
Anti-CD8 (15/15)
B
Stat4-/- mice
100
75
50
25
0
% tumor free
0
15
30
45
60
Days post-tumor challenge
PBS
AdBHG
AdhDCT
AdhDCT + anti-CD4
AdhDCT + anti-CD8
Control Ab (10/10)
Anti-CD4 (15/15)
Anti-CD8 (0/15)

FIGURE 3-

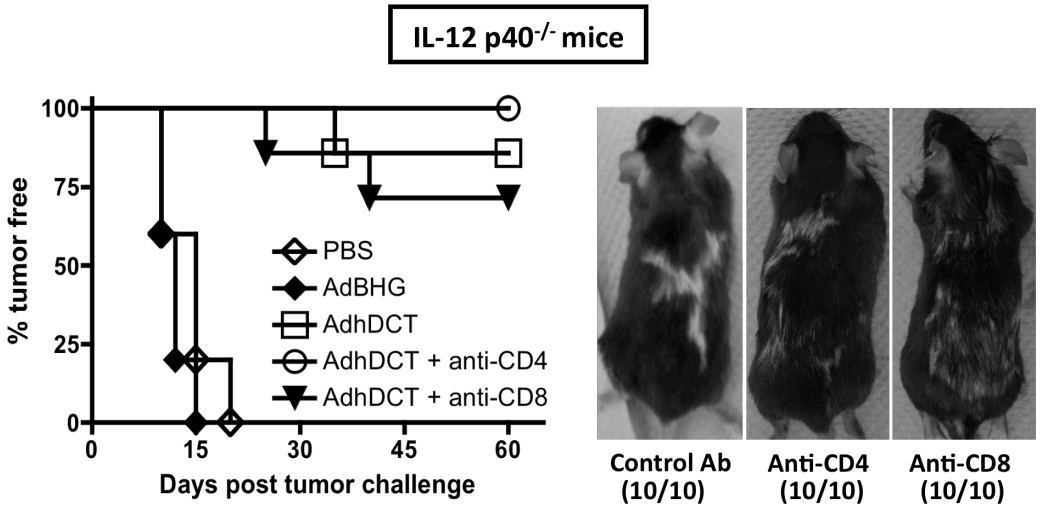
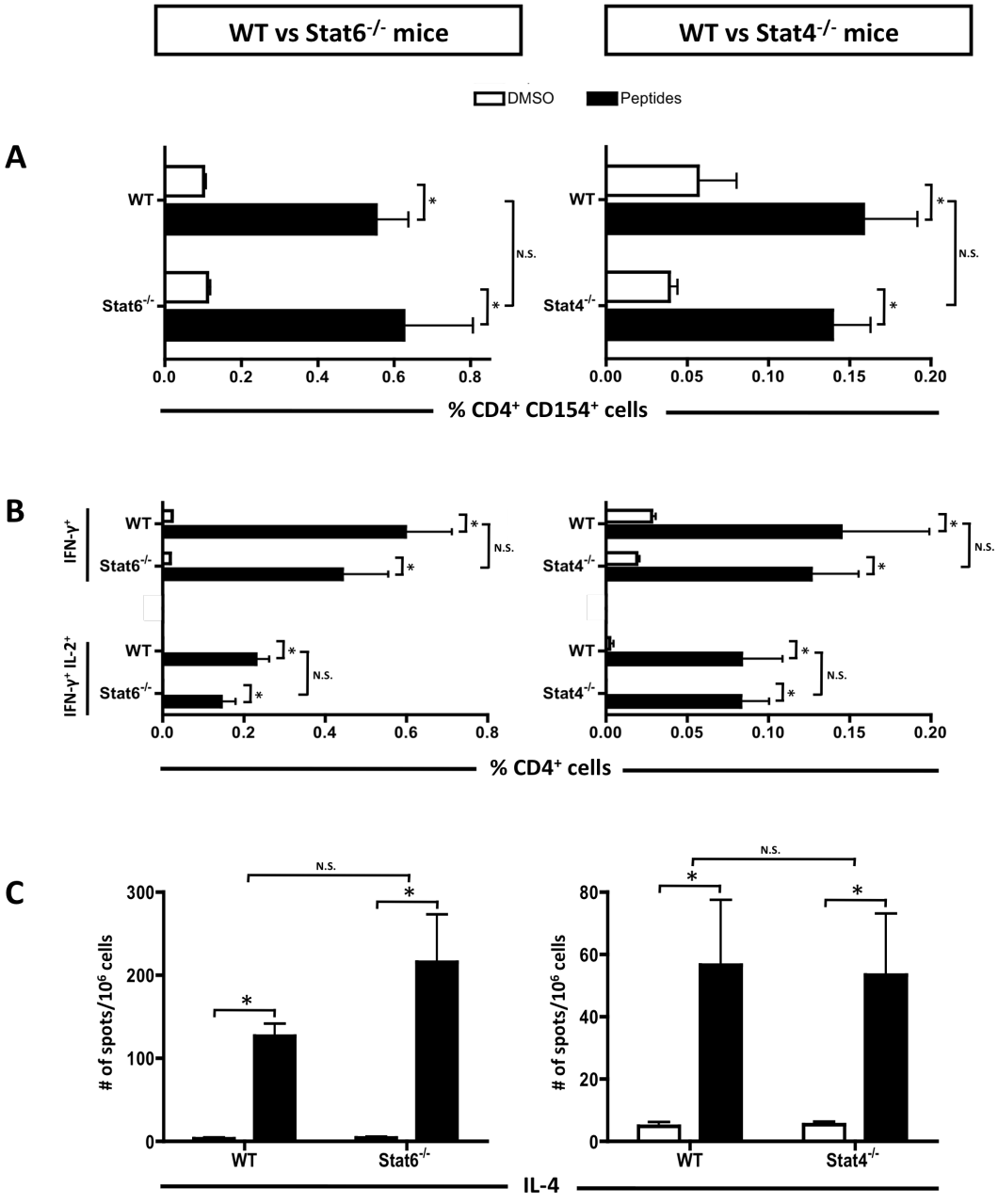

IL-12 p40-/- mice
% tumor free
100
75
50
25
0
0
15
30
45
60
Days post tumor challenge
PBS
AdBHG
AdhDCT
AdhDCT + anti-CD4
AdhDCT + anti-CD8
Control Ab (10/10)
Anti-CD4 (10/10)
Anti-CD8 (10/10)

FIGURE 4-


WT vs Stat6-/- mice
WT vs Stat4-/- mice
DMSO
Peptides
A
WT
Stat6-/-
Stat4-/-
N.S.
*
0.0
0.2
0.4
0.6
0.8
0.00
0.05
0.10
0.15
0.20
% CD4+ CD154+ cells
B
IFN-γ+
IFN-γ+ IL-2+
% CD4+ cells
C
# of spots/10^6 cells
300
200
100
0
80
60
40
20
IL-4

— CHAPTER 3 —

Processing of tumor antigen differentially impacts the development of helper and effector $CD4^+$ T cell responses

Processing of tumor antigen differentially impacts the development of helper and effector CD4$^+$ T cell responses

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Published in *Molecular Therapy*
Vol. 18(6), pp. 1224-1232, June 2010

Preface: *The research presented in this manuscript is the result of a collaboration between myself and Michael S. Ventresca, a former Master student in Dr. Bramson's laboratory. My participation to this experimental work spanned from 2006 to 2009. I carried out epitope mapping experiments, constructed one of the recombinant vaccine vectors used in this study and conducted the tumor challenge studies. Michael S. Ventresca constructed two recombinant vaccine vectors as well as performed Western blot and T cell analyses. Laura A. Marshall (undergraduate student) constructed one recombinant vaccine vector and Carole Evelegh (laboratory technician) provided technical assistance. Briefly, I contributed to figures 1-3 and generated the data presented in figure 4, supplementary figure 1 and supplementary tables 1 and 2. I also wrote the manuscript and addressed the reviewers' comments with the help of Dr. Bramson. This work was supervised by Dr. Bramson who, along with Dr. Wan, provided experimental guidance and contributed to the interpretation of the results.*

Title: Processing of tumor antigen differentially impacts the development of helper and effector $CD4^+$ T cell responses

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Running title: Post-translational modifications of CD4 epitopes

Key words: Dopachrome tautomerase, melanoma, adenovirus, vaccine, T lymphocyte

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ABSTRACT

$CD4^+$ T cells contribute to the antitumor T cell response as both effectors that promote tumor rejection and helpers that facilitate the activation of other antitumor effector cells, such as $CD8^+$ T cells. Maximal engagement of both effector and helper $CD4^+$ T cell responses is a desirable attribute of cancer vaccines. We have employed the B16F10 murine melanoma model and a series of recombinant adenovirus vaccines expressing mutant forms of the tumor antigen, dopachrome tautomerase, to investigate the relationship between antigen processing and the antitumor $CD4^+$ T cell response. Our results have revealed an unexpected dichotomy in the generation of helper and effector $CD4^+$ T cell responses where $CD4^+$ T effector responses are dependent upon protein processing and trafficking while $CD4^+$ T helper responses are not. The results have important implications for strategies aimed at augmenting antigen immunogenicity by altering intracellular processing and localization.

INTRODUCTION

$CD4^+$ T cells are a key component of the adaptive immune response against tumors and increasing evidence suggests that $CD4^+$ T cells can be highly potent antitumor effectors [1-5]. When tumor cells express MHC II, as in the case of B cell

leukemias, $CD4^+$ T cells are capable of recognizing tumor cells directly, leading to up-regulation of death-inducing ligands and secretion of cytotoxic granules [6-10]. $CD4^+$ T cells can also mediate rejection of MHC II-negative tumors through indirect mechanisms following *in situ* activation by APCs that have engulfed tumor-derived antigens. Activated $CD4^+$ T cells can promote tumor rejection indirectly through the release of tumor-suppressing cytokines [11-14] and the recruitment of innate effector cells [15,16]. This indirect pathway is an important facet of the tumor-specific effector $CD4^+$ T cells as this mechanism allows $CD4^+$ T cells to reject tumors which escape $CD8^+$ T cell recognition by down-modulation of MHC I [15-18]. Recent work from both animal and human studies has underscored the potency of effector $CD4^+$ T cells for cancer immunotherapy and even suggested that $CD4^+$ T cells may be more potent than $CD8^+$ T cells when compared on a cell-per-cell basis [19,20].

Previous investigations by our group have revealed an important role for $CD4^+$ T cells in the protective immunity produced by a prototype melanoma vaccine comprising a recombinant human adenovirus (Ad) type 5 vector expressing human dopachrome tautomerase (hDCT; vector name = AdhDCT). Immunization with AdhDCT can render immune competent mice completely protected against tumor challenge and can promote regression of established tumors when combined with cyclophosphamide [21-23]. Using a combination of antibody depletion and gene-deficient mice, we have shown that $CD4^+$ T cells play a significant role in the antitumor response produced by immunization with

AdhDCT [21,22,24]. Following AdhDCT immunization, DCT-specific $CD4^+$ T cells act as helpers for the $CD8^+$ T cell response and effectors that are capable of effectuating tumor rejection and skin depigmentation [21]. Using synthetic peptides, we identified a heteroclitic $CD4^+$ T cell epitope ($hDCT_{89-101}$) in the hDCT that functions as a target for helper $CD4^+$ T cells that promote $CD8^+$ T cell immunity [22]. Immunization with the murine homolog of DCT (mDCT) produces only a weak $CD8^+$ T cell response. By converting Gln86 and Asn92 in mDCT to Leu and His, respectively, it is possible to engineer the mDCT protein to carry the heteroclitic epitope found in hDCT [22]. The $CD8^+$ T cell response produced by the mutant protein was 10-fold greater than the response to wild type mDCT and comparable to the response produced by hDCT, confirming the helper function of the $CD4^+$ T cells directed against this epitope [22]. Interestingly, $CD4^+$ T cells directed against $hDCT_{89-101}$ do not effectuate tumor rejection [22]. Therefore, we refer to $hDCT_{89-101}$-specific $CD4^+$ T cells as *helpers*. We have been unable to define a target for the $CD4^+$ T cells that promote tumor rejection but they can be identified functionally using tumor challenge studies in mice that lack $CD8^+$ T cells [21,24]. In the context of this manuscript, we will refer to the latter population as *effectors* since they effectuate tumor rejection. Further, we have uncovered an interesting dichotomy with regard to the processes involved in $CD4^+$ T cell-mediated antitumor immunity and autoimmunity whereby the former was dependent on IL-4/STAT6 signaling while the latter required IFN-γ/STAT4 signaling [24]. Our current efforts are directed at exploiting this dichotomy and

maximizing $CD4^+$ T cell-mediated tumor rejection while minimizing autoimmune sequelae.

In this manuscript, we describe an Ad vector that expresses a mutant form of hDCT lacking the dominant $CD8^+$ T cell epitope, SVYDFFVWL (AdhDCTΔVYD). This vector was created to facilitate studies of $CD4^+$ T cell-dependent antitumor immunity without the mitigating effects of hDCT-specific $CD8^+$ T cells. Surprisingly, this mutant failed to elicit protective $CD4^+$ T cell immunity. Characterization of the hDCTΔVYD protein and analysis of additional mutants revealed that the protective $CD4^+$ T cell response was dramatically affected by intracellular processing of hDCT whereas the helper $CD4^+$ T cell response was not. These results have important implications for vaccine design and indicate that care must be taken when manipulating antigens in an effort to increase their immunogenicity.

RESULTS

Immunization with a mutant hDCT that lacks the immunodominant CD8 epitope, SVYDFFVWL, results in loss of both $CD8^+$ and $CD4^+$ T cell-mediated tumor protection

Previous reports have demonstrated that the hDCT epitope, SVYDFFVWL ($DCT_{180\text{-}188}$), is alone sufficient to produce protective immunity [25,26]. Therefore,

we created a variant of hDCT (hDCTΔVYD, **Fig. 1a**) which lacked this epitope in an effort to generate an antigen that would only evoke protective $CD4^+$ T cell immunity. The serine residue at position 180 was not removed in order to preserve a putative N-linked glycosylation motif (N-X-S) found immediately upstream of $DCT_{180\text{-}188}$ ($^{178}NCS_{180}$). Mice were immunized with Ad vaccines expressing either wild type hDCT (AdhDCT) or the mutant (AdhDCTΔVYD) to examine immunogenicity via ICS analysis (see **Fig. S1** for example of the flow cytometry data). As expected, the $hDCT_{180\text{-}188}$-specific response was fully abrogated following immunization with AdhDCTΔVYD while $CD8^+$ T cell immunity to the subdominant epitope, $hDCT_{342\text{-}351}$, was intact (**Fig. 1b**). In fact, immunity to $hDCT_{342\text{-}351}$ was significantly increased ($p<0.05$) in mice immunized with AdhDCTΔVYD. We previously demonstrated that the magnitude of the $CD8^+$ T cell response to $hDCT_{342\text{-}351}$ was dependent upon helper $CD4^+$ T cells responsive to the heteroclitic epitope $hDCT_{89\text{-}101}$ [22]. The robust $CD8^+$ T cell response to $hDCT_{342\text{-}351}$ suggests that the mutation in AdhDCTΔVYD did not impair the development of helper $CD4^+$ T cells. Indeed, examination of $CD4^+$ T cell immunity to the dominant and subdominant $CD4^+$ T cell epitopes in hDCT, $hDCT_{89\text{-}101}$ and $hDCT_{242\text{-}254}$, revealed no differences between the wild type and mutant vaccines (**Fig. 1c**).

Our previous work demonstrated that both $CD4^+$ T cells and $CD8^+$ T cells were capable of independently protecting against lethal challenge with B16F10 following immunization with AdhDCT [21,22] and it was necessary to ablate both

cell populations to abrogate the protective effect [21]. Indeed, we show that $CD8^+$ T cell depletion prior to tumor challenge does not impair protective immunity imparted by AdhDCT (compare **Figs. 1d,e**). Therefore, we expected AdhDCTΔVYD to provide robust protection against B16F10 challenge through a $CD4^+$ T cell-dependent mechanism. Surprisingly, the protective response produced by AdhDCTΔVYD was largely attenuated relative to AdhDCT (**Fig. 1d**). The modest protective immunity afforded by AdhDCTΔVYD was lost following depletion of $CD8^+$ T cells, indicating that the protection was provided by immunity elicited by subdominant $CD8^+$ T cell epitopes, like $hDCT_{342\text{-}351}$ (**Fig. 1e**).

We have previously determined that CD8-deficient mice can be used to study $CD4^+$ T cell protective immunity generated by immunization with AdhDCT [22,24]. Therefore, we also evaluated protection against B16F10 following immunization with AdhDCTΔVYD in CD8-deficient mice. Similar to the scenario in CD8-depleted wild type mice, immunization with AdhDCT provided complete protection against B16F10 whereas no protection was afforded by immunization with AdhDCTΔVYD (**Fig. 1f**). The surprising observation that removal of a $CD8^+$ T cell epitope attenuated the protective $CD4^+$ T cell response prompted us to further investigate the mechanisms underlying the generation of helper and effector $CD4^+$ T cell responses following AdhDCT immunization.

Comprehensive epitope mapping of the region surrounding the deletion in AdhDCTΔVYD did not reveal any previously unidentified CD4 epitopes

It seemed paradoxical that the helper $CD4^+$ T cell response elicited by hDCT was unaffected by the deletion of residues 181 – 188 while the protective $CD4^+$ T cell response was fully attenuated. The simplest explanation for this observation is that the deletion also affects a $CD4^+$ T cell epitope. Using a combination of IFN-γ, IL-4 and IL-10 ELISPOT assays, we have conducted extensive mapping of the DCT T cell epitopes in both CD8-deficient mice (data not shown) and wild type mice [22]. We could not identify a $CD4^+$ T cell epitope that correlated with tumor rejection. Based on the current results, we prepared a comprehensive set of synthetic peptides that spanned the entire region deleted in hDCTΔVYD (**Table S1**). ICS analysis of these peptides using splenocytes from CD8-deficient mice immunized with AdhDCT failed to reveal new reactivities (**Table S1**). These results argue against the likelihood that removal of residues 181 – 188 disrupted a $CD4^+$ T cell epitope in hDCT.

Mutation of the putative glycosylation site $^{178}NCS_{180}$ results in impaired protein trafficking and attenuated $CD4^+$ T cell-mediated protection

Protein conformation can significantly impact the presentation of epitopes on MHC II. As an example, Golgher *et. al.* determined that recognition of gp90 by $CD4^+$ T cells was dependent on the peptide conformation which was controlled by glycosylation [27]. Since the deletion in hDCTΔVYD was adjacent to a putative N-

linked glycosylation site, we suspected that glycosylation at this site may have been disrupted. On Western blot analysis, hDCT resolves as two distinct glycoforms of 69 and 80 kDa [28] (**Fig. 2a, lane 1**). When we compared the migration pattern of hDCTΔVYD to hDCT by Western blot, we observed that the higher molecular weight form (80 kDa) was lacking from hDCTΔVYD (**Fig. 2a, lane 4**) and that minor, smaller molecular weight forms (<69 kDa) were appearing. These results were reminiscent of a previous report where glycosylation of TRP-1 was disrupted [29]. Based on the proximity of the deletion in hDCTΔVYD to a putative glycosylation site ($^{178}NCS_{180}$), we suspected that this mutation may have disrupted glycosylation at N178. We, therefore, created a hDCT mutant where N178 was replaced with D178 (hDCT-N178D, **Fig. 2b**) to determine whether lack of glycosylation at this site was the determining factor in the impaired immunogenicity of AdhDCTΔVYD. The hDCT-N178D mutant, similarly to hDCTΔVYD, also failed to produce the higher molecular weight form (**Fig. 2a, lane 7**) and both mutants seemed to give rise to the development of minor glycoforms that were <69 kDa but greater than 59 kDa (the estimated molecular weight of the protein).

The 69 kDa glycoform of hDCT is found in the endoplasmic reticulum (ER) following translocation of the protein from the cytoplasm and addition of initial N-linked glycans; this glycoform appears to represent the majority of the hDCT when expressed from recombinant Ad (**Fig. 2a, lane 1**). The 80 kDa glycoform is found in the Golgi and represents a more mature form of the protein

that harbors complex glycan structures as well as additional post-translational modifications [28]. This glycoform is only a minor component of the hDCT produced by AdhDCT infection, suggesting that the majority of the virally-expressed protein remains in the ER. To confirm the localization of the various glycoforms of hDCT, the proteins were subjected to digestion with glycosidases [28]. $EndoH_f$ specifically cleaves N-linked glycans that are attached in the ER whereas PNGaseF cleaves all sugar chains, including complex structures added in the Golgi. The fully de-glycosylated proteins migrated at an approximate weight of 59 kDa (**Fig. 2a, lanes 3, 6, 9**), which is consistent with the estimated molecular weight of hDCT. As predicted, $EndoH_f$ treatment caused the 69 kDa band to appear as 59 kDa but did not affect the 80 kDa band (**Fig. 2a, lanes 2, 5, 8**) supporting the assertion that the 80 kDa represents a more mature form of the protein that has migrated to the Golgi. Since neither hDCT-N178D nor hDCTΔVYD gave rise to the 80 kDa glycoform of DCT, we conclude that these proteins do not migrate to the Golgi.

We next evaluated the immunogenicity of AdhDCT-N178D. As in the case of AdhDCTΔVYD, the $CD4^+$ T cell responses to $hDCT_{89\text{-}101}$ and $hDCT_{242\text{-}254}$ were comparable to those elicited by AdhDCT (**Fig. 2c**). To confirm that the helper functions were unaffected, we also examined the $CD8^+$ T cell responses and found that $CD8^+$ T cell immunity was intact as well (**Fig. 2d**). Thus, despite the protein trafficking defect, AdhDCT-N178D evokes a helper T cell response comparable to AdhDCT. To examine the effector $CD4^+$ T cell response produced

by AdhDCT-N178D, we immunized CD8-deficient mice as described above [22,24]. Similar to AdhDCTΔVYD, protective immunity produced by AdhDCT-N178D was significantly impaired compared to AdhDCT (**Fig. 2e**). Thus, although the helper $CD4^+$ T cell response produced by AdhDCT-N178D was unimpaired relative to AdhDCT, the effector $CD4^+$ T cell response was significantly attenuated.

De-glycosylation of hDCT putative N-linked glycosylation sites does not generate any new CD4 epitopes

Since the presence of the 80 kDa molecular weight glycoform of hDCT correlated with the generation of protective $CD4^+$ T cell immunity, we considered a previous report involving tyrosinase where a T cell epitope was generated by cytoplasmic de-glycosylation of N-linked glycans, which resulted in the conversion of asparagine to aspartic acid [30]. We hypothesized that the generation of the CD4 effector epitope(s) may be dependent upon a similar event. So, we synthesized peptides covering all putative N-linked glycosylation motifs (N-X-C/S/T) and substituted asparagine residues for aspartic acid residues (**Table S2**). Potential reactivity to these peptides was evaluated via ICS analysis. Results obtained from these experiments failed to uncover any new CD4 reactivities, indicating that conversion of asparagines to aspartic acid following de-glycosylation of hDCT does not give rise to measurable T cell responses following immunization with AdhDCT (**Table S2**).

Immunization with a trafficking-defective variant of hDCT results in abrogated $CD4^+$ T cell-mediated tumor protection

The melanosomal transport signal present in melanoma-associated antigens (gp100, TRP-1/gp75, TRP-2/DCT) can direct antigens to the MHC II compartment and enhance $CD4^+$ T cell stimulation [31,32]. Therefore, the impaired effector $CD4^+$ T cell response observed following immunization with AdhDCTΔVYD and AdhDCT-N178D may reflect improper intracellular trafficking rather than the inability to generate a conformational or glycosylation-dependent effector $CD4^+$ T cell epitope. To address the possibility that targeting of hDCT is important for the generation of the effector $CD4^+$ T cell responses, we removed two key targeting elements from the protein: the ER signal sequence (SS, residues deleted: 2-23) [33] and the putative melanosomal transport signal (MTS, residues deleted: 491-496) (**Fig. 3a**). hDCTΔSS lacks the ER signal sequence and thus is expected to remain cytoplasmic. hDCTΔMTS lacks the melanosomal targeting signal, but retains the transmembrane domain and is expected to traffic through the Golgi without being re-directed to melanosomes. As expected, Western blot analysis shows that hDCTΔSS completely lacks N-linked glycosylation (**Fig. 3b, lanes 4-6**). Interestingly, hDCTΔMTS actually appeared to be more stable than hDCT and gave rise to higher levels of the mature 80 kDa glycoform (**Fig. 3b, lane 7**). With regard to the generation of $CD4^+$ T cell immunity, both AdhDCTΔSS and AdhDCTΔMTS were able to elicit $hDCT_{89\text{-}101}$- and $hDCT_{242\text{-}254}$-specific $CD4^+$ T cell responses of a similar magnitude to

AdhDCT (**Fig. 3c**). In fact, the response to $hDCT_{89-101}$ was significantly higher in mice immunized with AdhDCTΔMTS compared to AdhDCT ($p<0.05$), likely due to the fact that this protein variant appears to accumulate to higher levels. $DCT_{180-188}$-specific $CD8^+$ T cell responses produced by AdhDCTΔSS were reduced relative to AdhDCT ($p<0.005$; **Fig. 3d**), consistent with the observation that this mutant is expressed at lower levels than the wild type protein; however, the $CD8^+$ T cell response to $hDCT_{342-351}$ was intact and the response to $DCT_{180-188}$ was still markedly higher than the response to the murine form of the protein (mDCT) which cannot elicit $CD4^+$ T cell help [22] indicating that the helper response produced by AdhDCTΔSS was intact. Likewise, the $CD8^+$ T cell response produced by AdhDCTΔMTS was comparable to the wild type vaccine indicating that the helper $CD4^+$ T cell response evoked by this vaccine was intact as well (**Fig. 3d**).

Generation of protective $CD4^+$ T cell immunity was next evaluated in CD8-deficient mice. As shown in **Figure 3e**, $CD4^+$ T cell-mediated tumor protection was completely abrogated in mice immunized with AdhDCTΔSS whereas mice immunized with AdhDCTΔMTS displayed tumor protection comparable to AdhDCT. We can conclude from these latter observations that the melanosomal transport signal is not critical for the production of either helper or effector $CD4^+$ T cell responses. Deletion of the ER signal sequence, similar to the ΔVYD and N178D mutations, impaired the generation of effector $CD4^+$ T cell immunity whereas the helper response remained unaffected.

AdhDCTΔVYD fails to elicit protective CD4$^+$ T cell immunity in DCT-deficient mice

We have previously demonstrated that CD4$^+$ T cell immunity to DCT is more stringently regulated than the CD8$^+$ T cell response [22]. Therefore, this dichotomy between helper and effector CD4$^+$ T cell responses may not be a general property of the DCT antigen, but rather simply reflects a lack of CD4$^+$ T cell effectors that can respond to DCT in wild type mice. To address this possibility, we have immunized DCT-deficient mice with the mutant viruses to determine whether a similar dichotomy exists under conditions where CD4$^+$ T cell immunity to hDCT is less stringently regulated. Inoculation of DCT-deficient mice with AdhDCT evokes a more robust CD4$^+$ T cell response than wild type mice [22]. Interestingly, reactivity to a dominant CD4 epitope, DCT_{70-82}, appears only in DCT-deficient mice. This epitope is fully conserved between the murine and human forms of the protein [22] suggesting that it could be targeted for rejection by DCT-specific CD4$^+$ T cells. We immunized DCT-deficient mice with either AdhDCT or AdhDCTΔVYD and depleted the CD8$^+$ T cell prior to tumor challenge to visualize CD4$^+$ T cell-dependent tumor rejection. The immune response elicited by AdhDCTΔVYD to the dominant CD4$^+$ T cell epitopes and subdominant CD8$^+$ T cell epitope was comparable to AdhDCT (**Fig. 4a,b**). However, only the mice immunized with AdhDCT were protected from challenge following CD8$^+$ T cell depletion (**Fig. 4c**).

