12-2011

The Cytoplasmic Tail Of Mhc Class I Molecules Plays A Critical Role In Dendritic Cell-Induced T Cell Immunity

Tania G. Rodriguez-Cruz

THE CYTOPLASMIC TAIL OF MHC CLASS I MOLECULES PLAYS A CRITICAL ROLE IN DENDRITIC CELL-INDUCED T CELL IMMUNITY

by

Tania G. Rodríguez-Cruz, B.S.

APPROVED:

Gregory Lizee, Ph.D.
(Supervisory Professor)

Patrick Hwu, M.D.

Stephanie Watowich, Ph.D.

Stephen Ullrich, Ph.D.

Willem Overwijk, Ph.D

APPROVED:

Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

THE CYTOPLASMIC TAIL OF MHC CLASS I MOLECULES PLAYS A CRITICAL ROLE IN DENDRITIC CELL-INDUCED T CELL IMMUNITY

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

M.D. Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHYLOSOPHY

By

Tania G. Rodríguez-Cruz, B.S.

Houston, Texas
December, 2011

DEDICATION

I dedicate this dissertation to my parents, Carmen and Ricardo, and to my brother, Omar, for all their support throughout my life and for inspiring me to achieve my goals, even though it meant being physically away from them.

I would also like to dedicate this dissertation to my grandparents for their infinite love, kindness, life lessons, and for the great food. I also want to dedicate this dissertation to all my aunts, uncles, and cousins for the humor, comfort and for the wonderful childhood memories. Thanks for helping me to become the person I am today. This is for all of you…

ACKNOWLEDGEMENTS

I want to acknowledge with great gratitude, several people whose involvement and support made it possible to complete this journey. First and foremost, my deepest appreciation goes to my advisor, Dr. Greg Lizee for accepting me in his laboratory, challenge me, and believing in me. His guidance and supervision throughout all these years have been instrumental for my career development. I would also like to express my sincere appreciation to Dr. Patrick Hwu for all his support and scientific advice throughout these years.

My sincere appreciation also goes to my supervisory committee members, Dr. Stephanie Watowich, Dr. Stephen Ullrich and Dr. Laurence Cooper, for their invaluable intellectual guidance, support and feedback regarding my research work and for constantly challenging me to become a better scientist. I would also like to extend my appreciation to other members of my advisory and examination committees, Dr. Michel Gilliet, Dr. Gilbert Cote, Dr. Willem Overwijk, for their important contribution to my training.

I would like to also acknowledge all the present and past members of the laboratory. In particular, to Dr. Shujuan Liu, Dr. Jahan Khalili, Dr. Weiyi Peng, Mayra Cantu, Dr. Brian Rabinovich, Dr. Yanyan Lou, Yan Yang, Jessica Chacon, for their support, and helpful scientific discussions.

My genuine appreciation goes to the University of Texas Health Science Center Graduate School of Biomedical Sciences and M.D. Anderson Cancer Center for providing me with cutting-edge education that will serve as the foundation of my career as a scientist.

I want to thank the National Institute of Health for providing the financial support during my PhD training through a ROI supplement (R01-CA111999-S1). I am also grateful to our collaborators Dr. Willem Overwijk and Dr. Laura Johnson for providing with mice and cell lines.

I firmly believe that no Ph.D. can be achieved without friends who provided support and encouragement through these years. I want to thank my friends, Dr. Guadalupe Reyes, Dr. Guillermo Armaiz, Dr. Luis Vence, Dr. Chantal Bernabetch and Jessica De Orbeta for their emotional support during my training and for their many valuable life and career advices.

Last, but not least, I would like to thank all my friends who stood by my side all these years. First, I want to thank my high school and college friends Emy, Odemaris, Rina, Rebeca, and Carissa, for the many wonderful memories, the laughs and for still being part of my life. I also want to thank my friends and family from Houston for helping me in maintaining a balanced and fun life. I will be forever thankful for having you in my life.

THE CYTOPLASMIC DOMAIN OF MHC CLASS I MOLECULES PLAYS A CRITICAL ROLE IN DENDRITIC CELL-INDUCED T CELL IMMUNITY

Publication No. _____

Tania Giselle Rodríguez-Cruz, Ph.D.

Supervisory Professor: Gregory Lizee, Ph.D.

The presentation of MHC class I (MHC-I)/peptide complexes by dendritic cells (DCs) is critical for the maintenance of central tolerance to *self* and for the regulation of cytotoxic T lymphocytes (CTL)-mediated adaptive immune responses against pathogens and cancer cells. Interestingly, several findings have suggested that the cytoplasmic tail of MHC class I plays a functional role in the regulation of CTL immune responses. For example, our previous studies demonstrated that exon 7-deleted MHC-I molecules (Δ7) not only showed extended DC cell surface half-lives but also induced significantly increased CTL responses to viral challenge *in vivo*. Although the Δ7 splice variant of MHC class I does not occur naturally in humans, the animal studies prompted us to examine whether exon 7-deleted MHC-I molecules could generate augmented CTL responses in a therapeutic DC-based vaccine setting. To examine the stimulatory capacity of Δ7 MHC class molecules, we generated a lentivirus-mediated gene transfer system to induce the expression of different MHC-I cytoplasmic tail isoforms in both mouse and human DCs. These DCs were then used as vaccines in a melanoma mouse tumor model and in a human *invitro* co-culture system.

In this thesis, we show that DCs expressing exon 7-deleted MHC class I molecules (Δ7-D^b), stimulated remarkably higher levels of T-cell cytokine production and significantly increased the proliferation of melanoma-specific (Pmel-1) T cells compared with DCs expressing wild type MHC-I. We also demonstrate that, in combination with adoptive transfer of Pmel-1 T-cell, DCs expressing Δ7-D^b molecules induced greater anti-tumor responses against established B16 melanoma tumors, significantly extending mouse survival as compared to DCs expressing WT- D^b molecules. Moreover, we also observed that human DCs expressing Δ7-HLA-A*0201 showed similarly augmented CTL stimulatory ability. Mechanistic studies suggest that Δ7 MHC-I molecules showed impaired lateral membrane movement and extended cell surface half-lives within the DC/T-cell interface, leading to increased spatial availability of MHC-I/peptide complexes for recognition by CD8+ T cells. Collectively, these results suggest that targeting exon 7 within the cytoplasmic tail of MHC class I molecules in DC vaccines has the potential to enhance $CD8^+$ T-cell stimulatory capacity and improve clinical outcomes in patients with cancer or viral infections.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1- INTRODUCTION

1.1 The immune system: Overview

The immune system has evolved many different mechanisms to make rapid, highly specific and often protective responses against a wide variety of pathogens, including bacteria and viruses. In fact, the tragic example of severe immunodeficiencies as seen in some congenitally-determined diseases and in acquired immunodeficiency syndrome (AIDS), illustrates the critical role of the immune system in protection against pathogenic microorganisms [1; 2]. After a century of much debate as to whether the immune system can eliminate tumor cells, strong evidence now suggests that the cellular immune system plays a critical role in the control of tumorigenesis [3; 4] The cellular immune system not only plays a role in the elimination of pathogens and cancer cells, but it also plays a role in the development of several clinical conditions, including autoimmune diseases (i.e. Type 1 diabetes, Lupus), allergies and rejection of transplanted tissues [5; 6; 7; 8]

The cells of the immune system originate from hematopoetic stem cells and are constantly re-circulating throughout the body for detection of invading pathogens or tumor cells. In vertebrates, immune defenses consist of two connected systems: innate and adaptive immunity. Cells from the innate immune system express receptors that are evolutionary conserved and recognize pathogen-associated molecular patterns (PAMPs), which are shared by common microorganisms. This conserved receptor-mediated recognition leads to the capture and fragmentation of pathogens into small antigenic proteins, accompanied by inflammation and in many cases pathogen clearance. The cells mainly responsible for these functions are macrophages and dendritic cells [9; 10]

Cells from the adaptive immune system are characterized by their ability to specifically recognize a wide range of pathogens and to generate long-lasting memory that provides improved protection against re-infection. The adaptive immune responses are mediated by T and B lymphocytes, which express highly diverse antigen specific receptors. In contrast to the evolutionary-conserved receptors found in innate immune cells, the T-cell receptor (TCR) and the B-cell receptor (BCR) are generated by gene rearrangements, which provide each single lymphocyte with a unique specificity. B lymphocytes eliminate extracellular pathogens by producing antibodies [9; 10]. T lymphocytes are divided into two major categories: $CD4^+$ T cells and $CD8^+$ T cells. These types of lymphocytes are known to specifically recognize antigens bound to the groove of MHC proteins, which are encoded by a large complex of genes called the major histocompatibility complex (MHC) [11]. There are two main structurally and functionally distinct classes of MHC proteins: class I MHC proteins and class II MHC proteins. $CD4^+$ T cells recognize antigens bound to MHC class II. This type of T-cell lymphocyte have the ability to help or inhibit the development of specific types of immune responses, including antibody production and increased microbidicial activity of macrophages. In contrast, $CD8^+$ T lymphocytes recognize antigens bound to MHC class I molecules. This type of T-cell lymphocyte is involved in direct effector functions such as the lysis of virus-infected cells or cancer cells [11; 12]. For the purpose of this thesis, the biological functions of $CD8^+$ T cells and their roles in cancer immunity will be discussed in more details.

1.2 T cells eliminate tumors and virally infected cells

The development of T cells begins with the migration of hematopoetic precursors from the bone marrow to the thymus. At the thymus, hematopoetic precursors commit to the T-cell lineage, which then undergoes thymic selection. Thymic selection of the T-cell lineage is based on the recognition of self-peptide/MHC class I complexes at the cell surface of thymic dendritic cells, and thymic medullary epithelial cells by the TCR of T cells [13]. The binding properties of TCRs for their peptide/MHC ligands (i.e. affinity and avidity rates) are critically important in not only T-cell thymic selection but also for full activation of peripheral T-cell responses [14]. During thymic selection, TCRs with low affinity for self-peptide/MHC leads to weak interactions promoting T-cell survival (positive selection). However, TCRs with high affinities for self-peptide MHC complexes leads to strong interactions promoting T-cell apoptosis (negative selection). Thymic selection is important because it induces central tolerance by eliminating autoreactive T-cells with high affinity TCRs for self peptides and by selecting T-cells with low affinities TCRs for self-peptides [14]. Having succeeded thymic selection, naive T cells (i.e. CD8+ T cells) then exit the thymus and migrate to peripheral lymph nodes, where they become activated into effector T cells that can kill target cells such as tumor cells and virally-infected cells.

Naïve T cells are mainly found in peripheral lymphoid organs, and their activation is initiated following the recognition of pathogen or tumor-associated antigens on the surface of an antigen-presenting cell (APC), usually a dendritic cell. The antigen is in the form of peptide fragments which are generated by the degradation of foreign protein antigens within the proteosome or acidic compartments (i.e. endosomes) of the APC. The

peptide fragments are then transported into the endoplasmic reticulum (ER) for binding to nascent MHC class I proteins. The affinity of the peptide for a specific MHC class I molecule is critical for the formation of stable peptide/MHC class I complexes that can be recognized by the TCR of the cognate CD8+ T cell. For example, it has been demonstrated that peptides bound to MHC class I molecules with low affinity leads to low affinity/avidity or weak interactions between the TCR-pMHC complexes resulting in incomplete activation of the cognate T-cell. It has also been demonstrated that low affinity binding of peptide to MHC can mediate the escape of autoreactive T cells from negative selection [15].

T-cell activation requires two major types of signals: The first signal involves the recognition of the peptide/MHC complex located on the surface of an APC by the TCR on T cells. The second signal involves the triggering of co-stimulatory molecules (i.e. CD28, CD80) and adhesion molecules (i.e. integrins, LFA-1) expressed on the cell surface of both the APC and T-cell [12]. These signals lead to the stimulation of the TCR, which in turn induces a signaling cascade (i.e. MAPK signaling) that promotes the differentiation of naïve T cells into potent effector antigen-specific T cells, such as cytotoxic T cells (CTL) [16].

The antigen recognition event for the generation of the first signal is a very sensitive process since it has been demonstrated that as few as 1-50 pMHC complexes are sufficient to activate target-cell lysis by CTLs [17]. Two models have been proposed to explain how TCR-pMHC interactions result in T cell activation: the serial TCR engagement and the kinetic proofreading model. The serial TCR engagement model proposes that a single pMHC complex is able to serially trigger multiple TCR molecules

since T cell activation can be accomplished at low physiological density of specific pMHC complexes on the target cell surface [18]. This model is consistent with different studies that have demonstrated that the TCR-pMHC interactions are of low affinity and have rapid association and dissociation rates [19]. In conclusion, in order for few pMHC complexes to efficiently trigger multiple TCRs, each individual TCR needs to bind and dissociate from its ligand with fast kinetics to allow the next TCR to be triggered resulting in a sustained TCR signal. On the other hand, the kinetic proofreading model proposes that a minimal half-life for the TCR-pMHC interaction is an additional requirement for a complete TCR signal [20]. According to this model, fast dissociation of the TCR from the pMHC complex would result in incomplete TCR signaling preventing T cell activation. By contrast, a more sustained interaction (slow dissociation rates) would allow complete TCR signaling and T cell activation. In support of this model, different studies have reported that T cell activation correlates with the half-life of the TCR-pMHC interaction [20]. However, it has been recently demonstrated that for T-cell activation, both shortened and prolonged TCR-pMHC interaction half-lives have detrimental effects on T cell activation [21]. Therefore, it has been suggested that efficient T cell activation occurs within an optimal dwell-time range of TCR-pMHC interaction. It has been described that such dwell-time for triggering efficient T cell activation needs to be sufficiently long to complete the TCR signal cascade but also short enough to allow serial engagement of multiple TCRs by a few cognate pMHC complexes.

Once naïve CD8+ T cells are activated, they undergo clonal expansion and migrate to peripheral tissues expressing the same peptide/MHC class I complex that induce their activation. During clonal expansion, the initial numbers of antigen-specific precursors

increase more than 1000-fold, generating a pool of effector T cells, which can directly kill pathogen-infected cells or tumor cells [22]. The expansion phase usually lasts for 7 to 9 days. After the effector phase, the majority (90-95%) of antigen-specific T cells die through a mechanism called apoptosis in order to maintain homeostasis. The surviving cells form a stable memory pool that can generate more rapid and greater responses upon secondary encounter with the antigen [23]. Activated T cells also secrete cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN- γ), which are known to not only promote T cell expansion, but also enhance anti-viral and anti-tumor responses by up-regulating the expression of MHC proteins on cancer cells or viral-infected cells [23; 24; 25]. Once activated, CTLs migrate through the peripheral tissues where they may encounter cells expressing the same foreign antigen that induced their activation and differentiation [12].

Upon antigen recognition on the target cell, CTLs utilize at least two mechanisms to kill the target cell; both mechanisms operate by inducing the target cell to undergo apoptosis, which is a highly evolutionary conserved mechanism of cell death that ensures the elimination of "unwanted" cells from the body [26]. In order to kill target cells, CTLs begin to secrete a pore-forming protein called perforin, which is known to polymerize at the target cell surface to form transmembrane pores. Perforin is found in secretory vesicles (i.e. CD107a+ vesicles) of the CTLs and is released by exocytosis at the point of contact with the target cell [26; 27]. The secretory vesicles also contain *granzyme B*, which is a protease that enters the target cell through the perforin-induced transmembrane pores and activates caspase-induced apoptosis [27; 28]. A second strategy that CTL utilize to kill target cells is through the interaction of Fas and Fas ligand. Fas is expressed on the target cells while Fas ligand is expressed on the surface of CTLs. The interaction of Fas and

FasL leads to a signaling cascade that activates downstream caspases and subsequent apoptosis of the target cell [29].

Because of their extraordinary capacity to specifically recognize foreign antigens, to migrate toward infected areas and to directly kill target cells, CTLs show great promise in the development of therapeutic treatment for different diseases, including cancer and viral infections. In fact, many studies have used CTLs to treat cancer patients and although proven to induce anti-tumor responses, complete responses have been relatively rare [30; 31; 32]. However, one limitation is that the affinity of most TCRs specific for shared tumor antigens is usually very low. Thus, different strategies have been developed to increase the affinity and avidity of TCRs. For example, one study has demonstrated that removing N-glycosylation sites in the constant region of the TCR leads to enhanced avidity and improved recognition of tumor cells by T cells [33]. Other studies have demonstrated that specific mutations in the CDR3 domain of the TCR can control the TCR-pMHC interaction half-lives leading to optimal T cell activation [21]. Another limitation is that CTLs do not persist *in vivo* long enough to eradicate tumors [32]. In addition, cells within the tumor microenvironment express or secrete inhibitory factors that downregulate the effector functions of CTLs. To overcome these limitations, different studies have developed new strategies. For example, in melanoma, the transfer of CTLs into lymphodepleted melanoma patients results in objective clinical response rates as high as 50% [34]. Other strategies such as vaccines plus adjuvants are currently being developed to increase T-cell proliferation and effector functions [35; 36; 37].

1.3 Dendritic cells are master regulators of T-cell immunity

The processing and presentation of peptides by dendritic cells (DCs) is critical for the activation and differentiation of naïve T cells into effector cells [38]. DCs are professional antigen presenting cells located in most tissues, where they capture, process and display antigen-derived peptides on MHC molecules. Following antigen capture and processing, DCs migrate to lymphoid organs, where they present peptide-MHC complexes to T-cell antigen receptors (TCR) on naïve T cells. In conjunction with TCR triggering, DCs can also engage co-stimulatory and cytokine receptors to influence T cell proliferation, survival and effector activity.

There are different DC subsets with distinct cell surface markers and biological functions. Three of the major DC subsets have been described in mouse: (1) $CD11c^{+}CD8^{-}$ DCs, (2) $CD11c^{+}CD8^{+}$ DCs and (3) $CD11c^{+}CD45RA^{+}Gr1^{+}$ plasmacytoid DCs (PDCs) [39]. The $CD8^{-}$DCs have been described to be more efficient at capturing antigens and displaying them on MHC class II molecules for recognition of $CD4^{+}$ T cells. On the other hand, the $CD8^{+}$ DCs were demonstrated to be more efficient at capturing antigens and displaying them on MHC class I molecules to promote $CD8^{+}$ T cell responses [40; 41]. The $CD8^{+}$ DCs also have the unique capacity to capture dying cells and display exogenously-derived antigens on MHC class I molecules, a process termed cross-presentation [41]. Because of their capacity to initiate CTL responses *in vivo*, $CD8^{+}$DCs are the most logical targets for vaccine development. In contrast to $CD8^{-}$DCs and $CD8^{+}$DCs, PDCs are the main producers of Type I interferons, in particular interferon-α (IFN- α), which has been shown to be important for the recruitment and activation of other immune cells into the infected area to control disease [42]. In humans, two main DCs subsets have been described: myeloid-derived DCs ($CD11c^{+}CD123^{-}$) and lymphoid-derived DCs or

plasmacytoid DCs ($CD11c^{-}CD123^{+}$) [39]. However, in contrast to mouse DC subsets, the study of human DC subsets has been more challenging mainly because they only represent less than 2% of total cell numbers in tissues, and blood monocytes are the main reliable source of precursor cells [35; 43]. Both challenges are being overcome, and adequate DCs numbers can now be generated from progenitors. One method to generate sufficient DC numbers is by culturing hematopoetic stem cells from bone-marrow ($CD34^{+}$) in the presence of different cytokines [44]. Another method is to differentiate DCs derived from human blood monocytes that have been cultured with GM-CSF and IL-4, followed by activation in a monocyte-conditioned medium.

DCs need to undergo a process of activation in order to initiate potent T-cell immune responses [38]. During steady state, DCs are mostly found in peripheral tissues as immature DCs. Immature DCs (iDCs) are professional phagocytes that can internalize and process antigens from pathogens for example. They express low levels of MHC molecules (MHC class I and MHC class II), the glycoprotein CD83 and the co-stimulatory molecules, CD86 and CD80, which are critical for efficient activation of T cells [38]. Because they express low levels of CD80 and CD86, iDCs play an important role in the induction of peripheral tolerance, a process required for protecting self tissues. However, upon infection, danger signals from pathogens induce iDCs to undergo a differentiation program called maturation. This maturation process transforms iDCs into potent activators of T-cell mediated immune responses [45]. Danger signals are recognized by evolutionary conserved receptors such as Toll like receptors (TLRs), which are abundantly expressed in DCs [46]. Upon recognition of danger signals, TLRs initiate a signaling cascade that induces iDCs to upregulate MHC and co-stimulatory molecules at their cell surface and to secrete cytokines

[38; 46]. Inflammatory cytokines, bacterial and viral products can also induce dendritic cell maturation through direct interaction with receptors expressed on DCs [47]. In contrast to immature DCs, mature DCs have low phagocytic activity but are remarkably efficient in antigen presentation [38]. Mature DC are the most potent antigen presenting cell for activating naïve T cells, in particular CD8+ T cells, to differentiate into cytotoxic antigen specific T cells (i.e CTLs). This is mainly because mature DCs express high levels of antigen presenting molecules (MHC class I and MHC class II) and co-stimulatory molecules (CD80, CD86 and CD83) at their cell surface. They also secrete high levels of interleukin-12 (IL-12), which is known to mediate the differentiation of CTLs [38].