DISCUSSION

In the present report, we provide evidence that the generation of helper and effector $CD4^+$ T cell responses can be markedly affected by antigen structure and processing. Variants of the hDCT antigen that harbor mutations within the region of a putative glycosylation motif ($^{178}NCS_{180}$) show altered protein processing. Consistent with this observation, a previous report has suggested that the N178 glycosylation site in hDCT, which is highly conserved, is associated with protein translocation [34]. As a result, it is likely that glycan maturation and other post-translational modifications on the mutant hDCT proteins, which occur in the Golgi, are impaired in cells infected with AdhDCT-N178D and AdhDCTΔVYD. Protective immunity correlated with the ability of the hDCT protein to mature to the higher molecular weight glycoform, pointing to a role for protein trafficking and subsequent post-translational modifications in the generation of protective hDCT-specific $CD4^+$ T cell immunity. While the exact nature of the epitope generated from this higher molecular weight form remains unknown, it is clear that it must have a more complex structure than can be estimated from the linear protein sequence. A variety of protein modifications have been shown to influence epitope processing and presentation, including: isoaspartylation, phosphorylation, glycosylation and deamination [30,35-38]. While some post-translational modifications are enzymatic in nature, others arise at physiological conditions and can be more frequent in a stressed environment resulting from

disease, inflammation and trauma for instance [39]. Our lab continues to investigate the nature of the $CD4^+$ T cell effector epitope, but it remains elusive. Strikingly, neither the helper $CD4^+$ T cell response nor the $CD8^+$ T cell response appeared to be influenced in the same way. Results obtained from DCT-deficient mice demonstrate that the dichotomy in the generation of helper and effector $CD4^+$ T cell responses following immunization with AdhDCT is not a reflection of the available $CD4^+$ T cell repertoire but is actually an intrinsic property of the hDCT protein.

It is unclear at this time how the lack of glycan maturation influences the generation of antitumor $CD4^+$ T cells. A number of reports have demonstrated the importance of glycosylation in the generation of T cell epitopes. Specific glycosylated MUC-1 epitopes are recognized by $CD4^+$ T cells that do not cross-react with the corresponding unglycosylated peptides [38]. Similarly, $CD4^+$ T cells reactive with the envelope protein (gp90) from an endogenous murine leukemia virus were only reactive with the glycosylated form of the protein [27]. In the latter case, the T cells did not appear to recognize glycosylated residues. Rather, glycosylation appeared to be important for establishing the correct orientation of the peptide for $CD4^+$ T cell recognition [40]. Whether a similar phenomenon can explain the generation of DCT epitopes that promote tumor rejection remains to be established. It has previously been reported that impairment of glycosylation and redirection of glycosylated proteins to the cytosol can enhance $CD8^+$ T cell immunity [29,41], however, our results do not support these strategies as being a

universal approach. We conducted extensive epitope mapping on all of the mutant proteins described in this manuscript (data not shown) and did not uncover any novel T cell epitopes. Therefore, according to our results, disruption of trafficking and processing of hDCT does not improve immunogenicity and, as observed for the effector $CD4^+$ T cell response, such disruption can actually impair protective immunity. Thusly, the impact of protein mutations must be considered on an empirical basis and cannot be broadly assumed.

MHC II loading and presentation has classically been linked to the uptake of exogenous antigens. However, endogenous proteins which traffic through the endocytic pathway, such as DCT, can be loaded directly onto MHC II [42,43]. Given our results, it is possible that the different $CD4^+$ T cell responses may actually be influenced by the endogenous and exogenous routes for MHC II loading. Perhaps, effector epitopes can only be generated via the endogenous pathway and, therefore, these epitopes are not produced from the mutant proteins which fail to exit the ER. In contrast, the helper epitopes may be produced by either pathway and, thus, are generated from all of the mutant proteins. In an effort to capitalize on endogenous loading of MHC II following uptake of recombinant virus vaccines by APCs, a number of strategies have been developed to selectively target tumor-associated antigens to MHC II compartments for enhanced antigen presentation to $CD4^+$ T cells. For instance, Wu *et. al.* have used the sorting signal of lysosomal-associated membrane protein 1 (LAMP-1) fused to an antigen to direct it towards lysosomes, which then intersect with MHC II compartments [44].

A similar approach developed by another group involves coupling of the trafficking signal contained in the invariant chain Ii to a tumor antigen [45]. The application of such targeting strategies have been associated with elevated $CD4^+$ T cell immunity and corresponding increased $CD8^+$ T cell immunity, presumably as a result of elevated $CD4^+$ T cell help. Our results failed to establish a linkage between antigen targeting and the development of $CD4^+$ T cell help for $CD8^+$ T cell immunity. Indeed, targeting antigen to the endocytic pathway is not a universal method to augment $CD4^+$ T cell immunity and other studies utilizing similar strategies failed to show improved T cell immunity [46-48].

Overall, our studies offer novel insight into the processes required to elicit $CD4^+$ T cell immunity and demonstrate that distinct pathways can exist to generate helper and effector $CD4^+$ T cells. Indeed, mature glycosylation, and likely post-translational modifications, appear to be involved in the generation of protective $CD4^+$ T cell antitumor immunity following immunization with hDCT, a clinically-relevant tumor-associated antigen, whereas helper $CD4^+$ T cell responses were not influenced by these post-translational events. These observations have important implication for the design of cancer vaccination platforms aimed at eliciting $CD4^+$ T cell immunity.

MATERIALS AND METHODS

Mice and cell culture

Six- to 8-week-old female C57BL/6 mice were purchased from Charles River Breeding Laboratory (Wilmington, MA). CD8-deficient mice [49] and DCT-deficient mice [50] were bred in the pathogen-free Central Animal Facility at McMaster University. All of our investigations have been approved by the McMaster Animal Research Ethics Board. C57BL/6 mouse-derived melanoma B16F10 cell line was cultured in MEM-F11 medium supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies), 1X concentration of sodium pyruvate, non-essential amino acids and vitamin solution (Gibco). RPMI medium containing fetal bovine serum, L-glutamine, penicillin and streptomycin was used for ICS assays.

Recombinant adenoviruses

All replication-deficient adenoviruses (Ad) used in this study contain deletions in their E1 and E3 regions [22]. The expression cassettes were inserted in the E1 region under the control of the murine cytomegalovirus (CMV) promoter and the SV40 polyadenylation sequence. Ad vectors were created according to the Two-Plasmid Rescue Method using 293 cells. AdhDCT expresses the full-length human DCT gene. AdhDCTΔVYD was created using standard PCR techniques

and encodes a mutated form of hDCT lacking the immunodominant CD8 epitope $hDCT_{181-188}$ (VYDFFVWL). AdhDCT-N178D encodes hDCT in which a point mutation at position 178, where an asparagine (N) was mutated to an aspartic acid (D), was introduced via site-directed mutagenesis. AdhDCTΔSS and AdhDCTΔMTS were created by standard PCR techniques in which hDCT harbors deletions within the intracellular localization sequences previously identified [33] (SS, endoplasmic reticulum signal sequence [hDCTΔ2-23]; MTS, melanosomal targeting sequence [hDCTΔ491-496]). AdLCMV-$GP_{33/61}$ encodes the sequences corresponding to residues 33-41 and 61-80 of the lymphocytic choriomeningitis virus glycoprotein and was used as a negative control (referred to as AdControl in figures).

Immunization, immunodepletion and tumor challenge

10^8 pfu of Ad vector was prepared in 100µl sterile PBS and injected in both rear thighs (50µl/thigh) of each mouse. Mice were challenged subcutaneously on day 7 post-immunization with $2x10^4$ B16F10 cells. Tumor growth was monitored daily and measured twice a week over a period of 60 days. Immunodepletion studies were conducted using the 2.43 monoclonal antibody (anti-CD8, American Type Culture Collection), which was prepared in our laboratory. A rat IgG (Sigma, St-Louis, MO) was used as control. The depletion efficiency of $CD8^+$ T cells was >98% by flow cytometry using antibodies of a different clone (data not shown).

Peptides

$DCT_{180-188}$ (immunodominant CD8 epitope) was purchased from Dalton Chemical Laboratories Inc (Toronto, Canada). The following peptides were ordered from Biomer Technologies (Pleasanton, CA): $hDCT_{342-351}$ (subdominant CD8 epitope), $hDCT_{89-101}$ (immunodominant CD4 epitope) and $hDCT_{242-254}$ (subdominant CD4 epitope). 15-mer overlapping peptides surrounding the $hDCT_{180-188}$ epitope (**Table S1**) are part of a previously described hDCT library [22]. 15-mer peptides overlapping putative N-linked glycosylation sites (N-X-C/S/T) within hDCT were synthesized in which asparagine (N)-to-aspartic acid (D) mutations were incorporated (**Table S2**; PepScan Systems, Lelystad, The Netherlands). All peptides were dissolved in DMSO and stored at -20˚C.

Monoclonal Antibodies

The following monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA): anti-CD3ε-PE (clone 145-2C11), anti-CD8α-PE-Cy5 (clone 53-6.7), anti-CD4-PE-Cy7 (clone RM4-5), anti-IFN-γ-APC (clone XMG1.2), anti-CD16/CD32 (Fc Block; clone 2.4G2) and anti-CD28 (clone 37.51).

Intracellular Cytokine Staining (ICS)

ICS was performed as described previously [22] with the following modifications: 1) splenocytes were harvested 10 days post-immunization, 2) cells were stimulated with 1μg/ml and 10μg/ml peptide concentrations to detect $CD8^+$ and

$CD4^+$ T cell responses respectively and 3) no anti-CD49d was added to the stimulations. DMSO was used as negative control. Lymphocytes were examined by flow cytometry (BD FACSCanto) and analyzed using FlowJo software. Results are shown as absolute numbers of antigen-specific cells per spleen and background values have been subtracted. T cell responses were considered meaningful when the percentage of DCT-specific cells was 2X background values and >0.02%. Results reported as frequencies are also available in **Supplementary Figure 2**.

Western immunoblotting and glycosidase assays

293T cells transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) were harvested after 24hrs in modified RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) containing protease inhibitors (PMSF and protease inhibitor cocktail (Sigma)). Protein concentration was determined via a Bradford assay (BioRad). For glycosidase assays, 20μg of proteins were treated with $EndoH_f$ or PNGaseF according to recommended conditions. The glycosidase-treated lysate was diluted 1:2 in SDS gel loading buffer (BioRad), boiled for 5 mins, electrophoresed on 10% SDS-PAGE gels, transferred to nitrocellulose membranes and probed for DCT using α-TRP2(C-9) (Santa Cruz Biotechnology) and polyclonal goat α-mouse IgG-HRP (Cedarlane). GAPDH was used as a loading control and the membranes were probed with α-GAPDH antibodies (Sigma) following stripping of the blot (50mM TRIS, pH6.8, 2% SDS, 40 μL/mL

β-mercaptoethanol for 1hr at RT). Development was done using ECL.

Statistical Analysis

A two-tailed, unpaired Student's *t* test was used for the analysis. Differences between means were considered significant at $p<0.05$. Results were generated using GraphPad Prism 4.0b software (Graph Pad Software, LaJolla, CA).

ACKNOWLEDGMENTS

Funding for this work was provided by the Ontario Cancer Research Network. The authors thank Chuyan Ying for preparing the antibodies used in these experiments. D.B. was supported by a research studentship from the Canadian Institutes for Health Research. M.V. was supported by research studentships from the National Sciences and Engineering Research Council and the Ontario Graduate Scholarships program.

SUPPLEMENTARY MATERIAL

Figure S1. Example of FACS analysis. Splenocytes from wild-type mice immunized with AdhDCT were stimulated *in-vitro* with peptides corresponding to the CD4 and CD8 epitopes of hDCT and assayed for IFN-γ production via ICS (DMSO: negative control).

Figure S2. Compiled intracellular cytokine staining results reported as T cell frequencies. Splenocytes were harvested from (**a,b,d,f,h**) WT and (**c,e,g**) CD8-deficient mice 10 days post-immunization with the various DCT antigens and stimulated *in-vitro* with the (**b,c,e,g**) CD4 and (**a,d,f,h**) CD8 epitope peptides. Results are reported as IFN-γ-secreting antigen-specific T cell frequencies (*p<0.05 and **p<0.0005 compared to AdhDCT; n.s.: no significance).

Table S1. List of hDCT peptides surrounding the sequence deleted[a] in the AdhDCTΔVYD vector and peptide reactivity as measured by ICS[b]. [a]Deleted sequence is indicated in bold. [b]Peptide reactivity was tested via ICS analysis with splenocytes from CD8-deficient mice immunized with AdhDCT. Results shown correspond to the average from 3 mice and are representative of two separate experiments (s.d.: standard deviation). $hDCT_{89\text{-}101}$ was used as a positive control and DMSO as a negative control (N/A: not applicable).

Table S2. List of hDCT peptides containing an asparagine (N)-to-aspartic acid (D) mutation[a] of putative N-linked glycosylation sites (motif: N-X-C/S/T) and peptide reactivity as measured by ICS[b]. [a]Asparagine (N)-to-aspartic acid (D) mutations are indicated in bold. [b]Peptide reactivity was tested via ICS analysis with splenocytes from CD8-deficient mice immunized with AdhDCT. Results shown correspond to the average from 3 mice and are representative of two separate experiments (s.d.: standard deviation). $hDCT_{89\text{-}101}$ was used as a positive control and DMSO as a negative control (N/A: not applicable).

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FIGURE LEGENDS

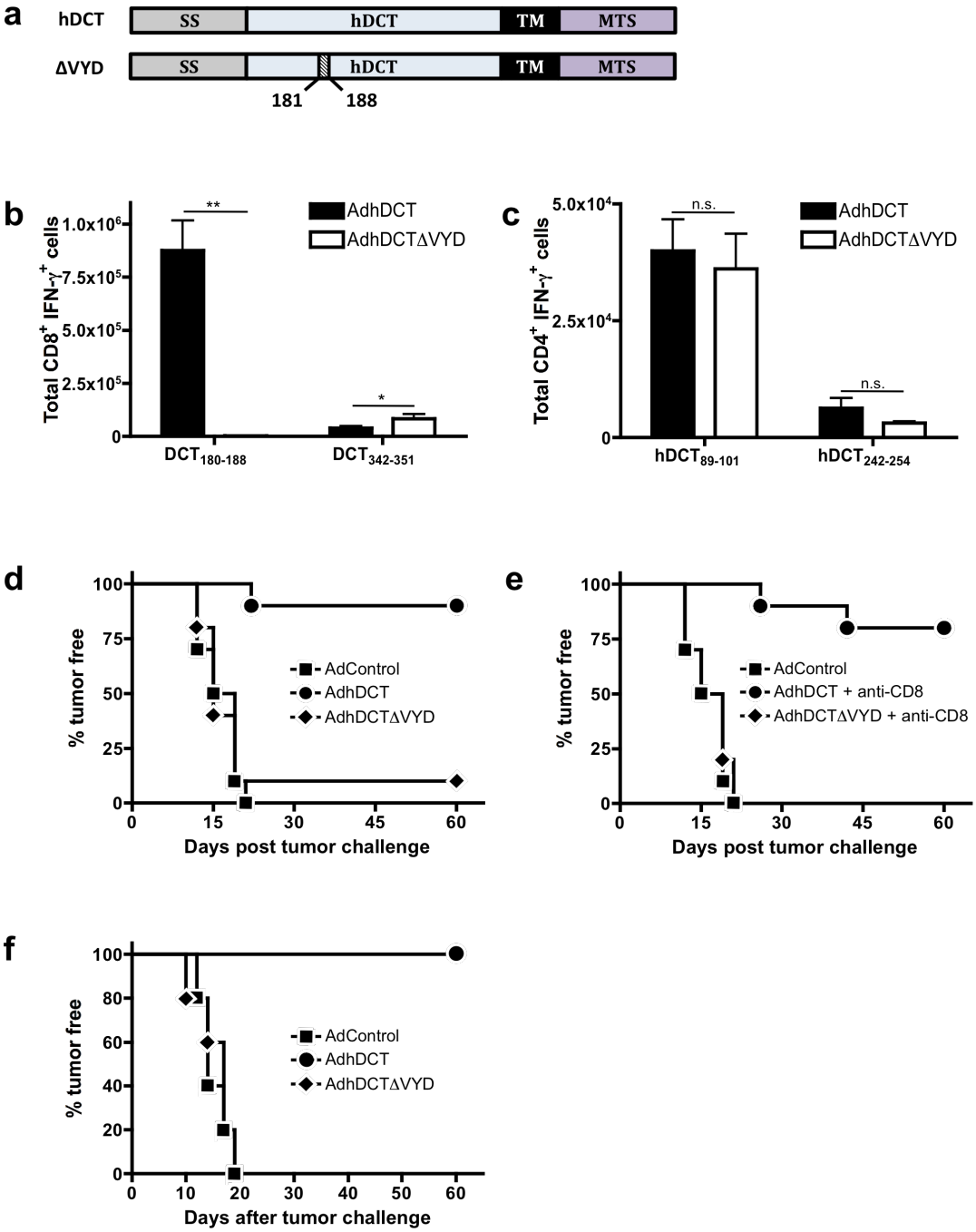
Figure 1. Immunization with AdhDCTΔVYD abrogates $CD4^+$ T cell-mediated tumor protection while T cell responses to the known helper CD4 epitopes are maintained. (**a**) Schematic representation of the hDCTΔVYD variant (SS: endoplasmic reticulum *S*ignal *S*equence; TM: *T*ransmembrane *D*omain; MTS: *M*elanosomal *T*ransport *S*ignal). (**b,c**) T cell responses to the known (**b**) CD8 epitopes ($DCT_{180-188}$ and $hDCT_{342-351}$) and (**c**) CD4 epitopes ($hDCT_{89-101}$ and $hDCT_{242-254}$) were assessed via intracellular cytokine staining. Results are shown as mean±SEM from 10 mice pooled from 2 separate experiments and represent the absolute number of antigen-specific cells per spleen (*p<0.05 and **p<0.0005 compared to AdhDCT; n.s.: no significance). (**d-f**) Mice (n=10) were immunized intramuscularly with 10^8 pfu of AdControl, AdhDCT or AdhDCTΔVYD. (**d**) Wild type mice, (**e**) $CD8^+$ T cell-depleted wild type mice and (**f**) CD8-deficient mice were challenged subcutaneously with $2x10^4$ B16F10 cells 7 days post-immunization. Depletion of $CD8^+$ T cells was performed by intraperitoneal injection of purified 2.43 antibodies starting on days -3 and -1 prior to tumor challenge and subsequently administered once a week until tumors developed. A rat IgG was used as control treatment.

Figure 2. Immunization with hDCT variants lacking mature N-linked glycosylation results in impaired $CD4^+$ T cell-mediated tumor protection while T cell responses to the known helper CD4 epitopes are maintained. (**a**) Western blot analysis reveals lack of mature N-linked glycosylation in hDCTΔVYD and hDCT-N178D protein variants. 293T cells were transfected for 24hrs with plasmid DNA expressing hDCT, hDCTΔVYD or hDCT-N178D. Lysates were prepared in RIPA lysis buffer and equal amounts of protein (~20μg) were treated with the glycosidase enzymes $EndoH_f$ or PNGaseF for 24hrs at 37°C. The entire reaction was diluted in 2X gel loading buffer and resolved by 10% SDS-PAGE. DCT was probed for using α-TRP-2 (C-9) and developed by ECL, followed by re-probing with α-GAPDH as a loading control. (**b**) Schematic representation of the hDCT-N178D variant (SS: endoplasmic reticulum *S*ignal *S*equence; TM: *T*ransmembrane *D*omain; MTS: *M*elanosomal *T*ransport *S*ignal). (**c,d**) T cell responses to the known (**c**) CD4 epitopes ($hDCT_{89\text{-}101}$ and $hDCT_{242\text{-}254}$) and (**d**) CD8 epitopes ($DCT_{180\text{-}188}$ and $hDCT_{342\text{-}351}$) were assessed via intracellular cytokine staining. Results are shown as mean±SEM from 10 mice pooled from 2 separate experiments and represent the absolute number of antigen-specific cells per spleen (n.s.: no significance). (**e**) CD8-deficient mice (n=10-15) were immunized intramuscularly with 10^8 pfu of AdControl, AdhDCT or AdhDCT-N178D. Mice were challenged subcutaneously with $2x10^4$ B16F10 cells 7 days post-immunization.

Figure 3. Immunization with AdhDCTΔSS which is glycosylation-defective, but not AdhDCTΔMTS, abrogates $CD4^+$ T cell-mediated tumor protection while T cell responses to the known helper CD4 epitopes are maintained. (**a**) Schematic representation of the hDCTΔSS and AdhDCTΔMTS variants (SS: endoplasmic reticulum *S*ignal *S*equence; TM: *T*ransmembrane *D*omain; MTS: *M*elanosomal *T*ransport *S*ignal). (**b**) Western blot analysis reveals lack of mature N-linked glycosylation in hDCTΔSS variant, but not AdhDCTΔMTS variant. 293T cells were transfected with plasmid DNA expressing hDCT, hDCTΔSS, hDCTΔMTS or GFP for 24hrs. Lysates were prepared in RIPA lysis buffer and equal amounts of protein (~20μg) were treated with the glycosidase enzymes $EndoH_f$ or PNGaseF for 24hrs at 37°C. The entire reaction was diluted in 2X gel loading buffer and resolved by 10% SDS-PAGE. DCT was probed for using α-TRP-2 (C-9) and developed by ECL, followed by re-probing with α-GAPDH as a loading control. (**c,d**) T cell responses to the known (**c**) CD4 epitopes ($hDCT_{89\text{-}101}$ and $hDCT_{242\text{-}254}$) and (**d**) CD8 epitopes ($DCT_{180\text{-}188}$ and $hDCT_{342\text{-}351}$) were assessed via intracellular cytokine staining. Results are shown as mean±SEM from 10 mice pooled from 2 separate experiments and represent the absolute number of antigen-specific cells per spleen (*p<0.05 compared to AdhDCT; n.s.: no significance). (**e**) CD8-deficient mice (n=10-15) were immunized intramuscularly with 10^8 pfu of AdControl, AdhDCT, AdhDCTΔSS or AdhDCTΔMTS. Mice were challenged subcutaneously with $2x10^4$ B16F10 cells 7 days post-immunization.

Figure 4. Immunization of DCT-deficient mice with AdhDCTΔVYD abrogates $CD4^+$ T cell-mediated tumor protection while T cell responses to the known helper CD4 epitopes are maintained. (**a,b**) T cell responses to the known (**a**) CD4 epitopes ($hDCT_{89-101}$ and $hDCT_{242-254}$) and (**b**) CD8 epitopes ($DCT_{180-188}$ and $hDCT_{342-351}$) were assessed via intracellular cytokine staining. Results are shown as mean±SEM from 10 mice pooled from 2 separate experiments and represent the absolute number of antigen-specific cells per spleen (*p<0.05 and **p<0.0005 compared to AdhDCT; n.s.: no significance). (**c**) DCT-deficient mice (n=10-15) were immunized intramuscularly with 10^8 pfu of AdControl, AdhDCT or AdhDCTΔVYD. Mice were challenged subcutaneously with $2x10^4$ B16F10 cells 7 days post-immunization. Depletion of $CD8^+$ T cells was performed by intraperitoneal injection of purified 2.43 antibodies starting on days -3 and -1 prior to tumor challenge and subsequently administered once a week until tumors developed. A rat IgG was used as control treatment.

FIGURE 1-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hDCT
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hDCT
TM
MTS
ΔVYD
SS
hDCT
TM
MTS
181
188
b
Total CD8+ IFN-γ+ cells
1.0x10^6
7.5x10^5
5.0x10^5
2.5x10^5
0
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*
AdhDCT
AdhDCTΔVYD
DCT180-188
DCT342-351
c
Total CD4+ IFN-γ+ cells
5.0x10^4
2.5x10^4
0
n.s.
n.s.
AdhDCT
AdhDCTΔVYD
hDCT89-101
hDCT242-254
d
% tumor free
100
75
50
25
0
0
15
30
45
60
Days post tumor challenge
AdControl
AdhDCT
AdhDCTΔVYD
e
% tumor free
100
75
50
25
0
0
15
30
45
60
Days post tumor challenge
AdControl
AdhDCT + anti-CD8
AdhDCTΔVYD + anti-CD8
f
% tumor free
100
80
60
40
20
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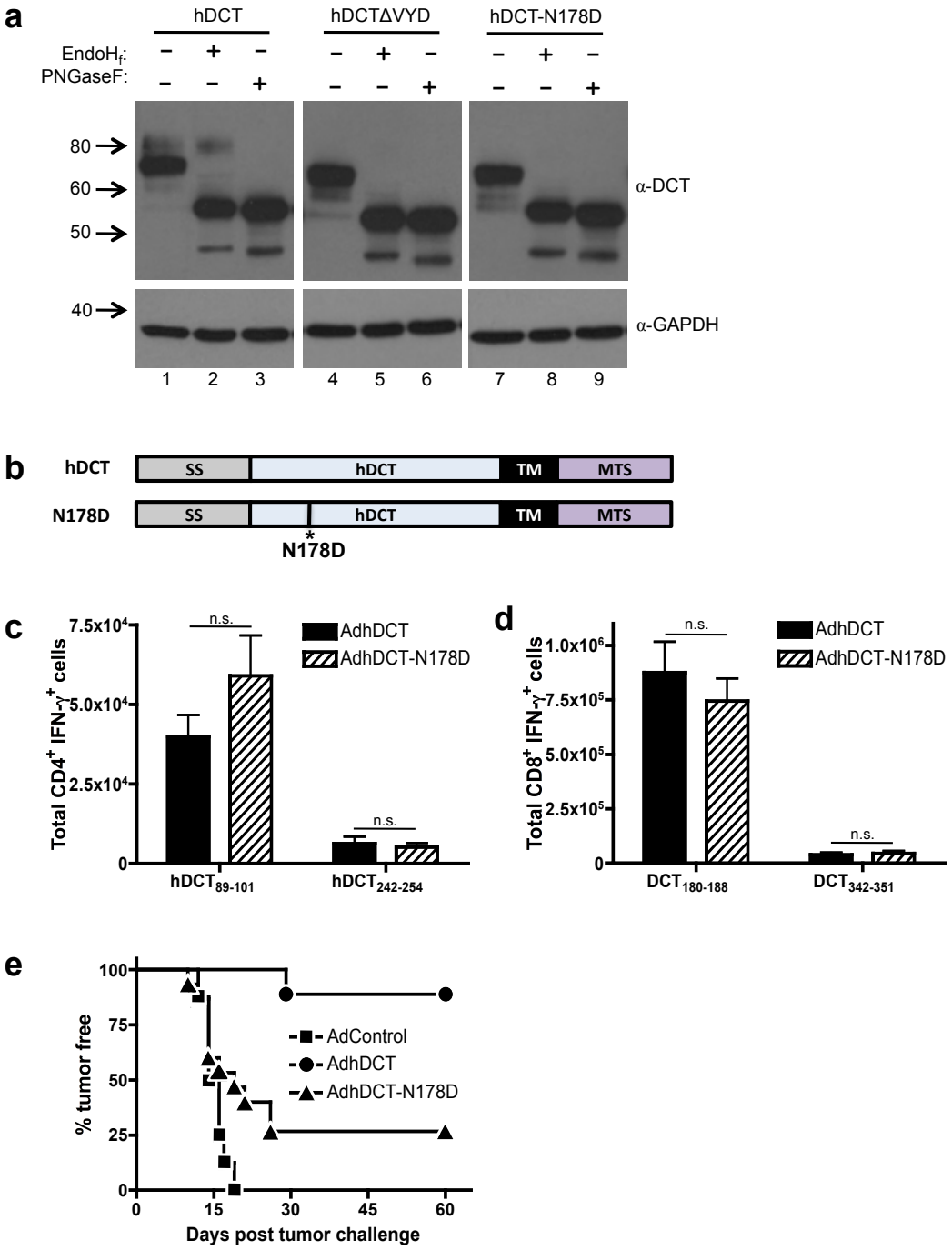
40
50
60
Days after tumor challenge
AdControl
AdhDCT
AdhDCTΔVYD

FIGURE 2-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hDCT
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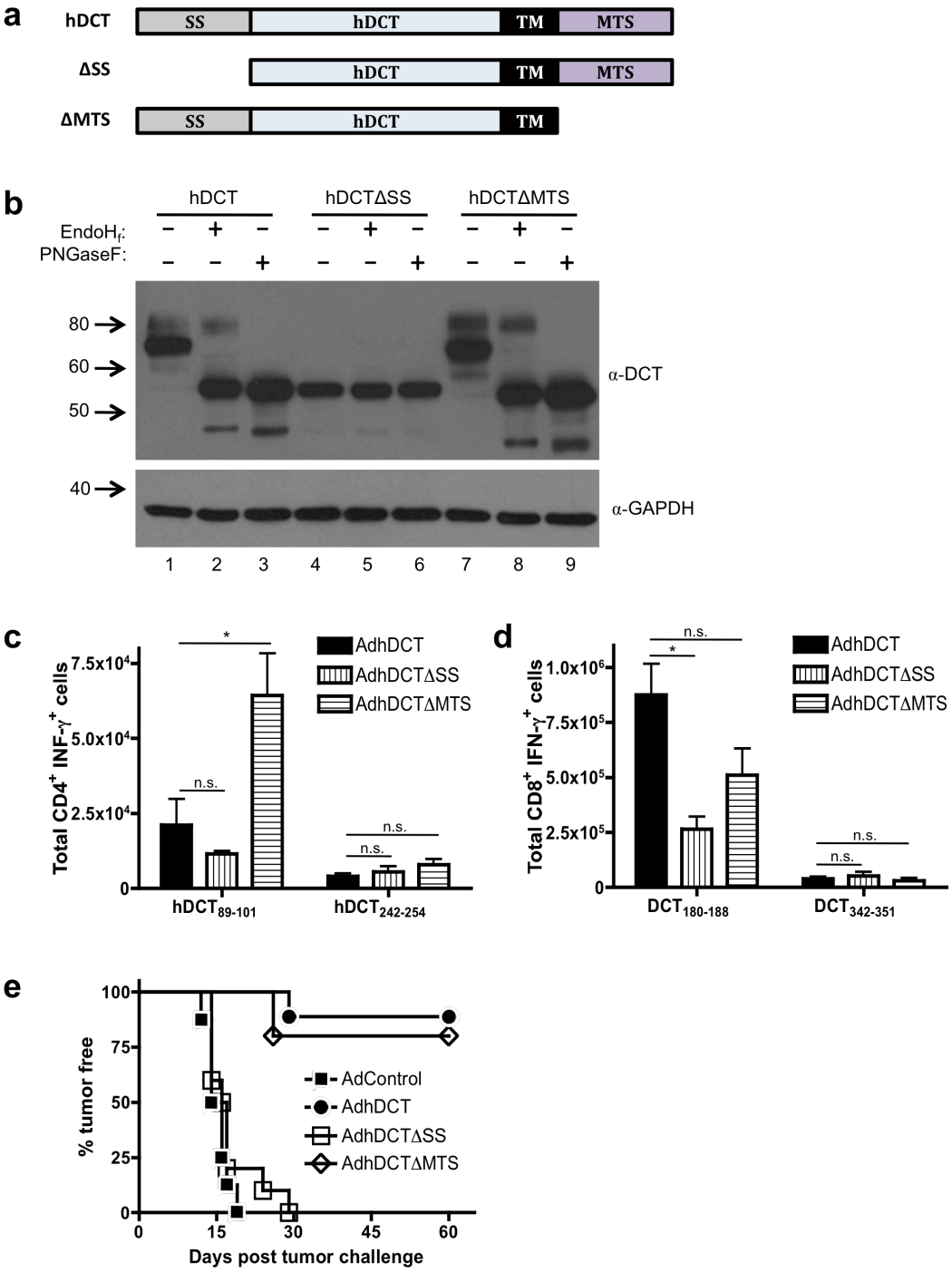
hDCT-N178D
EndoHf:
PNGaseF:
80
60
50
40
α-DCT
α-GAPDH
1 2 3 4 5 6 7 8 9
b
hDCT
N178D
SS
hDCT
TM
MTS
N178D
c
Total CD4+ IFN-γ+ cells
7.5x10^4
5.0x10^4
2.5x10^4
0
n.s.
AdhDCT
AdhDCT-N178D
hDCT89-101
hDCT242-254
d
Total CD8+ IFN-γ+ cells
1.0x10^6
7.5x10^5
5.0x10^5
2.5x10^5
DCT180-188
DCT342-351
e
% tumor free
100
75
50
25
0
AdControl
AdhDCT
AdhDCT-N178D
0 15 30 45 60
Days post tumor challenge

FIGURE 3-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SS
hDCT
TM
MTS
ΔSS
hDCT
TM
MTS
ΔMTS
SS
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hDCT
hDCTΔSS
hDCTΔMTS
EndoHf:
PNGaseF:
80
60
50
40
α-DCT
α-GAPDH
1 2 3 4 5 6 7 8 9
c
Total CD4+ INF-γ+ cells
7.5x10⁴
5.0x10⁴
2.5x10⁴
0
AdhDCT
AdhDCTΔSS
AdhDCTΔMTS
n.s.
*
hDCT89-101
hDCT242-254
d
Total CD8+ IFN-γ+ cells
1.0x10⁶
7.5x10⁵
5.0x10⁵
2.5x10⁵
0
DCT180-188
DCT342-351
e
% tumor free
100
75
50
25
0
0 15 30 45 60
Days post tumor challenge
AdControl
AdhDCT
AdhDCTΔSS
AdhDCTΔMTS

FIGURE 4-

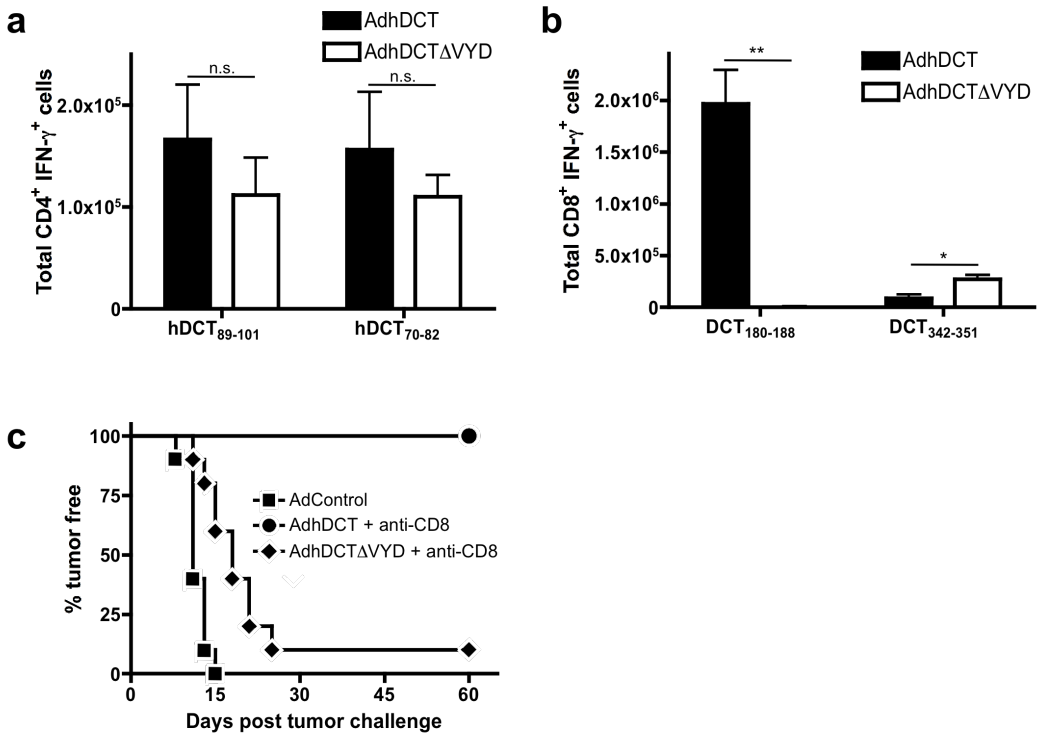
a
AdhDCT
AdhDCTΔVYD
n.s.
n.s.
Total CD4+ IFN-γ+ cells
2.0x10^5
1.0x10^5
0
hDCT89-101
hDCT70-82
b
**
*
AdhDCT
AdhDCTΔVYD
Total CD8+ IFN-γ+ cells
2.0x10^6
1.5x10^6
1.0x10^6
5.0x10^5
0
DCT180-188
DCT342-351
c
% tumor free
100
75
50
25
0
0
15
30
45
60
Days post tumor challenge
AdControl
AdhDCT + anti-CD8
AdhDCTΔVYD + anti-CD8

FIGURE S1-

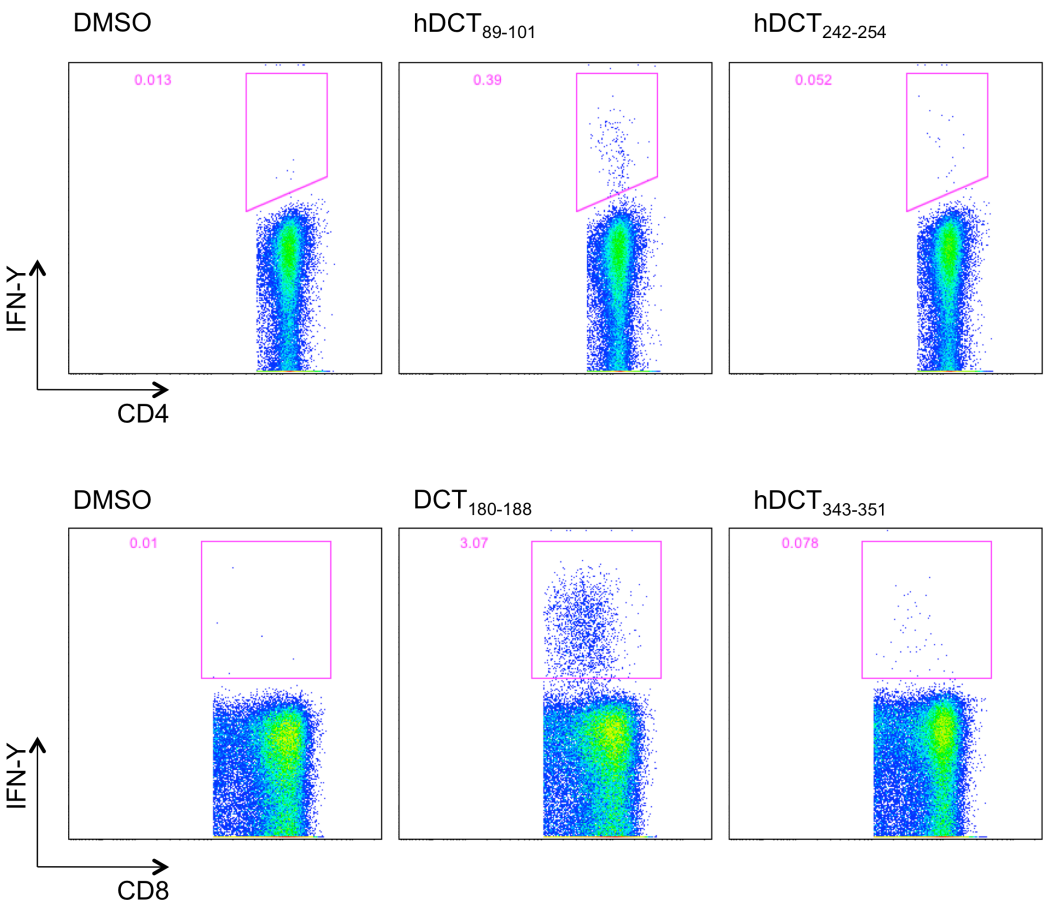
DMSO
hDCT89-101
hDCT242-254
0.013
0.39
0.052
IFN-Y
CD4
DMSO
DCT180-188
hDCT343-351
0.01
3.07
0.078
IFN-Y
CD8

FIGURE S2-

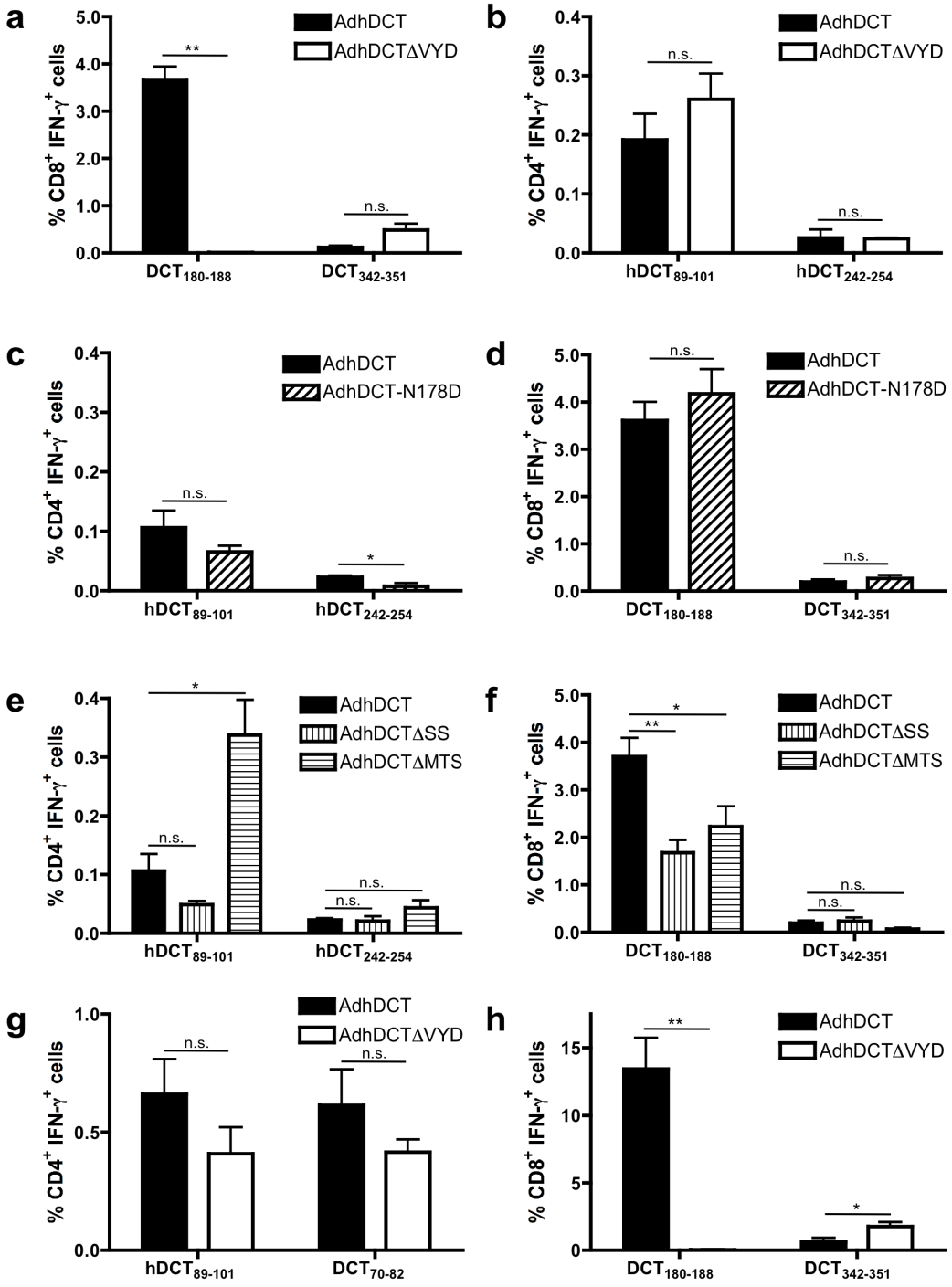
a
% CD8+ IFN-γ+ cells
AdhDCT
AdhDCTΔVYD
**
n.s.
DCT180-188
DCT342-351
b
% CD4+ IFN-γ+ cells
AdhDCT
AdhDCTΔVYD
n.s.
n.s.
hDCT89-101
hDCT242-254
c
% CD4+ IFN-γ+ cells
AdhDCT
AdhDCT-N178D
n.s.
*
hDCT89-101
hDCT242-254
d
% CD8+ IFN-γ+ cells
AdhDCT
AdhDCT-N178D
n.s.
n.s.
DCT180-188
DCT342-351
e
% CD4+ IFN-γ+ cells
AdhDCT
AdhDCTΔSS
AdhDCTΔMTS
*
n.s.
n.s.
n.s.
hDCT89-101
hDCT242-254
f
% CD8+ IFN-γ+ cells
AdhDCT
AdhDCTΔSS
AdhDCTΔMTS
*
**
n.s.
n.s.
DCT180-188
DCT342-351
g
% CD4+ IFN-γ+ cells
AdhDCT
AdhDCTΔVYD
n.s.
n.s.
hDCT89-101
DCT70-82
h
% CD8+ IFN-γ+ cells
AdhDCT
AdhDCTΔVYD
**
*
DCT180-188
DCT342-351

TABLE S1-

Peptide	Sequence	Mean % $CD4^+$ IFN-γ^+ cells (Mean ± s.d.)
$hDCT_{150-164}$	KRVHPDYVITTQHWL	0.020 ± 0.011
$hDCT_{156-167}$	YVITTQHWLGLL	0.026 ± 0.006
$hDCT_{159-173}$	TTQHWLGLLGPNGTQ	0.038 ± 0.011
$hDCT_{165-177}$	GLLGPNGTQPQFA	0.010 ± 0.014
$hDCT_{169-182}$	PNGTQPQFANCSVY	0.025 ± 0.004
$hDCT_{169-183}$	PNGTQPQFANCS**VYD**	0.009 ± 0.016
$hDCT_{173-187}$	QPQFANCS**VYDFFVW**	0.007 ± 0.012
$hDCT_{174-188}$	PQFANCS**VYDFFVWL**	0.020 ± 0.000
$hDCT_{177-191}$	ANCS**VYDFFVWL**HYY	0.011 ± 0.020
$hDCT_{180-194}$	S**VYDFFVWL**HYYSVR	0.019 ± 0.005
$hDCT_{181-195}$	**VYDFFVWL**HYYSVRD	0.006 ± 0.010
$hDCT_{185-199}$	**FVWL**HYYSVRDTLLG	0.005 ± 0.009
$hDCT_{186-198}$	**VWL**HYYSVRDTLL	0.020 ± 0.004
$hDCT_{190-204}$	YYSVRDTLLGPGRPY	0.032 ± 0.012
$hDCT_{196-209}$	TLLGPGRPYRAIDF	0.032 ± 0.002
$hDCT_{201-215}$	GRPYRAIDFSHQGPA	0.021 ± 0.003
$hDCT_{207-221}$	IDFSHQGPAFVTWHR	0.024 ± 0.013
$hDCT_{89-101}$	KFFHRTCKCTGNFA	0.260 ± 0.057
DMSO	N/A	0.028 ± 0.011

TABLE S2-

Peptide	Sequence	Mean % $CD4^+$ IFN-γ^+ cells (Mean ± s.d.)
$hDCT_{157-171}$	VITTQHWLGLLGP**D**G	0.006 ± 0.010
$hDCT_{160-177}$	TQHWLGLLGP**D**GTQPQFA	0.005 ± 0.008
$hDCT_{167-181}$	LGP**D**GTQPQFANCSV	0.004 ± 0.007
$hDCT_{167-181}$	LGPNGTQPQFA**D**CSV	0.003 ± 0.006
$hDCT_{167-181}$	LGP**D**GTQPQFA**D**CSV	0.004 ± 0.007
$hDCT_{171-185}$	GTQPQFA**D**CSVYDFF	0.009 ± 0.008
$hDCT_{175-188}$	QFA**D**CSVYDFFVWL	0.01 ± 0.011
$hDCT_{178-191}$	**D**CSVYDFFVWLHYY	0.01 ± 0.009
$hDCT_{225-240}$	LCLERDLQRLIG**D**ESF	0.005 ± 0.009
$hDCT_{230-244}$	DLQRLIG**D**ESFALPY	0.004 ± 0.008
$hDCT_{234-248}$	LIG**D**ESFALPYWNFA	0.004 ± 0.007
$hDCT_{241-256}$	ALPYWNFATGR**D**ECDV	0.014 ± 0.003
$hDCT_{246-261}$	NFATGR**D**ECDVCTDQL	0.009 ± 0.008
$hDCT_{251-264}$	R**D**ECDVCTDQLFGA	0.012 ± 0.002
$hDCT_{288-303}$	SLDDYNHLVTLC**D**GTY	0.007 ± 0.012
$hDCT_{293-307}$	NHLVTLC**D**GTYEGLL	0.003 ± 0.006
$hDCT_{297-312}$	TLC**D**GTYEGLLRRNQM	0.003 ± 0.006
$hDCT_{330-345}$	SLQKFDNPPFFQ**D**STF	0.007 ± 0.006
$hDCT_{335-348}$	DNPPFFQ**D**STFSFR	0.004 ± 0.008
$hDCT_{338-351}$	PFFQ**D**STFSFRNAL	0.004 ± 0.008
$hDCT_{341-355}$	Q**D**STFSFRNALEGFD	0.008 ± 0.007
$hDCT_{366-381}$	MSLHNLVHSFL**D**GTNA	0.005 ± 0.008
$hDCT_{371-384}$	LVHSFL**D**GTNALPH	0.005 ± 0.008
$hDCT_{374-387}$	SFL**D**GTNALPHSAA	0.004 ± 0.007
$hDCT_{377-391}$	**D**GTNALPHSAANDPI	0.007 ± 0.006
$hDCT_{89-101}$	KFFHRTCKCTGNFA	0.093 ± 0.012
DMSO	N/A	0.010 ± 0.010

— CHAPTER 4 —

Early IFN-γ production by vaccine-induced T cells provokes tumor adaptation and PD-1 ligand-mediated suppression of local T cell activity

Early IFN-γ production by vaccine-induced T cells provokes tumor adaptation and PD-1 ligand-mediated suppression of local T cell activity

A. J. Robert McGray[1], Dannie Bernard[1], Ryan Kelly[1], Mayank Jha[1], Caitlin Gregory[1], Jennifer D. Bassett[1], Yonghong Wan[1] and Jonathan L. Bramson[1]

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Preface: *The research presented in this manuscript is the result of a collaboration between myself and A.J. Robert McGray, a PhD candidate in Dr. Bramson's laboratory. My participation to this experimental work spanned from July 2010 to April 2011. With technical assistance from Ryan Kelly, Mayank Jha, Caitlin Gregory (undergraduate students) and Jennifer D. Bassett (PhD candidate), A.J. Robert McGray conducted initial transcriptional analyses, tumor infiltrating lymphocyte characterization as well as 4-1BB dose titration. Together with A.J. Robert McGray, I worked on in-vivo studies, ex-vivo T cell assays and transcriptional analyses pertaining to T cell subset depletion, PD-1 blockade and 4-1BB agonist treatment experiments. Briefly, I contributed to the generation of major part of the results presented in all the figures and I also collaborated to writing the introduction, discussion and materials and methods sections of the following manuscript. This work, which remains in progress in our laboratory, was supervised by Dr. Bramson who, along with Dr. Wan, provided experimental guidance and contributed to interpretation of the results.*

Title: Early IFN-γ production by vaccine-induced T cells provokes tumor adaptation and PD-1 ligand-mediated suppression of local T cell activity

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Running title: Vaccine-induced adaptation suppresses TIL activity

Key words: T lymphocyte, vaccine, immune suppression, PD-1, 4-1BB

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ABSTRACT

We have reported that a recombinant adenovirus vaccine encoding dopachrome tautomerase (rHuAd5-hDCT) produces robust DCT-specific immunity, but only modest suppression of melanoma growth. Analysis of the tumor microenvironment following vaccination revealed rapid, but transient, immune attack that diminished before peak infiltration of vaccine-induced T cells. Inclusion of a 4-1BB agonist antibody (α4-1BB) with rHuAd5-hDCT partially restored intratumoral immune attack and further suppressed tumor growth, but immune function waned and tumors relapsed. We noted that vaccine-induced T cells within the tumor expressed PD-1 and that PD-1 ligands were significantly elevated in the tumor following vaccination. Strikingly, both tumor growth suppression and PD-1 ligand expression were IFN-γ dependent, revealing the complex biology of this cytokine. PD-1 blockade in combination with rHuAd5-hDCT + α4-1BB produced a synergistic enhancement in intratumoral immune activity leading to clearance of most tumors. Enhanced anti-tumor efficacy did not correlate with the frequency or functionality of DCT-specific $CD8^+$ T cells within the tumor. Rather, PD-1 blockade enhanced local immune activity, despite elevated levels of PD-1 ligands. Our results demonstrate that cancer vaccines are limited by the rapid adaptation of the local environment, which must be overcome to achieve tumor regression.

INTRODUCTION

T cells play a key role in immune surveillance and tumor rejection. Although central and peripheral tolerance limits the availability of tumor-reactive T cells, strong anti-tumor T cell responses can be generated using various cancer immunization platforms [1]. Overall, however, there has been a lack of clinical efficacy, prompting the need to re-evaluate the design of tumor-targeting immunotherapies [2]. Much research is currently focused on enhancing vaccine immunogenicity to elicit higher numbers of tumor-specific T cells [3] and overcoming the immune suppressive tumor microenvironment [4]. The latter is a highly complex homeostatic process that involves multiple pathways. To this point, however, immune suppression has been considered largely in static terms and the dynamic nature of the events within the tumor microenvironment are poorly characterized.

Previous studies by our group demonstrated that vaccination with a recombinant human adenovirus serotype 5 (rHuAd5) vector expressing the human dopachrome tautomerase antigen (hDCT; vector: rHuAd5-hDCT) elicited robust protection against the B16F10 murine melanoma in prophylactic and neo-adjuvant settings [5-8]. The same vaccine, however, only provided modest therapeutic benefit against growing tumors [7,9]. We observed that vaccine-induced tumor infiltrating lymphocytes (TILs) displayed impaired cytokine production and cytotoxic

functions compared with peripheral T cells, leading us to conclude that the lack of therapeutic effect was due to lack of sufficient T cell function [9].