DCs present peptides in the context of MHC class I molecules to $CD8^+$ T-cells by using two major pathways: (1) direct presentation, in which the cell itself is infected with the antigen it presents on MHC-I; and (2) cross-presentation, in which the DCs engulfs components of an infected cell and then processes and present the associated antigen bound to MHC-I. [48]. However, a third mechanism -cross-dressing- has also been recently postulated. This mechanism of antigen presentation involves DCs (acceptor) to acquire peptide/MHC-I complexes directly from another infected APC allowing antigen presentation by acceptor DC to occur rapidly, without the need of any antigen processing. Moreover, Wakim and Bevan have recently reported that cross-dressing can activate memory but not naive CD8+ T cells. One possible explanation for this may be that naïve T cells, with their high activation threshold, disfavor cross-dressing because it involves the presentation of vanishingly small number of peptide/MHC class I complexes. A more detailed molecular description of these pathways is discussed in the MHC molecules section.

DCs have held great promise for use in vaccines aimed at enhancing pathogen or tumor antigen-specific CTLs because of their high potency for initiating T cell-mediated immune responses. However, several questions need to be addressed in the development of effective vaccines that utilize DCs. These questions require a deep understanding of the biological functions of DCs. For example: the first question to be addressed should be what type T-cell mediated immune response is desired: CD4+ T cells or CD8+ T cells? Another question to be addressed would be what type of DC subset to use for eliciting such an immune response? Finally, what maturation signals are appropriate for activating DCs in particular settings? In cancer settings, several DC vaccines have proven to be effective at generating potent CTL-mediated antitumor responses [35]. Some of these vaccines formulations are discussed below in the DC cancer vaccines section.

1.4 The role of MHC class I molecules in DC-induced T-cell immunity

MHC class I molecules are one of the two primary classes of proteins encoded by the major histocompatibility complex (MHC), which were discovered to play a critical role in tissue rejection and immunity against pathogens and tumors [25]. MHC class I molecules are expressed by almost all nucleated cells and their main function is to display peptides from intracellular proteins (i.e. viral or self proteins) to $CD8^+$ T cells. However, MHC class I molecules can also bind and present peptides from extracellular proteins (i.e. apoptotic tumor cells) to $CD8^+$ T cells, a process known to as cross-presentation [49]. Once the TCR of $CD8^+$ T cells recognizes peptide/MHC class I complexes at the cell surface of DCs, a T-cell signaling cascade is initiated that promotes the elimination of abnormal or virus-infected cells.

MHC class I molecules present peptides through two main pathways: the endogenous or classical pathway and the cross-presentation pathway (See figure 1). The presentation of intracellular-peptides through the endogenous pathway begins in the cytosol of antigen presenting cells with the degradation of proteins, self proteins as well as those of pathogens. Degradation occurs in the proteosome, which is a macromolecule with proteolytic activity known to degrade cytosolyc proteins into small peptides fragments. The resulting peptide fragments are then transported from the cytosol into the lumen of the endoplasmic reticulum (ER) through the transporter associated with antigen processing proteins (TAP). In the ER, peptides combine with nascent class I MHC proteins with the help of ER-resident chaperons, such as calnexin, tapasin, calreticulin and Erp57 [49]. These proteins are involved in the proper assembly of a complete receptor complex suitable for transport to the cell surface. For example, calnexin is known to stabilize the MHC class I α chains prior to β2-microglobulin binding [50]. Tapasin binds to MHC class I molecules and facilitates the binding of MHC class I molecules to TAP, thus leading to enhanced peptide loading and colocalization. MHC class I molecules lacking a bound peptide are extremely unstable and requires the binding of the chaperones, calreticulin and Erp57. Once assembled, the peptide/MHC class I complexes are then released from the quality-control machinery of the ER and transported to the plasma membrane through the default secretory pathway. The transport of MHC class I molecules through the secretory pathway involves different prost-translational modifications of the MHC class I molecule, including glycosylation [51]. Once the glycosylation process occurs, the MHC class I molecules are transported to the plasma membrane, where they present peptides to $CD8^+$ T cells, followed by internalization, recycling and degradation through different endosomal

compartments [52; 53]. On the other hand, cross-presentation is based on the uptake of exogenous antigens by DCs via phagocytosis. It has been demonstrated that phagocytosis of exogenous antigens (i.e. apoptotic tumor cells) is mediated by Cdc42 and Rac GTPases, which trigger actin rearrangements, protrusions of membrane around the apoptotic body and engulfment into a phagosome that then fuses with early endosomes for antigen fragmentation [54] [49; 55; 56]. Once in early endosomes, exogenously-derived peptides can be loaded onto recycling MHC class I molecules and then transported to the plasma membrane via Rab dependent mechanisms as demonstrated by different studies [49; 54; 55]. Once at the cell surface, exogenously-derived peptides can be recognized by CD8+ T cells. The fact that antigen loading occurs in endosomal compartments during cross-presentation, it has been suggested that the classical and cross-presentation pathways of MHC class I are spatially separated [52] [56].

1.5 MHC class I internalization and endocytosis

The molecular mechanisms of MHC class I internalization via endocytosis have been described by differing studies demonstrating that MHC class I molecules can be internalized either via clathrin-coated pits or in a clathrin-independent manner [57; 58]. For example, different electron microscopy studies have demonstrated that MHC class I molecules can be found in clathrin coated vesicles (CCVs) [57]. Also other reports have suggested that the cytoplasmic domain of MHC class I molecules contains a putative tyrosine-based endocytic motif (YXXΦ, Y=tyrosine, X= any amino acid and Φ = hydrophobic amino acid) at position 320 of the cytoplasmic tail [59]. In fact, tyrosine 320

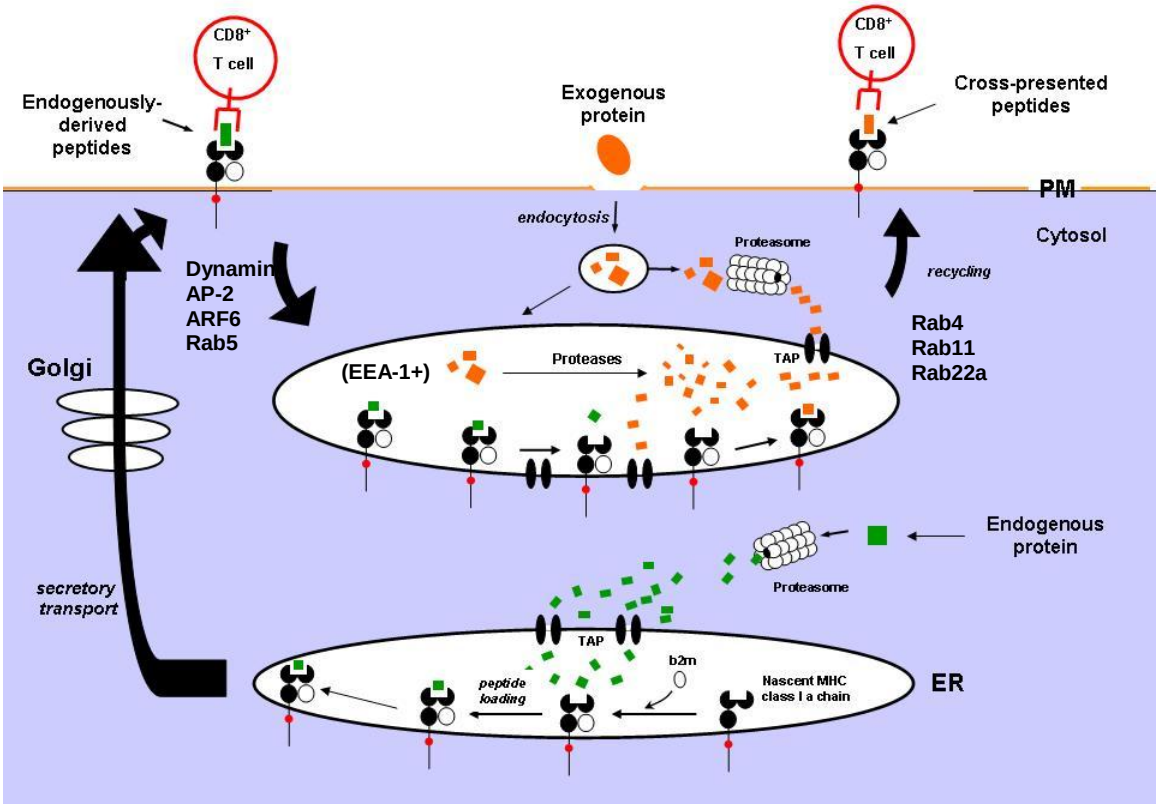
point mutants of MHC class I showed internalization defects [60]. It has been demonstrated that tyrosine-based endocytic motifs as well as dileucine-based motifs can be recognized by specific adaptor and accessory proteins involved in the initial formation of clathrin-coated vesicles (CCVs) [61]. The molecular players that regulate clathrin-mediated endocytosis have been described. For example, it has been demonstrated that the adaptor protein-2 complex (AP-2) can recognize both tyrosine and dileucine-based motifs on the cytoplasmic domain of cargo molecules (i.e. LDL, MHC-class I, transferrin receptor, CD3-γ) [62]. Such interactions promote the formation of peripheral plasma membrane invaginations that are then coated with clathrin. Several studies have demonstrated that clathrin polymerization at sites of membrane invaginations is regulated by the adaptor molecule, AP180. AP180 is known to recruit clathrin to PtdIns (4, 5)P_2 zones in the membrane. Clathrin polymerization is important because it initiates the formation of clathrin-coated vesicles (CCVs) neck, which is then cut by the membrane accessory scission protein, dynamin [63]. Dynamin is a large GTPase that forms a helical polymer around the CCV neck and upon GTP hydrolysis, mediates the fission of the vesicle from the plasma membrane promoting the release and intracellular transport of the CCVs containing cargo molecules (i.e. MHC class I) [64]. The CCVs then fuse to early endosomal compartments, a process that has been demonstrated to be Rab5 dependent [65]. Rab 5 is a small GTPase protein found at the plasma membrane and in CCVs. It has been suggested that Rab5 directs the trafficking of CCVs from the peripheral membrane to early endosomal compartments by binding to specific motor proteins of the cytoskeleton (i.e myosin) and by interacting with the early endosomal antigen (EEA-1) [65]. Such interaction leads the fusion of Rab-5 expressing CCVs to early endosomes. Once in the early endosome compartment, clathrin-dependent cargoes can be

sorted into lysosomes for degradation or into recycling endosomes for trafficking back to the plasma membrane [64; 66; 67]. It has been demonstrated that membrane recycling of clathrin-dependent cargoes is regulated by small the Rab GTPases proteins, Rab4 and Rab11. While Rab 4 mediates the rapid recycling of clathrin-dependent cargoes from early endosomes to the plasma membrane (rapid recycling), Rab11 mediates the trafficking of clathrin-dependent cargoes from early endosomes into recycling endosomes. The recycling endosomes then translocate to and fused with the plasma membrane in a Rab11a dependent mechanism [67; 68].

However, internalization and endocytic recycling of MHC class I molecules can also be regulated by a clathrin-independent pathway since inhibitors of the clathrin-dependent pathway (i.e. AP180 mutants, dynamin mutants) did not completely impair MHC class I endocytosis [63]. Contrary to the clathrin-dependent pathway, clathrin-independent endocytic pathway requires the ADP-ribosylation factor 6 (ARF6), actin polymerization and Rab22a GTPases for the initial formation of tubular membrane invaginations and intracellular trafficking of vesicles containing cargo molecules (MHC class I) [Radhakrishna, 1999 #15][69; 70]. ARF6 is a small GTPase that regulates peripheral plasma membrane/endosomal trafficking by switching between its GDP and GTP form [71; 72]. Previous studies have demonstrated that ARF6 is found to be in its GTP form at the inner space of plasma membrane and that ARF6 GTP initiates the formation of actin rich surface protrusions, which have been shown to be regulated by the actin regulatory protein, HS1 [58; 73]. Inactivation of ARF6-GTP through hydrolysis, signals ARF6-GDP expressing vesicles to be transported from the plasma membrane to early endosomal compartments via membrane intermediates that are yet to be defined. One

study demonstrates that excision of the ARF6 vesicles occurs in a dynamin-independent process [69]. However, the molecules that regulate the excision of ARF6 vesicles from the plasma membrane has not been described, but is very likely that it could be small GTPases. Once in the early endosomal compartment, ARF6-dependent cargoes, encounter clathrin dependent cargoes, however, their routes for membrane recycling are regulated by distinct Rab proteins. For example, while Rab 4 and Rab 11 have been involved in clathrin-dependent endocytosis, Rab22a has been shown to allow the correct delivery of MHC class I containing vesicles from recycling endosomes to the plasma membrane in a clathrin-independent manner [70]. The recycling of MHC class I containing vesicles to the plasma membrane is also regulated by the conversion of ARF6 GDP to ARF6 GTP through nucleotide change [68; 69]. Nevertheless, different internalization and recycling routes regulate peptide-MHC class I complex cell surface expression leading to controlled T cell activation. The different antigen presentation pathways and trafficking of MHC class I molecules are summarized in Figure 1.

Figure 1. Antigen presentation and endocytosis of MHC class I molecules. Endogenous peptides (green squares) enter the ER via TAP and bind to nascent MHC class I molecules. Stable peptide-MHC class I complexes exit the ER and are transported to the plasma membrane through the secretory transport pathway. At the plasma membrane, pepitde-MHC class I complexes are recognized by cognate CD8+ T cells and then internalized via clathrin-dependent (mediated by AP-2, dynamin and Rab5) and/or ARF6-dependent endocytosis. The peptide-MHC class I complexes then recycle to the plasma membrane via different Rab proteins. On the other hand, exogenous antigens (orange) are phagocytosed via Rho-a GTPases into early endosomal compartments, where they can be degraded by early endosomal proteases (i.e. cathepsins) and loaded onto recycling MHC-I. They can also be degraded by the proteosome and transported into early endosomes via endosomal TAP. The pMHC complexes are then transported to the plasma membrane for cross-presentation to CD8+ T cells.


CD8+
T cell
Endogenously-
derived
peptides
Exogenous
protein
CD8+
T cell
Cross-presented
peptides
PM
Cytosol
endocytosis
Proteasome
recycling
Dynamin
AP-2
ARF6
Rab5
Rab4
Rab11
Rab22a
Golgi
(EEA-1+)
Proteases
TAP
Endogenous
protein
Proteasome
secretory
transport
TAP
b2m
peptide
loading
Nascent MHC
class I a chain
ER

1.6 Role of the MHC-I cytoplasmic tail

Many distinct MHC class I loci have been described in both humans and mice. In humans, functional genes have been found for the HLA-A, -B, -C, -E, -F, and –G in the short-arm of chromosome 6 [74]. In mice, MHC class I molecules are encoded by the H-2K, D and L loci on chromosome 17. The products of the different MHC class I loci show distinct tissue distributions and regulation of expression. For example, the HLA-A, -B and –C products are expressed on a wide variety of somatic cells, with the highest expression in hematopoetic cells. HLA-G is expressed by throphoblasts in the fetus [75]. Each human gene is highly polymorphic. For example, there are more than 800 known variants of HLA-B genes, more than 500 variants of HLA-A genes, and more than 100 variants of HLA-C genes. The high diversity of MHC class I molecules is of critical significance because it ensures the presentation of a very wide variety of peptides to $CD8^+$ T cells. Expression of MHC class I molecules can be regulated by several cytokines such as IFN-γ and TNF-α. For example, several studies have shown that MHC class I molecules can be highly upregulated in several different cell lines following the addition of IFN-γ and/or TNF- α to the culture [49].

The crystal structure of a MHC class I molecule (HLA-A2) was first described in 1987 by Bjorkman and co-workers [76]. The three-dimensional structure of class I molecules demonstrated that MHC class I are heterodimers consisting of a 44-kd α chain noncovalently bound to a 12-kd soluble polypeptide called β_2-*microglobulin,* which is not encoded by the MHC locus (See figure 2). The class I α chain is composed of three extracellular domains (α_1, α_2, and α_3), a transmembrane segment, and a short cytoplasmic

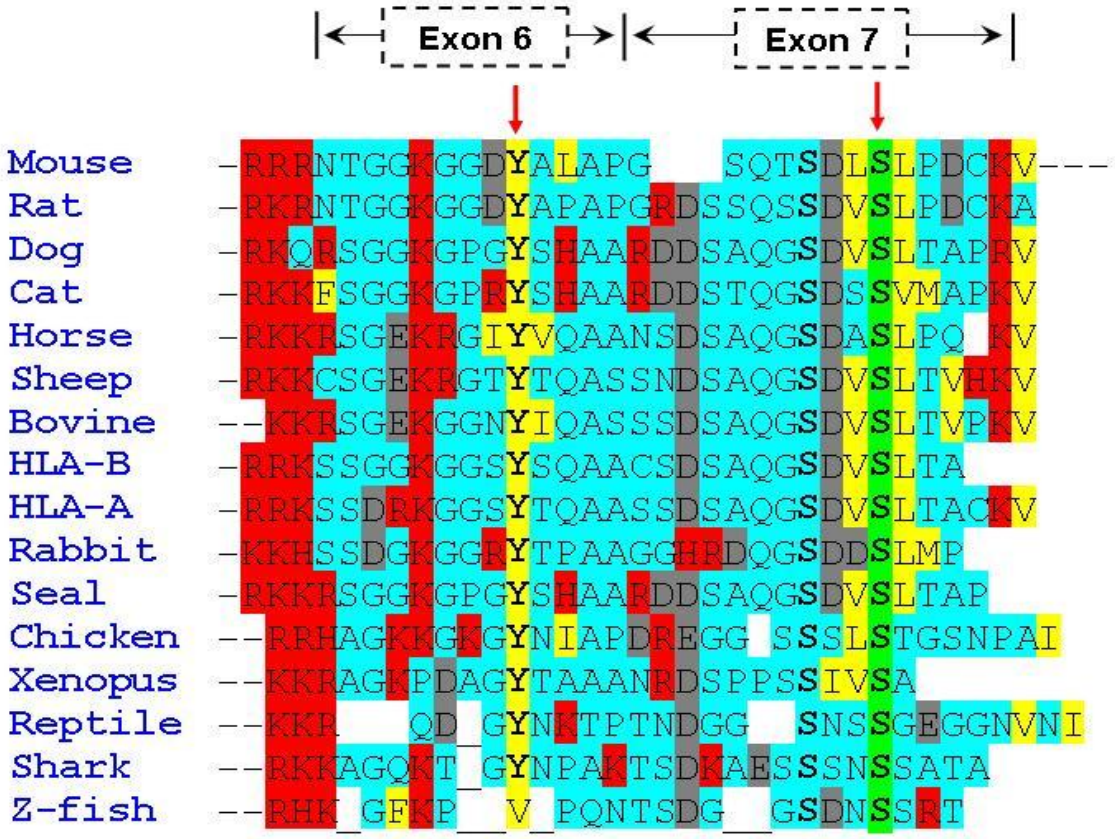
tail. Portions of the alpha chain are highly variable, with the polymorphisms located mainly in the in the α 1 and α 2 domains. The α_1 and α_2 domains associate closely to form a deep groove that constitutes the binding sites for antigenic peptides. They also constitute the binding sites for the T-cell receptor [77]. By contrast, the amino acid sequence of the α_3 domain is mostly conserved. It interacts with β_2-*microglobulin* and also serves as binding site for the CD8 co-receptor, which facilitates the interaction of T cells with MHC class I molecules. The peptides that associate with MHC class I molecules are characterized by having preferential anchor residues, which are important for binding to specific MHC class I alleles [78; 79]. For example, HLA-A2 binds peptides that almost always have a leucine residue in the second position and a valine residue in the last position, while the other residues are highly variable [76]. Many different peptides can be presented by a particular class I MHC protein since a few of the residues are crucial for MHC-I binding. Thus, a tremendous repertoire of peptides can be presented by these molecules to T cells.

Different studies have demonstrated that the ~35 amino acid short cytoplasmic tail of MHC-I plays a critical role not only in intracellular trafficking but also in DC-mediated antigen presentation and CTL activation [80; 81]. The cytoplasmic tail of MHC class I is encoded mainly by two separate exons (6 and 7), both containing a number of highly conserved motifs (See figure 3) [80]. The biological function of the MHC class I cytoplasmic tail was demonstrated by different groups using various cytoplasmic tail mutants of MHC class I [80; 82; 83]. One study revealed that deletion of the entire MHC-I cytoplasmic tail resulted in a complete abrogation of anti-viral CTL responses *in vivo [51]*. Other studies demonstrated that the tyrosine residue (Tyr-320) located in exon 6 is a putative endocytic motif that is required for appropriate MHC-I trafficking through DC

endosomal compartments, cross-presentation of exogenous antigens, and anti-viral CTL priming [60; 80]. By contrast, deletion of exon 7-encoded cytoplasmic amino acids results in increased anti-viral CTL responses *in vivo* [80]. Interestingly, it has been shown that several species including mice, chickens and bovine, naturally express exon 7-deleted MHC class I variants [84; 85; 86]. Such splice variants lack at least one conserved serine phosphorylation site, Ser-335, and have been shown to exhibit delayed internalization in a number of cell types, including DCs [80; 87]. However, in contrast to Tyr-320-mutated MHC-I, MHC class I molecules lacking exon 7 maintain their ability to recycle through DC endosomal compartments, and appear to be fully functional at binding and presenting both endogenous and exogenous antigens [80; 88].