In this manuscript, we have re-evaluated the events within the tumor microenvironment following vaccination with rHuAd5-hDCT. Our new data demonstrated that the regulation of T cell activation within the tumor was more complex and dynamic than previously appreciated. Examination of local events within the tumor revealed that the lack of efficacy was not due to insufficient functionality of the tumor infiltrating cells but rather it was due to an adaptive process within the tumor that inhibited local T cell activity. Further, blockade of PD-1, a negative regulatory receptor expressed on activated T cells, restored T cell activity within the tumor leading to complete tumor destruction without influencing T cell functionality. These results offer important new insights into the dynamic nature of the tumor microenvironment following vaccination and emphasize the need to develop methods of immune monitoring that permit repeated analysis of T cell activity within the tumor.

RESULTS

$CD8^+$ T cells suppress B16F10 tumor growth following vaccination through production of IFN-γ

In accordance with our previous reports [7,9-11], immunization of mice bearing small B16F10 tumors with either rHuAd5-hDCT or rHuAd5-hgp100 yielded a robust antigen-specific $CD8^+$ T cell response (**Fig. 1a**) that peaked around 2 weeks after immunization. While vaccination with rHuAd5-hDCT resulted in a modest, yet significant, suppression of tumor growth (**Fig. 1b**), treatment with rHuAd5-hgp100 had no significant impact on tumor growth (**Fig. 1b**), consistent with our previous report [10]. To investigate the relative contribution of $CD8^+$ and $CD4^+$ T cell subsets in mediating this anti-tumor effect, we conducted depletion studies following rHuAd5-hDCT administration. We observed that $CD8^+$ T cells, but not $CD4^+$ T cells, were required for growth inhibition by the rHuAd5-hDCT vaccine (**Fig. 1c**). Further, we also determined that IFN-γ was critical for tumor growth suppression by rHuAd5-hDCT (**Fig. 1d**).

Vaccine-induced $CD8^+$ T cells mount a transient immune attack that is attenuated prior to peak infiltration

Given the importance of IFN-γ to tumor suppression following rHuAd5-hDCT vaccination, we assayed IFN-γ expression within the treated tumors at various times post-vaccination as a measure of local T cell activation based on evidence

that IFN-γ is rapidly upregulated following TCR stimulation and extinguished with equal rapidity when contact with the MHC/peptide complex is disrupted [12]. Whole tumor RNA was prepared from mice that were vaccinated with rHuAd5-hDCT, rHuAd5-hgp100, or left untreated and IFN-γ expression was measured by qRT-PCR. Expression of IFN-γ was relatively low in untreated tumors (**Fig. 2a**) whereas immunization with rHuAd5-hDCT led to a rapid, but transient, increase in intratumoral IFN-γ (**Fig. 2a**). Little expression of IFN-γ was measured in tumors from mice immunized with rHuAd5-hgp100, despite a robust gp100-specific $CD8^+$ T cell response (**Fig. 1a**), although expression was significantly elevated relative to untreated mice at days 9 - 11, indicating some activation of gp100-specific T cells within these tumors. To confirm that IFN-γ expression in the tumor was due to T cell activation, mice bearing B16F10 tumors were immunized with rHuAd5-hDCT and depleted of $CD8^+$ or $CD4^+$ T cells. $CD8^+$ T cell-depletion reduced IFN-γ expression to the level of untreated mice while $CD4^+$ T cell-depletion had no impact on cytokine expression within the tumor (**Fig. S1a**). Thus, our results demonstrate that transcriptional analysis can provide a direct measure of vaccine-induced $CD8^+$ T cell activity within the tumor microenvironment. Further, these data demonstrate that DCT-specific $CD8^+$ T cells are only transiently activated within the tumor following rHuAd5-hDCT immunization.

TNF-α is another pro-inflammatory cytokine that is produced by effector $CD8^+$ T cells and is associated with anti-tumor immunity [13,14]. Following

rHuAd5-hDCT immunization, TNF-α expression displayed kinetics similar to IFN-γ, albeit delayed (**Fig. 2a**); however, the relative increase in expression of TNF-α did not achieve the same magnitude as IFN-γ. Similar to IFN-γ, the expression of TNF-α was wholly dependent upon $CD8^+$ T cells (**Fig. S1b**). Again, TNF-α expression in tumors from mice immunized with rHuAd5-hgp100 was only measurable at late time points and did not achieve peak levels comparable to the expression in tumors from mice immunized with rHuAd5-hDCT. It is notable that the period of heightened IFN-γ and TNF-α expression (**Fig. 2a**) within the tumor corresponded to the period of rHuAd5-hDCT-mediated tumor growth suppression (**Fig. 1b**).

The short course of cytokine production within the tumor following rHuAd5-hDCT was inconsistent with the vaccine-induced T cell response, which escalated progressively over the first 2 weeks following vaccination (**Fig. 1a**). We next questioned whether the expansion of DCT-specific TILs peaked at day 5, corresponding to the interval at which IFN-γ expression was maximal. Analysis of DCT-specific $CD8^+$ TILs revealed markedly higher numbers at day 10 post-immunization compared to day 5 (**Fig. 2b,** left panel). Similarly, gp100-specific $CD8^+$ TILs were also higher at day 9 compared to day 5 (**Fig. 2b**, right panel). These observations suggested that the reduction in cytokine expression in the tumor was not due to fewer infiltrating $CD8^+$ T cells. Instead, the small number of early infiltrating DCT-specific T cells were initially highly active, based on the

transcriptional analysis, but became progressively less active in the tumor over time despite increasing in number.

Immune attack following vaccination is associated with elevated expression of antigen presenting machinery

Based on the importance of IFN-γ for tumor suppression in this model, we next sought to assess whether downstream IFN-γ inducible genes (STAT-1 & CIITA) and antigen presentation machinery (MHC I & II) could also be enhanced following vaccination with rHuAd5-hDCT. Indeed, we observed that STAT-1 expression displayed kinetics similar to IFN-γ, although the STAT-1 levels did not diminish to the same extent as IFN-γ (**Fig. 2c**). Likewise, CIITA expression was robustly induced following treatment with rHuAd5-hDCT and, similar to STAT-1, remained elevated for a prolonged period (**Fig. 2c**). It is interesting to note that expression of both STAT-1 and CIITA are significantly elevated at later time points following rHuAd5-hgp100 immunization, consistent with a delay in immune attack. Upregulation of both STAT-1 and CIITA in the tumor was observed to be mediated by $CD8^+$ T cells and was dependent on IFN-γ production (**Fig. S1c,d and S2**). Upregulation of both MHC I and MHC II displayed a delayed and protracted expression relative to IFN-γ following rHuAd5-hDCT immunization (**Fig. 2c**) and was dependent on IFN-γ (**Fig. S2**). While MHC I upregulation was completely dependent upon $CD8^+$ T cells (**Fig. S1e**), MHC II expression was markedly diminished in the absence of $CD8^+$ T cells (reduced by

91% following depletion of $CD8^+$ T cells), but was still above the level observed in untreated mice (**Fig. S1f**). Strikingly, immunization with rHuAd5-hgp100 did not result in any change in MHC I or II expression within the tumor when compared to untreated mice (**Fig. 2c**), indicating that although the immune response elicited by rHuAd5-hgp100 (**Fig. 2a**) was able to alter intratumoral signaling (**Fig. 2c**), it was insufficient to increase antigen presentation within the tumor.

Increased 4-1BB signaling can partially recover the local immune attack resulting in enhanced rHuAd5-hDCT efficacy

The data in **Figures 2a,b** indicate that the DCT-specific $CD8^+$ T cells present at day 5 were more functional than those present at day 10, suggesting that the tumor environment became progressively more immune suppressive. We investigated whether an agonist monoclonal antibody against 4-1BB (α4-1BB) could enhance local immune function based on reports that this agonist could enhance genetic vaccines [15-18] and recover TIL function [19]. The α4-1BB antibody was administered 5 days after immunization to coincide with the peak expression of 4-1BB on $CD8^+$ T cells (data not shown). As predicted, treatment of tumor-bearing mice with rHuAd5-hDCT followed by α4-1BB partially recovered immune attack within the tumor, as defined by elevation in IFN-γ and TNF-α expression in comparison to tumors from mice treated with rHuAd5-hDCT alone (**Fig. 3a**). Interestingly, TNF-α production was recovered to a higher level than IFN-γ. The

elevation in cytokine expression was not due to the α4-1BB alone since tumors from mice treated with rHuAd5-LCMV-GP, which does not express a tumor antigen, in combination with α4-1BB exhibited cytokine expression comparable to untreated tumors (**Fig. 3b**). Consistent with the elevated local immune attack, the combination of rHuAd5-hDCT with α4-1BB caused tumor regression (**Fig. 3c**) and improved overall survival (**Fig. 3d**), although most tumors ultimately relapsed. It is notable that autoimmune vitiligo was observed in mice that experienced complete tumor regression (**Fig. S3a**). Treatment with α4-1BB in combination with the irrelevant vaccine rHuAd5-LCMV-GP had no impact on either tumor growth or survival relative to untreated mice (**Fig. 3d** and data not shown).

Vaccine-induced tumor-specific CD8$^+$ T cells display elevated PD-1 expression selectively in the tumor

Although expression of both IFN-γ and TNF-α persisted for a prolonged period when rHuAd5-hDCT was combined with α4-1BB, cytokine expression again began to decline (**Fig. 3a**), suggesting that T cell effectors within the tumor were being functionally suppressed. The immunosuppressive receptor PD-1 is often upregulated on CD8$^+$ T cells faced with a high antigen burden, as in the case of the tumor microenvironment [20-22], so PD-1 expression was measured on vaccine-induced CD8$^+$ T cells in the peripheral blood (PBL) and within the tumor (TIL). While PD-1 expression was largely absent on DCT-specific CD8$^+$ PBLs (**Fig. 4**,

left panels, solid black lines), PD-1 was significantly upregulated on DCT-specific $CD8^+$ TILs, irrespective of treatment with α4-1BB (**Fig. 4**, left panels, grey histograms, rHuAd5-hDCT MFI = 879.5 +/- 74.8, rHuAd5-hDCT + α4-1BB MFI = 838.0 +/- 87.4). To determine if PD-1 expression by $CD8^+$ TILs required cognate interaction with tumor-associated antigen, tumor-bearing mice were immunized with rHuAd5-LCMV-GP. The LCMV-GP-specific $CD8^+$ PBLs and TILs were both largely PD-1-negative, in the presence or absence of α4-1BB, demonstrating a requirement for cognate recognition of tumor antigen for PD-1 upregulation (**Fig. 4**, middle panels, grey histograms, rHuAd5-LCMV-GP MFI = 202.7 +/- 47.6, rHuAd5-LCMV-GP + α4-1BB MFI = 238.8 +/- 41.7). Interestingly, PD-1 expression was also elevated on gp100-specific $CD8^+$ TILs, similar to the DCT-specific $CD8^+$ TILs, indicating that PD-1 is upregulated on TILs following low-level cognate interactions [10] (**Fig. 4**, right panel, grey histograms, MFI = 839.8 +/- 44.7).

Early infiltration of vaccine-induced tumor-specific $CD8^+$ T cells results in rapid upregulation of PD-1 ligands

The data in **Figure 4** suggested that PD-1 signaling was a likely contributor to the apparent dysfunction of tumor-reactive $CD8^+$ TILs. Therefore, we examined the expression of the PD-1 ligands, PD-L1 and PD-L2 in tumors following immunization. Similar to the observed kinetics for the effector cytokines, the expression of the PD-1 ligands displayed rapid induction following vaccination,

peaking 5 days post-vaccination (**Fig. 5a**). These results demonstrate that the tumor rapidly adapted to the immune attack following vaccination. Interestingly, the kinetics of PD-L1 and PD-L2 were quite different, suggesting that they may have distinct regulation. PD-L1 mirrored IFN-γ production (**Fig. 5a** compare with **Fig. 2a**) and, indeed, PD-L1 expression was fully abrogated in IFN-γ-deficient mice and in mice depleted of $CD8^+$ T cells (**Figs. S1g and S2**). Similarly, maximal PD-L2 expression required $CD8^+$ T cells (**Fig. S1h**) and, although early expression of PD-L2 (day 5) was completely dependent upon IFN-γ, later expression (day 8) revealed an IFN-γ-independent mechanism (**Fig. S2**). Immunization with rHuAd5-hgp100 only induced weak expression of PD-L1 at later time points post-vaccination and minimal upregulation of PD-L2 at any time point (**Fig. 5a**). As observed for IFN-γ and TNF-α, expression of PD-L1 and PD-L2 was further enhanced when rHuAd5-hDCT vaccination was combined with α4-1BB (**Fig. 5b**). Again, PD-L1 expression paralleled IFN-γ expression (compare **Fig. 5b** with **Fig. 3a**). These observations were specific to the combination of rHuAd5-hDCT + α4-1BB, as immunization with rHuAd5-LCMV-GP + α4-1BB had no impact on PD-L1 or PD-L2 expression in comparison to untreated control mice (**Fig. 5c**).

PD-1 blockade acts synergistically with 4-1BB co-stimulation to dramatically enhance immune attack within the tumor leading to complete tumor regression

Our data indicate that rapid expression of PD-1 ligands in the tumor following vaccination leads to progressive impairment of local T cell activity. To address this possibility, we employed an antagonist monoclonal antibody to block PD-1 signaling (αPD-1) in combination with rHuAd5-hDCT + α4-1BB immunotherapy. As predicted, PD-1 blockade enhanced cytokine production within the tumor (**Fig. 6a**). Strikingly, the combination of α4-1BB and αPD-1 produced a synergistic enhancement of T cell activation within the tumor as measured by both IFN-γ and TNF-α expression (**Fig. 6a**) indicating a profound reversal of immune suppression. Further, cytokine production in the tumors treated with rHuAd5-hDCT + α4-1BB + αPD-1 escalated throughout the entire time series until the tumors became too small to reliably retrieve RNA (around day 15 post-vaccination). We also observed a synergistic enhancement in the expression of PD-L1 and PD-L2, further demonstrating the reciprocity between immune attack and upregulation of immune suppressive pathways in the tumor (**Fig. 6b**). Yet, despite increased expression of PD-1 ligands, PD-1 blockade was able to effectively reduce the suppressive pressure placed on DCT-specific $CD8^+$ TILs.

The synergistic enhancement of local immune attack by the combination rHuAd5-hDCT + α4-1BB + αPD-1 (**Fig. 6c**, closed triangles) manifested as near complete regression of all treated tumors (n=13) and approximately 70% of the

mice remained tumor-free (**Fig. 6d**), which was a significant improvement over all other treatments ($p<0.0001$). Again, the therapeutic effect required tumor-specific T cells produced by the rHuAd5-hDCT vaccine because mice immunized with rHuAd5-LCMV-GP + α4-1BB + αPD-1 displayed no change in tumor growth (data not shown) or long-term survival (**Fig. 6d**, closed diamonds). The improved outcome absolutely required 4-1BB stimulation because mice treated with rHuAd5-hDCT + αPD-1 only displayed transient tumor regression and all tumors relapsed within 2 weeks (**Figs. 6c and 6d**, closed squares). Again, autoimmune vitiligo developed in all tumor-free mice (**Fig. S3b**).

The therapeutic benefit of PD-1 blockade is not the result of increased numbers of DCT-specific CD8$^+$ TILs or expanded polyfunctionality

The benefit of 4-1BB co-stimulation and PD-1 blockade following rHuAd5-hDCT vaccination could have been due to elevated numbers of DCT-specific TILs, an alteration in the functionality of DCT-specific TILs or an increase in the ability of DCT-specific TILs to respond to tumor antigen. Immunization with rHuAd5-hDCT + α4-1BB did yield an 8.7-fold increase in the frequency of circulating DCT-specific CD8$^+$ T cells compared to mice immunized with the vaccine administered in combination with rat IgG (**Fig. 7a**) and a 6.9-fold increase in the number of DCT-specific CD8$^+$ TILs (**Fig. 7b**). However, the addition of αPD-1 to the α4-1BB did not further enhance the frequency of DCT-specific CD8$^+$ T cells in the periphery (**Fig. 7a**) and actually decreased the number of DCT-

specific $CD8^+$ TILs (**Fig. 7b**), presumably through some form of antibody-mediated depletion since the DCT-specific $CD8^+$ TILs were also PD-1-positive (**Fig. 4**).

We examined the polyfunctional properties of the effector DCT-specific $CD8^+$ PBLs and TILs to determine whether PD-1 blockade reversed the previously described functional defects manifest in DCT-specific TILs [9]. Similar to our previous report, the DCT-specific $CD8^+$ T cells in the PBL were capable of producing multiple cytokines (IFN-γ and TNF-α) and undergoing degranulation (measured by mobilization of CD107a), while the DCT-specific $CD8^+$ TILs were compromised in their ability to produce TNF-α and degranulate (**Figs. 7c-f**). Treatment with the α4-1BB or αPD-1 individually did not seem to impact $CD8^+$ T cell functionality to a significant degree, but we did note that the DCT-specific $CD8^+$ PBLs had greater capacity for TNF-α production when both α4-1BB and αPD-1 were administered (**Fig. 7e**), indicating that this combination elicited $CD8^+$ T cells with greater functional attributes; however, this enhancement did not manifest remarkably within the TILs. An increase in the frequency of IFN-γ-negative CD107a-positive $CD8^+$ TILs was also observed upon inclusion of αPD-1 treatment (**Figs. 7e,f**). However, this cell population was present in both peptide stimulated and control samples (data not shown) and was therefore not indicative of DCT-specific $CD8^+$ TILs. When the data in **Figures 6a** and **7** are taken together, it appears that the DCT-specific $CD8^+$ TILs in the mice treated with both

α4-1BB and αPD-1 were fewer in number, but much more active on a per-cell basis, presumably due to the reversal of PD-1-mediated negative signaling.

DISCUSSION

In the current study, we have addressed the limited efficacy of a prototypic cancer vaccine, rHuAd5-hDCT, against growing B16F10 melanomas. Despite producing a robust immune response, the vaccine only resulted in a modest and brief suppression of tumor growth, which was dependent on $CD8^+$ T cells and IFN-γ. Anecdotally, it has been suggested that the kinetics of the immune response elicited by vaccination may be too slow to significantly impact rapidly growing tumors like B16F10. However, our findings suggest that the true hurdle is the rapid adaptation of the tumor to vaccine-mediated immune attack.

Transcriptional analysis provides a useful tool to monitor biological events within the tumor microenvironment [23,24]. Using this approach, our studies revealed that the tumor adapts rapidly to the immune attack produced by vaccination. Within the period of one week, the tumor environment went from being conducive to T cell attack to an environment that was completely refractory to T cell attack. Strikingly, intratumoral T cell activity, as defined by IFN-γ and TNF-α expression, could be measured within 5 days of rHuAd5-hDCT administration, but was extinguished within the following 5 days, despite

continued recruitment of additional DCT-specific T cells. The extinction of T cell activity within the tumor was due, in large part, to upregulation of PD-1 on the T cells and $CD8^+$ T cell-dependent upregulation of PD-1 ligands within the tumor. These results indicate that early infiltrating T cells were more active within the tumor than T cells that infiltrated at later times post-vaccination due to an adaptive response of the tumor triggered by the early infiltrating T cells. Although the exact mechanisms of PD-1 upregulation remain to be determined, the process required cognate antigen within the tumor microenvironment, similar to a previous report [21]. IFN-α can promote PD-1 expression in the absence of specific antigen [25], which may explain the slight elevation in PD-1 expression noted on the LCMV GP-specific TILs. We also found that both PD-L1 and PD-L2 were rapidly upregulated in the tumor as a consequence of IFN-γ production by tumor-specific $CD8^+$ T cells; however, regulation of PD-L2 expression at later time points was IFN-γ-independent. This outcome is in agreement with previous reports demonstrating a role for IFN-γ in PD-1 ligand induction [26-29]. Our findings, however, point to a reciprocal relationship between IFN-γ and PD-1 ligand expression that has not been previously appreciated. With each manipulation of our vaccine strategy, elevated IFN-γ within the tumor was paralleled by a comparable elevation of PD-L1 and PD-L2. Once the IFN-γ expression, and, by extension, T cell activity, was suppressed and the tumor began to relapse, PD-L1 expression returned to baseline levels. In contrast, PD-L2 expression was more sustained, demonstrating that IFN-γ is not the only driver of

this adaptive response. Interestingly, the late kinetics of PD-L2 were more similar to those of TNF-α, suggesting a commonality to the expression pattern of these molecules. This proportional and transient adaptive response emphasizes the dynamic nature of immune escape following vaccination. Interestingly, tumor protection afforded by the rHuAd5-hDCT vaccine was abrogated in the absence of IFN-γ, highlighting this cytokine as a "double-edged sword" capable of mediating anti-tumor activity while simultaneously activating immune suppressive counter-regulatory mechanisms [29-31].

In 2011, two other groups also reported that PD-1 blockade could enhance the activity of distinct vaccination platforms (peptide-based and lentivirus-based) in the B16F10 model [31,32]; however, neither group examined the local events following immune blockade, nor did they examine the changes in TIL number or polyfunctionality. Our study provides novel insight into the mechanisms of immune enhancement following PD-1 blockade, by revealing that treatment with αPD-1 led to a dramatic elevation in local T cell activity on a per-cell basis (as measured by IFN-γ and TNF-α expression). At a functional level, the TILs from all treatment groups displayed similar properties, indicating that αPD-1 did not dramatically alter the functional capacity of the TILs. Rather, it appears that PD-1 blockade released the DCT-specific TILs from the local restraint imposed by the elevated expression of PD-1 ligands allowing more of the DCT-specific T cells to elaborate their effector function in response to tumor antigen.

Overall, our findings further elucidate the limitations of cancer vaccines, notably the ability of tumors to rapidly adapt to immune attack and suppress intratumoral activity of T cells prior to peak T cell expansion. This study also reinforces the concept that optimal delivery of cancer vaccines will require maximizing vaccine immunogenicity and suppressing negative regulators of T cell function [33]. Moreover, our data indicate that *ex vivo* analyses of peripheral blood cells and TILs should be interpreted with caution since they do not accurately reflect the true activity of immune cells within the tumor. Lastly, transcriptional analysis represents a powerful technique capable of revealing the status of vaccine-induced T cell immunity and immune suppression within the tumor microenvironment [34].

MATERIALS AND METHODS

Mice

Female C57BL/6 mice were purchased from Charles River Breeding Laboratory (Wilmington, MA). IFN-γ-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME). All of our investigations have been approved by the McMaster Animal Research Ethics Board.

Recombinant adenoviruses

The E1,E3-deleted recombinant human adenovirus serotype 5 (rHuAd5) vectors [35] used in this study have been described previously [8,10]. rHuAd5-hDCT expresses the full-length human dopachrome tautomerase (DCT) gene. rHuAd5-hgp100 expresses the full-length human gp100 gene. rHuAd5-LCMV-GP encodes the dominant $CD8^+$ T cell epitope of the lymphocytic choriomeningitis virus glycoprotein.

Tumor challenge and immunization

Mice were challenged intradermally with 10^5 B16F10 cells. 10^8 pfu of Ad vector was prepared in 100µl sterile PBS and injected in both rear thighs (50µl/thigh) 5 days after tumor challenge. Tumor growth was monitored daily and measured with calipers every other day. Tumor volume was calculated as width x length x depth.

Isolation of tumor infiltrating lymphocytes (TILs)

TILs were isolated as previously described [9]. Briefly, tumors were digested in a mixture of 0.5 mg/mL collagenase type I (Gibco), 0.2 mg/mL DNase (Roche) and 0.02 mg/mL hyalorunidase (Sigma) prepared in Hank's Buffered Saline (10ml/250mg of tumor). The digested material was passed successively through 70µm and 40µm nylon cell strainers and lymphocytes were purified using a mouse CD90.2 positive selection kit (EasySep, Stemcell Technologies).

Monoclonal antibodies

Anti-PD-1 (clone RMP1-14) was purchased from BioXcell and administered 3 days following vaccination using a schedule of 250μg/mouse every 3 days [36] for a total of 4 injections. Anti-4-1BB was produced at McMaster University from the 3H3 hybridoma (kindly provided by Robert Mittler, Emory University) and administered to mice 5 days after vaccination at a dose of 200μg/mouse. Total rat IgG (Sigma) was used as control. CD4 (clone GK1.5) and CD8 (clone 2.43) depleting monoclonal antibodies were produced in our laboratory from hybridomas obtained from the American Type and Culture Collection. All flow cytometry antibodies (anti-CD16/CD32, anti-CD28, anti-CD4-PE-Cy7, anti-CD8α-PerCP-Cy5.5, anti-PD-1-PE, anti-CD107a-FITC, anti-IFN-γ-APC and anti-TNF-α-FITC) were purchased from BD Biosciences.

Intracellular cytokine staining (ICS)

$CD8^+$ T cell epitope peptides ($DCT_{180-188}$, $hDCT_{342-351}$, LCMV-GP_{31-43} and LCMV-GP_{34-41}) were purchased from Biomer Technologies, dissolved in DMSO and stored at -20˚C. $CD8^+$ T cell epitope peptides specific for gp100 were identified from epitope mapping assays (unpublished data) and pooled for ICS analysis. The ICS method has been described previously [7]. Briefly, lymphocytes were stimulated with either the DCT, gp100 or LCMV-GP peptides (1μg/mL) for 5 hrs at 37°C in the presence of 8μg/mL anti-CD28 and 5μg/mL brefeldin A (BD Pharmingen). The CD107a mobilization assay was performed by adding anti-

CD107a-FITC at the beginning of the peptide stimulation as described [9]. Data were acquired on a FACSCanto (BD Biosciences) and analyzed using FlowJo software (TreeStar).

RNA extraction from solid tumors and quantitative real-time PCR

Tumors were excised, snap-frozen in liquid nitrogen and stored at -80°C. Tumors were homogenized in Trizol (Invitrogen) using a Polytron PT 1200C (Kinematica) and total RNA was extracted according to the manufacturer's specifications. RNA samples were further purified using an RNeasy mini kit (Qiagen) and treated with Ambion's DNA-free kit. Reverse transcription was performed with Superscript III First-Strand (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was carried out on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using Perfecta SYBR Green SuperMix, ROX (Quanta Biosciences). Reaction efficiency was determined for individual primer sets using a minimum of 5 serial dilutions to ensure similar efficiency between target and endogenous control reactions. Data were analyzed via the delta/delta CT method using the Sequence Detector Software version 2.2 (Applied Biosystems). Primer sequences were as follows: IFN-γ (FWD CTTGAAAGACAATCAGGCCATC; REV CAGCAGCGACTCCTTTTCC), TNF-α (FWD AAATAGCTCCCAGAAAAGCAAG; REV CTGCCACAAGCAGGAATGAG), STAT-1 (FWD AGAAAAACGCTGGGAACAGA; REV

GGGTTCAGGAAGAAGGAGAGA), CIITA (FWD GAAGACGCTAAGGGCAACAC; REV TGAAGGTGAAGCCAGAAGAAC), MHC I (FWD CATCTGTGGTGGTGCCTCTT; REV GCATAGTCCCCTCCTTTTCC), MHC II (FWD CTTATTAGGAATGGGGACTGGA; REV CCTGTGACGGATGAAAAGG), PD-L1 (FWD AACCCGTGAGTGGGAAGAG; REV CCTGTTCTGTGGAGGATGTG) and PD-L2 (FWD ATAGGCAAGGAGCCCAGAAC; REV AACCCGGACTTCCCCTACAC). GAPDH (FWD AGGAGCGAGACCCCACTAAC; REV GGTTCACACCCATCACAAAC) was used as an endogenous control.

Statistical analysis

Two-tailed, unpaired Student's *t* tests were used to compare two treatment groups. One and two way Analysis of Variances (ANOVA) were used for data analysis of more than two groups and a Bonferroni post test was utilized to determine significant differences between treatment groups. Survival data was compared using a logrank test. Results were generated using GraphPad Prism 4.0b software. Differences between means were considered significant at $p<0.05$: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NS: not significant.

ACKNOWLEDGEMENTS

This work was supported by funds from the Terry Fox Foundation. D.B. was supported by a scholarship from the Canadian Institutes for Health Research. The authors disclose no conflict of interest.

SUPPLEMENTARY MATERIAL

Figure S1: DCT-specific $CD8^+$ T cells mediate transcriptional changes observed within the tumor. Expression of IFN-γ **(a)**, TNF-α **(b)**, STAT-1 (**c**), CIITA (**d**), MHC I (**e**), MHC II (**f**) PD-L1 **(g)**, and PD-L2 **(h)** in tumors from mice treated with rHuAd5-hDCT and depleted of $CD4^+$ (αCD4), $CD8^+$ (αCD8), both cell subsets (αCD8/αCD4) or left non-depleted (NT) (n =4). Data points correspond to peak expression of individual genes. The grey area corresponds to the mean $^+$/- SEM of untreated tumors. Data presented as mean $^+$/- SEM.

Figure S2: IFN-γ mediates the expression of genes associated with antigen presentation, as well as PD-L1, and early PD-L2 expression within tumors following immunization. Expression of STAT1, CIITA, MHC I, MHC II, PD-L1, and PD-L2 in tumors from WT (day 5 or 7, corresponding to peak expression)

or IFN-$\gamma^{-/-}$ (day 5 & 8) mice treated with rHuAd5-hDCT. Data presented as mean $^{+}$/- SEM.

Figure S3: Mice that experience full tumor regression develop progressive autoimmune vitiligo. (a) Progressive vitiligo observed in a representative mouse treated with rHuAd5-hDCT + α4-1BB where complete tumor regression was observed. **(b)** Vitiligo originating at the site of tumor inoculation in mice treated with rHuAd5-hDCT + α4-1BB + αPD-1 where complete tumor regression was observed. Vitiligo was progressive as shown in **(a).**

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FIGURE LEGENDS

Figure 1: $CD8^+$ T cells and IFN-γ mediate vaccine-induced tumor growth suppression. **(a)** DCT and gp100-specific $CD8^+$ PBLs were measured post-rHuAd5-hDCT or rHuAd5-hgp100 immunization (n=5-12) **(b)** Tumor-bearing mice were immunized with either rHuAd5-hDCT (□), rHuAd5-hgp100 (△), or left untreated (○). **(c)** Tumor-bearing mice were treated with rHuAd5-hDCT and depleted of $CD4^+$ (■), $CD8^+$ (▼), or both cell subsets (◆) or left non-depleted (NT) (□). **(d)** Tumor-bearing WT (□) or IFN-$\gamma^{-/-}$ (◇) mice were treated with rHuAd5-hDCT. Tumor volumes in **b - d** reflect individual representative experiments (n = 4-5). Data presented as mean $^+$/- SEM.

Figure 2: Transcriptional analysis reveals dynamic changes within the tumor prior to peak infiltration by vaccine induced $CD8^+$ TILs. **(a)** Expression of IFN-γ and TNF-α in tumors from mice treated with either rHuAd5-hDCT (□), rHuAd5-hgp100 (△) or left untreated (○) (n=4). **(b)** DCT and gp100-specific $CD8^+$ TILs were measured post-rHuAd5-hDCT or rHuAd5-hgp100 immunization (n=8-10). Day 5 TILs were pooled prior to flow cytometry due to low cell recovery. **(c)** Expression of STAT-1, CIITA, MHC I, and MHC II in tumors from mice treated with either rHuAd5-hDCT (□) or rHuAd5-hgp100 (△) or left untreated (○) (n=4). Data presented as mean $^+$/- SEM.

Figure 3: Stimulation of 4-1BB partially restores intratumoral T cell activity following vaccination resulting in transient tumor regression and improved survival. **(a)** Expression of IFN-γ and TNF-α in tumors from mice immunized with rHuAd5-hDCT $^{+}$ α4-1BB (△; n=4-8). Data from rHuAd5-hDCT mice reproduced from Figure 2 for reference (□). **(b)** Expression of IFN-γ and TNF-α in tumors 9 days after treatment with rHuAd5-hDCT $^{+}$ α4-1BB (n=7-9), rHuAd5-LCMV-GP $^{+}$ α4-1BB (n=4) or untreated (n=4). **(c,d)** Tumor-bearing mice were immunized with rHuAd5-hDCT and treated with α4-1BB (△; n=20) or Rat IgG (□; n=9). As controls, mice were immunized with rHuAd5-LCMV-GP $^{+}$ α4-1BB (◇; n =8). Tumor volumes were calculated from a single representative experiment (n=4-5) and survival data was compiled from independent experiments. Data presented as mean $^{+}$/- SEM.

Figure 4: PD-1 is upregulated on tumor-specific CD8^{+} TILs following tumor infiltration. PD-1 was measured on antigen-specific CD8^{+} PBLs (black line) and TILs (grey histograms) 9 or 10 days following immunization with rHuAd5-hDCT $^{+}$/- α4-1BB, rHuAd5-LCMV-GP $^{+}$/- α4-1BB or rHuAd5-hgp100. Dashed lines correspond to controls without PD-1 staining. Data presented from a single representative sample (n=5-8).

Figure 5: Immunosuppresive PD-1 ligands are rapidly induced in tumors following vaccination and are further enhanced through 4-1BB co-stimulation. (a) Expression of PD-L1 and PD-L2 in tumors from mice treated with either rHuAd5-hDCT (□), rHuAd5-hgp100 (△) or left untreated (○) (n=4). **(b)** Expression of PD-L1 and PD-L2 in tumors from mice immunized with rHuAd5-hDCT $^{+}$ α4-1BB (△; n=4-8). Data from rHuAd5-hDCT mice reproduced from Figure 5a for reference (□). **(c)** Expression of PD-L1 and PD-L2 in tumors 9 days after treatment with rHuAd5-hDCT $^{+}$ α4-1BB (n=7-9), rHuAd5-LCMV-GP $^{+}$ α4-1BB (n=4) or untreated (n=4). Data presented as mean $^{+}$/- SEM.

Figure 6: Vaccination combined with 4-1BB stimulation and PD-1 blockade elicits a synergistic increase in local T cell activity within the tumor, resulting in complete tumor regression. (a) Expression of IFN-γ and TNF-α in tumors from mice treated with rHuAd5-hDCT in combination with α4-1BB (△), α4-1BB $^{+}$ αPD-1 (▲), or αPD-1 (■) (n=4-8). α4-1BB data reproduced from figure 3a for reference. **(b)** Expression of PD-L1 and PD-L2 in tumors from mice treated with rHuAd5-hDCT in combination with α4-1BB (△), α4-1BB $^{+}$ αPD-1 (▲), or αPD-1 (■) (n=4-8). α4-1BB data reproduced from figure 5b for reference. **(c,d)** Tumor-bearing mice were immunized with rHuAd5-hDCT $^{+}$ α4-1BB $^{+}$ αPD-1 (▲; n=10), rHuAd5-hDCT $^{+}$ αPD-1 (■; n=10) or rHuAd5-LCMV-GP $^{+}$ α4-1BB $^{+}$ αPD-1 (◆; n=5) and rHuAd5-LCMV-GP $^{+}$ αPD-1 (○) (n=5) as controls. Tumor volumes were calculated from a single representative experiment (n=4-5) and

survival data was compiled from independent experiments. Data presented as mean $^{+}$/- SEM.

Figure 7: 4-1BB co-stimulation $^{+}$/- PD-1 blockade following vaccination increases the DCT-specific immune response in both the peripheral blood and tumor without altering the functionality of DCT-specific CD8^{+} TILs. (a, b) Tumor-bearing mice were immunized with rHuAd5-hDCT and treated with α4-1BB, α4-1BB + αPD1, αPD-1 alone, or Rat IgG. As controls, mice were immunized with rHuAd5-LCMV-GP + α4-1BB, α4-1BB + αPD1, or αPD1 alone. **(a)** DCT-specific CD8^{+} PBLs were quantified 10 days post-vaccination (n=5-20). **(b)** DCT-specific CD8^{+} TILs were quantified 10 days post-vaccination (n=5-8). TILs from rHuAd5-hDCT + α4-1BB + αPD-1 and rHuAd5-hDCT + αPD-1 treated mice were pooled prior to flow cytometry due to low cell recovery. **(c-f)** Flow cytometric analysis of IFN-γ and TNF-α production and CD107a mobilization from rHuAd5hDCT + αPD-1 samples described in **b**. Values correspond to mean values calculated from compiled data. Data presented as mean $^{+}$/- SEM.

FIGURE 1-

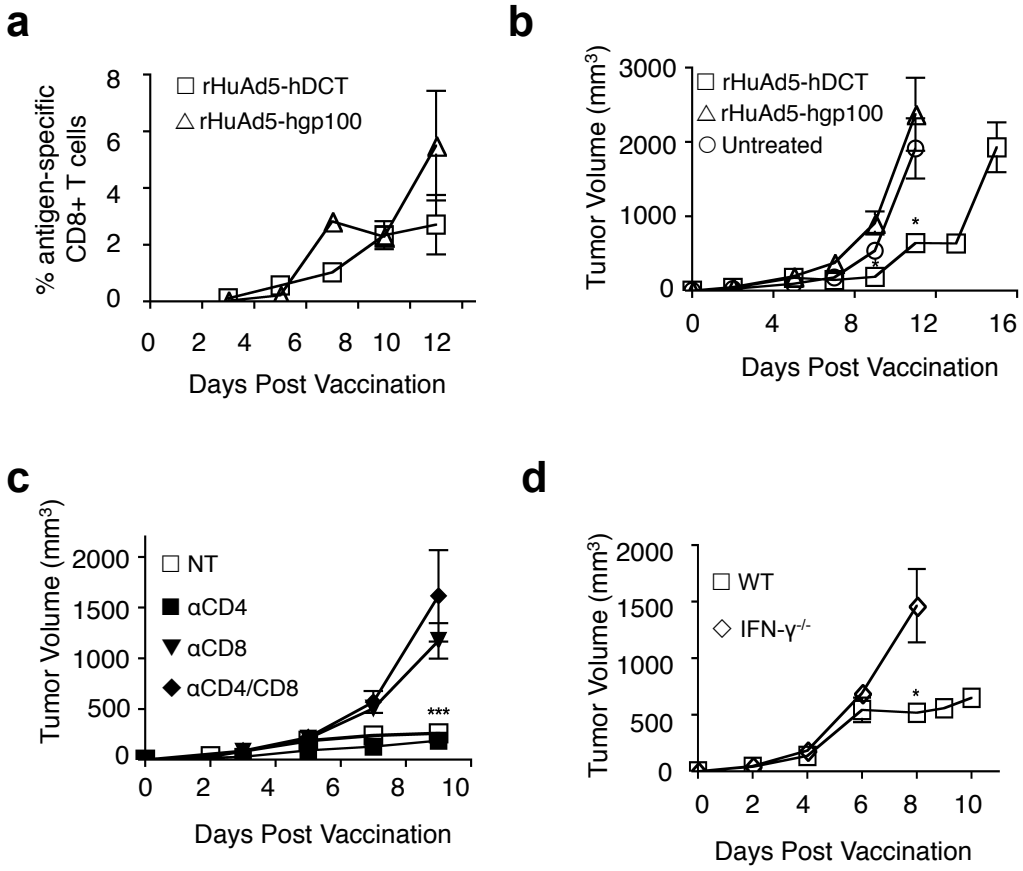

a
% antigen-specific CD8+ T cells
□ rHuAd5-hDCT
△ rHuAd5-hgp100
Days Post Vaccination
b
Tumor Volume (mm3)
□ rHuAd5-hDCT
△ rHuAd5-hgp100
○ Untreated
Days Post Vaccination
c
Tumor Volume (mm3)
□ NT
■ αCD4
▼ αCD8
◆ αCD4/CD8
Days Post Vaccination
d
Tumor Volume (mm3)
□ WT
◇ IFN-γ-/-
Days Post Vaccination

FIGURE 2-

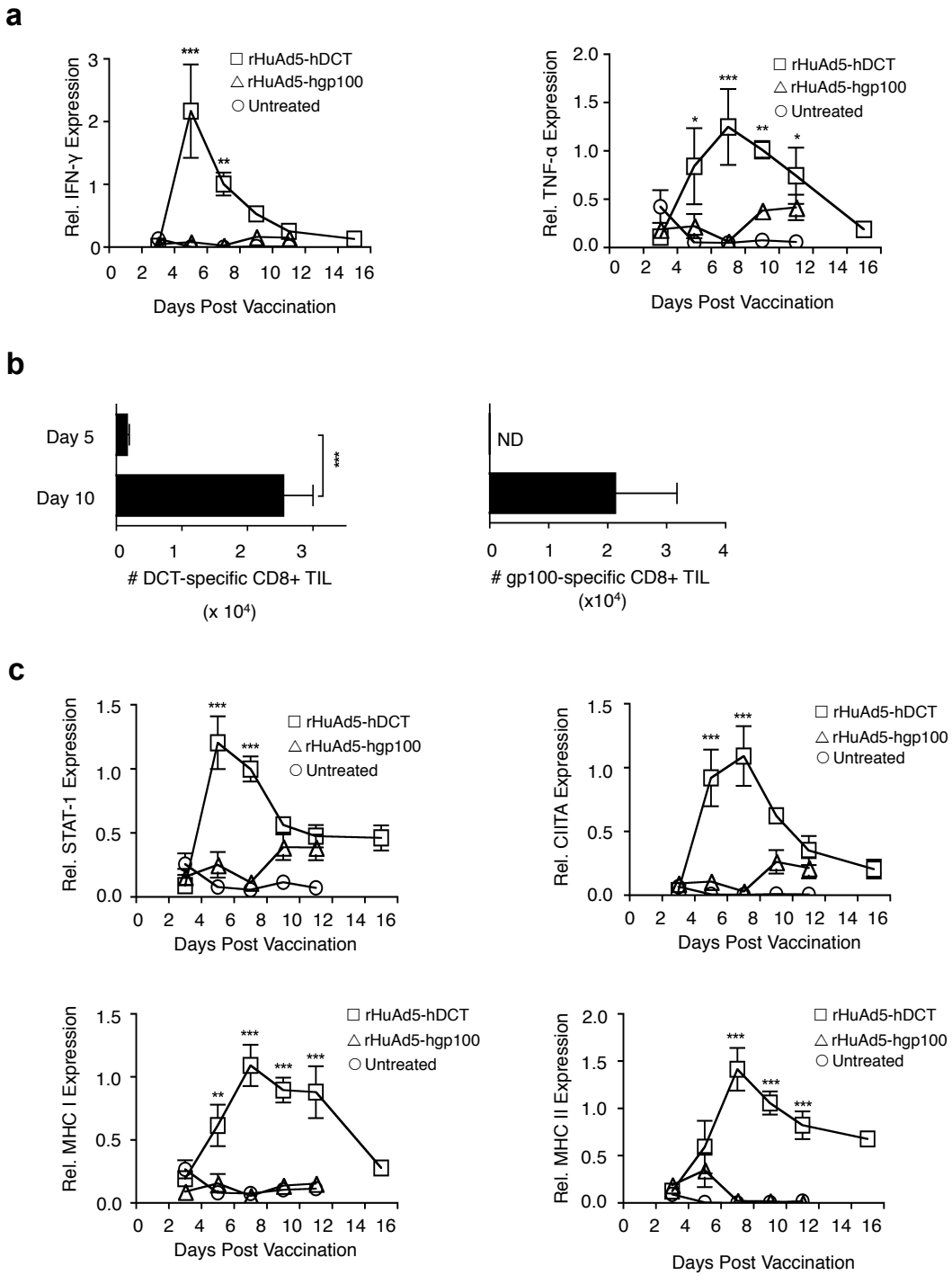

a
Rel. IFN-γ Expression
Days Post Vaccination
□ rHuAd5-hDCT
△ rHuAd5-hgp100
○ Untreated
Rel. TNF-α Expression
Days Post Vaccination
b
Day 5
Day 10
DCT-specific CD8+ TIL
(x 10⁴)
ND
gp100-specific CD8+ TIL
(x10⁴)
c
Rel. STAT-1 Expression
Rel. CIITA Expression
Rel. MHC I Expression
Rel. MHC II Expression
Days Post Vaccination

FIGURE 3-

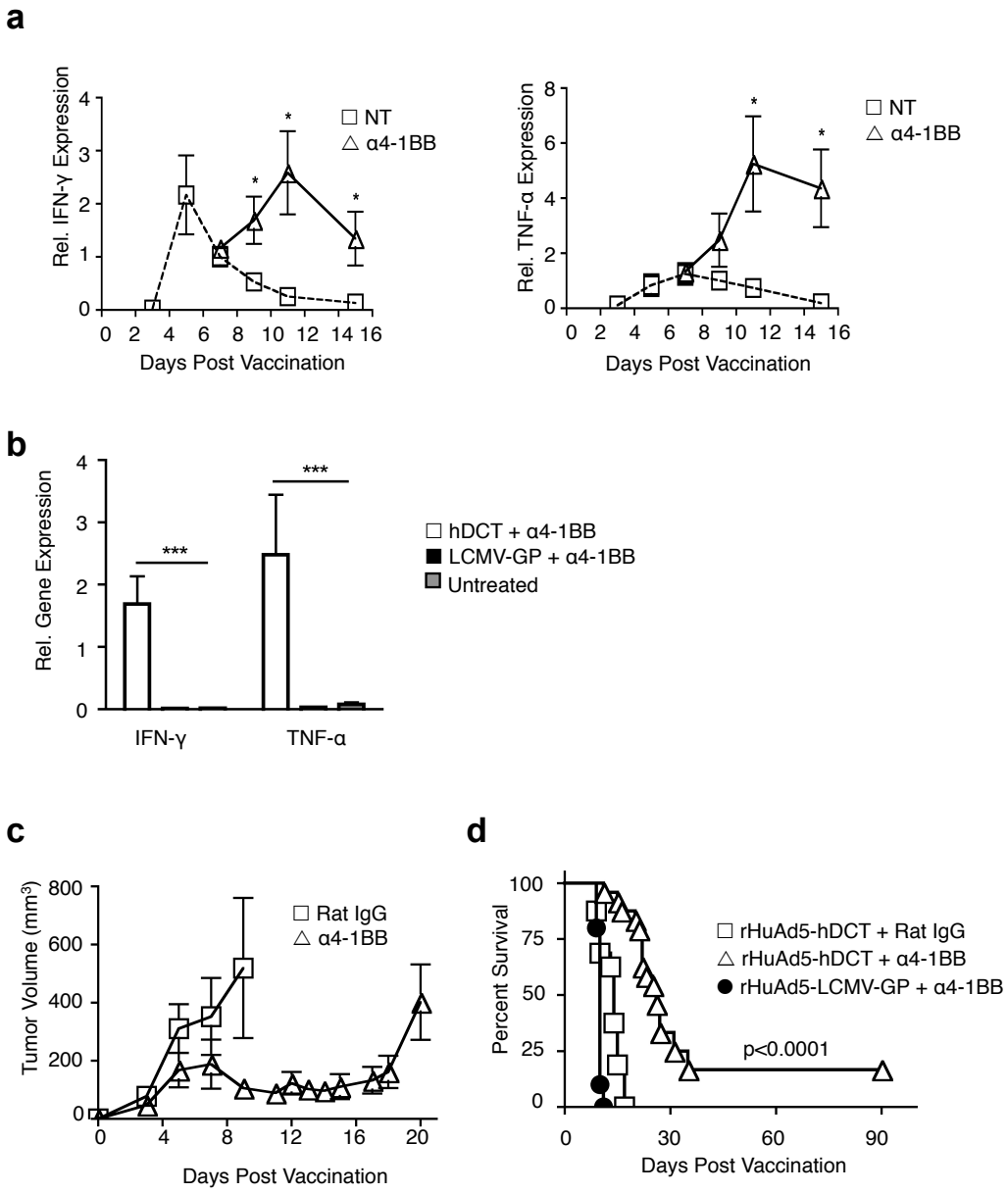

a
Rel. IFN-γ Expression
Days Post Vaccination
□ NT
△ α4-1BB
Rel. TNF-α Expression
Days Post Vaccination
□ NT
△ α4-1BB
b
Rel. Gene Expression
***
***
IFN-γ
TNF-α
□ hDCT + α4-1BB
■ LCMV-GP + α4-1BB
Untreated
c
Tumor Volume (mm3)
Days Post Vaccination
□ Rat IgG
△ α4-1BB
d
Percent Survival
Days Post Vaccination
□ rHuAd5-hDCT + Rat IgG
△ rHuAd5-hDCT + α4-1BB
● rHuAd5-LCMV-GP + α4-1BB
p<0.0001

FIGURE 4-

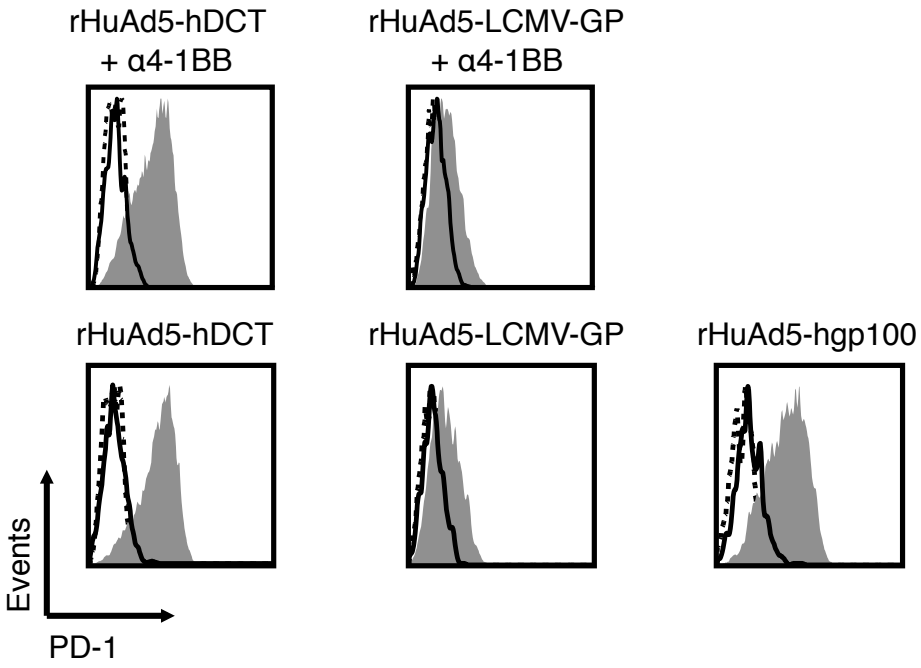

rHuAd5-hDCT
+ α4-1BB
rHuAd5-LCMV-GP
+ α4-1BB
rHuAd5-hDCT
rHuAd5-LCMV-GP
rHuAd5-hgp100
Events
PD-1

FIGURE 5-

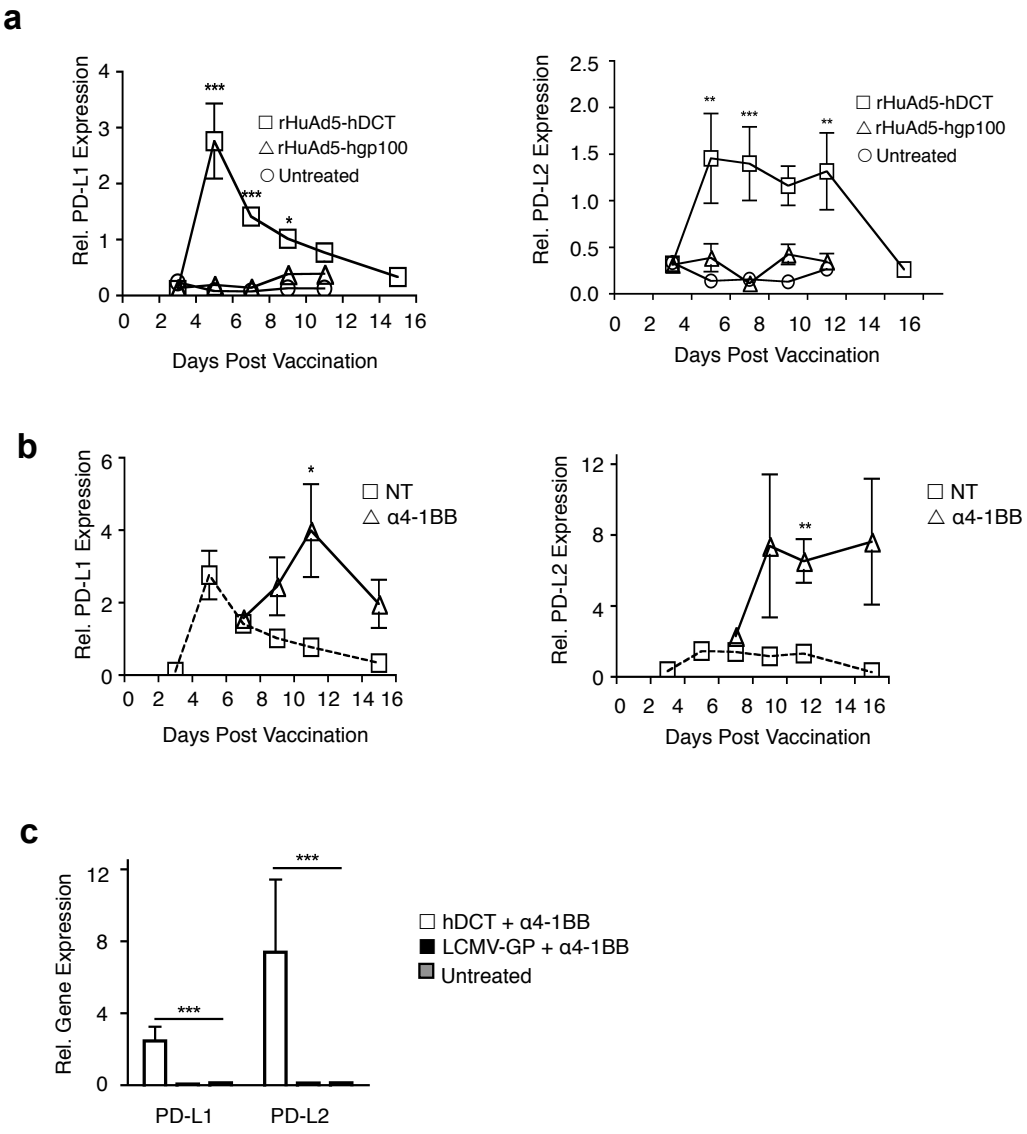

a
Rel. PD-L1 Expression
Days Post Vaccination
□ rHuAd5-hDCT
△ rHuAd5-hgp100
○ Untreated
Rel. PD-L2 Expression
Days Post Vaccination
□ rHuAd5-hDCT
△ rHuAd5-hgp100
○ Untreated
b
Rel. PD-L1 Expression
Days Post Vaccination
□ NT
△ α4-1BB
Rel. PD-L2 Expression
Days Post Vaccination
□ NT
△ α4-1BB
c
Rel. Gene Expression
PD-L1
PD-L2
□ hDCT + α4-1BB
■ LCMV-GP + α4-1BB
Untreated