The intracellular trafficking of MHC class I molecules in different cell types is regulated by its own cytoplasmic tail. For example, different studies have shown that wild-type (WT) surface MHC class I proteins are rapidly internalized and recycled through endocytic compartments of both human and mouse DCs. This process is cytoplasmic tail-dependent and limits the surface half-life of MHC-I/peptide complexes for recognition by $CD8^+$ T cells [88]. Since MHC class I molecules lacking exon 7 were demonstrated to have increased cell surface half-lives in DCs, we **hypothesized** that expression of MHC class I molecules lacking exon 7 might lead to superior antigen presentation and therefore more efficient CTL activation. In this thesis we tested this hypothesis by comparing the relative capacity of WT and Δ7 MHC-I molecules to stimulate antigen-specific CTL responses in a DC-based vaccine setting.

Figure 2: Elements of the MHC class I cytoplasmic tail show a high degree of evolutionary conservation. The cytoplasmic tail of MHC class-I is mostly composed of exon 6 and exon 7. Alignment of the amino acid sequences derived from the MHC class I cytoplasmic tail of widely divergent species reveals absolute conservation of amino acids (red arrows). Tyrosine 320 (highlighted in yellow) and Serine 335 (highlighted in green) are two highly conserved amino acids. Colors depict acid R-group charge characteristics: yellow, hydrophobic; blue, polar and uncharged; red, positively charged; and grey, negatively charged.


Exon 6
Exon 7
Mouse
Rat
Dog
Cat
Horse
Sheep
Bovine
HLA-B
HLA-A
Rabbit
Seal
Chicken
Xenopus
Reptile
Shark
Z-fish

1.7 Skin cancer: Melanoma

Cancer is a complex disease that causes millions of deaths worldwide and therefore has been classified as a major problem in public health [89]. Many cancers develop when DNA damage leads to different mutations that promote cells to grow out-of-control [90]. Among all types of cancers, non-melanoma skin cancer is the most common type of cancer in the United States with more than 2 million people being diagnosed every year [91]. Non-melanoma skin cancer is divided into basal cell carcinoma and squamous cell carcinoma; both being less life-threatening and significantly more common than melanoma. Although melanoma only represents approximately 4% of all skin cancers, epidemiological studies have demonstrated that it causes approximately 75% of deaths related to skin cancer mostly due to its increased metastatic potential [92]. In the past 3 decades, the incidence of melanoma has increased dramatically, with Caucasians and men older than 50 years old being at a higher risk of developing metastatic melanoma.

Melanoma usually appears as an existing mole that begins to change color, size or morphology. This type of skin cancer develops from the uncontrolled growth of melanocytes, which are mostly located in the bottom layer of the skin's epidermis but can also be found in the middle layer of the eye, the inner ear, bones, intestines, and heart [93]. Melanocytes are the major producers of melanin, which is a ubiquitous dark pigment responsible for the color of skin and hair. The main function of melanin is to protect the skin from ultraviolet light-induced radiation which can cause serious mutations in the DNA. If the mutations are not repaired by the DNA repair machinery, melanocytes begin to

grow abnormally and may become malignant cells that can eventually lead to the development of melanoma [94].

Although there are different melanoma risk factors including family history and genetics, the largest melanoma risk factor is the prolonged exposure to ultraviolet rays (UV) from the sunlight and tanning beds. Ultraviolet rays are classified into UV-A (400 nm–315 nm), UV-B (315 nm–280 nm) and UV-C (280 nm–100 nm). However, UV-associated skin carcinogenesis studies have revealed that UV-B radiation accounts for about two-thirds of melanoma cases [94]. Mechanistic studies have shown that UV-B radiation causes mutations in genes involved in cell proliferation, differentiation and death. For example, specific mutations in the BRAF gene (i.e. mutation V600E), which is involved in cell growth, lead to the progression of melanoma as demonstrated by DNA screening analyses in human samples These DNA screening studies determined that approximately 60% of melanoma cases are associated with V600E BRAF [95]

Melanoma can be detected by using different methods including X-rays, PET scans, CT scans and ultrasound. Detection at earlier stages is highly important for better prognosis [96]. The progression of melanoma is divided into different stages based on tumor size, ulceration, metastatic potential and survival rates [97]. Patients diagnosed with stage 0 or melanoma in situ have abnormal melanocytes in their epidermis that may spread to adjacent normal tissues if not surgically removed. If the disease is diagnosed and treated at this early stage, there is almost 100% chance of survival. Patients with stage I have developed melanoma in the epidermis and dermis of their skin but it has not spread to nearby lymph nodes The tumor size at this stage is approximately less than 2mm and the

survival rate is 85-99%. The most common treatment for this stage is surgical removal of the tumor. If the tumor is not surgically removed during stage I, then the tumor may continue to grow and develop ulceration. Ulceration is a negative prognostic factor for patients with stage II melanoma and is associated with the presence of epidermis in the tissue overlying the tumor [96; 97]. Patients diagnosed with this stage have a 40-85% survival chance after treatment. Stage III melanoma is characterized by tumors that have spread or metastasize to nearby lymph nodes. Patients diagnosed with stage III melanoma have about 30-60% survival chance after treatment. During stage IV melanoma, cancer cells have spread to other organs including distant lymph nodes, skin, liver, brain and lungs. Patients diagnosed with stage IV have only a 10-20% survival chance after treatment. The chance of survival is increased when the tumor is diagnosed and treated at early stages [96; 97].

Melanoma, in contrast to other types of cancer, is highly immunogenic. In the past two decades, many different melanoma-associated antigens have been described and demonstrated to be specifically recognized by the immune system [32]. Based on these findings, many studies aim to use the cellular immune system for the treatment of melanoma since conventional chemotherapy regimens only leads to 10% of clinical responses [98]. The treatment for melanoma includes surgical removal of the tumor, chemotherapy, radiotherapy, immunotherapy or a combination of these treatments [96]. Different immunotherapy regimens for the treatment of melanoma and other types of cancers are discussed in the next section.

1.8 Cancer Immunotherapies

The concept of Immunotherapy evolved from early murine studies demonstrating that the immune system can recognize and eliminate pathogens and tumors with high specificity. Immunotherapy refers to any approach that utilizes the patient's natural immune defense system as a treatment for different diseases, including autoimmune disorders, infectious diseases and cancer [30]. Immunotherapy for cancer was first introduced by William B. Coley in the nineteenth century. In his studies, he observed tumor regression and even disappearance following injections of a vaccine containing bacterial extracts in and around tumors [99]. Based on many of his seminal observations, different cancer immunotherapy methods are currently under development with the main purpose of enhancing the body's natural immune defense to specifically recognize and eliminate tumor cells. The main advantage of using immunotherapies, is that they are specifically targeted to only eliminate malignant cells and not normal cells resulting in lower toxicity levels compared to those induced by many chemotherapy regimens [100].

However, there are many barriers to be overcome in the development of cancer immunotherapy. The concept of Immune survilliance was not widely accepted until experimental models demonstrated that lymphocytes spontaneously migrate to tumors and there, they collaborate to control tumor growth in inbred mice [30]. However, tumor cells and their microenvironment have evolved different mechanisms to favor tumor outgrowth despite immune survilliance. One strategy is that tumor cells, in contrast to normal cells, become more resistance to cell death or apoptosis in conditions where nutrition and oxygen supplies are limited [101; 102]. Another strategy is that tumor cells have the ability to regulate the expression of critical molecules involved in the induction of immune

responses. For example, tumor cells downregulate the expression of MHC and co-stimulatory molecules while upregulating co-inhibitory molecules such as PD-L1 and PD-L2, which suppress the effector function of T cells [103; 104; 105]. Also, cells within the tumor microenvironment, including stromal cells and fibroblasts, secrete high levels of inhibitory factors, such as interleukin-10 (IL-10) and tumor growth factor-β (TGF- β) [101; 102]. Inhibitory immune cells including T regulatory cells (Tregs), immature dendritic cells and myeloid-derived suppressor cells (MDSC) have also been found in the tumor microenvironment and play a critical role in the regulation of immune responses. All of these barriers within the tumor microenvironment suppress the function of anti-tumor specific T cells, leading tumor escape and progression of tumorigenesis. However, many different clinical strategies have now been developed to block or suppress the negative activity of the tumor microenvironment, and therefore boost antigen-specific T cell mediated anti-tumor immunity [105].

Immunotherapy for cancer is divided into two categories: Passive immunotherapy and active immunotherapy. Passive immunotherapy involves the generation of immune components in the laboratory that are eventually administered into patients in order to provide immunity against tumors. One advantage of using passive immunotherapy is that it does not require the patient's immune system to take an active role in the elimination of tumors. Thus, such immunotherapy is preferred when the patient's immune system is highly compromised due to large tumor burdens or previous therapies. One disadvantage of this approach is that tumors may become resistant to this therapy as the cancer cells mutate or downregulate surface expression of targeted antigens [30].By contrast, active immunotherapy usually involves the generation of vaccines to stimulate the patient's own

immune system to fight cancer. One major advantage of using this type of immunotherapy is that it can induce both therapeutic immunity (in the form of tumor-specific effector T cells) and protective immunity (in the form of tumor specific memory T cells that can control tumor relapse) [100]. Different examples of current immunotherapy regimens are illustrated below.

CANCER IMMUNOTHERAPY	
PASSIVE	**ACTIVE**
Monoclonal antibodies: • Anti-CD20 (Rituximab) for Non-Hodgkin's Lymphoma. • Anti-CTLA-4 (Ipilimumab) for Melanoma • Anti-HER2 (Herceptin) for breast cancer **Adoptive T-cell Transfer:** • Ex vivo–engineered T cells (CARs) • TILs	**Antigen-specific vaccines:** • Peptide vaccines • **DC vaccines** • Tumor lysates **Antigen non-specific vaccines:** • IFN-α • IL-2 • TNF-α

The use of monoclonal antibodies is an example of passive immunotherapy. Monoclonal antibodies are molecules engineered in the laboratory that specifically recognize particular targets on cancer cells (i.e. tumor antigens) [106]. Monoclonal antibodies can be administered into patients intravenously. Once monoclonal antibodies recognize cancer cells it can lead to disruption of cancer cell growth, or to increase anti-tumor immunity. One example of a monoclonal antibody currently used to treat B-cell lymphomas resistant to other chemotherapy approaches is Rituximab [107]. Rituximab was approved by the FDA in 1997 based on its safety and effectiveness in clinical trials. This monoclonal antibody binds to the CD20 molecule on B cells. CD20 has been described to regulate cell proliferation, and blocking its activity using rituximab leads to elimination of

malignant B cells. Another example of a FDA-approved monoclonal antibody to treat the most aggressive type of breast cancer ($HER2^+$ breast cancer) is Herceptin [108; 109].

The use of immune cells such as T cells with anti-tumor activity is another example of passive immunotherapy. Since the discovery that T cells can kill tumors, different approaches have been developed for adoptively transferring tumor specific T cells into cancer patients [31; 32]. This approach requires the isolation of T cells from the patient's tumor followed by the progressive *ex vivo* expansion of those tumor-specific autologous T cells using high concentrations of IL-2. The main purpose of the *ex vivo* expansion is to increase the number of tumor-specific T cells with potent anti-tumor function for subsequent infusions into patients. Although early clinical studies were disappointing, recent reports have demonstrated that prior lymphodepletetion using chemotherapy increases the anti-tumor activity of the infused T cells, resulting in approximately 50% objective clinical responses in patients with metastatic melanoma [110].

One example of active immunotherapy is the use of vaccines. Vaccines have been widely used to boost the immune system's natural ability to protect the body against pathogens that may cause disease. Based on the same principles used to generate vaccines against infectious agents, several therapeutic anti-cancer vaccines have been developed for the treatment of different cancers, including melanoma, prostate, breast, lung and other cancers [111]. Cancer vaccines are biological preparations designed to stimulate immune cells, mainly $CD8^+$T cells, and direct them to recognize and kill specific types of cancer. One approach to generate a cancer vaccine is to isolate tumor antigens from cancer cells and immunize cancer patients directly with the main purpose of inducing greater immune

responses against those specific antigens [112; 113]. Although many tumor antigens have been reported for different cancers, not all of them can boost immune responses against cancer cells. Therefore, many clinical cancer vaccine trials aim to select antigens that are specifically recognized by $CD8^+$T cells. Some examples of tumor antigens recognized by $CD8^+$T cells and have been demonstrated to boost anti-tumor responses following vaccination are the melanoma-associated antigens: Melan-A/MART-1, NY-ESO-1 and gp100 [114; 115; 116].

Cytokines have also been widely used as an active immunotherapy approach. Cytokines are substances that are naturally produced by white blood cells and their main role is to regulate immune responses. Some cytokines are known to increase the effector functions of CD8+ T cells (i.e. IL-2), whereas other cytokines inhibit the activity of these T cells (i.e. IL-10) [117]. Cytokines such as IL-2, IFN-α and GM-CSF have been frequently used in the clinic [118]. GM-CSF and IL-2 have proven to be effective adjuvants to boost the activity of cancer vaccines by either improving antigen presentation of tumor antigens or by supporting the effector function of vaccine-induced T-cell immune responses [118]. IFN-α has already been approved by the FDA to treat many cancers, including melanoma [119].

1.9 Dendritic Cell cancer vaccines

The generation of effective cancer vaccines depends not only on the discovery of target antigens but also on strategies to deliver those antigens to elicit tumor-specific T cell immune response. To elicit potent T-cell mediated immune responses against cancer, several different cancer vaccines have now been designed and tested clinically to treat

different types of cancers, including metastatic melanoma and lymphomas. These have included immunizing patients with defined tumor-associated antigens (i.e. single peptides or whole tumor proteins), naked plasmid DNA, attenuated viral vectors, and dendritic cells (DCs) [120; 121]. Although early studies demonstrated that the use of each vaccine as single therapy regimen induced low objective clinical responses, more recent studies have shown that cancer vaccines are significantly more effective when used in combination with adoptive cell transfer of tumor-specific T cells [36].

Of all the different vaccine formulations tested clinically, vaccines that utilize DCs have demonstrated the best combination of safety and efficacy [43]. The main goal of DC-based cancer vaccines is to boost T cell-mediated antitumor immunity by introducing dendritic cells loaded with specific tumor-associated antigens to patients. Different methods to develop DC cancer vaccines have been used in both animal models and clinical trials. One method is the ex-vivo generation of antigen-loaded DCs. This method involves (1) the isolation of dendritic cells or precursors from the peripheral blood of cancer patients, (2) the ex-vivo DC culture and loading with a specific antigen obtained from the patient's own tumor and (3) the re-infusion of the antigen-loaded dendritic cells back into the patients to boost T cell-mediated anti-tumor immune responses [35]. The use of these ex-vivo generated DC cancer vaccines has generated effective anti-tumor responses in different studies. For example, clinical studies have demonstrated that vaccination with DCs pulsed with a melanoma antigen results in long term tumor regressions in patients with late stage metastatic melanoma as compared to other vaccine approaches (i.e viral vectors, plasmid DNA) [44; 122]. Also, our group has previously reported that giving a combination of tumor antigen-specific CD8+ T-cells and DCs pulsed with a tumor peptide

results in significant anti-tumor responses in a melanoma animal model [36]. Another method to deliver antigens for the generation of DC cancer vaccines is the *in vivo* DC targeting using antibodies against specific DC surface receptors, such as DEC205 [123; 124; 125; 126]. One advantage of this method is that is does not require the ex-vivo generation of DCs, which can be an expensive process. Although clinical trials need to be performed to demonstrate the effectiveness of the *in vivo* DC targeting method, preclinical studies in mice demonstrate that such strategies promote substantial augmentation of antigen-specific T cell immunity.

Understanding the biology of DCs is critical for the generation of effective DC cancer vaccines. DCs have a number of important advantages over other vaccines. For example, one advantage is that they have the ability to take up antigens from different tissues, such as the skin, and process them for subsequent presentation to T cells. Another advantage is that DCs express high levels of antigen presenting molecules (MHC molecules) and co-stimulatory receptors (i.e. CD80, CD86) and secrete large amounts of cytokines (i.e. IL-12) which are important for T cell activation, proliferation and differentiation [35; 38; 127]. But perhaps the most important advantage is that they can induce both therapeutic and protective anti-tumor immunity. Thus, DCs possess a number of important attributes that make them highly suitable as vaccines for generating antitumor immunity.

Although DC vaccine approaches have shown much promise, clinical responses remain relatively rare. Recently, the FDA has approved the use of PAP-GM-CSF fusion protein (Provenge) developed by Dendreon as the first therapeutic cancer vaccine for the

treatment of prostate cancer [128; 129]. The approval of Provenge not only demonstrates that efficacy of DC-based cancer vaccines in treating advance prostate cancer but it has also opened the doors for the development of vaccines for other types of cancer, including melanoma and breast [111].

Several limitations need to be overcome to increase the potency of current DC vaccines. One limitation is that the presentation of peptides (i.e. tumor- associated peptides) by DCs is limited due to rapid internalization of peptide/MHC class I complexes into acidic intracellular compartments (i.e. lysosomes) for degradation [88; 130; 131]. This rapid internalization process is regulated by the MHC class I cytoplasmic tail and limits the cell surface half lives of peptide/MHC class I complexes for recognition by $CD8^+$ T cells. To overcome this limitation, we generated a modified DC vaccine with enhanced antigen presentation capacity by introducing specific mutations into the MHC class I cytoplasmic tail. One of the MHC class I modified DC vaccines demonstrated substantially increased anti-tumor T cell responses *in vivo* as compared with a DC vaccine expressing the wild type MHC class I variant [132]. These studies provide a strong rationale and method of improving DC-based therapeutic vaccines for use in cancer therapy.

1.10 Long term goal, objectives and hypothesis of the present work

The **long term goal** of this dissertation is to better understand the regulation of MHC class I antigen presentation in DCs so that we can identify key target proteins that are involved in MHC class I trafficking and antigen presentation at the molecular level. The identification of these molecules may lead to new insights that will provide rationale for improved design of DC-based vaccines. The generation of such vaccines may be used to manipulate T-cell immune responses in different clinical settings including autoimmunity, chronic infectious diseases and cancer. In particular, the **main objectives** of this dissertation are to evaluate the role of the MHC class I cytoplasmic tail in DC-induced T-cell immune responses and to understand the role of the cytoplasmic tail in trafficking, cell surface half-lives, clustering and polarization of peptide/MHC-I complexes, which are critical for the initiation of CD8+ T cell-mediated immune responses.

We **hypothesized** that exon 7 encoded by the MHC-I cytoplasmic tail may regulate DC antigen presentation and modulate T-cell immune responses. For this purpose, we have evaluated the stimulatory capacity of DCs expressing exon 7-deleted MHC-I molecules at inducing CD8+ T cell immune responses *in vivo* and *invitro.* In addition, we have evaluated the cell surface half-lives and clustering of exon 7-deleted MHC class I molecules in human DCs.

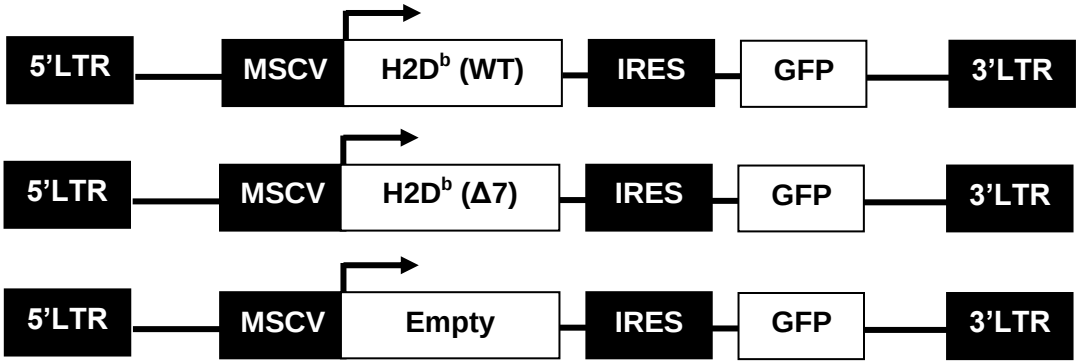
CHAPTER 2-RESULTS

2.1 Mouse DCs express similar levels of wild type and Δ7 variants of H-2D^b

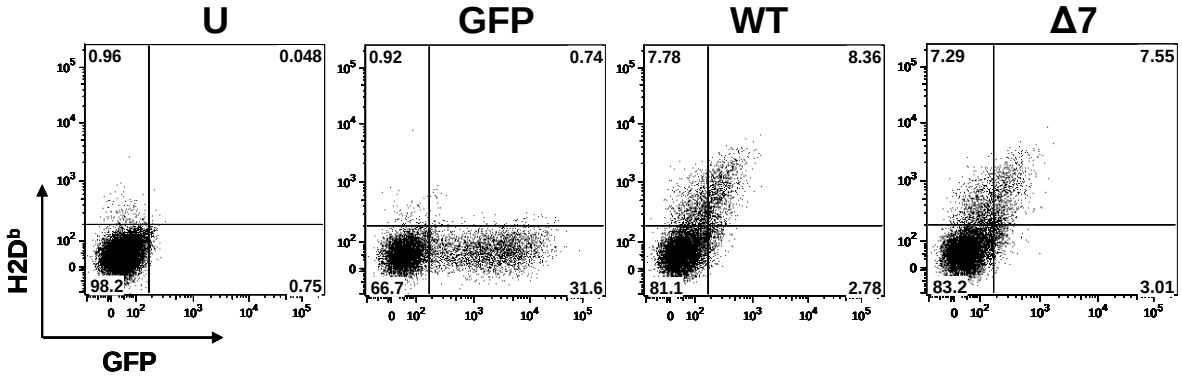
To test the capacity of DCs expressing exon 7-deleted MHC class I at inducing CTL- mediated immune responses, we first developed a PCR-based site-directed mutagenesis protocol to specifically remove exon 7 (encoding 13 amino acids of the cytoplasmic tail, including the serine phosphorylation site) of the mouse H-2D^b cytoplasmic domain [86]. Following sequencing analysis, bicistronic lentiviral vectors under the regulation of the Mouse Stem Cell Virus (MSCV) promoter were generated to induce the expression of WT H-2D^b or Δ7 H-2D^b molecules in mouse bone-marrow derived DCs (BM-DCs) from DBA/2 mice (haplotype H-2^d). Green fluorescent protein (GFP) was also expressed from a downstream internal ribosome entry site (IRES) (Figure A). To confirm the expression levels of WT H-2D^b or Δ7 H-2D^b molecules in DCs, we stained surface H2-D^b molecules using a fluorescently labeled H2-D^b monoclonal antibody and performed flow cytometry analysis at 72 h after transduction. The flow cytometry analysis showed that DC transduction efficiencies range from ~15 to 30% using a MOI of 10. In addition, such analysis demonstrated that transduced BM-DCs express comparable levels of WT H-2D^b and Δ7 H-2D^b molecules at the cell surface (Figure B). These data indicated that peptide binding and trafficking of newly synthesized H-2D^b molecules from the endoplasmic reticulum to the plasma membrane was not affected by the mutations generated in the cytoplasmic tail of H-2D^b.