FIGURE 6-

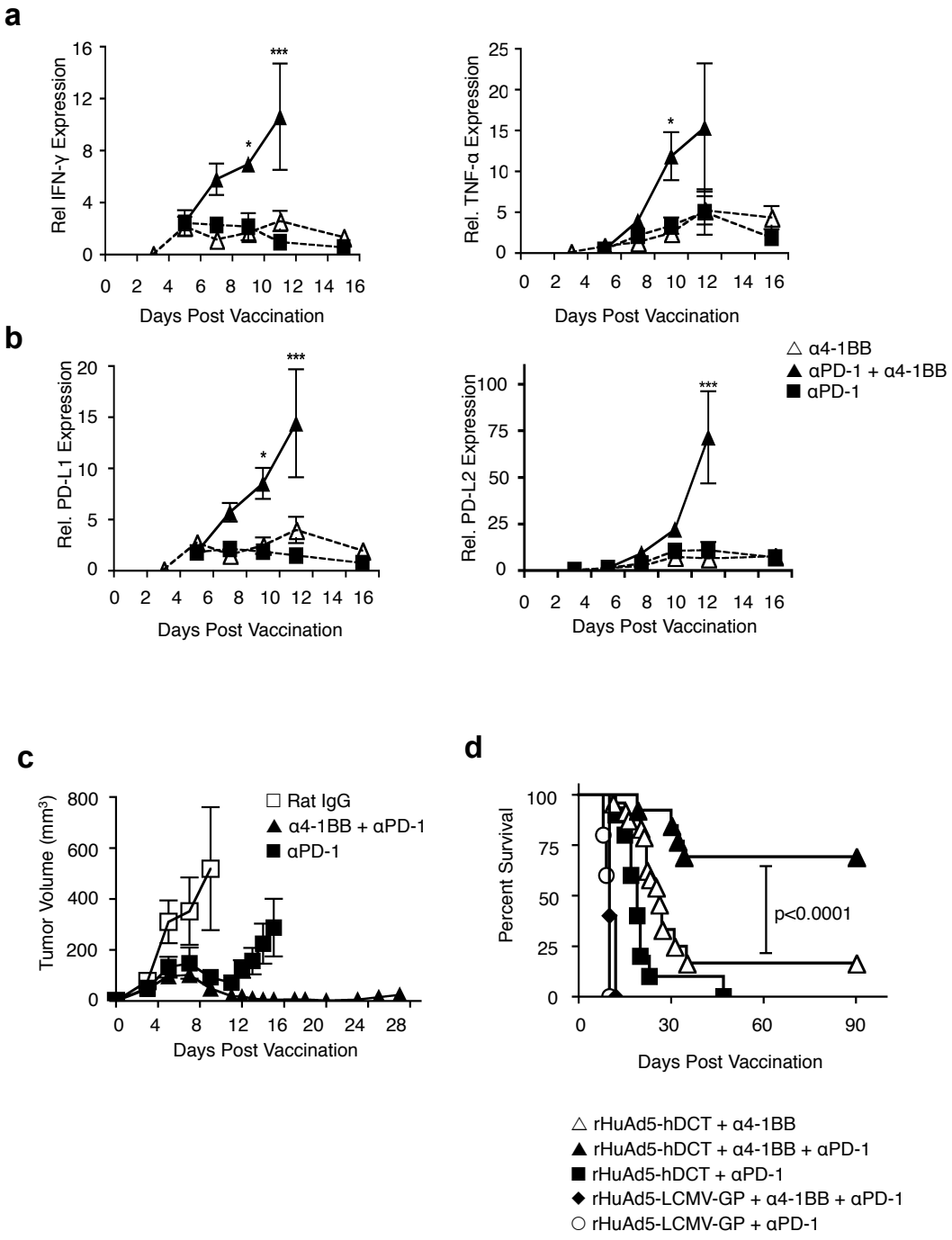

a
Rel IFN-γ Expression
Days Post Vaccination
Rel. TNF-α Expression
Days Post Vaccination
b
Rel. PD-L1 Expression
Days Post Vaccination
Rel. PD-L2 Expression
Days Post Vaccination
△ α4-1BB
▲ αPD-1 + α4-1BB
■ αPD-1
c
Tumor Volume (mm³)
□ Rat IgG
▲ α4-1BB + αPD-1
■ αPD-1
Days Post Vaccination
d
Percent Survival
p<0.0001
Days Post Vaccination
△ rHuAd5-hDCT + α4-1BB
▲ rHuAd5-hDCT + α4-1BB + αPD-1
■ rHuAd5-hDCT + αPD-1
◆ rHuAd5-LCMV-GP + α4-1BB + αPD-1
○ rHuAd5-LCMV-GP + αPD-1

FIGURE 7-

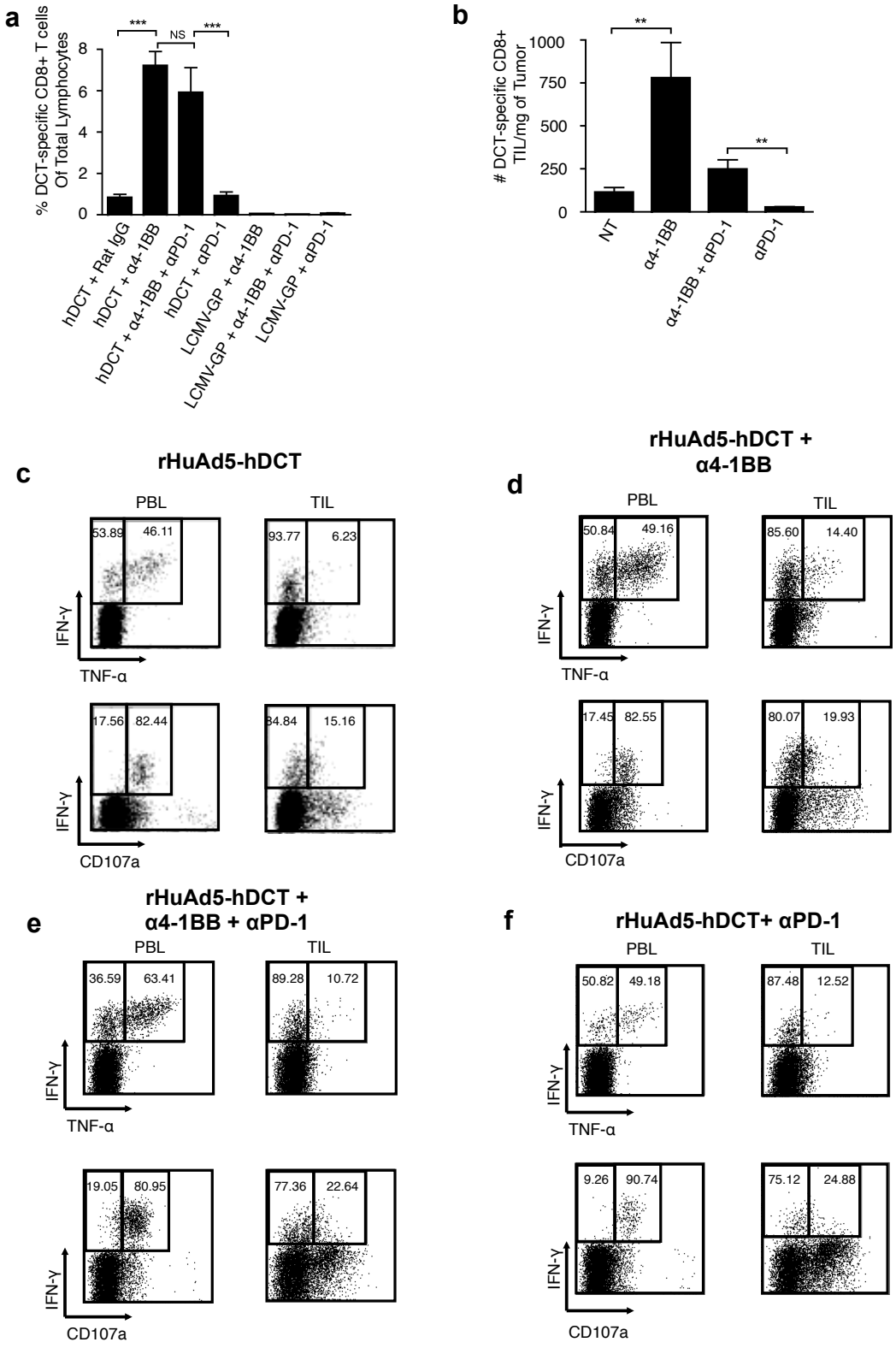

a
% DCT-specific CD8+ T cells Of Total Lymphocytes
***
NS
***
hDCT + Rat IgG
hDCT + α4-1BB
hDCT + α4-1BB + αPD-1
hDCT + αPD-1
LCMV-GP + α4-1BB
LCMV-GP + α4-1BB + αPD-1
LCMV-GP + αPD-1
b
DCT-specific CD8+ TIL/mg of Tumor
**
**
NT
α4-1BB
α4-1BB + αPD-1
αPD-1
c
rHuAd5-hDCT
PBL
TIL
53.89
46.11
93.77
6.23
17.56
82.44
84.84
15.16
IFN-γ
TNF-α
CD107a
d
rHuAd5-hDCT + α4-1BB
50.84
49.16
85.60
14.40
17.45
82.55
80.07
19.93
e
rHuAd5-hDCT + α4-1BB + αPD-1
36.59
63.41
89.28
10.72
19.05
80.95
77.36
22.64
f
rHuAd5-hDCT+ αPD-1
50.82
49.18
87.48
12.52
9.26
90.74
75.12
24.88

FIGURE S1-

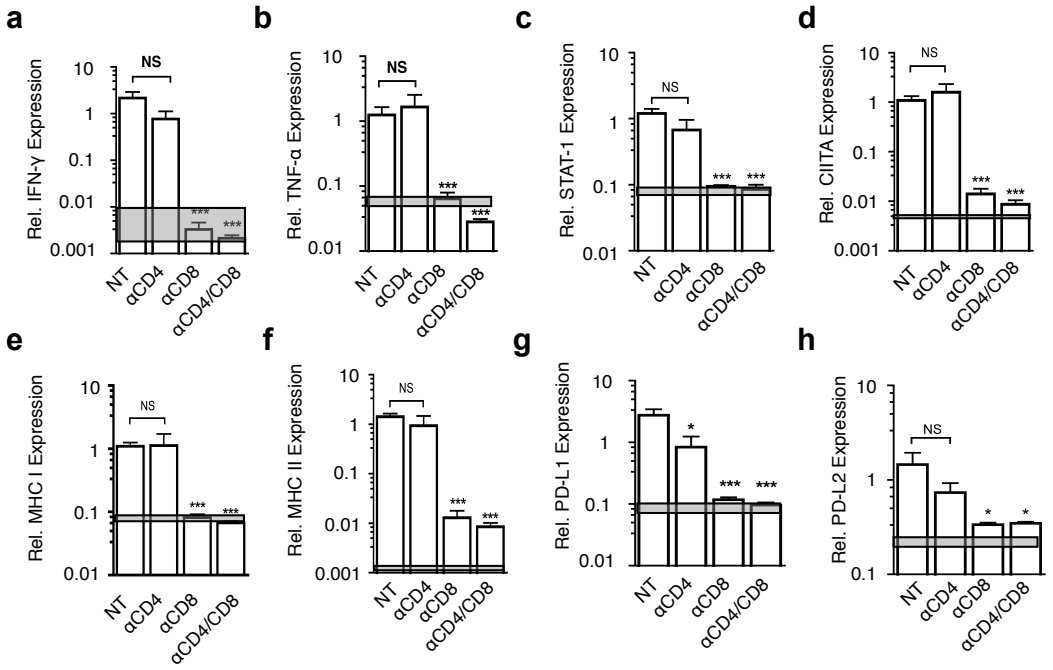

a
b
c
d
e
f
g
h
Rel. IFN-γ Expression
Rel. TNF-α Expression
Rel. STAT-1 Expression
Rel. CIITA Expression
Rel. MHC I Expression
Rel. MHC II Expression
Rel. PD-L1 Expression
Rel. PD-L2 Expression
NS
*
***
NT
αCD4
αCD8
αCD4/CD8

FIGURE S2-

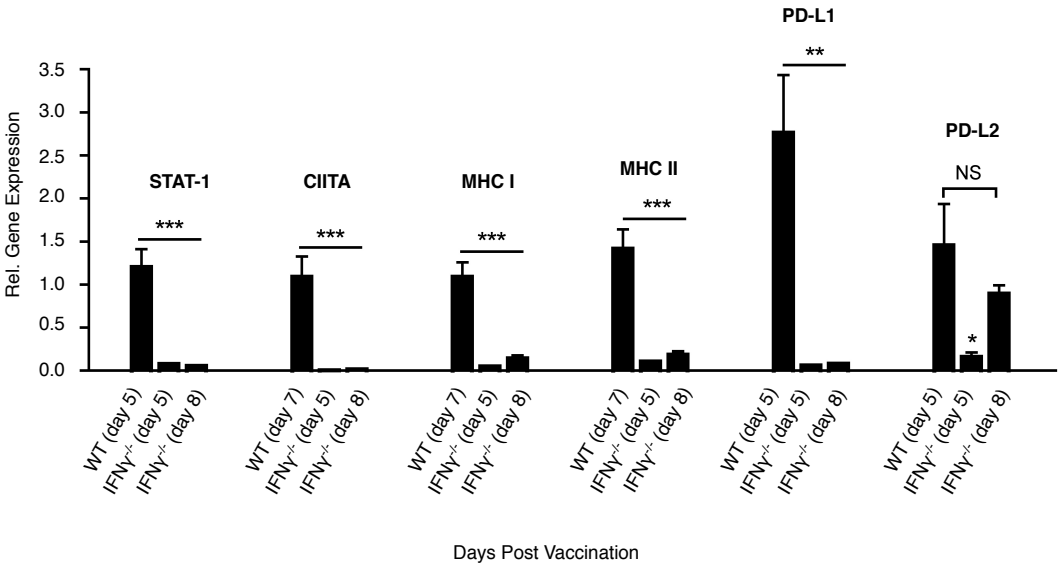
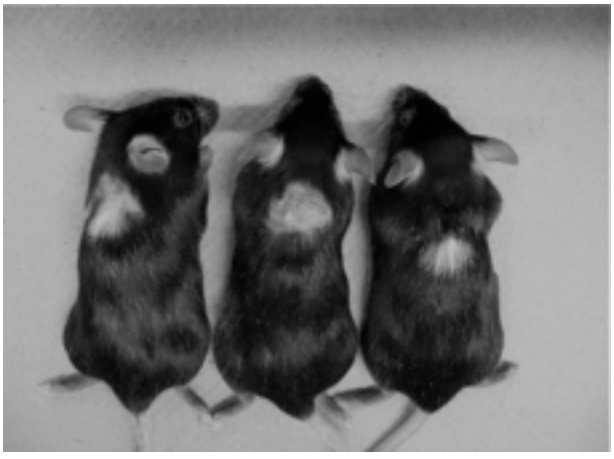

PD-L1
**
PD-L2
NS
STAT-1
***
CIITA
***
MHC I
***
MHC II
***
*
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Rel. Gene Expression
WT (day 5)
IFNγ-/- (day 5)
IFNγ-/- (day 8)
WT (day 7)
IFNγ-/- (day 5)
IFNγ-/- (day 8)
WT (day 7)
IFNγ-/- (day 5)
IFNγ-/- (day 8)
WT (day 7)
IFNγ-/- (day 5)
IFNγ-/- (day 8)
WT (day 5)
IFNγ-/- (day 5)
IFNγ-/- (day 8)
WT (day 5)
IFNγ-/- (day 5)
IFNγ-/- (day 8)
Days Post Vaccination

FIGURE S3-

a

b



— CHAPTER 5 —

Discussion

In this last chapter, I will summarize briefly the key findings of my work, as they have previously been discussed individually. In light of the data presented in Chapter 4, the discussion will focus on strategies to improve antitumor T cell immune responses and overcome tumor immunosuppression, hurdles which currently significantly limit the success of cancer-specific vaccination.

1.0 Summary of findings

The research that I carried out during my PhD training focused on characterizing mechanisms by which vaccination against DCT, a clinically relevant TAA, mediates tumor protection and autoimmunity.

The results presented as part of **Chapter 2** revealed that $CD4^+$ T cell-mediated tumor protection and autoimmunity appear to be dictated by distinct mechanisms in this model. Indeed, our data showed that protection required STAT-6 and IL-4 while autoimmunity depended on STAT-4 and partially IFN-γ. T cell analyses performed following vaccination of wild type as well as STAT-4- and STAT-6-deficient mice indicated that DCT-specific $CD4^+$ T cells harbored the same cytokine profile. These latter results strongly suggested that the dichotomy between the signaling requirements of tumor protection and autoimmunity are extrinsic of $CD4^+$ T cell differentiation. The effector mechanisms implicated in tumor protection and autoimmunity downstream of $CD4^+$ T cell activation are still elusive at this point. Whether such difference can

be exploited to minimize autoimmune pathology in the context of cancer immunotherapy remains to be investigated; of note however, our results showed that this dichotomy is not observed at the level of $CD8^+$ T cell responses. Cumulatively, these findings support the idea that cancer vaccine-specific $CD4^+$ T cells can take part in antitumor immune responses

The work undertaken as part of **Chapter 3** aimed to identify the epitope(s) recognized by DCT-specific effector $CD4^+$ T cells. Our data set uncovered an important role for post-translational modification of the DCT protein in the generation of effector $CD4^+$ T cell responses. In spite of detailed epitope mapping of DCT, we were unable to identify any potential effector epitopes. Such finding, or lack thereof, draws attention to an inherent limitation associated with the use of overlapping synthetic peptide libraries for epitope mapping. Given that much fewer TAA-specific CD4 epitopes have been identified comparatively to CD8 epitopes, one might wonder to which extent the less constrained nature of the MHC II binding groove can accommodate CD4 epitopes which retain post-translational modifications. Our vaccination studies conducted with glycosylation-mutant forms of DCT further supported the need for DCT to traffic to the Golgi apparatus where additional post-translational modifications take place. To this date, the exact nature of the epitope recognized by DCT-specific effector $CD4^+$ T cells remains unknown. Overall, our data demonstrate that $CD4^+$ T cell helper and effector epitopes can be generated via independent processes.

The results generated in the therapeutic vaccination setting, which are presented in **Chapter 4**, emphasize the rapid kinetics of tumor adaptation following vaccination with DCT. It is widely thought that tumors are inherently suppressive and tumor-specific TILs are generally dysfunctional. Contrary to this belief, our data clearly demonstrate that tumor immunosuppression is a progressive and dynamic phenomenon that is incited by T cell attack, more specifically from $CD8^+$ T cell-derived IFN-γ in our model. Indeed, transcriptional analysis showed that early-infiltrating DCT-specific $CD8^+$ T cells are initially highly active and that the tumor microenvironment subsequently becomes refractory to T cell killing, via upregulation of PD-L1, prior to peak infiltration by DCT-specific T cells. Inclusion of both anti-PD-1 and anti-41BB mAbs significantly increased the functionality of $CD8^+$ TILs, on a per-cell basis, allowing for complete regression of tumors and survival in a large proportion of the mice. Notably, tumor regression was entirely dependent on DCT-specific $CD8^+$ T cells, indicating that DCT-specific $CD4^+$ T cells played a minor role, if any, in this therapeutic setting. Overall, these results call attention to the rapidity by which tumors adapt to vaccine-specific T cell insult and illustrate the need for combinatorial immunotherapeutic approaches directed at overcoming tumor immunosuppression.

Taken as a whole, the research presented in my thesis has significantly contributed to our understanding of how DCT-specific $CD4^+$ T cell responses are generated (Chapters 2 & 3) and, although much remains to be learned with regard

to overcoming the limitations encountered in the therapeutic vaccination setting, offers preliminary insight into the mechanisms at play (Chapter 4).

2.0 Improving cancer vaccination

The occasional cures obtained with cancer immunotherapies attest of the biological feasibility of this treatment approach. Adoptive T cell transfer therapy is currently thought to be the most effective cancer immunotherapeutic approach for metastatic disease [374]. The significant rate of objective cancer regression reported with adoptive cell transfer therapy likely reflects the fact that cancer patients typically receive multiple injections of high numbers of tumor-specific autologous T cells, i.e. up to ~10^{11} cells/injection [374]. Presumably, such bolus of *ex-vivo* expanded T cells, which also undergo homeostatic proliferation, exerts antitumor effects before tumors have had a chance to counteract and suppress the immune attack. As previously mentioned however, adoptive T cell transfer therapy is limited by the costs associated with cell production and technical difficulties related to the broad implementation of such patient-customized treatment [301]. In contrast, provided that limitations such as tumor immunosuppression are overcome, therapeutic cancer vaccination has the potential to be a more cost-effective and easier cancer immunotherapeutic approach to put into clinical practice. In this section, I will review a selected

number of strategies, pertinent to our findings, which are currently being tested in pre-clinical and clinical settings to improve cancer immunotherapy.

2.1 Cancer-related inflammation

The resolution phase of inflammation promotes tissue repair/regeneration following infection or non-infectious tissue injury/irritation. A link between inflammation and cancer was first proposed in the 1860s [375] and has since been associated with increased susceptibility to cancer formation. Indeed, it is estimated that over 15% of the world's cancer incidence is linked to microbial infections (e.g. hepatitis B and C viruses, human papillomavirus, *H. pylori*, etc) [376]. Additionally, it is well known that inflammatory bowel disease and exposure to lung irritants such as asbestos and silica are underlying causes of colon and lung carcinomas [377,378]. The fact that aspirin and other non-steroidal anti-inflammatory drugs exhibit anticancer properties [379] also provides further evidence that inflammation and cancer are intimately linked. Notably, inflammatory gene signatures have also been found in various types of cancer, for which microbial infections or chronic inflammation are not predisposing factors, and were shown to correlate with poor prognosis [380]. This intrinsic development of cancer-related inflammation originates from the multiple genetic alterations that accumulate in cancer cells, which in turn promote the production of innate cell recruiting molecules including colony-stimulating factors (CSFs), inflammatory chemokines and cytokines [381]. This ensuing inflammation further

contributes to genotoxic stress and cancer progression by sustaining tumor cell proliferation and angiogenesis [382]. Cancer-related inflammation has therefore recently been pinpointed as one of the "enabling characteristics" supporting the hallmarks of cancer enumerated previously [5]. Targeting underlying causes and consequences of pro-tumoral inflammation represents a potentially valuable anticancer strategy. Tumor-associated macrophages (TAMs) are regarded as being the main orchestrators and perpetuators of cancer-related inflammation [383,384]. Furthermore, several lines of evidence have linked tumor inflammation with the recruitment of myeloid-derived suppressor cells (MDSCs) and the maintenance of their suppressive activity [385]. Both of these immune inhibitory cell populations play significant roles in promoting tumor immunosuppression and immune escape (introduction, section 4.4). Notably, microarray analyses (data not shown) performed on tumors as part of the work presented in Chapter 4 have revealed strong upregulation of CCL2/CCR2 (involved in monocyte recruitment towards inflamed tissues) as well as a myeloid cell gene signature indicative of TAM and/or MDSC infiltration. It is therefore of interest to our group to characterize these cell populations and determine whether their role in tumor immunosuppression, if any, can be overcome.

2.2 Circumventing the accumulation and suppressive activity of tumor-associated macrophages (TAMs)

Approaches designed to block the accumulation and protumoral functions of TAMs are still in the early stages of development and pre-clinical studies have

yielded encouraging results. Using gene-deficient mice and blocking mAbs, 2 groups have demonstrated a critical role for CCL2/CCR2 signaling in TAM recruitment and ensuing angiogenesis in different tumor models [386,387]. Another pre-clinical study has also reported a synergistic effect and improved survival when combining anti-CCL2 mAbs with a chemotherapeutic agent [388]. The tolerability and clinical activity of anti-CCL2 mAbs (CNTO 888) are currently being tested in patients with solid tumors in early phase clinical trials [389]. Another strategy, initially used by Coley [12], is to stimulate "good" inflammation. One such approach is for instance aimed at re-polarizing TAMs towards a tumoricidal M1 phenotype. Two pre-clinical studies have indeed shown that this can be achieved upon intratumoral administration of the TLR9 ligand CpG in combination with intraperitoneal anti-IL-10R [390] or, alternatively, following systemic IL-12 treatment [391]. Such findings have however yet to be demonstrated in the clinic. Overall, it is safe to assume that therapeutic drugs targeting macrophage-mediated protumoral inflammation will not be sufficient as single agents to achieve significant antitumoral activity, but they do however have the capability to synergize with other cancer immunotherapeutic approaches.

2.3 Circumventing the generation and function of myeloid-derived suppressor cells (MDSCs)

Given the strong connection between poor prognosis and prevalence of MDSCs in cancer patients, several approaches are aimed at circumventing their accumulation and suppressive functions. For instance, both pre-clinical and clinical studies

have reported improved tumor-specific T cell responses following treatment with all-trans retinoic acid, a vitamin A metabolite which induces the differentiation of MDSCs [392,393]. Using a mouse model, Pan *et al* demonstrated that tumor-secreted stem cell factor (SCF) promoted the recruitment of MDSCs, an event which required the cell surface expression of c-kit on MDSCs [394]. Upon *in-vivo* blocking of c-kit with mAbs, these authors were able to prevent MDSC accumulation, angiogenesis and Treg development as well as reverse tumor-specific T cell anergy [394]. Such result is clinically relevant as various small molecule tyrosine kinase inhibitors that inhibit c-kit signaling among others are used as anticancer drugs (e.g. imatinib, sunitinib, sorafenib). For example, it has been shown recently that sunitinib, approved for the treatment of renal cell carcinoma patients, mediates its effect in part via reduction of the MDSC population, which correlates with reduced Treg-mediated suppression [395]. Functional inhibition of MDSC-mediated iNOS and arginase immunosuppression is also being considered. Indeed, via downregulation of nitric oxide synthase and arginase, sildenafil (Viagra®) treatment was shown to improve the efficacy of adoptive T cell transfer therapy in a mouse tumor model [396]. Three clinical trials are currently testing whether tadalafi (Cialis®) can help stimulate antitumor immune responses in various cancer treatment scenarios [389]. Lastly, inhibition of both iNOS and arginase has also been observed following oral administration of nitric oxide-releasing aspirin, which was associated with enhanced therapeutic cancer vaccination efficacy [397]. While it is known that aspirin and other non-

steroidal anti-inflammatory drugs can prevent the formation of certain cancer types [379], agents such as nitric oxide-releasing aspirin have yet to be evaluated in combination with cancer immunotherapeutic approaches.

3.0 Concluding remarks

The next generation of cancer immunotherapies will undoubtedly require an all-encompassing approach, challenged by the need for affordable translation of scientific discoveries into the clinic. Many studies have highlighted the importance of $CD4^+$ T cells not only in promoting $CD8^+$ T cell recruitment, effector functions and memory responses, but also in mediating significant tumor clearance themselves. Optimal design of future cancer vaccination platforms should therefore ideally include CD4 determinants. Perhaps more importantly, we must remember that tumors employ numerous stratagems allowing them to thrive while dynamically interacting with and manipulating immune cells. Generating strong antitumor immunity as well as defeating immunological barriers are essential goals of upcoming anticancer strategies. To this end, novel cancer immunotherapic regimens should involve combinatorial approaches. As previously mentioned, highly effective cancer immunotherapy will most likely be accompanied by autoimmunity; methods to better predict and treat such collateral damage should therefore be concurrently investigated. Lastly, I believe that, as our knowledge of tumor and vaccine immunology progresses, cancer immunotherapies will become increasingly successful in the clinic.

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— APPENDIX 1 —

Investigating the impact of autophagy modulation on dendritic cell vaccines

Investigating the impact of autophagy modulation on dendritic cell vaccines

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Preface: *The research presented in this appendix reflects a side project that I had entertained in an effort to enhance $CD4^+$ T cell activation based on the premise that DC vaccines elicit better $CD4^+$ T cell immunity than direct immunization with a virus (Wan et al, Cancer Res. 2000 Jun 15;60(12):3247-53). This experimental work was carried out from July 2009 to June 2010. Unfortunately, the project was not leading us in the direction we had hoped and, therefore, was left in a semi-complete form. As the primary researcher, I designed and executed all experiments described in Figures 1-6. Jeanette E. Boudreau (PhD candidate), performed the NK:DC coculture assay presented as Figure 7. Dr. Julian J. Lum (collaborator) generated the transgenic mice and provided bones for dendritic cell culture. This work was supervised by Dr. Bramson who, along with Dr. Wan, provided experimental guidance and contributed to the interpretation of the results.*

ABSTRACT

Autophagy is linked to extended survival when cells face cellular stress. *Ex-vivo* derived dendritic cells (DCs) undergo substantial stress upon antigen loading and *in-vivo* delivery and the importance of autophagy in protecting DCs from these stressors is poorly understood. We have employed 2 strategies to investigate the impact of autophagy on DC vaccines. In the first case, we have investigated the role of basal autophagy in the context of DC vaccination. We have crossed $ATG5^{fl/fl}$ mice with CD11cCRE mice to generate conditional knockout mice in which DCs are autophagy-deficient. Results obtained from *in-vitro* phenotyping experiments of DCs transduced with recombinant adenovirus (Ad) and vesicular stomatitis virus (VSV) vectors indicate that autophagy deficiency does not impact maturation of the cells. However, lack of autophagy resulted in poor DC survivability in hypoxia. In the second case, we employed rapamycin as an inducer of autophagy in an effort to precondition DCs prior to viral transduction and immunization. Kinetic analysis showed that rapamycin effectively inhibited mammalian target of rapamycin (mTOR) within 5 hours and concurrently induced autophagy. Rapamycin preconditioning of DCs prior to viral transduction did not affect infectivity or maturation. In contrast, we noted a 10-fold reduction in type I IFN secretion following VSV infection, but not Ad infection. *In-vivo* studies conducted with rapamycin-preconditioned DC vaccines did not reveal a beneficial outcome. Our results indicate that autophagy is likely an important survival

mechanism allowing DCs to deal with changes in oxygen level, but does not seem to influence T cell or NK cell activation by DC vaccines. Other preconditioning methods, which could minimize the stress encountered by these cells, should be investigated.

INTRODUCTION

Dendritic cells (DCs) are central orchestrators of tumor-specific immunity [1,2]. DC functions, however, can be impaired due to the immunosuppressive state found in cancer patients [3]. To overcome this hurdle, methods have been developed to produce DCs *ex-vivo* to enable controlled DC maturation and antigen loading. Despite recent success obtained in this field [4], important facets of DC vaccines remain to be optimized. Recombinant viral vectors have proven to be a useful method for loading DCs with tumor-associated antigens (TAAs). This method allows native antigen processing and presentation [5] and does not rely on the identification of antigenic epitopes or knowledge of MHC restriction since the TAAs are expressed within the patient's own cells. Moreover, as viruses are highly immunogenic, they provide powerful inflammatory stimuli required for initiating an immune response [6].

Previous investigations by our group have highlighted the potential of virus-transduced DCs for cancer immunotherapy. Our studies have demonstrated

that immunization with DCs transduced with an adenoviral (Ad) vector *ex-vivo* prevents the development of virus-neutralizing antibodies, permitting repeated administration of virus-transduced DC vaccines [7]. We have also reported that virus-infected DC vaccines can expand and activate tumoricidal NK cells while DC vaccines activated by other means, such as TLR stimulation, do not activate tumoricidal NK cells [8,9]. These cumulative findings underscore the potential of virus-infected DC vaccines in the cancer setting, but several issues have yet to be fully addressed. A likely limitation of current DC vaccines pertains to the observation that few DCs, less than 1% of input, reach the draining lymph nodes following *in-vivo* delivery [10,11].

DC vaccines produced *ex-vivo* face abrupt changes in nutrient and oxygen concentrations upon *in-vivo* delivery. Autophagy is a process that allows cells to recycle cytoplasmic contents (proteins, macromolecules, organelles and microorganisms) during periods of cellular stress [12]. Autophagy is induced following growth factor withdrawal [13,14], infection [15], endoplasmic reticulum stress [16] and hypoxia [17]. Autophagy also plays important roles with regard to immunological functions of antigen-presenting cells (APCs) such as TLR triggering [18-20] and MHC II presentation of cytoplasmic antigens to $CD4^+$ T cells [21-24]. In light of these observations, we hypothesize that autophagy may help DCs cope with these stresses and inducing autophagy prior to transfer will enhance DC functionality *in-vivo*. In the current study, we describe the generation of autophagy-deficient DCs from bone marrow precursors of conditional knockout

ATG5$^{fl/fl}$CD11cCRE mice. These DCs displayed normal maturation phenotypes following *ex-vivo* viral transduction with recombinant Ad and VSV vectors. However, when compared to their wild type counterparts, autophagy-deficient DCs were highly susceptible to culture in hypoxic conditions. The latter finding supported the idea that autophagy preconditioning could improve the immunogenicity of virus-transduced DC vaccines. Pre-treatment of virus-transduced DCs with rapamycin, a known inducer of autophagy, did not significantly alter the rate of infection or maturation profile of the DCs *in-vitro*, but did result in a 10-fold reduction in type I IFN in response to VSV infection. Subsequent *in-vivo* studies failed to reveal any benefit of rapamycin preconditioning. Thus, although autophagy appears to be critical for DC survival following *in-vivo* administration, autophagy preconditioning with rapamycin does not confer enhanced immunogenicity as hypothesized.

RESULTS AND DISCUSSION

Bone marrow derived ATG5$^{fl/fl}$CD11cCRE DCs display impaired autophagy

The ATG5 protein is involved in key steps leading to the formation of autophagosomes and ATG5 deficiency renders cells incapable of autophagy [25]. ATG5-deficient (ATG5$^{-/-}$) mice die shortly after birth due to nutrient starvation during the neonatal period [26]. We therefore opted to create a conditional knock-

out mouse where ATG5 was specifically deleted from the DC lineage by breeding $ATG5^{fl/fl}$ mice [27] with mice expressing CRE recombinase under the control of the CD11c promoter [28] to generate $ATG5^{fl/fl}$CD11cCRE mice. As shown in **figure 1a**, ATG5 recombination was confirmed by PCR analysis performed on day 7 of DC cultures, at which time bone marrow cells have differentiated to DCs. The presence of flox-ATG5, which corresponds to un-excised ATG5, in cell samples positive for CRE likely reflects the fact that typically 15-20% of cells do not express CD11c. Comparison of the intensity of the PCR product corresponding to the floxed-ATG5 allele between CRE negative and CRE positive samples also suggests that ATG5 recombination occurs effectively in DCs. Autophagy induction was evaluated at the protein level by measuring LC3 protein following serum starvation using Western blot analysis (**fig. 1b**). LC3 is a widely used marker for monitoring autophagy and exists in two different forms, LC3-I and LC3-II, the latter form accumulates in autophagosomes and a shift in proportion from LC3-I to LC3-II is considered evidence of increased autophagy [29]. Western blot detection of LC3 conversion (LC3-I to LC3-II) correlates with the amount of autophagosomes present [29]. Although we cannot explain at this time why LC3-II levels in both $ATG5^{fl/fl}$CD11cCRE^{+} and $ATG5^{fl/fl}$CD11cCRE^{-} are reduced in comparison to wild type cell samples, autophagy does appear to be decreased in $ATG5^{fl/fl}$CD11cCRE^{+} versus $ATG5^{fl/fl}$CD11cCRE^{-} DC cultures. The observation that LC3-I is elevated in $ATG5^{fl/fl}$CD11cCRE^{+} DC sample indicates lack of LC3 conversion, also suggestive of impaired autophagosome formation [29]. These

combined results demonstrate that DCs generated from ATG5$^{fl/fl}$CD11cCRE mice are autophagy-deficient.

Autophagy-deficient DCs mature similarly to wild type DCs upon viral transduction

As previously mentioned, several studies have linked autophagy with antigen processing and subsequent MHC II presentation to CD4$^+$ T cells [21-24], but little is known about the potential roles of autophagy with regard to other important aspects of DC functions.

In order to appraise the possible implications of autophagy in DC functions, we assessed the *in-vitro* differentiation and maturation potential of autophagy-deficient DCs following transduction with recombinant adenovirus (Ad) and vesicular stomatitis virus (VSV) vectors expressing green fluorescent protein (GFP). Ad and VSV vectors were selected to conduct the following studies based on findings by our group showing that Ad- and VSV-transduced DC vaccines can elicit robust antitumor immunity [30,31]. On day 7 of DC cultures, non-transduced autophagy-deficient DCs displayed a morphology similar to wild type DCs and ~80% of the cells expressed CD11c, a frequency comparable to wild type DCs (data not shown). Susceptibility to virus infection, as indicated by GFP expression (**Fig. 2a**), was measured 24 hrs post-transduction. Interestingly, lack of autophagy resulted in slightly higher rates of infection/transgene expression, with ~5% and ~12% additional CD11c$^+$ cells being GFP$^+$ in the case of Ad and

VSV, respectively. We next examined the effect of autophagy deficiency on the maturation profile of DCs. There were no obvious changes in expression levels of MHC II, CD86 and CD40 compared to wild type DCs (**Fig. 2b**). A similar observation could be made for TNF-α and IL-12 production (**Fig. 2c**). Autophagy deficiency also did not alter type I IFN production as measured in DC supernatants 24 hrs post-transduction with either Ad or VSV (**Fig. 2d**). This latter finding, at least in the case of VSV, differs from results described by Lee *et al* [18] who reported that IFN-α production was considerably decreased in $ATG5^{-/-}$ plasmacytoid DCs. Lee *et al* argued that autophagy was required for delivery of cytosolic viral replication intermediates to TLR-7 located inside the lysosomes [18]. Since myeloid-derived DCs do not express TLR-7, it is not surprising that type I IFN production remained unchanged following VSV-transduction of autophagy-deficient DCs compared to wild type DCs. Cumulatively, our *in-vitro* results indicate that autophagy deficiency does not significantly impact DC differentiation or maturation following viral transduction, although susceptibility to virus infection may be increased.

Autophagy-deficient DCs are highly susceptible to cell death induced by hypoxia

Given the important role of autophagy in cell survival, we sought to compare cell viability in hypoxic conditions among the different DC cultures. Cell death was assessed via 7-AAD staining 24 hrs post-transduction with either Ad or VSV.

When DCs, virally transduced or not, were subsequently cultured in presence of 1% O_2 during a period of 24 hrs, autophagy-deficient cultures exhibited high levels of cell death, as evidenced by increased 7-AAD staining (**Fig. 3**). These results strongly suggest that lack of basal autophagy significantly impedes the ability of DCs to survive in hypoxic conditions. In agreement with this latter finding, several studies have highlighted the importance of autophagy in cardioprotection from ischemia [32-35].

Rapamycin treatment of DCs leads to inhibition of mammalian target of rapamycin (mTOR) within 5 hrs and concurrent induction of autophagy

mTOR is a serine/threonine kinase that is central to various cellular processes such as cell growth, transcription and translation [36]. Among other things, mTOR activates p70S6K and inhibits 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1), two translation regulators [36]. mTOR signaling is also known to negatively control autophagy [13]. Rapamycin is a macrolide antibiotic isolated from *S. hygroscopicus* which induces autophagy via mTOR inhibition [37]. In light of the survival role attributed to autophagy, we hypothesized that rapamycin treatment could precondition DCs prior to viral transduction to improve their survival following *in-vivo* delivery and enhance their immunogenicity. The concept of crosstalk between autophagy and apoptosis is in support of this hypothesis. Indeed, despite the fact that cellular stress can lead to autophagic cell death [38] or trigger apoptosis [39], it has been suggested that because the pathways

are partly shared between these two events, upstream signals may dictate the cell's fate depending on the circumstances [40].

Time course experiments were first carried out to determine the kinetics of action of rapamycin. The phosphorylation status of two downstream targets of mTOR, p70S6K and 4E-BP1, was assessed via Western blot analyses following rapamycin treatment of DCs with a low dose of 10 nM. As shown in **figure 4a**, rapamycin was effective at inhibiting mTOR activity within 5 hrs of treatment. This inhibition was also maintained for at least 24 hrs following virus infection, even when DCs were washed following 5 hrs of rapamycin preconditioning (**Fig. 4a**). We could therefore safely assume that rapamycin would remain active throughout subsequent *in-vitro* experiments and also during at least the first 24 hrs following *in-vivo* delivery. We also performed Western blot analyses to detect LC3 conversion to determine whether rapamycin treatment of DCs resulted in autophagy induction. After 6 hrs rapamycin preconditioning (10 or 100 nM), autophagy induction was observed as evidenced by enhanced levels of LC3-II in comparison to mock-treated samples (**Fig. 4b**).

Rapamycin preconditioning of virally transduced DCs does not significantly impact maturation, but does impair type I IFN production following VSV infection

Day 7 DCs were preconditioned with either 10 or 100 nM rapamycin or left untreated. Cells were then virally transduced with Ad or VSV vectors. As

seen in **figure 5a**, rapamycin pre-treatment did not significantly alter the frequency of GFP^+ DCs following Ad or VSV infection. In terms of DC maturation, levels of surface markers seemed conserved in presence of rapamycin in the context of both Ad and VSV transduction (**Fig. 5b**). These latter results are in agreement with a report showing that DCs differentiated in the absence of IL-4 retain their maturation ability when treated with rapamycin [41]. When cytokine production was evaluated, levels of IL-12 secretion following both Ad and VSV infection were unchanged when DCs were pre-treated with rapamycin (**Fig. 5c**). TNF-α production was however reduced in the context of VSV infection (**Fig. 5c**). Interestingly, we noted a 10-fold reduction in type I IFN production following VSV infection, but not Ad, when DCs were preconditioned with rapamycin (**Fig. 5d**). A similar effect has been reported by Cao *et al* [42] in the context of plasmacytoid DC activation via CpG treatment, whereby inhibited mTOR/downstream targets seemed to block the interaction of TLR-9 with MyD88, consequently interfering with activation of IRF-7 and downstream type I IFN production. This observation is in agreement with recent work by group showing the requirement for IRF-7 in type I IFN production by VSV-infected DCs [31]. As previously mentioned, there was no negative impact of rapamycin treatment on type I IFN production following Ad infection. This can be explained by the fact this modest production of type I IFN does not rely on IRF7 signaling. Together, the results obtained from *in-vitro* DC phenotyping experiments demonstrate that rapamycin preconditioning prior to viral transduction mostly

interferes with maturation of the cells at the level of the IRF7-dependent type I IFN response.

Rapamycin preconditioning of DC vaccines does not improve immune responses

To evaluate the effect of rapamycin preconditioning on the ability of DC vaccines to elicit immune responses, mice were immunized with DCs transduced with Ad or VSV vectors expressing human dopachrome tautomerase (hDCT), a melanoma-associated tumor antigen. hDCT-specific T cell responses were measured 14 days post-immunization via intracellular cytokine staining (ICS) following *in-vitro* stimulation of splenocytes with peptides corresponding to hDCT CD4 and CD8 epitopes (CD4 epitopes: KFF, LPY; CD8 epitopes: SVY, STF). Virus-specific $CD8^+$ T cell responses were also quantified using the same method (Ad epitope: FAL; VSV epitope: RGY). As seen in **Fig. 6a**, rapamycin-preconditioned DCs elicited virus-specific $CD8^+$ T cell responses that were similar in magnitude to the ones obtained from untreated DCs, if not lower as it was the case for Ad-transduced DCs preconditioned with 100 nM rapamycin. There were also no differences noted with regard to the hDCT-specific $CD8^+$ T cell response, which was only detectable following immunization with Ad-transduced DCs (**Fig. 6b**). $CD4^+$ T cell responses could sporadically be seen in mice immunized with Ad-transduced DCs, although background staining issues limited the interpretation of those results.

We have also investigated the effect of rapamycin preconditioning of DC vaccines on NK cell responses as we have previously shown that these cells actively participate in the antitumor effect of this vaccination platform [8,9]. This was achieved using an *in-vitro* coculture system whereby the ability of DCs to activate NK cells, defined by NK cell expression of IFN-γ and CD69, can be assessed by means of flow cytometry [31]. As seen in **figure 7**, rapamycin preconditioning of VSV-infected DCs prior to NK:DC coculture resulted in significantly impaired NK cell activation compared to coculture with VSV-infected DCs only. This result is not surprising, as we have shown that type I IFN production, which is critical for tumoricidal NK activation [31], is greatly diminished in presence of rapamycin in the context of VSV infection (**Fig. 5d**). Although the NK:DC coculture assay with Ad-transduced DCs was not conducted, we predict that NK activation would not be impacted by rapamycin treatment since the type I IFN production was maintained in these conditions. Overall, our *in-vivo* results indicate that rapamycin preconditioning does not significantly increase the immunogenicity of *ex-vivo* DC vaccines.

CONCLUDING REMARKS

We investigated the impact of autophagy modulation on DC vaccines. We successfully generated autophagy-deficient DCs from $ATG5^{fl/fl}$CD11cCRE

conditional knockout mice. Autophagy deficiency did not significantly impair the ability of the cells to mature upon viral transduction. However, autophagy-deficient DCs were highly susceptible to cell death when cultured in hypoxic conditions. Although *in-vivo* studies remain to be carried-out, the latter observation suggests that autophagy deficiency may interfere with cell viability following *in-vivo* delivery of DC vaccines, and therefore limit ensuing immune responses. When DCs were preconditioned with rapamycin to induce autophagy, no significant changes were observed in terms of maturation other than a reduced type I IFN production following VSV infection. $CD8^+$ T cell immunity elicited by rapamycin-preconditioned DC vaccines was unchanged. Moreover, *in-vitro* results indicate that NK cell immunity is likely to be impaired following immunization with VSV-transduced DCs pre-treated with rapamycin. Despite the fact that autophagy was indeed induced upon rapamycin treatment of DCs, it is difficult to delineate the effect of increased autophagy with the broad effects that mTOR inhibition has on cellular processes. In sum, rapamycin preconditioning of DC vaccines does not seem to enhance immunogenicity of the cells.

MATERIALS ANS METHODS

Mice and cell culture

Six- to 8-week-old female C57BL/6 mice were purchased from Charles River Breeding Laboratory (Wilmington, MA). Conditional knockout mice in which DCs are autophagy-deficient ($ATG5^{-/-}$) were kindly provided by Dr Julian J. Lum (Deeley research Center, BC). These mice were generated by crossing $ATG5^{flox/flox}$ mice [27] with CD11cCRE mice [28]. All of our investigations have been approved by the McMaster Animal Research Ethics Board. DCs were prepared as previously described [8,43]. Briefly, bone marrow cells were harvested from mouse femurs and tibias and cultured in the presence of 40 ng/ml of recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ). DCs were cultured in RPMI media supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies) as well as 1X sodium pyruvate, 1X non-essential amino acids and 0.1% β-mercaptoethanol (Gibco). Serum-free DC media was used to evaluate autophagy capability of cells where indicated. RPMI media containing FBS, L-glutamine, penicillin/streptomycin and β-mercaptoethanol was used for ICS assays. L929 cells used for IFN bioassays were cultured in α-MEM media containing 10% FBS, L-glutamine and antibiotics. Cells were maintained under normoxic conditions (21% O_2, 5% CO_2) at 37°C. Hypoxia treatment was performed by placing cells in a HERACell 150i incubator maintained at 1% O_2, 5% CO_2 and 37°C (Thermo Scientific).

Recombinant viruses

Replication-deficient adenoviruses (Ad) used in this study contain deletions in their E1 and E3 regions [44]. The expression cassettes were inserted in the E1 region under the control of the murine cytomegalovirus (CMV) promoter and the SV40 polyadenylation sequence. Ad vectors were created according to the Two-Plasmid Rescue Method using 293 cells [45]. AdEGFP-SVY (referred to as Ad-GFP in this manuscript), expresses the human $DCT_{180-188}$ CD8 epitope coupled to the C-terminal portion of enhanced-green fluorescent protein (EGFP) and AdhDCT expresses the full-length human DCT gene. VSV vectors were constructed using an M protein mutant of the Indiana serotype (ΔM51-VSV) and were created by subcloning PCR fragments between the XhoI and NheI sites of the plasmid pΔM51 [46]. VSVhDCT expresses the full-length human DCT gene and VSV-GFP carries the green fluorescent protein. ΔM51-VSV vectors were propagated in 293T cell cultures and purified by centrifugation on a sucrose gradient [47].

Rapamycin treatment, virus transduction and DC immunization

After a 7-day culture period, DCs were resuspended at a concentration of $2x10^6$ cells/ml in DC culture supernatant and subsequently treated with either 10 or 100nM rapamycin (Cedarlane, Burlington, ON, Canada) for 5 hrs. Ad and VSV transductions were carried out for 2 hrs using multiplicity of infections (MOIs) of 100 and 25 per cell, respectively. DCs were then washed twice with PBS prior to

footpad immunization with $1x10^6$ cells/mouse. Otherwise, media was added to dilute out viruses and DCs were left in culture 24 hrs for assessment of maturation phenotype by means of flow cytometry.

NK:DC coculture assay

DCs were treated with rapamycin and virally transduced as described above, and then resuspended in fresh media. NK cells were purified using a negative enrichment kit (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. NK cell purity was typically >85% (data not shown). NK cells and DCs were seeded at a 1:1 ratio ($5x10^4$ of each cell type) into a 96-well V-bottom plate and incubated for 12 hrs. GolgiPlug was added to cocultures during the last 6 hrs.

Monoclonal antibodies used in flow cytometry analyses

The following monoclonal antibodies were purchased from BD Pharmingen (Mississauga, ON, Canada): anti-CD11c-PE-Cy7 (clone HL3), anti-CD86-PE (clone GL1), anti-CD40 (clone 3/23), anti-TNF-α-PE (clone MP6-XT22), anti-IL-12p40/p70-APC (clone C15.6), anti-CD28 (clone 37.51), anti-CD16/CD32 (Fc Block; clone 2.4G2), anti-NK1.1-PE-Cy7 (clone PKH136), anti-CD69-PE (clone H1.2F3) anti-CD3ε-PE-Cy5.5 (clone 145-2C11), anti-CD4-PE-Cy7 (clone RM4-5), anti-CD8α-PE-Cy7 (clone 53-6.7) and anti-IFN-γ-APC (clone XMG1.2).

MHC II-PE-Cy5 (clone 25-9-17) was obtained from eBiosciences (Oakville, ON, Canada).

Flow cytometry

DC maturation was evaluated by analyzing surface marker expression and cytokine production via flow cytometry (BD FACSCanto, BD Biosciences, Mississauga, ON, Canada). Expression of MHC II, CD86 and CD40 was measured 24 hrs post-transduction. TNF-α and IL-12 production was detected in presence of GolgiPlug during the first 12 hrs or last 12 hrs of the maturation period following VSV and Ad transduction, respectively. Activation of NK cells following coculture with DCs was evaluated by staining for NK1.1, CD3 and IFN-γ. Immune responses following DC immunization were assessed on day 14 by means of *in-vitro* peptide stimulation and intracellular cytokine staining (ICS) analysis using mAbs to CD4, CD8α and IFN-γ. Splenocytes were stimulated, in presence of anti-CD28 mAb, with 1μg/ml and 10μg/ml peptide concentrations to detect $CD8^+$ and $CD4^+$ T cell responses respectively. All peptides were from Biomer Technology (San Fransisco, CA). DMSO was used as negative control. Results were analyzed using FlowJo 9.0.1 software (TreeStar, CA). Results from *in-vivo* studies are shown as absolute numbers of antigen-specific cells per spleen and background values have been subtracted.

IFN α/β bioassay

IFN bioassay was performed as previously described [9], with the exception that DC supernatants were collected 24 hrs post-transduction.

Western blotting

DCs were washed twice in ice-cold PBS and collected by centrifugation at 4°C. Cell pellets were lysed on ice for 15 mins in RIPA buffer containing protease and phosphatase inhibitors (1mM PMSF, 1X protease inhibitor cocktail and 1mM sodium orthovanadate (Sigma)) and then cleared by centrifugation for 10 mins at 14000 rpm at 4°C. Protein concentration was determined via a Bradford assay (BioRad). 50μg cell extracts were electrophoresed on 12% SDS-PAGE gels (15% for LC3 Western blotting) and then transferred to nitrocellulose membranes. Immunoblotting was performed using the following primary Abs from Cell Signaling Technology: p-p70S6K(Thr389), p-4E-BP1(Thr37/46) and LC3(G40). GAPDH was used as a loading control and mAb to GAPDH was acquired from Sigma. Blots were then incubated with anti-rabbit horseradish peroxidase secondary Ab prior to developing using an ECL system (Thermo Scientific).

ATG5 and CRE polymerase chain reaction

ATG5 flox recombination was assessed via polymerase chain reaction (PCR) in cultured DCs on day 7. DNA was isolated from $5x10^6$ DCs using a DNeasy kit (Qiagen). PCR settings were as follows: 95°C for 10 minutes, for 1 cycle, 95°C

for 30 seconds, 60°C for 30 seconds for 35 cycles and 72°C for 10 minutes. Primer sequences for ATG5 and CRE PCR reactions were as follows: wild type ATG5 (forward, gaatatgaaggcacacccctgaaatg), flox-ATG5 (forward, acaacgtcgagcacagctgcgcaagg), recombined ATG5 (forward, cagggaatggtgtctcccac), wild type ATG5/flox-ATG5/recombined ATG5 (reverse, gtactgcataatggtttaactcttgc) and CRE (forward, cgatgcaacgagtgatgagg; reverse, gcattgctgtcacttggtcct).

Statistical analysis

A two-tailed, unpaired Student's *t* test was used for the analysis. Differences between means were considered significant at $p<0.05$. Results were generated using GraphPad Prism 4.0b software (Graph Pad Software, LaJolla, CA).