Figure 3. Expression of WT and delta 7 variants of H2Db in mouse bone marrow derived DCs following transduction. (A) Bicistronic lentiviral vectors encoding either WT-H2D^b, Δ7-H2D^b or GFP molecules driven off by the Mouse Stem Cell Virus (MSCV) promoter were used to transduce mouse bone marrow derived dendritic cells. Lenti vectors also contain an IRES element to drive the expression of GFP. (B) Bone marrow-derived DCs from DBA2 mice were transduced to express either wild-type (WT) H-2D^b/GFP, exon 7-deleted (Δ7) H-2D^b/GFP, or GFP alone. Following transduction, DCs were stained for H2D^b and the levels of H2D^b and GFP molecules were analyzed by flow cytometry. U = untransduced DCs, GFP = DCs expressing GFP alone, WT = DCs expressing wild type H2D^b/GFP and Δ7 = DCs expressing Δ7-H2D^b/GFP. Reprinted from "Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity" Tania G. Rodriguez-Cruz, Shujuan Liu, Jahan Khalili, Mayra Whittington, Minyin Zhang, Willem Overwijk and Gregory Lizee. PLos ONE 2011 6(8):e22939.

A)


5'LTR
MSCV
H2D^b (WT)
IRES
GFP
3'LTR
5'LTR
MSCV
H2D^b (Δ7)
IRES
GFP
3'LTR
5'LTR
MSCV
Empty
IRES
GFP
3'LTR

B)


U
GFP
WT
Δ7
0.96
0.048
98.2
0.75
0.92
0.74
66.7
31.6
7.78
8.36
81.1
2.78
7.29
7.55
83.2
3.01
H2D^b
GFP

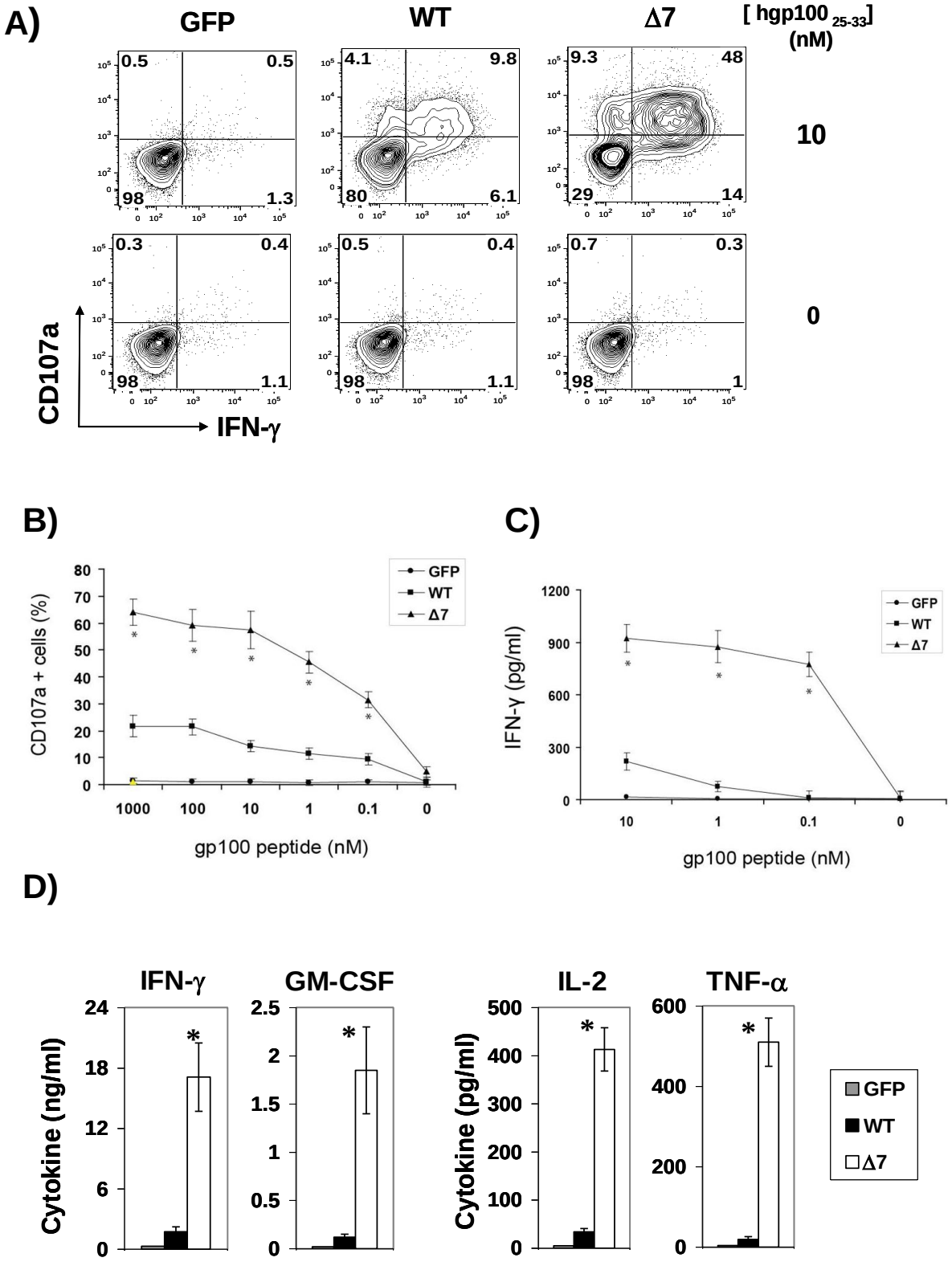
2.2 Δ7-D^b DCs induce enhanced $CD8^+$ T-cells effector functions *in vitro*.

The effector functions of melanoma specific CD8+ T-cells following stimulation with transduced DCs were also evaluated. For this, transduced BM-DCs were pulsed with titrated concentrations of human gp100 peptide and co-cultured with gp100-specific CD8+ T cells (Pmel-1 T cells) *in vitro*. Pmel-1 T cells were preferred in these studies because they can recognize gp100 peptide in association with $H2D^b$ molecules [133]. Following 4h of co-culture, flow cytometry analysis was performed to measure the surface expression of CD107a (degranulation marker) and intracellular IFN-γ in Pmel-1 T cells.

The analysis demonstrated that BM-DCs expressing Δ7-D^b and pulsed with 10nM of hgp100 peptide stimulated a much higher proportion of Pmel-1 T-cells to produce intracellular IFN-γ and express surface CD107a as compared to DCs expressing WT-D^b (71% vs. 20%) (Figure A). Similar results were also obtained at higher and lower peptide concentrations (Figure B). Interestingly, we observed that DCs expressing delta 7 induced strong Pmel-1 T-cell IFN-γ production at peptide amounts at least 10-fold lower than the lower limit of IFN-γ detection for DC expressing WT-$H2D^b$ (Figure C), suggesting that the binding properties of the TCR to its pMHC-I ligand may be modulated by modifications in the cytoplasmic tail of MHC-I. In addition, naïve Pmel-1 T cells produced significantly higher amounts of IFN-γ, IL-2, TNF-α, and GM-CSF following priming by DCs expressing delta 7 $H2D^b$ as measured by a Luminex assay (Figure D). These data indicated that exon 7 deleted $H2D^b$ molecules retained their antigen presentation function in our DC-vaccine settings. Also, these results suggested that deletion of exon 7 within the cytoplasmic tail of MHC-I may increase the TCR affinity and avidity for gp-100/H-2Db complexes resulting

in enhanced T-cell activation even at very low peptide concentrations. Based on these data, we hypothesized that exon 7 deleted MHC class I molecules may induce stronger T-cell anti-tumor responses *in vivo* and in clinical settings.

Figure 4. Δ7/H-2D^b DCs stimulate superior cytokine production and degranulation by CD8$^+$ T-cells. DCs expressing either WT-H2D^b/GFP, Δ7-H2D^b/GFP or GFP alone were pulsed with different concentrations of hgp100 peptide, and co-cultured with gp100-specific Pmel-1 CD8$^+$ T cells for 4hours. Unpulsed DCs (0nM of peptide) were used as controls. **(A)** A representative contour plot of intracellular IFN-γ production and CD107a expression in Pmel CD8+ T cells following stimulation with transduced DCs. **(B)** Percentage of intracellular CD107a in Pmel T cells following stimulation with transduced DCs pulsed with titrated amounts of gp100. This data is a representative of one triplicate experiment out of three. Data shows mean ± S.D. **(C)** Transduced DCs pulsed with titrated amounts of hgp100 peptide were co-cultured with Pmel CD8$^+$T cells for 18hrs. The levels of IFN-γ production in supernatants were analyzed by ELISA. **(D)** IFN-γ, GM-CSF, IL-2 and TNF-α release by Pmel-1 T cells following 72hrs stimulation with hgp100 peptide-pulsed (10 nM) DCs expressing either WT-H2D^b/GFP, Δ7-H2D^b/GFP or GFP alone, as determined by Luminex. All results are representative of one from four replicate experiments. Data shows mean ± S.D. p values were calculated using a Student t test comparing the effects of delta 7 with WT-H2Db. * = p values <0.05. This research was originally published in "Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity" Tania G. Rodriguez-Cruz, Shujuan Liu, Jahan Khalili, Mayra Whittington, Minyin Zhang, Willem Overwijk and Gregory Lizee. PLos ONE 2011 6(8):e22939.


A)
GFP
WT
Δ7
[hgp100 25-33] (nM)
10
0
CD107a
IFN-γ
B)
CD107a + cells (%)
GFP
WT
Δ7
gp100 peptide (nM)
C)
IFN-γ (pg/ml)
gp100 peptide (nM)
D)
IFN-γ
GM-CSF
IL-2
TNF-α
Cytokine (ng/ml)
Cytokine (pg/ml)
GFP
WT
Δ7

2.3 Δ7-D^b DCs stimulate improved Pmel-1 T-cell priming *in vitro and invivo*.

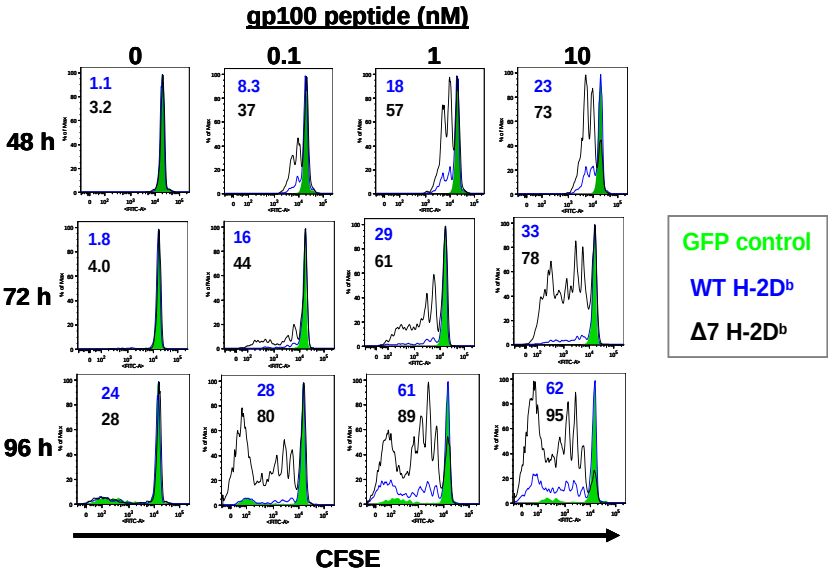
Proliferation of tumor-specific T cells is critical for the sustentation of potent anti-tumor immune responses [32]. Therefore, we next tested the ability of DC vaccines expressing exon 7-deleted $H2D^b$ molecules to induce the priming and proliferation of antigen-specific CD8+ T-cells. For this, naïve Pmel-1 T cells were labeled with CFSE and co-cultured for 4 days with hgp100-pulsed DCs expressing either WT-D^b or Δ7-D^b *in vitro*. To assess the relative priming abilities of exon 7-deleted Db mutant, flow cytometry was performed on day 2, 3, and 4 following DC stimulation. Flow cytometry analysis showed that DCs expressing Δ7-D^b molecules induced a significantly higher proportion of Pmel-1 T cells to undergo proliferation compared to DCs expressing WT-D^b molecules under all conditions tested. We also observed that the difference was particularly dramatic at limiting peptide concentrations, with Δ7-DCs clearly outperforming the WT-DCs at 1 nM and 0.1nM peptide (Figure A). In particular, the most striking difference was observed at 96 hr using 0.1nM of gp100 peptide. In this condition, Δ7-DCs improved the proliferation of Pmel-1 T cells more than 10 fold as compared to WT-DCs. These results suggested that deletion of exon 7 within the cytoplasmic tail of MHC-I may increase the antigen presentation and priming capacity of DCs as well as the affinity and avidity of the TCR for pMHC-I leading to a more sustained T-cell activation signaling.

Based on the results above, we then compared the capacity between WT and Δ7 DC vaccines at inducing CD8+T-cell priming *in vivo*. For this, we co-injected naïve Pmel T cells along with peptide-pulsed DCs expressing WT-D^b or Δ7 D^b into the tail-vein of Thy 1.2+ C57BL/6 recipient mice and analyzed T-cell proliferation. The percentage of Pmel-1 T cells in peripheral blood was analyzed by flow cytometry over time. Similar to the *in*

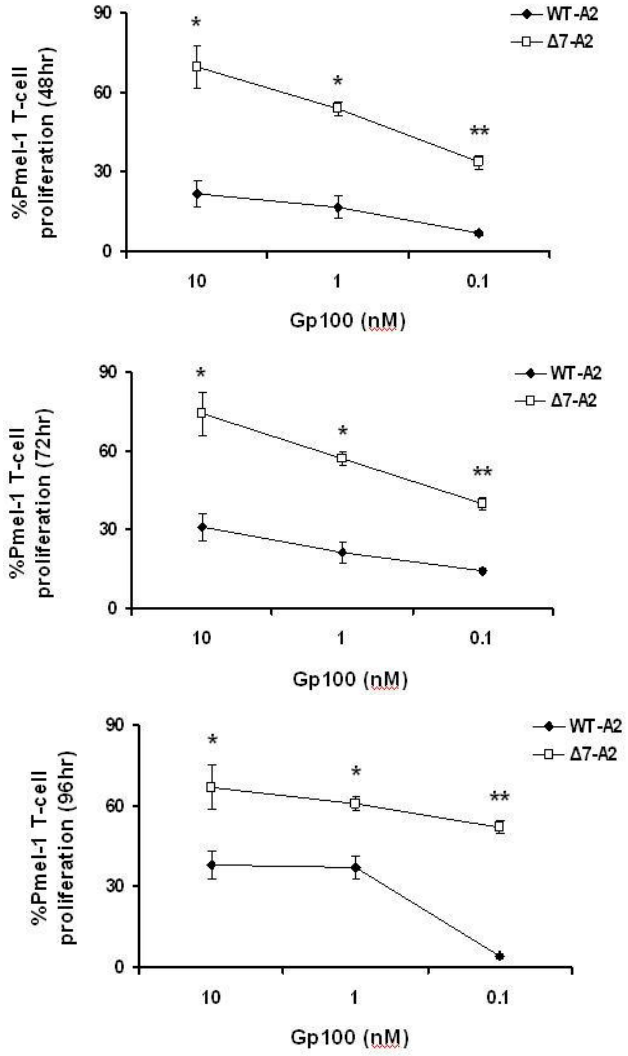
vitro results, DCs vaccines expressing Δ7 D^b molecules stimulated significantly better Pmel-1 T-cell priming than DC vaccines expressing WT D^b molecules (16% vs. 4% of total CD8+ T cells at the peak of the response), Figure C. However, we also observed that T cells from WT and Δ7 groups ceased proliferation by day 9 after injection. This may have resulted due to large amounts of T-cell death since previous studies have demonstrated that the majority of T-cells have short-half lives after priming. This result suggested that T-cell priming and proliferation may be sustained by increasing DC vaccine doses [36]

Figure 5. *In vitro* and *invivo* priming of CD8+ T cells following stimulation with transduced DCs. (A) WT-H2D^b/GFP, Δ7-H2D^b/GFP or GFP DCs were pulsed with titrated concentrations of hgp100 (25-33) peptide, and used to activate CFSE-labeled, naïve Pmel-1 CD8$^+$ T cells *invitro*. Proliferation of gated Pmel T cells was analyzed at 48 hours, 72 hours, and 96 hours of co-culture by flow cytometry based on CFSE dilution. Numbers on histograms indicates the percentage of divided Pmel T cells following priming with DCs expressing WT-H2D^b (blue) or Δ7-H2D^b (black). **(B)** Normalized data was plotted to analyze statistical differences between WT and Δ7 DCs by using a student Ttest. * = $p<0.05$, ** = $p<0.01$. Data was normalized to control samples (unpulsed DCs). GFP control sample was referred as 1. Error bars represent SEM from three individual samples. **(C)** Transduced DCs were pulsed with 300nM of hgp100 peptide, and adoptively transferred along with Pmel-1 CD8$^+$ T cells into C57BL/6 mice. DC-induced T-cell expansion *in vivo* was analyzed by measuring the percentage of Thy1.1$^+$ Pmel-1 T cells in peripheral blood at days 3, 6, 9 and 14 following adoptive transfer. U, untransduced DCs. Error bars represent SEM of three mice per group. This result is a representative of three independent experiments. This data was originally published in "Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity" Tania G. Rodriguez-Cruz, Shujuan Liu, Jahan Khalili, Mayra Whittington, Minyin Zhang, Willem Overwijk and Gregory Lizee. PLos ONE 2011 6(8):e22939.