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FIGURE LEGENDS

Figure 1. DCs generated from ATG5$^{fl/fl}$CD11cCRE^{+} mice are autophagy-deficient. Bone marrow-derived DCs were generated from wild type, ATG5$^{fl/fl}$CD11cCRE^{+} or ATG5$^{fl/fl}$CD11cCRE^{-}. DNA and protein extracts were prepared from DC cultures on day 7. **(a)** PCR analysis for wild type (WT) ATG5, flox-ATG5, recombined ATG5 and CRE confirmed the various genotypes. **(b)** LC3-II Western blot analysis showed reduced autophagy in DCs generated from ATG5$^{fl/fl}$CD11cCRE^{+} bone marrow compared to controls.

Figure 2. Autophagy deficiency does not significantly alter the ability of DCs to mature following viral transduction. Bone marrow-derived DCs were generated from wild type or ATG5$^{fl/fl}$CD11cCRE^{+} mice. On day 7, cells were transduced for 2 hrs with either recombinant adenovirus (Ad) or vesicular stomatitis virus (VSV) vectors expressing green fluorescent protein (GFP). **(a)** GFP expression, **(b)** surface maturation status (MHC II, CD86 and CD40) and **(c)** cytokine production (TNF-α and IL-12) were assessed via flow cytometry. **(d)** Type I IFN production was measured by bioassay.

Figure 3. Lack of autophagy in DCs may confer resistance to cell death resulting from VSV transduction, but is otherwise detrimental to DC survival in hypoxia. Bone marrow-derived DCs were generated from wild type or

$ATG5^{fl/fl}CD11cCRE^{+}$ mice. On day 7 of DC cultures, cells were transduced for 2 hrs with either recombinant adenovirus (Ad) or vesicular stomatitis virus (VSV) vectors expressing green fluorescent protein (GFP). DCs were then subsequently incubated in either **(a)** nomoxia or **(b)** hypoxia (1% oxygen) conditions for 24 hrs prior to assessment of cell viability via 7-AAD staining.

Figure 4. Rapamycin treatment of DCs leads to mTOR inhibition within 5 hours and concurrent autophagy induction. Bone marrow-derived DCs were generated from wild type mice. On day 7 of DC culture, cells were treated with either 10 or 100 nM rapamycin or left untreated. **(a)** Western blot analyses reveal inhibition of mTOR kinase activity on its downstream targets p70S6K and 4E-BP1 within 5 hrs of treatment and for at least 24 hrs in the context of virus infection (Ad: adenovirus; VSV: vesicular stomatitis virus). **(b)** Rapamycin treatment (6 hrs) leads to autophagy induction as indicated by LC3 Western blot analysis.

Figure 5. Pre-treatment of virus-transduced DCs with rapamycin does not significantly alter their maturation ability, but does reduce type I IFN production following VSV infection. Bone marrow-derived DCs were generated from wild type mice. On day 7 of DC culture, cells were treated for 5 hrs with either 10 or 100 nM rapamycin or left untreated. DCs were subsequently transduced with recombinant adenovirus (Ad) or vesicular stomatitis virus (VSV)

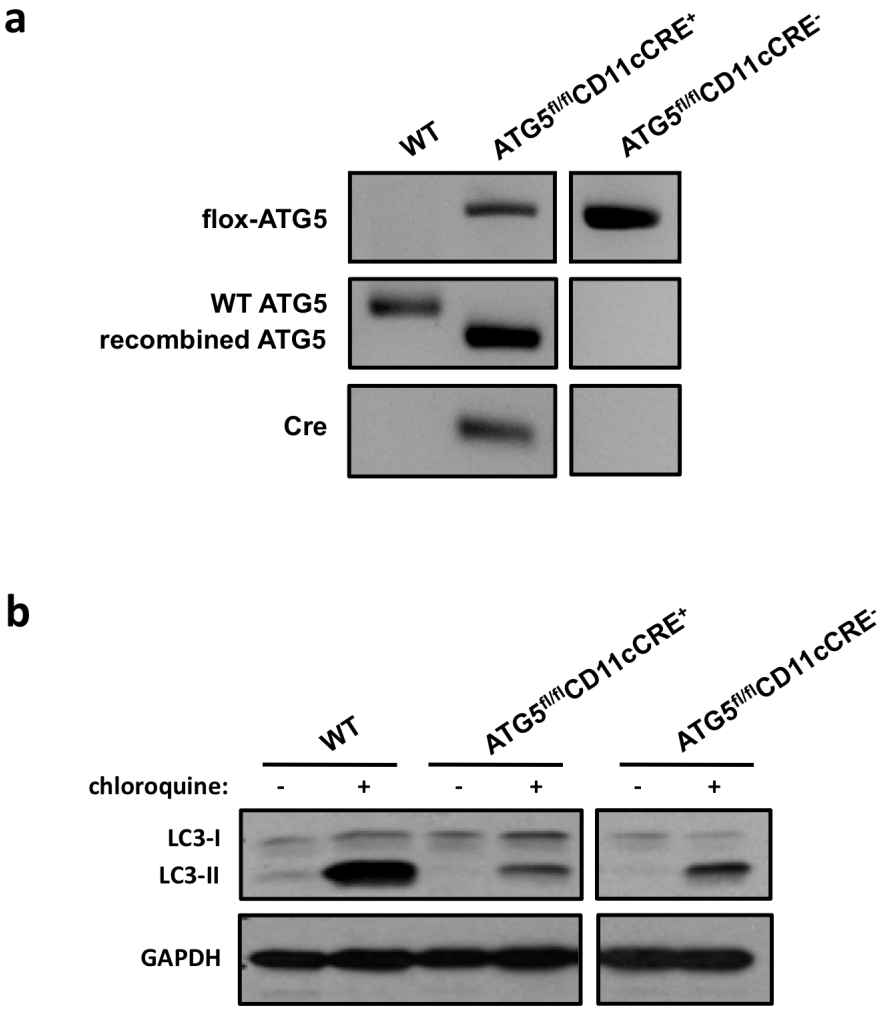
vectors expressing GFP. **(a)** GFP expression, **(b)** surface maturation status (MHC II, CD86 and CD40) and **(c)** cytokine production (TNF-α and IL-12) were assessed via flow cytometry. **(d)** Type I IFN production was measured by bioassay.

Figure 6. Prior rapamycin treatment of virus-transduced DCs does not improve T cell responses. Bone marrow-derived DCs were generated from wild type mice. On day 7 of DC culture, cells were treated for 5 hrs with either 10 or 100 nM rapamycin or left untreated. DCs were subsequently transduced with recombinant adenovirus (Ad) or vesicular stomatitis virus (VSV) vectors expressing the TAA human dopachrome tautomerase (hDCT). Mice were immunized via footpad injection with $1x10^6$ cells/mouse. On day 14 post-immunization, spleens were harvested for measurement of T cell responses elicited against **(a)** viral epitopes (Ad: FAL; VSV: RGY) and **(b)** hDCT epitopes (Ad: SVY/STF; VSV: not detected). Results are shown as mean±SEM from 10 mice pooled from 2 separate experiments and represent the absolute number of antigen-specific cells per spleen (*$p<0.05$).

Figure 7. Prior rapamycin treatment of virus-transduced DCs impairs the NK cell response. Bone marrow-derived DCs were generated from wild type mice. On day 7 of DC culture, cells were treated for 5 hrs with either 10 or 100 nM rapamycin or left untreated. DCs were subsequently transduced with

vesicular stomatitis virus (VSV) vector expressing the TAA human dopachrome tautomerase (hDCT). DCs were then placed in co-culture with purified naïve NK cells for 12 hours. NK cell activation was measured via flow cytometry following the addition of GolgiPlug during the last 6 hrs of co-culture.

FIGURE 1-


a
WT
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flox-ATG5
WT ATG5
recombined ATG5
Cre
b
WT
ATG5fl/flCD11cCRE+
ATG5fl/flCD11cCRE-
chloroquine: - + - + - +
LC3-I
LC3-II
GAPDH

FIGURE 2-

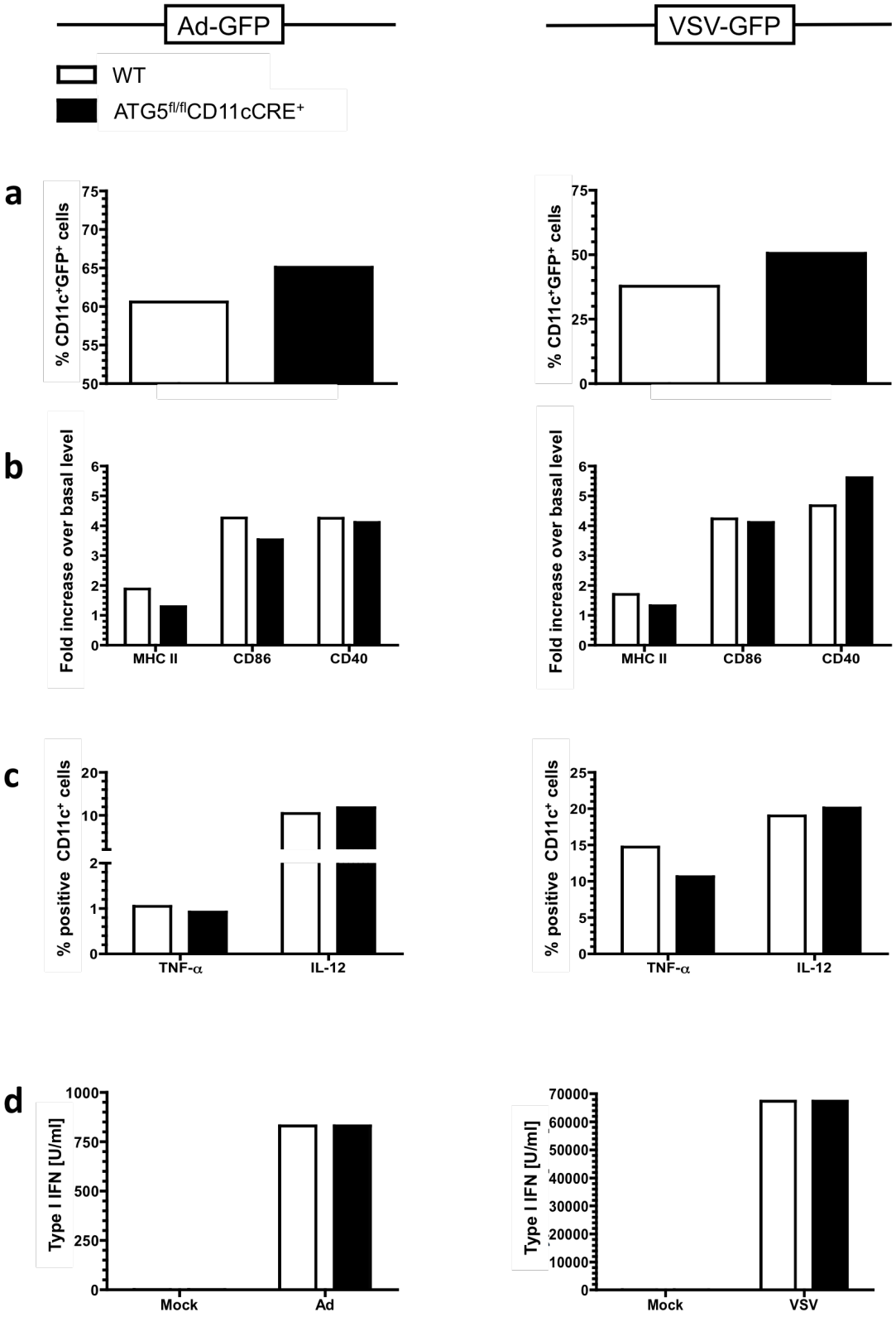

Ad-GFP
VSV-GFP
WT
ATG5fl/flCD11cCRE+
a
% CD11c+GFP+ cells
b
Fold increase over basal level
MHC II
CD86
CD40
c
% positive CD11c+ cells
TNF-α
IL-12
d
Type I IFN [U/ml]
Mock
Ad
VSV

FIGURE 3-

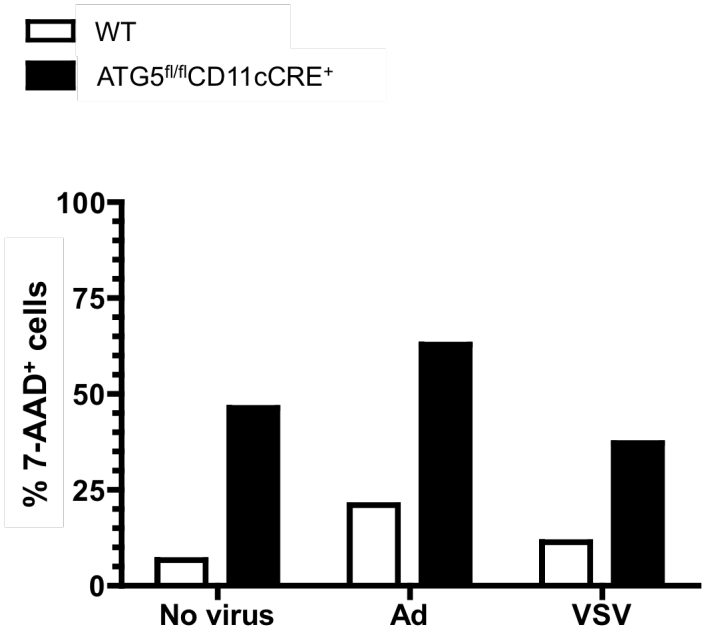


FIGURE 4-

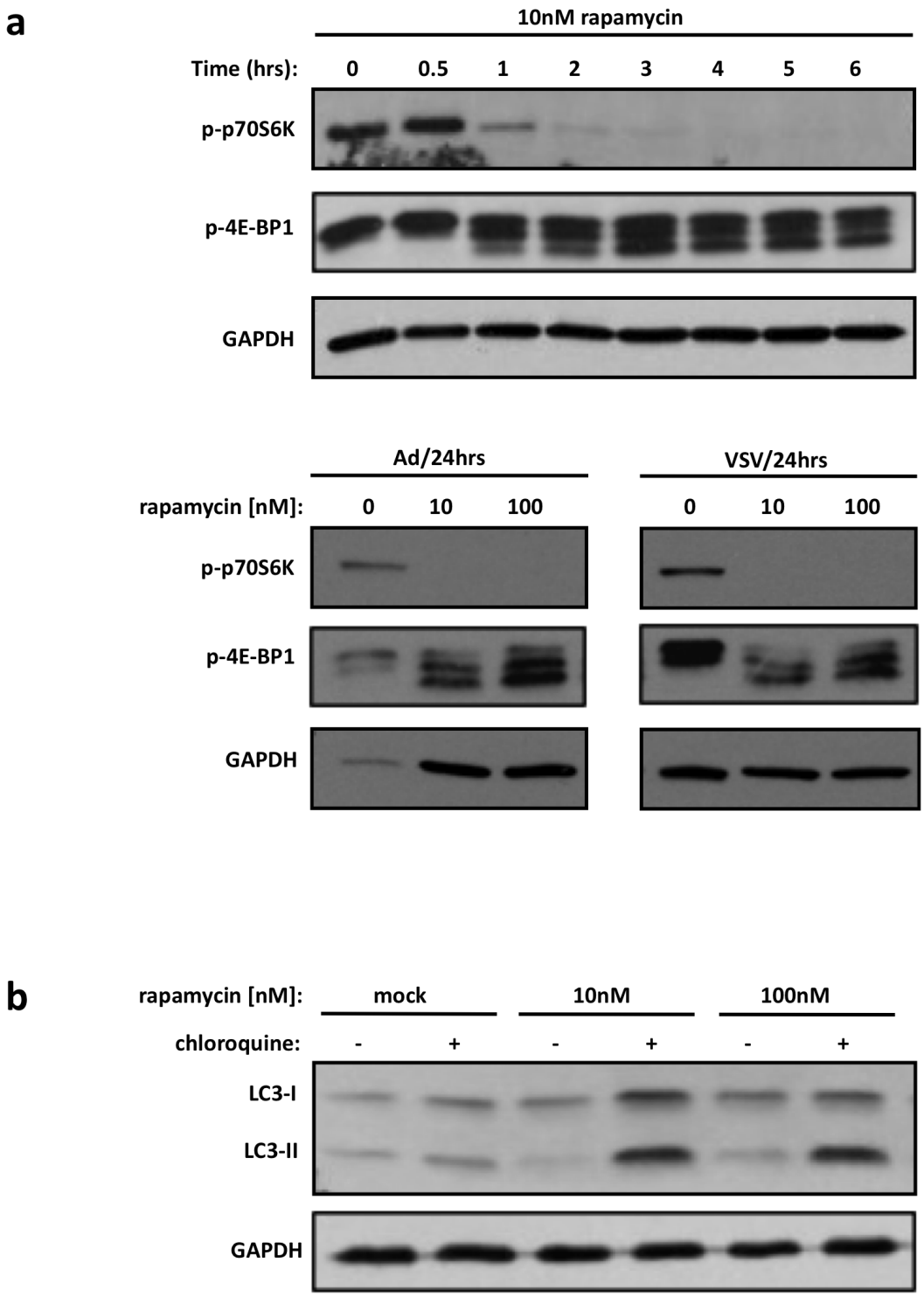

a
10nM rapamycin
Time (hrs):
0
0.5
1
2
3
4
5
6
p-p70S6K
p-4E-BP1
GAPDH
Ad/24hrs
VSV/24hrs
rapamycin [nM]:
0
10
100
0
10
100
p-p70S6K
p-4E-BP1
GAPDH
b
rapamycin [nM]:
mock
10nM
100nM
chloroquine:
-
+
-
+
-
+
LC3-I
LC3-II
GAPDH

FIGURE 5-

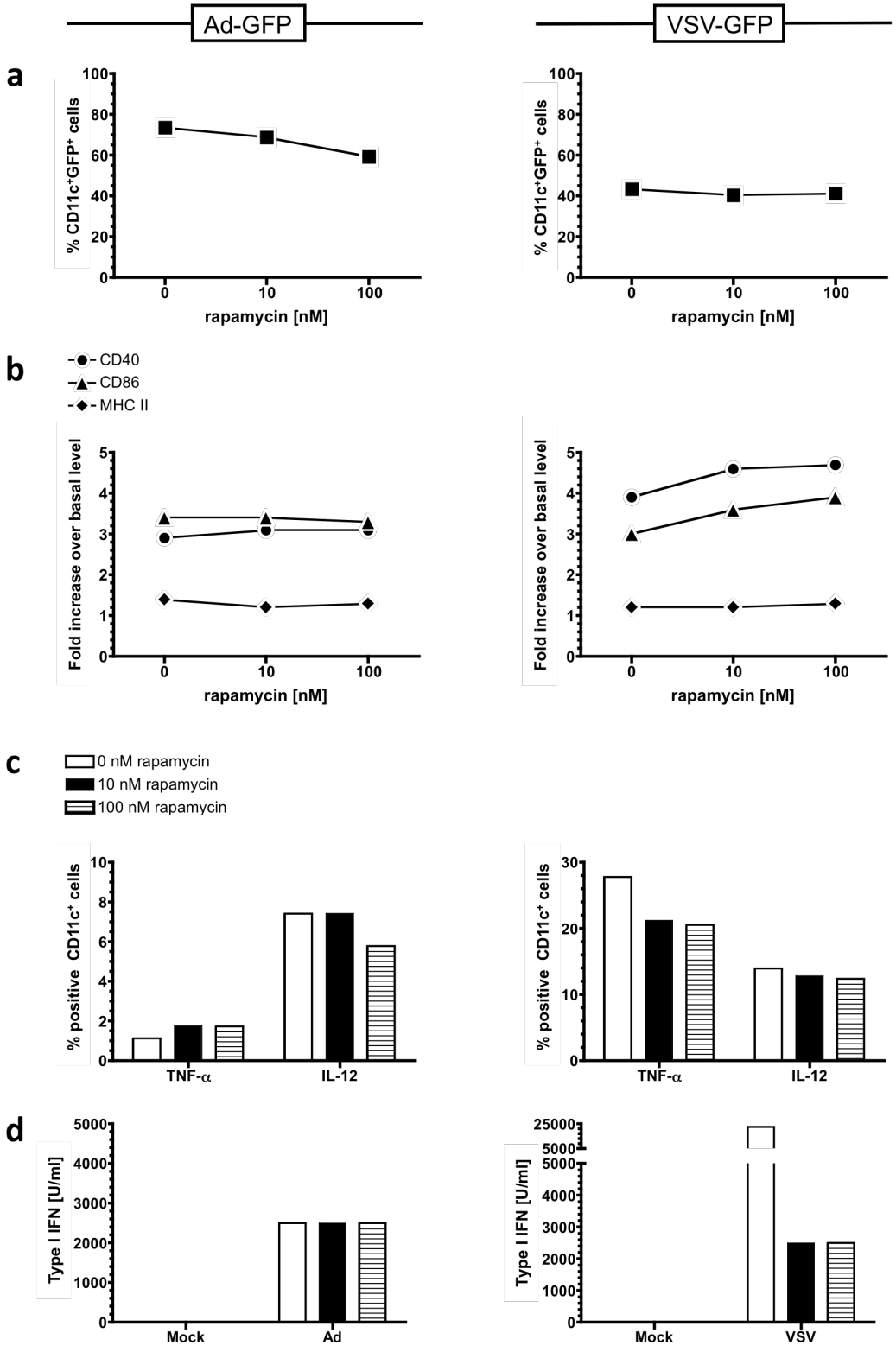

Ad-GFP
VSV-GFP
a
% CD11c+GFP+ cells
rapamycin [nM]
b
CD40
CD86
MHC II
Fold increase over basal level
rapamycin [nM]
c
0 nM rapamycin
10 nM rapamycin
100 nM rapamycin
% positive CD11c+ cells
TNF-α
IL-12
d
Type I IFN [U/ml]
Mock
Ad
VSV

FIGURE 6-

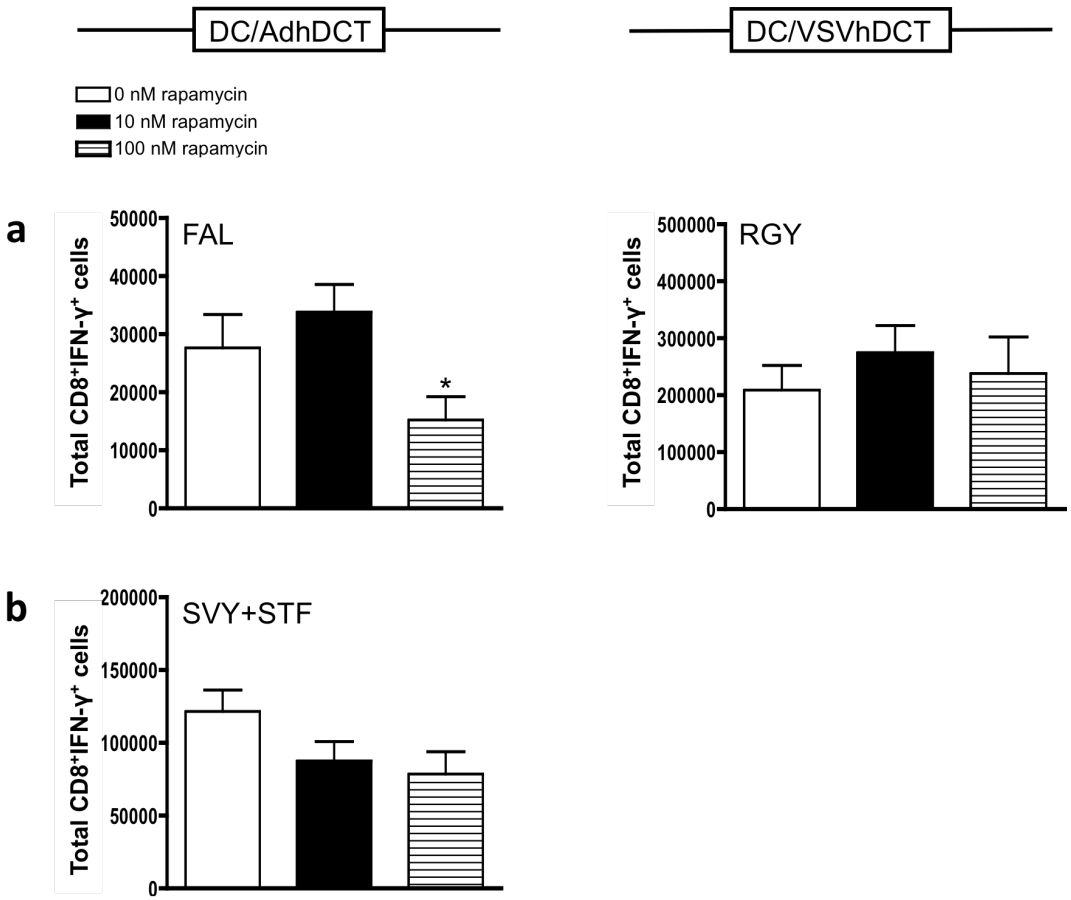
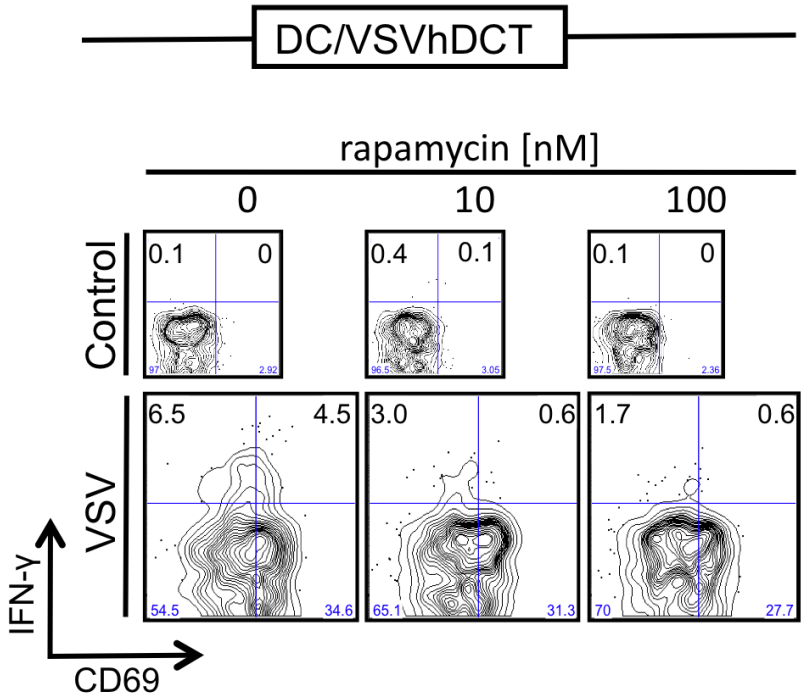

DC/AdhDCT
DC/VSVhDCT
0 nM rapamycin
10 nM rapamycin
100 nM rapamycin
a
FAL
RGY
Total CD8+IFN-γ+ cells
50000
40000
30000
20000
10000
0
500000
400000
300000
200000
100000
*
b
SVY+STF
200000
150000
100000
50000

FIGURE 7-


DC/VSVhDCT
rapamycin [nM]
0
10
100
Control
VSV
0.1
0
0.4
0.1
0.1
0
6.5
4.5
3.0
0.6
1.7
0.6
54.5
34.6
65.1
31.3
70
27.7
IFN-γ
CD69

— APPENDIX 2 —

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