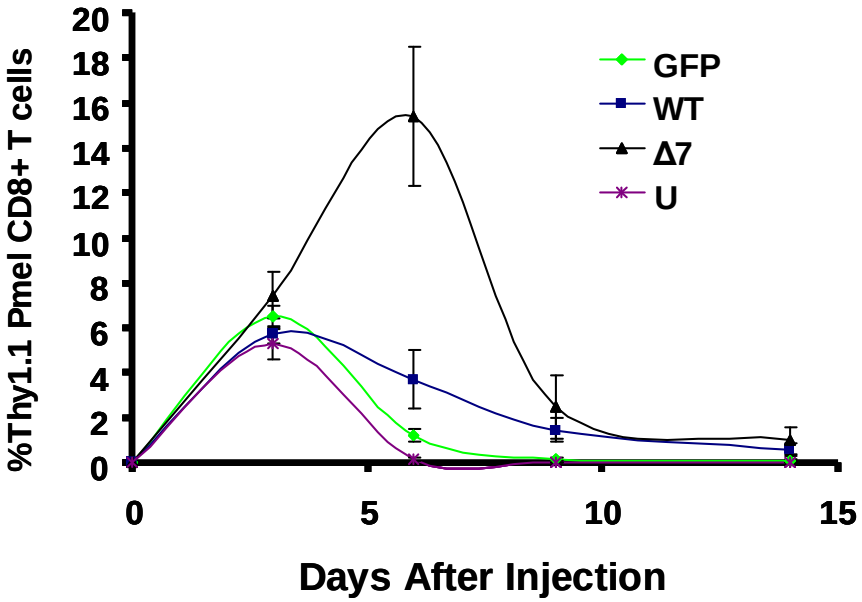
A)


gp100 peptide (nM)
0
0.1
1
10
48 h
72 h
96 h
1.1
3.2
8.3
37
18
57
23
73
1.8
4.0
16
44
29
61
33
78
24
28
28
80
61
89
62
95
CFSE
GFP control
WT H-2Db
Δ7 H-2Db

B)


%Pmel-1 T-cell proliferation (48hr)
%Pmel-1 T-cell proliferation (72hr)
%Pmel-1 T-cell proliferation (96hr)
WT-A2
Δ7-A2
Gp100 (nM)
10
1
0.1
0
30
60
90
48hrs
72hrs
96hrs

C)


%Thy1.1 Pmel CD8+ T cells
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
Days After Injection
GFP
WT
Δ7
U

2.4 Δ7-2D^b DC vaccines induce improved T-cell anti-tumor responses *in vivo*

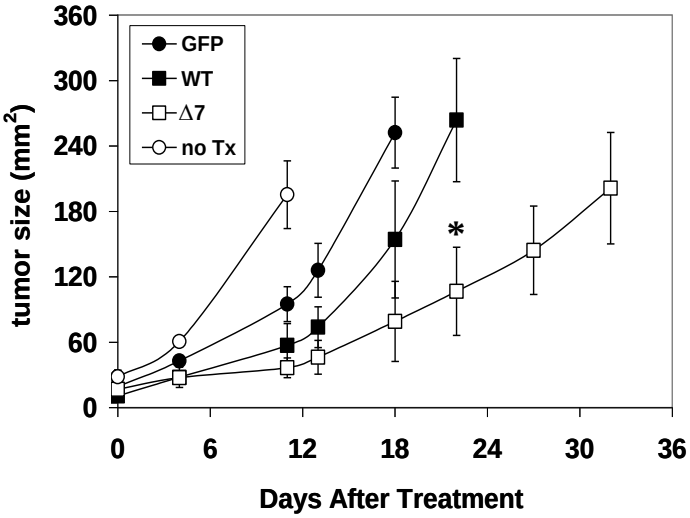
Because it has been shown that exon 7-deleted MHC class I molecules induced enhanced anti-viral T-cell responses and that Δ7 H-2Db DCs lead to improved Pmel T-cell proliferation [80] [and data presented above], we sought to determine whether DC vaccines expressing Δ7 H-2Db could lead to better T cell-mediated anti-tumor responses. To test this, we used an adoptive Pmel-1 T-cell transfer model in combination with DC vaccines to treat gp100-expressing B16 melanoma tumors as described previously [36]. Mice were first inoculated with B16 melanoma subcutaneously before given i.v. co-injection of Pmel-1 T cells and gp100-pulsed DCs expressing WT or Δ7 H2D^b molecules. Tumor size, levels of Pmel-1 T cells in the blood and survival levels were then analyzed over time following treatment.

We observed that the tumor size at day 23 post-treatment was significantly smaller in mice treated with a Δ7-D^b DC vaccine compared to mice treated with a WT-D^b DC vaccine ($p = 0.04$ at day 23) (Figure A). Also, the percentage of peripheral blood Pmel-1 T cells was greater in tumor bearing mice treated with Δ7 DC vaccines at days 7 and 21 (Figure B). In addition Δ7 DC vaccines along with Pmel-1 T cells also resulted in a significant survival benefit (mean survival 31d vs 20 d, p = *0.0004)* compared to mice administered with WT DC vaccines and Pmel-1 T cells (Figure C).

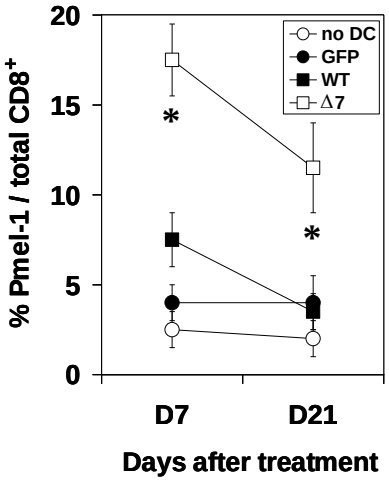
Collectively, these results indicated that Δ7 MHC class I molecules could provide a substantial advantage over WT MHC class I molecules for stimulating T-cell mediated anti-tumor responses in a DC-based cancer vaccine setting.

Figure 6: CD8+ T-cell anti-tumor responses following stimulation with transduced DCs. Transduced DCs pulsed with 300nM of hgp100 peptide were co-injected along with Pmel CD8+ T cells into tumor (B16 melanoma) bearing mice, followed by 3 days of intravenous IL-2. **(A)** B16 tumor size at different time points, following treatment with transduced DCs along with Pmel T cells. This data is a representative of one from three replicate experiments. Error bars represent mean ± S.D. * = $p < 0.05$**.** p values were calculated using a student t test. **(B)** Flow cytometry analysis of the relative percentages of Pmel-1 T-cells in the peripheral blood of treated animals at days 7 and 21 after treatment. This data is a representative of one from three replicate experiments. Error bars represent mean ± S.D. * = $p < 0.05$**.** p values were calculated using a student t test. **(C)** Survival analysis of treated mice as measured by the Kaplan Meier method. A Student T test was used to analyze the statistical significance of the groups. $p = 0.0004$ (mean survival 31d vs 20 d). All data are representative of a minimum of 3 replicate experiments. $N = 8$ mice per group. This data was originally published in "Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity" Tania G. Rodriguez-Cruz, Shujuan Liu, Jahan Khalili, Mayra Whittington, Minyin Zhang, Willem Overwijk and Gregory Lizee. PLos ONE 2011 6(8):e22939.

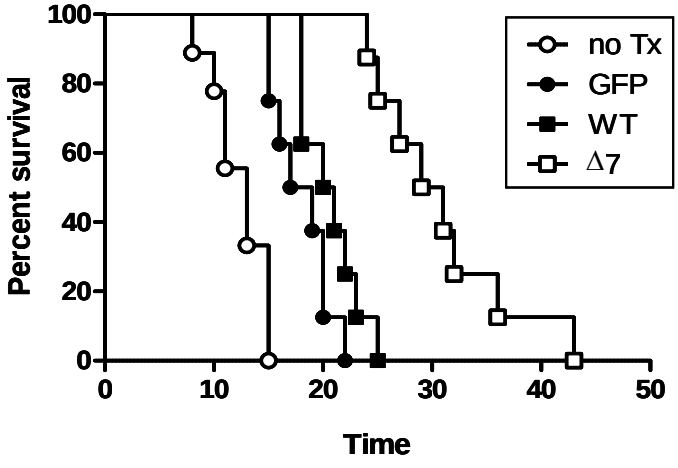
A)


GFP
WT
Δ7
no Tx
tumor size (mm2)
360
300
240
180
120
60
0
0
6
12
18
24
30
36
Days After Treatment
*

B)


no DC
GFP
WT
Δ7
% Pmel-1 / total CD8+
20
15
10
5
0
D7
D21
Days after treatment
*
*

C)

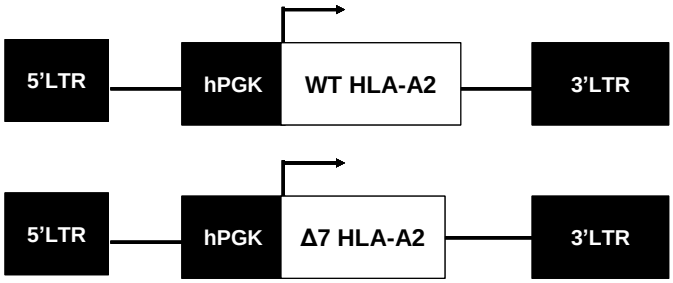

no Tx
GFP
WT
Δ7
Percent survival
100
80
60
40
20
0
0
10
20
30
40
50
Time

2.5 Transduced human DCs express comparable levels of wild type and Δ7 isoforms of HLA-A*0201

The mechanistic insights from the murine studies prompted us to next evaluate whether the Δ7 isoform of HLA class I could similarly enhance human CTL responses. First, lentiviral vectors encoding WT (WT-A2) or Δ7 (Δ7-A2) HLA-A2 molecules under the control of the human ubiquitous PGK promoter were developed and used to induce the expression of such HLA-A2 variants into primary human DCs (derived from CD34+HSC) and the human KG-1 DC-like cell line, (Figure A). Following 5 days post-transduction, surface levels of WT-A2 and Δ7-A2 were measured in both CD34+-derived DCs and KG-1 cells using a HLA-A2 fluorescently-labeled monoclonal antibody and by flow cytometry. The flow cytometric analysis demonstrated that transduced human DCs expressed comparable cell surface levels of WT-A2 or Δ7-A2 (Figure B). We also observed that those DCs expressed comparable levels of CD86, CD80, MHC class II, CD11c, B7H-1, B7-DC and CD70 (data not shown). This data suggested that deletion of exon 7 from the HLA-A2 cytoplasmic tail did not abrogate the molecule's transport from the endoplasmic reticulum to the cell surface of human DCs.

Figure 7: Expression of WT and Δ7 variants of HLA-A*0201 in human DCs. (A) Lentiviral vectors used to transduce human DC-like KG-1 cells and human CD34-derived DCs. The human phosphoglycerate kinase (hPGK) promoter was used to drive the expression of WT and Δ7 isoforms of HLA-A*0201. *Bottom*, Predicted amino acid sequences of the cytoplasmic domains of WT and Δ7 HLA-A*0201. Exon 7-encoding amino acids are depicted as red, and bold font indicates reported phosphorylation sites. **(B)** Human DC-like KG-1 cells and primary $CD34^{+}$-derived DCs were transduced to express comparable levels of surface HLA-A*0201, as determined by HLA-A2-specific mAb staining and flow cytometry. WT-A2, grey and blue histograms; Δ7-A2, black and green histograms. Light grey histograms represent untransduced DCs. Reprinted from"Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity" Tania G. Rodriguez-Cruz, Shujuan Liu, Jahan Khalili, Mayra Whittington, Minyin Zhang, Willem Overwijk and Gregory Lizee. PLos ONE 2011 6(8):e22939.

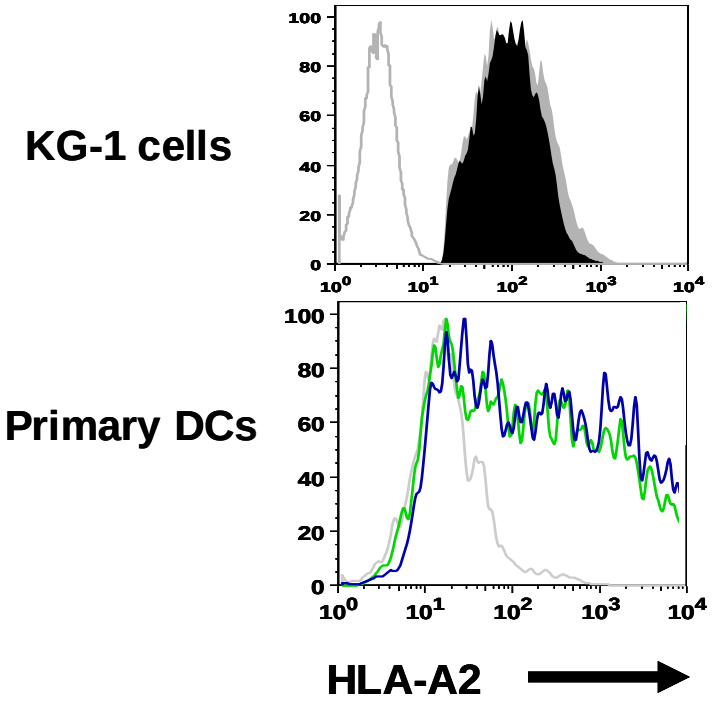
A)



·A*0201 (WT) Tm – RRKSSDRKGGS**Y**TQAASSDSAQGSDV**S**LTACKV

·A*0201 (Δ7) Tm – RRKSSDRKGGS**Y**TQAAV

B)



2.6 Δ7-A2/DCs facilitated augmented inflammatory cytokine production by human CTL.

We next evaluated the capacity of human DCs expressing Δ7-A2 to stimulate human antigen-specific CD8+ T cells effector functions. For this purpose, human DCs (KG-1 cells and CD34+-derived DCs) expressing either WT-A2 or Δ7-A2 were pulsed with different concentrations of MART-1 (melanoma antigen recognized by T cells) 27L peptide. Peptide pulsed-DCs were then used to stimulate MART-1 specific CD8+ T cells in an 18h *invitro* co-culture system. The levels of different inflammatory cytokines produced by the MART-1 specific T cells following stimulation were then analyzed in co-culture supernatants using ELISA and Luminex.

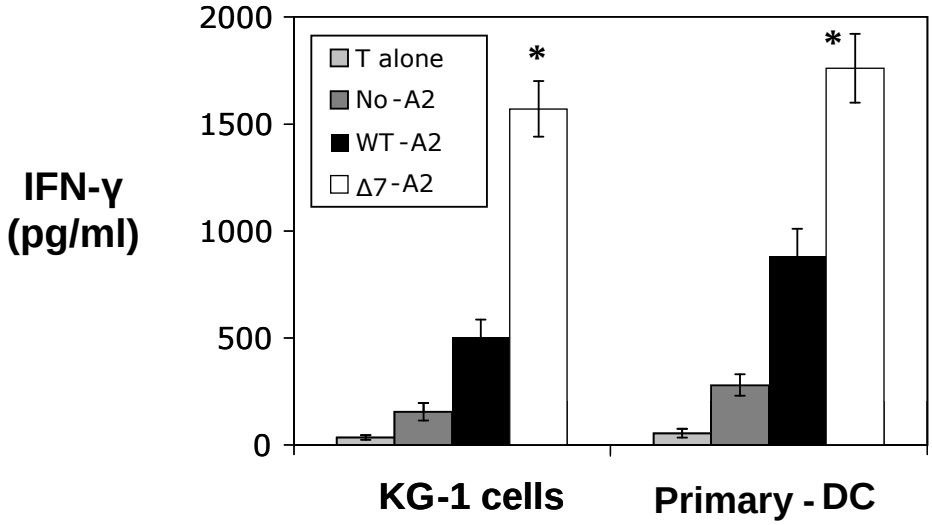
Similar to the murine studies, we observed that both human primary DCs and KG-1 cells expressing Δ7-A2 molecules and pulsed with 10nM MART-1 peptide significantly increased IFN-gamma production from MART-1 specific CD8+ T cells as compared to peptide-pulsed DCs expressing WT-A2. MART-1 specific T cells alone or co-cultured with untransduced DCs (No-A2) produced almost undetectable amounts of IFN-gamma as expected (Figure A). The difference in IFN-gamma production from MART-1 specific T cells was even more significant at lower peptide concentrations. We observed that MART-1 specific T cells produced undetectable amounts of IFN-gamma when DCs expressing WT-A2 were pulsed with 1nM of peptide. However, at the same peptide concentration, DCs expressing Δ7-A2 were able to stimulate MART-1 specific T cells to produce more than 4,000 pg/ml of IFN-gamma, Figure B. In addition, we also evaluated the capacity of DCs expressing Δ7-A2 to stimulate influenza matrix protein 1 (FluM1)-specific CD8+ T cells from the peripheral blood mononuclear cells (PBMC) of normal donors. Similar to the

studies using MART-1 specific T cells, KG-1 cells expressing Δ7-A2 molecules induced significantly enhanced FluM1-specific inflammatory cytokine and chemokine production compared to KG-1 cells expressing WT-A2 molecules. The levels of IFN-γ, MIP-1α and MIP-1β were 3 to 5-fold higher and the levels of IL-2, MCP-1, and TNF-α were typically >10-fold higher than WT-A2 stimulated T-cell cultures as measured by Luminex. The levels of VEGF produced by Flu specific T cells following stimulation with transduced DCs were almost undetectable in our *invitro* system, Figure C.

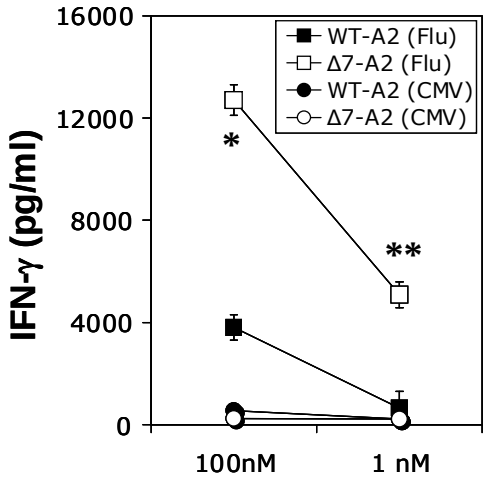
The fact that DCs expressing Δ7-A2 stimulated enhanced T-cell effector function at very low concentrations of peptide suggested that the affinity and avidity properties of the TCR may be modulated by specific motifs in the MHC-I cytoplasmic tail. Based on these results and previous studies that have demonstrated that the efficacy of CTLs cellular responses is highly dependent upon their functional avidity, we hypothesized that Δ7-A2 molecules may increase the functional avidity of effector T cells to recognize tumor cells or infected cells during the killing or efferent phase. We tested this hypothesis in the human T-cell expansion experiments.

Figure 8: $CD8^+$ T-cell inflammatory cytokine secretion following stimulation with transduced human DCs. (A) Transduced KG-1 cells and $CD34^+$-derived DCs were pulsed with the melanoma peptide, MART-1 (100 nM), and co-cultured with MART-1-specific $CD8^+$ T cells. Overnight culture supernatants were analyzed for IFN-γ secretion by ELISA. **(B)** Transduced KG-1 cells were pulsed with titrated concentrations of FluM1 peptide, and used to stimulate influenza-specific $CD8^+$ T cells. IFN-γ secretion in response to titrated amounts of FluM1 peptide was analyzed by ELISA. **(C)** Specific inflammatory cytokine release in response to DC stimulation for 18h, as determined by Luminex. All results are representative of a minimum of 3 replicate experiments. * = p values <0.05. P values were calculated using a student T test analysis comparing the effects of delta 7 with WT-H2Db. This data was originally published in "Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity" Tania G. Rodriguez-Cruz, Shujuan Liu, Jahan Khalili, Mayra Whittington, Minyin Zhang, Willem Overwijk and Gregory Lizee. PLos ONE 2011 6(8):e22939.

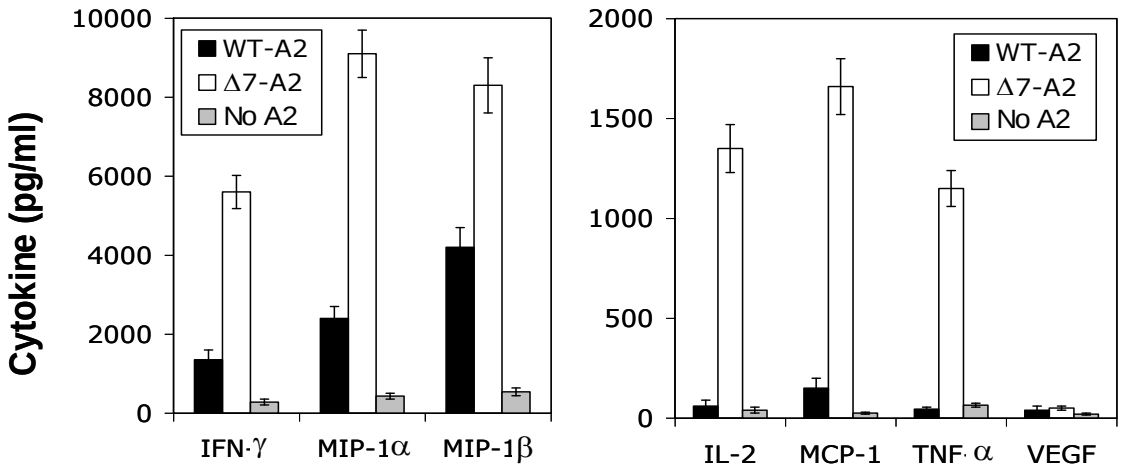
A)


IFN-γ (pg/ml)
2000
1500
1000
500
0
T alone
No-A2
WT-A2
Δ7-A2
*
*
KG-1 cells
Primary - DC

B)


IFN-γ (pg/ml)
16000
12000
8000
4000
0
WT-A2 (Flu)
Δ7-A2 (Flu)
WT-A2 (CMV)
Δ7-A2 (CMV)
*
**
100nM
1 nM

C)


Cytokine (pg/ml)
10000
8000
6000
4000
2000
0
WT-A2
Δ7-A2
No A2
IFN·γ
MIP-1α
MIP-1β
2000
1500
1000
500
0
WT-A2
Δ7-A2
No A2
IL-2
MCP-1
TNF·α
VEGF

2.7 Δ7-A2/DCs improved the expansion of antigen specific CD8+ T cells

Because one goal of DC vaccines is to stimulate the expansion of human antigen-specific T cells, we next went onto determine whether DC expressing Δ7-A2 molecules increase the expansion of HLA-A*0201 restricted CD8+T cells compared to DCs expressing WT-A2 in an *invitro* co-culture system. For this, we isolated CD8+ T cells from normal donor PBMCs (Flu specific T cells were <1% of the CD8+ T cell compartment) and co-culture them with Flu-pulsed KG-1 cells expressing either WT-A2 or Δ7-A2 for the period of 9 days. Following co-culture, the expansion of Flu-specific CD8+ T cells was measured by using a FITC-conjugated CD8+ specific antibody, an APC-conjugated Flu specific tetramer and flow cytometry.

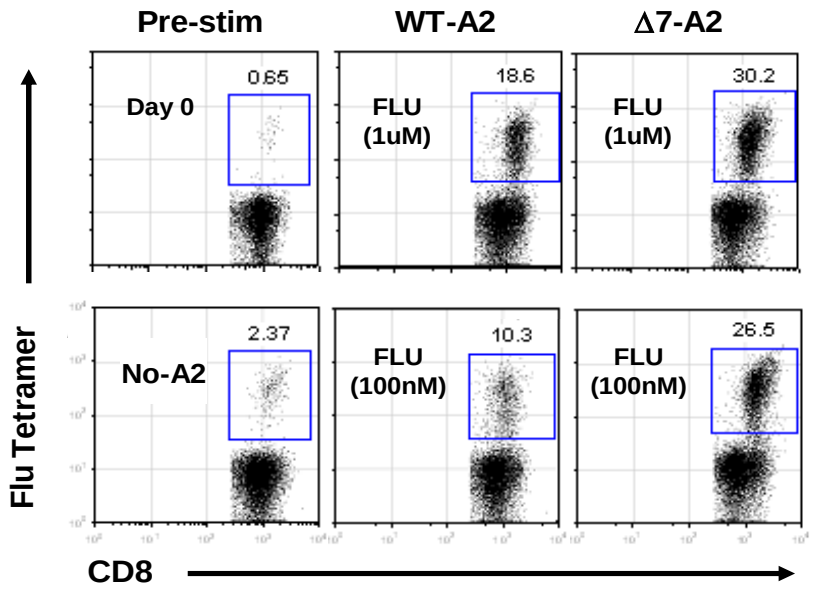
We found that FluM1 peptide-pulsed Δ7-A2 KG-1 cells facilitated superior expansion of FluM1-specific CD8+ T-cells compared with WT-A2 KG-1 cells (2 fold increased expansion) and that this effect was most remarkable at lower peptide concentrations (Figure A). However, we did not observe significant differences in the MFI of tetramer staining following T-cell expansion. These results led us to think that Δ7-A2 may not affect the affinity and avidity of the TCR following priming since previous studies have shown that the intensity of tetramer staining correlates with the affinity and avidity of TCRs. We also observed similar results for the expansion of CMV-specific T cells from normal donor PBMC following a single round of *in vitro* stimulation with transduced KG-1

cells (Figure B). As expected, antigen specific T cells co-culture with untransduced KG-1 cells showed a modest expansion.

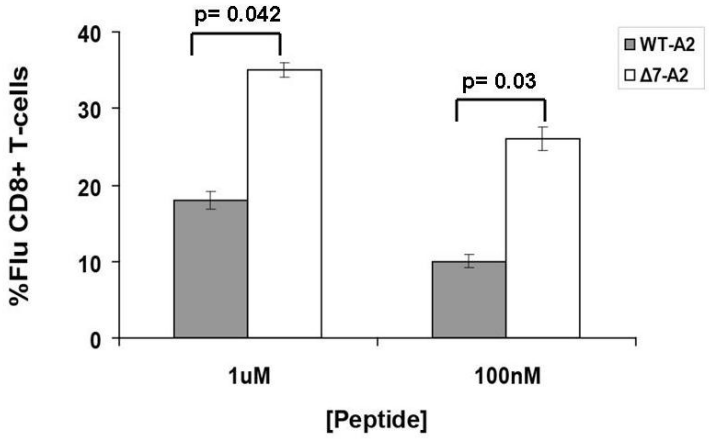
To test whether Δ7-A2 molecules may affect the functional avidity of the primed Flu specific T cells during the killing or efferent phase, T-cell intracellular IFN-γ and expression of CD107a was measured by flow cytometry following a second round of stimulation with peptide pulsed T2 cells. Although their distinct expansion indices, we found that $CD8^+$ T cells expanded by either Δ7-A2 or WT-A2-expressing DCs were equally functional with regard to functional avidity, intracellular IFN-γ levels, and degranulation capacity (Figure C). These results suggested that deletion of exon 7 within the MHC-I cytoplasmic tail increases the expansion and effector function of CTLs through mechanisms independent of affinity and functional avidity of antigen specific T cells. We also attempted to evaluate the *in vitro* expansion of melanoma-specific CD8+ T cells using a single round of DC stimulation but was found to be significantly less robust compared with virus-specific T cell expansion.

Figure 9: Expansion of human antigen-specific CD8+ T cells following stimulation with transduced KG-1 cells. KG-1 cells expressing WT-A2 or Δ7-A2 molecules were pulsed with 100nM of the influenza peptide (FluM1). Cells were then used to stimulate the expansion of influenza-specific $CD8^+$ T cells isolated from normal donors PBMCs *in vitro*. Following 8days stimulation, the expansion of influenza-specific CD8+ T cells was determined by tetramer analysis and flow cytometry. Numbers on top of blue squares represent the % of Flu specific CD8+ T cells (Figure A). The % of Flu specific CD8+ T cells and mean fluorescence intensity (MFI) of the Flu tetramer were analyzed to measure statistical differences between the WT and Δ7-A2 groups following stimulation (Figure B). Transduced KG-1 cells were pulsed with Flu peptide and CMV peptide (pp65 peptide) and used to stimulate both Flu and CMV specific CD8+ T cells *in vitro*. The total number of virus-specific CD8+ T cells was then analyzed by the tryptan-blue method following *invitro* co-culture with transduced KG-1 cells (Figure C). Following expansion, T cells were then analyzed for effector function. For this, expanded T cells were co-cultured with T2 cells pulsed with titrated amounts of Flu peptide. After 4h stimulation, intracellular IFN-γ and surface CD107a was analyzed by flow cytometry (Figure D). All data are representative of at least 4 replicate experiments. Error bars represent SEM. A student T test was used to calculate p values and evaluate statistical differences between the WT-A2 and Δ7-A2 experimental groups. Reprinted from "Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity" Tania G. Rodriguez-Cruz, Shujuan Liu, Jahan Khalili, Mayra Whittington, Minyin Zhang, Willem Overwijk and Gregory Lizee. PLos ONE 2011 6(8):e22939

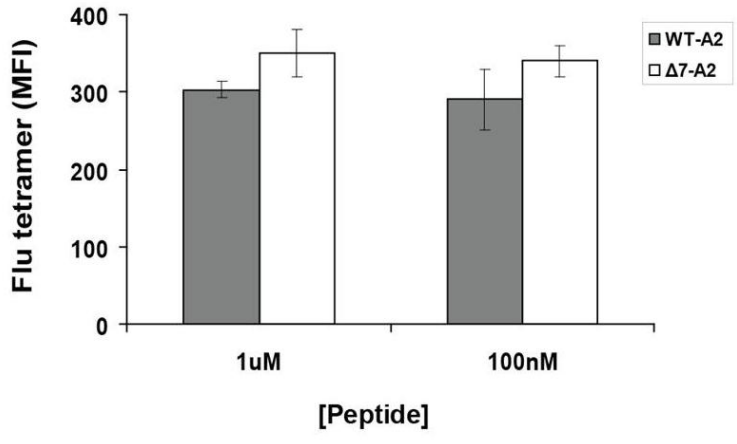
A)


Pre-stim
WT-A2
Δ7-A2
Day 0
0.65
FLU
(1uM)
18.6
FLU
(1uM)
30.2
No-A2
2.37
FLU
(100nM)
10.3
FLU
(100nM)
26.5
Flu Tetramer
CD8

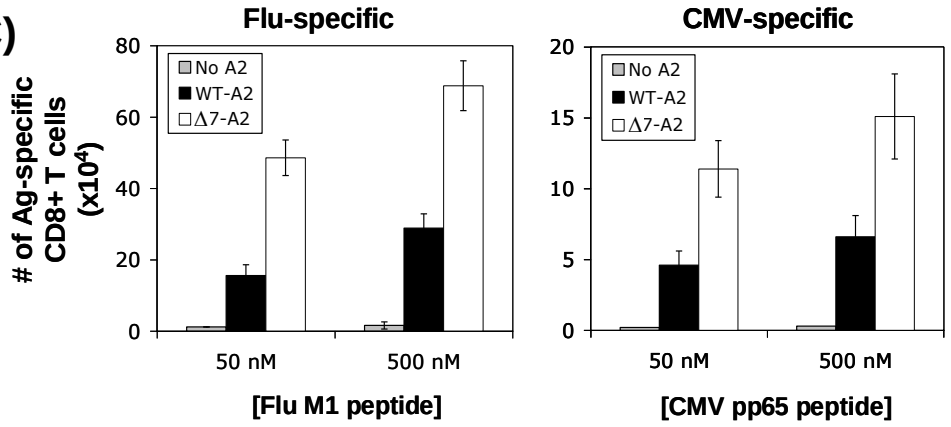
B)


p= 0.042
p= 0.03
WT-A2
Δ7-A2
%Flu CD8+ T-cells
40
30
20
10
0
1uM
100nM
[Peptide]

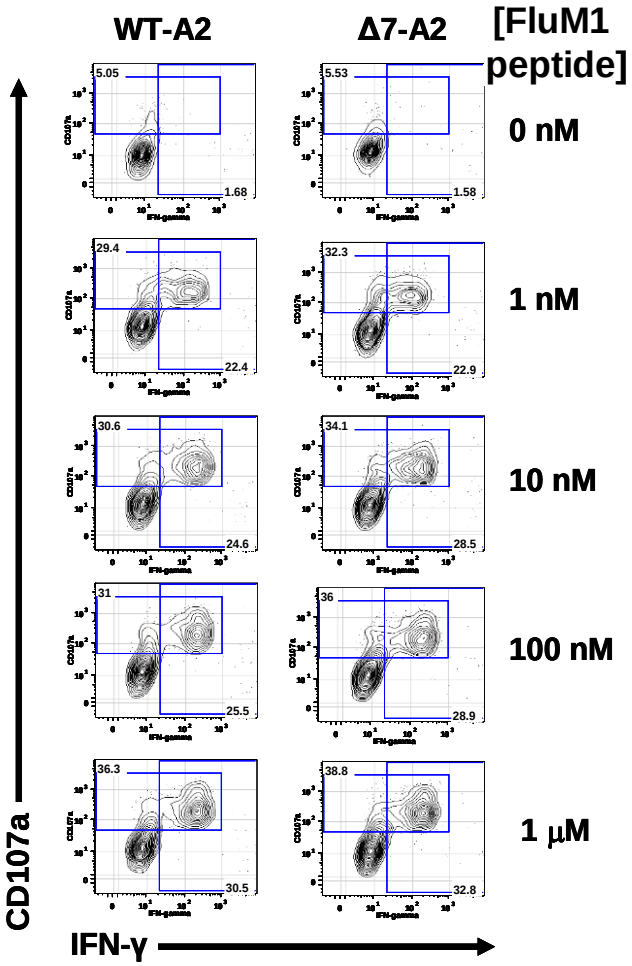
C)


WT-A2
Δ7-A2
Flu tetramer (MFI)
400
300
200
100
0
1uM
100nM
[Peptide]

C)


Flu-specific
CMV-specific
# of Ag-specific CD8+ T cells (x10⁴)
No A2
WT-A2
Δ7-A2
80
60
40
20
0
20
15
10
5
0
50 nM
500 nM
[Flu M1 peptide]
[CMV pp65 peptide]

D)


WT-A2
Δ7-A2
[FluM1 peptide]
0 nM
1 nM
10 nM
100 nM
1 μM
5.05
1.68
5.53
1.58
29.4
22.4
32.3
22.9
30.6
24.6
34.1
28.5
31
25.5
36
28.9
36.3
30.5
38.8
32.8
CD107a
IFN-γ

2.8. Δ7-HLA-A*0201 isoforms show increased bio-availability of MHC-I/peptide complexes for recognition by $CD8^+$ T cells.

Because previous studies have demonstrated that the duration of peptide/MHC complexes at the cell surface plays a critical role in the activation of T cells, we hypothesized that Δ7 MHC-I splice variant may show prolonged cell surface half-lives on APCs leading to improved T cell activation and cellular responses [21]. To test this hypothesis, we compared the surface half-lives of peptide/MHC class I complexes (WT or Δ7) over time by using a TCR-like monoclonal antibody that specifically recognizes MART-1/HLA-A*0201 peptide complexes. Consistent with murine studies [80] Δ7-A2/MART-1 peptide complexes demonstrated extended cell surface half-lives that were approximately twice that of WT-A2/peptide complexes in KG-1 cells (~16 h vs. ~8 h) as measured by flow cytometry (Figure A). Furthermore, confocal microscopy of transduced KG-1 cells revealed similar steady-state plasma membrane distributions of WT and Δ7 HLA-A*0201 when cells were incubated at 4°C. However when KG-1 cells were incubated at 37°C, Δ7-A2 molecules demonstrated substantially impaired lateral membrane movement and polar 'capping' compared with WT-A2. Instead, Δ7-A2 molecules remained relatively evenly dispersed at the plasma membrane (Figure B).

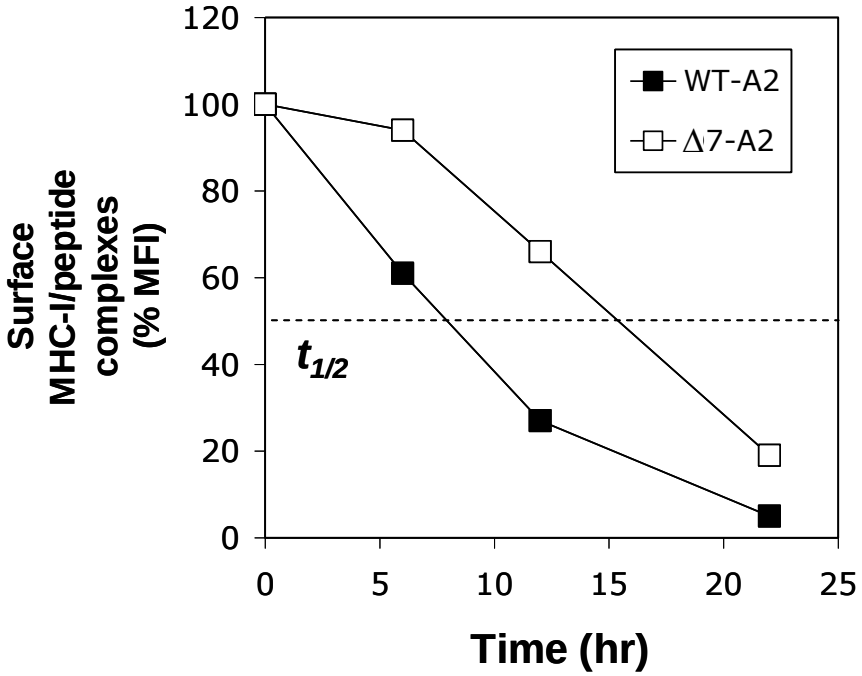
Moreover, MART-1-specific $CD8^+$ T cells co-cultured with peptide-pulsed KG-1 cells expressing WT-A2-GFP fusion proteins induced very similar clustering of WT-A2-GFP molecules that was invariably localized at the region of the DC/T-cell interface

(Figure C). However, Δ7-A2-GFP fusion proteins, showed little or no such membrane spatial clustering in response to $CD8^+$ T-cell contact (Figure C). As expected, the increased spatial distribution of membrane Δ7-A2/MART-1 complexes was also associated with an increase in the mean number of MART-1-specific T cells capable of forming conjugates with co-cultured Δ 7-A2 KG-1 cells on a per-APC basis following 2 h of co-culture (Figure D). Furthermore, larger order clusters of DC-T cell conjugates were formed after 6 hours of co-culture (Figure E) indicating that antigen presentation by Δ7-A2 molecules increases T-cell/DC interactions.

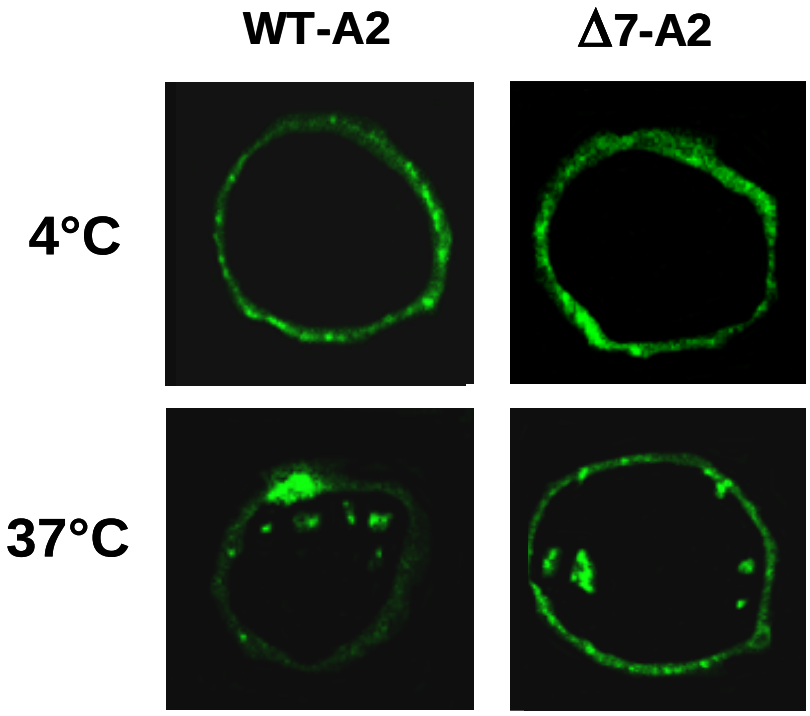
These data suggested that lateral movement and MHC-I clustering at the plasma membrane may be modulated by specific motifs within the cytoplasmic tail of MHC class I molecules and that such modulation may regulate T cell response outcomes. Also, these data suggested that delta 7 MHC class I molecules may show distinct signaling casacades that may affect antigen presentation, survival and cytokine production profiles of the APCs. However, how the MHC-I cytoplasmic tail modulate MHC-I clustering and signaling and how these parameters affect T cell activation at the molecular level need to be determined.

Figure 10: Surface mobilization and bioavailability of surface peptide/MHC class I complexes in KG-1 cells expressing WT-A2 or Δ7-A2 molecules. KG-1 cells expressing WT- or Δ7-HLA-A*0201 were pulsed with the melanoma-associated peptide (MART-1, 20nM) for 1hr at room temperature. Cells were then collected at different time points (0, 6, 12 and 24 hrs) following cell culture at 37°C, and stained at 4°C with a fluorescently-labeled TCR-like mAb that specifically recognizes HLA-A2/MART-1 complexes (Altor Biosciences). Graph depicts the mean fluorescence intensity (MFI) of surface WT-A2/MART-1 and Δ7-A2/MART-1 complexes on the cell surface of transduced KG-1 cells at each time point as analyzed by flow cytometry (Figure A). Transduced KG-1 cells were stained with a fluorescently-labeled HLA-A2-specific mAb (green) on ice to label surface HLA-A2 molecules. Cells were then incubated for 30 min at 4°C or 37°C and plated onto poly-L-lysine treated coverslips for confocal microscopy analysis (Figure B). KG-1 cells expressing WT-A2-GFP or Δ7-A2-GFP fusion proteins were pulsed with MART-1 peptide (100 nM) and co-incubated with MART-1-specific $CD8^+$ T cells (Figure C to E). Cells were then stained for CD3 (red), and analyzed by confocal microscopy. Arrows show augmented cell surface distribution of Δ7-A2/GFP fusion proteins (Figure C). Figure D shows quantification of the mean number of $CD8^+$ T-cells in contact with one KG-1 expressing either variant of HLA-A2 (WT or Δ7). Figure E demonstrates typical higher-order of APC/T-cell clusters as analyzed by light microscopy following 6h of co-culture. Reprinted from "Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity" Tania G. Rodriguez-Cruz,et al. PLos ONE 2011 6(8):e22939

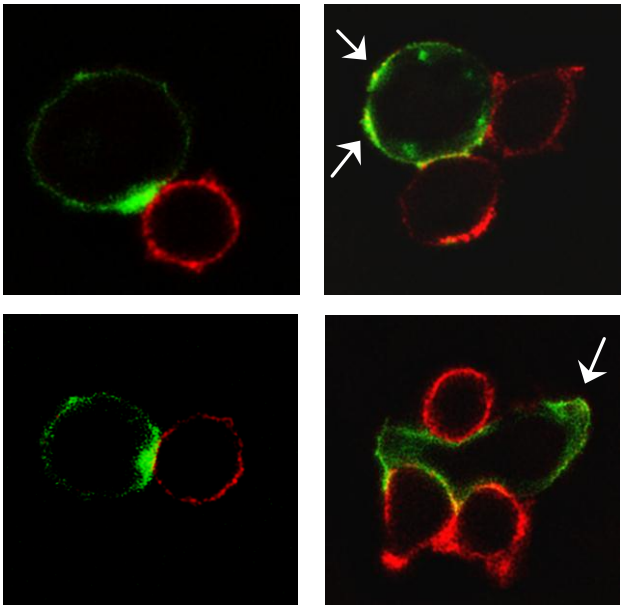
A)


120
100
80
60
40
20
0
0
5
10
15
20
25
Surface MHC-I/peptide complexes (% MFI)
Time (hr)
WT-A2
Δ7-A2
$t_{1/2}$

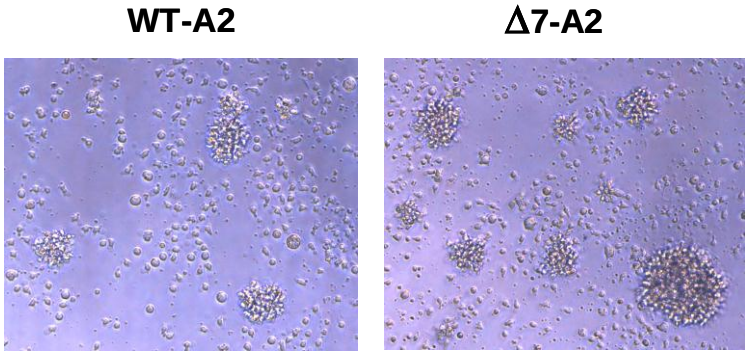
B)


WT-A2
Δ7-A2
4°C
37°C

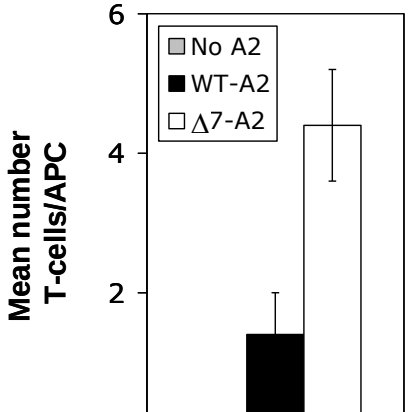
C)


WT-A2-GFP
Δ7-A2-GFP

D)


WT-A2
Δ7-A2

E)


Mean number T-cells/APC
6
4
2
No A2
WT-A2
Δ7-A2

CHAPTER 3

DISCUSSION AND FUTURE IRECTIONS

3.1 Summary

The major aims of this dissertation were to evaluate the function of the MHC class I cytoplasmic tail in DC-induced CTL anti-tumor and anti-viral responses, and in the regulation of surface internalization and polarization. We have addressed these aims by using both a melanoma mouse tumor model and a human *in vitro* DC/T-cell co-culture system. Specifically, we have demonstrated that DCs expressing exon 7-deleted MHC class I isoforms (Δ7-D^b), stimulated remarkably higher levels of CTL-cytokine production and also increased the proliferation of melanoma-specific (Pmel-1) T cells compared with DCs vaccines expressing wild type MHC-I *in vitro.* Additionally, we have demonstrated that, in combination with adoptive transfer of melanoma specific T-cells, DC vaccines expressing Δ7-D^b isoforms stimulated enhanced anti-tumor responses against established B16 melanoma leading to extension of mouse survival. Moreover, we showed that human DCs expressing Δ7-HLA-A*0201 molecules showed similarly augmented CTL stimulatory ability as compared to DCs expressing WT-HLA-A*0201 molecules. Finally, we have demonstrated that Δ7 MHC-I isoforms show impaired lateral membrane polarization and extended cell surface half-lives within the DC/T-cell interface, leading to increased spatial availability of MHC-I/peptide complexes for recognition by CD8+ T cells. Collectively,

this work demonstrates that the MHC class I cytoplasmic tail plays a key role in the generation of both mouse and human anti-tumor CTL responses.

3.2 MHC class I cytoplasmic tail splice isoform occur naturally in several species

The cytoplasmic tail of MHC-I shows an extraordinary high level of conservation of certain amino acids including Tyrosine 320 in exon 6, and Serine-335 in exon 7 [80; 86]. Interestingly, these amino acids have been described to be the potential phosphorylation sites of MHC class I [80; 87]. Several studies have reported different natural alternate splice isoforms of MHC class I heavy chains and that the generation of most of these isoforms involves the deletion of specific exons leading to truncated, elongated or soluble MHC class I molecules [84; 85; 134]. Of interest, previous studies have reported that exon 7 splicing isoforms of MHC class I occur naturally in mice, cows, and chickens resulting in a shorter cytoplasmic tail lacking the highly conserved Serine 335 phosphorylation site [84; 85; 86; 135]. One possible reason why alternative splicing occurs around exon 7 may be because it is composed of less than 50 base pairs as demonstrated by previous studies, which indicated that the length of internal exons plays a critical role in splice site selection [136]. However, how these splice variants isoforms of MHC-I affect immune responses is not well understood. Sequence analysis of more than 10,000 human expressed sequence tags for HLA-A, -B, and –C have suggested that exon 7-deleted splice variants of classical HLA alleles may not exist. Nevertheless, exon 7-deleted isoform of HLA-A*0201 showed a significantly enhanced capacity to stimulate human CD8+ T cells, suggesting that a potential loss of exon 7 splicing in humans during evolution may have had functional

implications for adaptive immunity, potentially providing protection from CTL-mediated autoimmunity or excessive inflammatory responses.

3.3 The role of the MHC-I cytoplasmic tail in the generation of T-cell responses

Because the presentation of peptides bound to MHC class I molecules is essential for the activation of CD8+ T cells, we examined whether the cytoplasmic tail of MHC class I molecules plays a functional role in the activation of both mouse and human CTL responses. We first hypothesized that exon 7 encoded by the cytoplasmic tail of MHC-I could regulate T-cell priming and effector function. To test this hypothesis, we generated DCs expressing either wild type or exon 7-deleted mutants of both H-2Db and HLA-A2 and evaluated their stimulatory capacity to induce priming and effector function of antigen-specific T cells *in vitro* and *invivo*. We found that exon 7 of MHC class I is completely unnecessary for the transport to the cell surface and as well as for the ability of MHC class I to acquire peptides since these molecules were able to be to recognized by cognate CD8+ T cells. Interestingly, we found that exon 7-deleted MHC class I molecules significantly induced superior T-cell immune responses as compared to native MHC-I in both murine and human systems. Our findings are consistent with previous studies supporting the idea that the MHC class I cytoplasmic tail contains motifs that can regulate T-cell activation resulting in increased or decreased CTL immune responses [80; 88]. For example, one study demonstrated that MHC class I molecules containing a point mutation in Tyr 320 (ΔY MHC-I) encoded by exon 6 of the cytoplasmic tail induced suboptimal CTL responses

after viral challenge *in vivo* [80]. The same study also demonstrated that MHC-I molecules lacking exon 7, in contrast to ΔY MHC-I molecules, induced superior CTL responses after viral challenge [80]. Moreover, another study showed that HLA-A2 molecules lacking the entire cytoplasmic tail demonstrated diminished recognition by EBV-specific T cells *in vitro* [137]. However, our recent preliminary data indicated that H-2Db molecules lacking the entire cytoplasmic tail induced T-cell proliferation similarly to native molecules *in vivo* (Figure 13). Taken together these results suggested that the MHC class I cytoplasmic tail can regulate T cell immune responses. The potential mechanisms by which MHC-I cytoplasmic tail may regulate such immune responses are discussed below.

3.4 Role of MHC-I cytoplasmic tail in internalization and endocytosis

This and previous studies have clearly demonstrated that MHC-I molecules lacking exon 7 induced superior CTL responses *in vitro* and *in vivo [80]* However, the potential mechanisms of action that may contribute to such effect are poorly understood. Because it has been demonstrated that internalization can affect the duration of antigen presentation by MHC-I molecules, we first hypothesized that exon 7 of MHC-I molecules may regulate internalization and that such regulation may lead to different T cell response outcomes [130]. We found that Δ7 HLA-A2 isoforms show delayed internalization rates as compared to WT-HLA-A2, suggesting that peptide/Δ7 HLA-A2 complexes are more stable at the cell surface than peptide/WT-HLA-A2 complexes. These results were consistent with previous studies demonstrating that specific mutations in the cytoplasmic tail of mouse MHC-I alleles can impair MHC-I internalization and endocytosis [59; 60; 80; 88; 138]. The results presented in this study suggested that internalization of MHC class I molecules can be

modulated by exon 7 of the MHC class I cytoplasmic tail. However, how exon 7 of the MHC-I cytoplasmic tail regulates internalization and trafficking of MHC-I molecules is not very well understood at the molecular level.

Since Δ7 MHC-I lacks two highly conserved serine residues and one being the phosphorylation site of MHC-I (Ser 335), it could be possible that serine phosphorylation in exon 7 may initiate a signal cascade that promotes MHC-I internalization [87]. However, how phosphorylation of MHC-I affect internalization at the molecular level has yet to be elucidated. Previous studies have reported that MHC-I molecules can undergo phosphorylation at the cell surface and in endosomal compartments [139]. Studies on cytoplasmic tail mutants of MHC class I molecules have suggested that phosphorylation of the cytoplasmic tail may play an important role in antigen presentation and trafficking of MHC-I molecules. For example, independent studies have demonstrated that mutations in the highly conserved tyrosine encoded by exon 6 of the MHC-I cytoplasmic tail impairs MHC-I trafficking into endosomal compartments and abrogated the ability of DCs to cross-present antigens [60; 80]. In addition, it has been suggested that phosphorylation of MHC-I molecules may tag MHC-I to undergo endocytic trafficking since inhibition of MHC-I phosphorylation by primaquine resulted in impaired recycling [139]. Moreover, it has been suggested that the cytoplasmic tail of MHC-I contains endocytic motifs that may mediate internalization possibly through interactions with different adaptor proteins, such as AP-2, ARF-6, RhoA and Rab family members which are known to regulate internalization and trafficking of different cell surface receptors by interacting with cytoskeletal components [54; 65; 70]. Therefore, it is possible that serine phosphorylation in exon 7 of the MHC-I may lead to the recruitment of AP-2 and/or ARF6 for example, and that such interactions

may allow the recruitment of other molecules perhaps clathrin, Rab proteins or different components of the cytoskeleton leading to rapid internalization and degradation [62; 71; 130; 138; 139]. It would be interesting to perform co-immunoprecipitation as well as mutagenesis experiments to elucidate whether AP-2, ARF-6 and Rab proteins can bind to exon 7 of the MHC-I cytoplasmic tail. These experiments may help to elucidate the molecular mechanisms leading to MHC-I internalization and provide a better understanding on how antigen presentation can be regulated.

However, we also found that although the reduced MHC-I DC surface internalization contributed to enhanced CTL immune responses, much of the increased stimulatory capacity by Δ7 was observed at early time points of DC-T cell interactions (1 to 3 hours), when WT MHC-I molecules had yet to undergo significant internalization. These results suggested that other cellular mechanisms such as cytoskeleton-induced polarization and clustering of MHC class I molecules towards the immune synapse may also contribute to the greater stimulatory capacity of Δ7 MHC class I molecules. In fact, it has been demonstrated that MHC-I clustering can affect the sensitivity ofT-cell recognition and that the DC cytoskeleton is required for the polarization and the formation of a functional immunological synapse but the molecular events are yet to be elucidated [140] [141; 142].

3.5 Role of the cytoplasmic tail in MHC-I clustering

To evaluate whether the cytoplasmic tail plays a role in cytoskeleton-induced polarization and clustering, we compared cell surface clustering of WT versus Δ7 MHC class I molecules in DCs following TCR ligation by confocal microscopy studies. We

found that recognition of peptides at the cell surface of APCs by cognate CD8+ T cells lead to rapid 'super clustering' of WT MHC-I molecules at the site of T-cell contact. This result supported previous studies and our data suggesting that rapid internalization of MHC-I molecules is initiated following 'super clustering' at the cell surface [141; 143]. We observed that this 'super clustering' of WT MHC class I molecules greatly limited the bio-availability of MHC-I/peptide complexes for recognition by $CD8^+$ T cells. However, unlike WT MHC class I molecules, Δ7 MHC-I molecules showed significantly impaired 'super clustering' at the site of T-cell contact, leading to increased MHC-I/peptide complex bio-availability and enabling APCs to stimulate more CD8+ T cells on a per-cell basis.

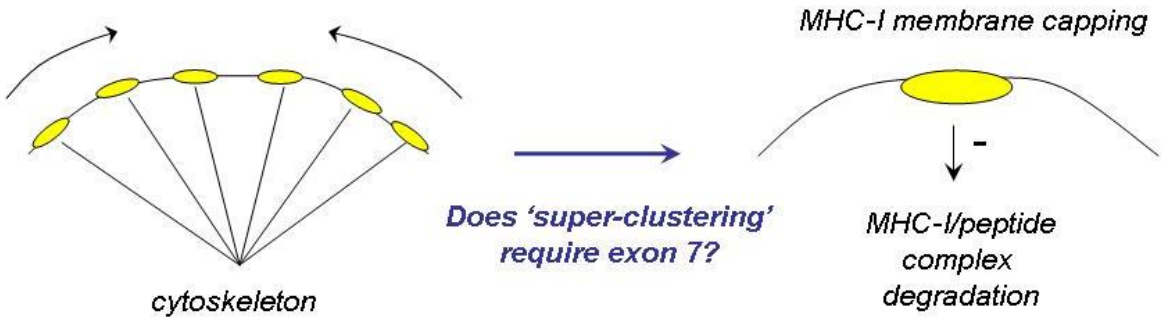
These results are consistent with previous studies demonstrating that MHC class I clustering at the cell surface of APCs modulates sensitivity of T cell recognition [141]. In addition, these results revealed that exon 7 in the cytoplasmic tail of MHC-I may regulate MHC-I clustering at the immunological synpase. However, how the cytoplasmic tail of MHC-I influences clustering at the molecular level is not well understood. Biophysical studies on lateral mobility and diffusibility have suggested that MHC-I molecules must cross the barriers imposed by the membrane lipid raft domains in the sub-membrane cytoskeleton in order to accumulate in the immunological synapse [141; 144; 145]. Studies by Damjanovich demonstrated that MHC class I clustering at the immune synapse involves dynamic homoassociations of MHC class I molecules as well as dynamic heteroassociations between MHC-I molecules and other proteins, including adhesion molecules and co-stimulartory molecules [146; 147]. Moreover, it has been demonstrated that the diffusion of MHC-I molecules in the plasma membrane is influenced by structural features of the MHC-I molecule and by the underlying cytoskeleton [148]. For example,

fluorescence photobleaching and recovery experiments on truncation mutants of MHC-I have demonstrated that the cytoplasmic tail restricts MHC-I mobility on the membrane by interacting with the cytoskeleton, which restricts the molecule's lateral diffusion in the membrane [148]. For example, studies by Capps have demonstrated that H-2Ld mutant having a cytoplasmic tail of seven amino acids was as restricted in its lateral mobility as the WT molecule with a full length of 31 amino acids. In contrast, H-2Ld mutants having a cytoplasmic tail of 4 amino acids or no cytoplasmic tail had a higher mobile fraction and a longer barrier-free path that did the WT molecules [145; 149; 150]. These studies suggested that the length and charge of the MHC class I cytoplasmic tail affects lateral diffusion. Consistent with those studies, we found that HLA-A2 molecules with full length cytoplasmic tail showed T-cell induced restricted lateral mobility in human DCs. By contrast, exon 7-deleted HLA-A2 molecules showed impaired clustering suggesting that these variant of HLA-A2 may have a longer barrier-free path. These results indicated that exon 7 of the MHC-I cytoplasmic tail may contain specific motifs that can regulate MHC-I clustering and diffusion.

Exon 7 encoded by the MHC cytoplasmic tail contains two serine phosphorylation sites and it has been suggested that phosphorylation of the cytoplasmic tail of MHC class I has a role in the interactions between class I MHC molecules and cytoskeletal structures [87]. Moreover, it has been suggested that the cytoplasmic tail of MHC class I molecules could bind proteins of the membrane barrier through electrostatic or stereospecific interactions, suggesting that specific post-translational modifications in the MHC-I cytoplasmic tail may affect MHC-I cell surface clustering and distribution [150] . Based on our data, it is possible that serine phosphorylation in exon 7 may initiate clustering of

MHC-I at the APC/T cell contact area by perhaps leading the recruitment of cytoskeletal components that mediate receptor mobility at the cell surface. In fact, it has been demonstrated that ligation with antibodies induces phosphorylation of the MHC-I cytoplasmic tail leading to rapid MHC-I clustering, which then promotes the activation of signaling molecules involved perhaps in internalization and degradation [139]. Delta 7 molecules may have showed impaired clustering perhaps due to differences in sequence charges, length of the molecule and steric forces that may affect interactions with the cytoskeleton and other membrane proteins [150]. These results suggested that serine phosphorylation of MHC-I may regulate T cell responses by inducing MHC clustering. However, the factors that may initiate MHC-I phosphorylation (i.e. TLR signals, kinases) and the potential cytoskeletal components that interact with the cytoplasmic tail of MHC-I are yet to be identified. The identification of such elements (kinases and cytoskeletal components) may provide insights on how MHC-I clustering is regulated at the molecular level. The understanding of MHC-I clustering at the molecular levels may be important for the generation of therapies that can modulate T cell responses in different clinical settings. A proposed model for the regulation of MHC-I clustering is described in Figure 12.

Figure 12: Proposed model for the regulation of MHC-I clustering. Surface MHC-I molecules found in lipid raft domains (small yellow circles) may become phosphorylated in exon 7 resulting in the recruitment of cytoskeletal components (i.e. actin filaments) to the cytoplasmic tail of MHC-I. Such interactions may results in plasma membrane mobility (arrows show mobility direction) and polarization of MHC-I towards specific membrane domains through the cytoskeleton. Once 'super clustering' of MHC-I (big yellow circle) occurs, it delivers a signal for MHC-I/peptide complex internalization and degradation. Exon 7-deleted MHC-I molecules do not show 'super clustering' and therefore they do not undergo rapid internalization and degradation.


MHC-I membrane capping
cytoskeleton
Does 'super-clustering' require exon 7?
MHC-I/peptide complex degradation

3.6 The role of the MHC class I cytoplasmic tail in signal transduction

Based on the results presented in this dissertation, we first hypothesized that deletion of exon 7 in MHC-I molecules may have significantly enhanced T cell priming by impairing MHC-I internalization, lateral movement and clustering and that such defects resulted in significantly increased bioavailability of MHC-I/peptide complexes for cognate CD8+ T cells. However, more recent preliminary results from our laboratory have suggested that this hypothesis may not fully explain how the cytoplasmic tail of MHC-I influences T cell activation. Specifically, we found that MHC-I mutants lacking the entire cytoplasmic tail showed similar internalization defects and cell surface distribution as Δ7 MHC-I molecules but, however, such Tail-less molecules did not affect the DC-induced T-cell proliferation *invivo*. Instead, DC expressing Tail-less molecules showed similar stimulatory capacity as DCs expressing WT MHC class I molecules (Figure 13). Therefore, increased bioavailability of MHC-I at the cell surface may not be the only explanation for why Δ7 MHC-I molecules enhanced T cell priming. Nevertheless, these data suggested that the cytoplasmic tail of MHC class I molecules contain domains that may differentially regulate T cell responses perhaps by providing positive and negative signals to T cells or even to the DCs. Based on these data, it is tempting to speculate that exon 6 contain motifs that may play a role as a *positive* regulators of CTL immune responses whereas exon 7

contain motifs that may play a role as negative regulators of CTL responses and that such regulation by these domains could maintain homeostatic T cells responses. Because exon 6 contains a highly conserved tyrosine residue and exon 7 contains two highly conserved serine residues, it is possible that phospshorylation of these residues may differentially influence signaling perhaps in both T cells and APCs leading to different immune outcomes (see below). We are currently generating cytoplasmic tail mutants lacking exon 6 in order to test whether this portion of the MHC-I cytoplasmic tail plays a positive regulatory role in T-cell responses. If our hypothesis is correct then we would expect that exon 6-deleted MHC-I molecules will not induce T cell responses while exon 7-deleted MHC-I molecules will increase T cell responses as compared to WT MHC-I after challenge.

One possible mechanism could be that specific motifs encoded by the cytoplasmic tail of MHC class I may differentially regulate signal transduction pathways involved in cell differentiation, survival and proliferation of both DCs and T cells. In fact, MHC class I molecules have been involved in signal transduction in T cells, endothelial cells and smooth muscle cells [151; 152; 153] For example, previous studies have demonstrated that cross-linking of MHC class I on the surface of T cells activates the ZAP70 and p56 tyrosine kinases and induces ζ-chain phosphorylation leading to an increase in intracellular free calcium concentration, IL-2 production and proliferation, as well as an increase in the expression of costimulatory receptors [154]. Moreover, it has been demonstrated that cross-linking HLA class I molecules in human endothelial cells (EC) resulted in signal transduction pathways that stimulated both EC proliferation and survival. Specifically, these studies reported that engagement of MHC class I molecules in EC induces tyrosine

phosphorylation of Src family protein kinases and activation of focal adhesion kinase (FAK). Moreover, they showed that MHC-I mediated activation of FAK triggers a pro-survival signaling cascade, resulting in the activation of PI3K/Akt-signaling pathway and upregulation of anti-apoptotic proteins such as Bcl-2 and Bcl-xL [151; 153]([155; 156]. These studies suggested that Class I-mediated upregulation of anti-apoptotic proteins renders EC resistance to complement mediated lysis. Thus, it is possible that engagement of MHC class I molecules by the TCR may play an important role not only in T cell activation but also in DC survival and function. However, the role of the MHC class I cytoplasmic tail in signal transduction pathways in DCs is not well understood.

Previous studies have demonstrated that cytoplasmic tail of MHC class I molecules is constitutively phosphorylated *in vivo* on a serine residue encoded in exon 7, suggesting that such exon may be involved in signal transduction pathways [87]. However, other studies have revealed that the cytoplasmic tail of class I molecules is not involved in T cell activation since cross-linking truncated MHC class I molecules (no cytoplasmic tail) induce IL-2 production similar to that induced by the native molecules, suggesting that the cytoplasmic tail of class I MHC molecules is not involved in signaling [157]. However, our results indicated that deletion of exon 7 leads to enhanced T-cell responses suggesting that it may regulate signaling cascades. Interestingly, it has been demonstrated that cross-linking of MHC class I molecules leads to phosphorylation events which resulted in the association of MHC class I molecules with other signaling receptors including insulin receptor (IR) and GLUT-4, on the cell surface of DCs suggesting that association of MHC-I with other receptors may propagate a signaling cascade leading to perhaps increased DC activation, survival and antigen presentation [131; 158]. However, the role of the MHC

class I cytoplasmic tail in the association of MHC-I with other receptors (i.e. IR) has not been reported. It is possible that specific motifs encoded by the cytoplasmic tail of MHC-I and their phosphorylation status may regulate interactions with other receptors leading to cell survival through mechanisms that may involve steric forces. In fact, it has been demonstrated that steric forces between the cytoplasmic tail of different receptors regulate the formation of different heterodimers at the cell surface [146]. Based on this, it is possible that MHC class I molecules associate with other signaling receptors at the cell surface of DCs and that such associations may be interrupted following serine phosphorylation in exon 7 of MHC-I leading to pro-survival signaling shutdown. This could be tested by analyzing the expression levels of anti-apoptotic molecules including Bcl-2 and Bcl-xL in DCs expressing either WT or Δ7 MHC class I molecules before and after co-culture with T cells. Increasing the survival rates of DCs may be beneficial for the generation of prolonged T cell immune responses. The proposed model describing a potential role of conserved motifs in the cytoplasmic tail of MHC class I is shown in Figure 14.

Figure 13. The cytoplasmic tail of MHC-I can influence T-cell priming. Naïve Thy1.1+ CD8+T cells from Pmel mice were co-injected along with gp100 pulsed DCs expressing either WT (blue), Δ7 (black) or Tail-less (orange) variants of H-2Db into C57 mice. T cell proliferation in peripheral blood over time was analyzed as readout for T cell priming. Error bars represent SEM of three mice per group.

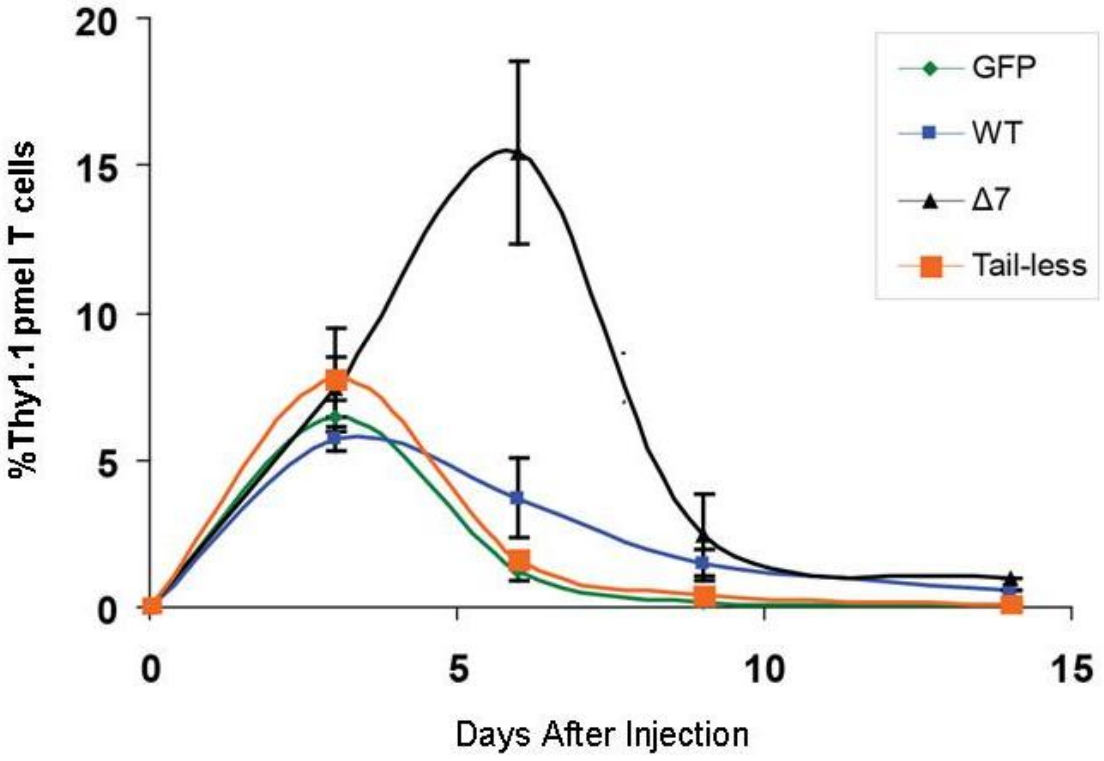
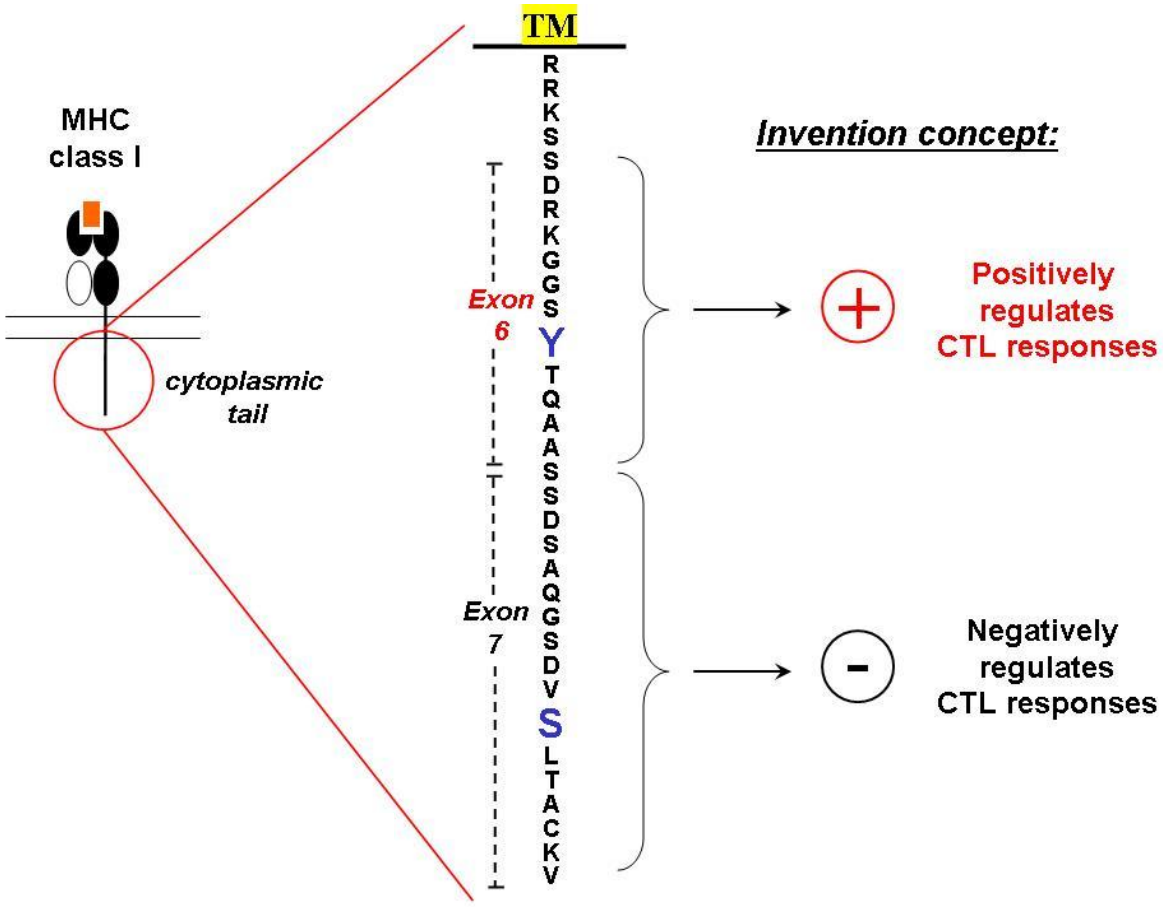

20
15
10
5
0
%Thy1.1 pmel T cells
0
5
10
15
Days After Injection
GFP
WT
Δ7
Tail-less

Figure 14. Hypothetical model of the role of conserved motifs in the cytoplasmic tail of MHC class I. The cytoplasmic tail of MHC class I molecules is composed mainly of two exons. Exon 6 (red) encodes a highly conserved tyrosine at position 320 (blue). This exon may function as a positive regulator of CTL responses following post-translational moifications (i.e. phosphorylation). Exon 7 (black) encodes a highly conserved serine motif at position 335 (blue). Based on this and other studies, we speculate that this exon may negatively regulate CTL responses following phosphorylation. TM = transmembrane domain.


TM
MHC
class I
cytoplasmic
tail
Exon
6
Exon
7
R R K S S D R K G G S Y T Q A A S S D S A Q G S D V S L T A C K V
Invention concept:
Positively
regulates
CTL responses
Negatively
regulates
CTL responses

3.7 Does the cytoplasmic tail of MHC-I affect cross-dressing?

Recent studies have demonstrated that cross-dressing is another antigen presentation mechanism by which DCs induce the activation of T cells. Cross-dressing refers to the transfer of membrane peptide/MHC class I molecules from one DCs (donor DC) to another DC (recipient DCs), which then presents the peptide to CD8+ T cells without the need of any antigen processing [159] [160]. This antigen presentation mechanism has been elegantly demonstrated by Wakim and Bevan to occur via cell-cell contact or trogocytosis and not through the secretion of exosomes [159]. These studies also showed that antigen presentation through cross-dressing of peptide/MHC-I complexes is very inefficient for the activation of naïve T cells possibly due to the presentation of vanishingly small number of peptide/MHC class I complexes. However, the mechanisms that regulate trogocytosis are not understood. Because we observed more peptide/MHC class I complexes at the cell surface of DCs expressing Δ7 MHC class I molecules as compared to DCs expressing WT MHC-I, it is possible that the cytoplasmic tail of MHC-I may regulate trogocytosis of peptide/MHC-I complexes from one DC to another DC, which then results in T cell activation. In particular, we speculate that Δ7 MHC class I molecules, lacking 13 amino acids including two potential serine phosphorylation sites, may be transferred from one DC to another more efficiently than WT MHC-I molecules maybe because such molecules are less heavy or/and contain the appropriate net charge allowing more transfer. Such increment in trogocytosis of peptide/MHC-I complexes may

allow DCs to present higher quantities of antigens to cognate T cells as well as to induce a more sustained signaling cascade leading to survival, proliferation and differentiation in both cross-dressed DCs and T cells. Also, it is possible that phoshorylation of MHC-I may affect trogocytosis, however, this need to be determined. These ideas could be tested by analyzing the transfer of WT and cytoplasmic tail mutants of MHC class I (i.e. Δ7) from one DC to another DC. For example, we could introduce the expression of either WT or Δ7 HLA-A2 fused to GFP into donor DCs and then co-culture these donor DCs with HLA-A2 negative recipient DCs to allow trogocytosis. Then the cross-dressed DCs expressing WT or Δ7 HLA-A2 could be analyzed for surface expression of transferred MHC-I and for their capacity to stimulate T cells. In addition, the capacity of transferred MHC-I molecules to stimulate signaling cascades could be also tested by analyzing the expression levels of pro-survival molecules in cross-dressed DCs as well as analyzing T cell signaling by measuring the levels of phosphorylated ERK in T cells, for example. Understanding how cross-dressing is regulated at the molecular level may provide new insights for the generation of new DC vaccines.

3.8 Pharmacological implications

Although the cellular and molecular mechanisms that regulate internalization, clustering and turnover of MHC-I/peptide complexes in DCs are mostly unknown, understanding such mechanisms is an important first step for the development of efficient therapies. In this study, we demonstrated that DCs induced to express Δ7 MHC-I enhanced T-cell mediated anti-tumor responses and significantly prolonged mouse survival. These findings suggested that similar strategies could increase the efficacy of human DC cancer vaccines developed to induce anti-viral or anti-tumor specific CTL immune responses.

However, we encountered two main limitations of using DCs vaccines engineered to expressed different MHC class I isoforms over the course of this study. The first limitation was the very often poor transduction efficiencies obtained following DCs infection with lentiviral vectors encoding different MHC class I isoforms. The second limitation was that CTL responses were restricted against one single MHC class I allele (H-2D^{b} or HLA-A*0201). Based on this, we speculated that it may be more effective to directly target exon 7-encoded determinants pharmacologically in order to counteract their negative effect on CTL priming. Such an approach may be superior because it could simultaneously improve antigen presentation by all endogenously-expressed HLA alleles.

Although the protein binding partners (i.e. kinases and phosphatases) of the MHC class I cytoplasmic tail are still unidentified, exon 7 does encode a highly conserved serine phosphorylation site (Ser-335) that may serve to regulate the trafficking and antigen presentation capacity of MHC-I molecules. Additionally, exon 6 is composed of at least two other possible motifs for protein post-translational changes. One motif is the highly-conserved putative phosphorylation site at Tyr-320, and the second motif is the highly-conserved ubiquitination site at Lys-316 [161]. If these post-translational modifications in the cytoplasmic tail influence MHC-I function *in vivo*, then we speculate that inhibitors of the kinases, phosphatases, or ubiquitin ligases that target the MHC-I tail may function as highly immunomodulatory therapies. These studies will not only address how MHC class I is regulated at the molecular level, but they might open a door toward the generation of new pharmacological drugs that may serve to manipulate CTL priming responses at the level of antigen presentation via the direct targeting of the MHC-I cytoplasmic tail.

CHAPTER 4

EXPERIMENTAL PROCEDURES

4.1 Mice and B16 melanoma

DBA/2, C57BL/6 and Thy1.1+ Pmel-1 transgenic mice were maintained in a pathogen-free facility at the MD Anderson Cancer Center. $CD8^+$ T cells expressing a TCR restricted to $H\text{-}2D^b$ molecules presenting the melanoma tumor antigen, gp100 ($gp100_{25\text{-}33}$) were isolated from the spleens of $Thy1.1^+$ Pmel-1 transgenic mice. The majority of the $CD8^+$ T cells from these transgenic mice were $VB13^+$ $Thy1.1^+$ (>95%) as measured by flow cytometry. All of the protocols conformed to MD Anderson Cancer Center guidelines for the care and use of laboratory animals. The murine melanoma cell (B16) was obtained from the National Cancer Institute tumor repository (Bethesda, MD) and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.03% L-glutamine, 100ug/ml streptomycin, 100ug/ml penicillin, and 50ug/ml gentamicin sulfate. All cell culture reagents were obtained from Invitrogen.

4.2 Human cell lines

The human DC-like cell line, KG-1, was obtained from ATCC (CCL-246™) and maintained in Iscove's Modified Dulbecco's Medium containing 20% Fetal Bovine Serum, Glutamax, 100ug/ml Penicillin and 100ug/ml streptomycin. 293METR cells were a kind gift from Brian Rabinovich (MD Anderson Cancer Center). These cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum, HEPES, Glutamax, Penicillin/Streptomycin and Normocin (all from Invitrogen). The MART-1

specific T cell line, DMF5, was kindly provided by Laura A. Johnson from the NIH. These MART-1 specific T cells were cultured in RPMI containing 10% FBS, HEPES, Glutamax, Penicillin/Streptomycin. In addition, the human recombinant IL-2 (300 IU/ml) was added every other day. Human recombinant IL-2 was obtained from ProLeukin (Novartis).

4.3 General cloning strategy for HLA-A2 and H-2D^b molecules

We utilized the Gateway system (Invitrogen) to clone different HLA-A2 and H-2D^b variants into lentiviral expression vectors. First, we designed gateway-adapted primers to amplify HLA-A*0201 and H-2D^b from pcDNA 3.1 HLA-A2 (NCBI) and pcDNA 3.1 H-2Db (James Gibbs, NIAID), respectively. The primers were designed to insert highly specific recombination sites (attB1 and attB2) at the 5' and 3' end of each molecule (see cloning strategy and primer list below). We also added EcoR1 and Cla-I restriction sites at the 5' and 3' end of the primers, respectively. The amplified PCR products flanked by attB1 and attB2 sites were then purified and cloned into the gateway entry vector, pDONR 222. pDONR 222 was used because it contains recombination sites that specifically recognize the attB1 and attB2 sites during BP recombination reactions. Following BR reactions, the products were then used for bacterial transformations and DNA from different clones was analyzed by sequencing. Validated clones were named pDONR222 HLA-A2 and pDONR222 H-2D^b. pDONR 222 HLA-A2 and pDONR222 H-2D^b entry vectors were then used to modify the cytoplasmic tail of both mouse and human MHC class I molecules. The different MHC class I variants were then transferred from the entry vector to different gateway-adapted lentiviral expression vectors via LR reactions. The

gateway-adapted lentiviral expression vectors were used because they specifically recognize the recombination sites located in pDONR 222.

Figure 15: Cloning different MHC class I molecules into Gateway-adapted entry vectors. (A) MHC class I molecules (orange) were amplified using primers containing the recombination sites attp1 and attp2 (patterned arrows). The PCR products were then cloned between the B1 and B2 sites of the gateway-adapted entry vector, pDONR 222 via BP reactions. The resulting vector was selected by kanamicyn (KanR) resistance and used for transferring the gene into gateway-adapted lentiviral expression vectors. **(B)** Recombination of pDONR 222 MHC class I (left) into a lentivirus expression vector containing a mammalian promoter (i.e. human PGK or MSCV promoter) was performed using LR reactions.

A)

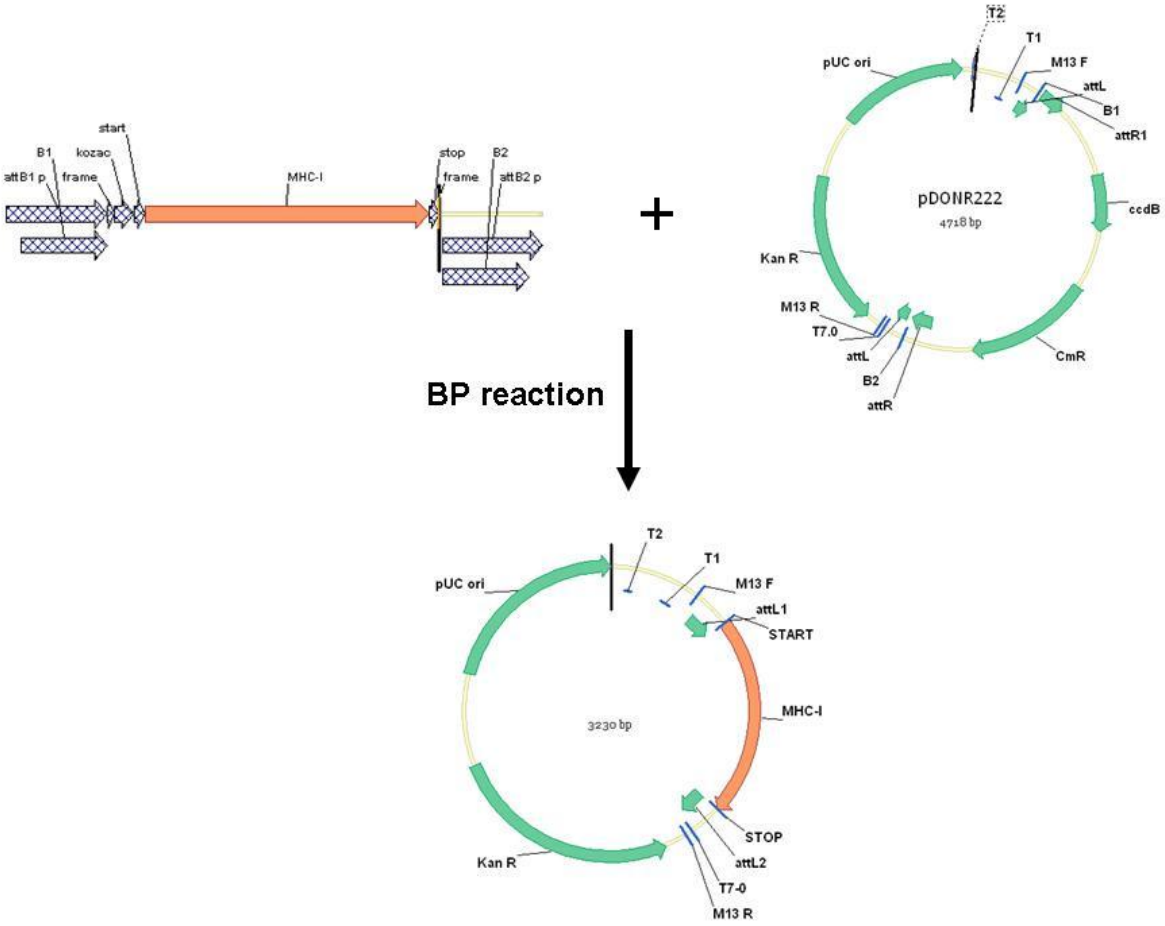
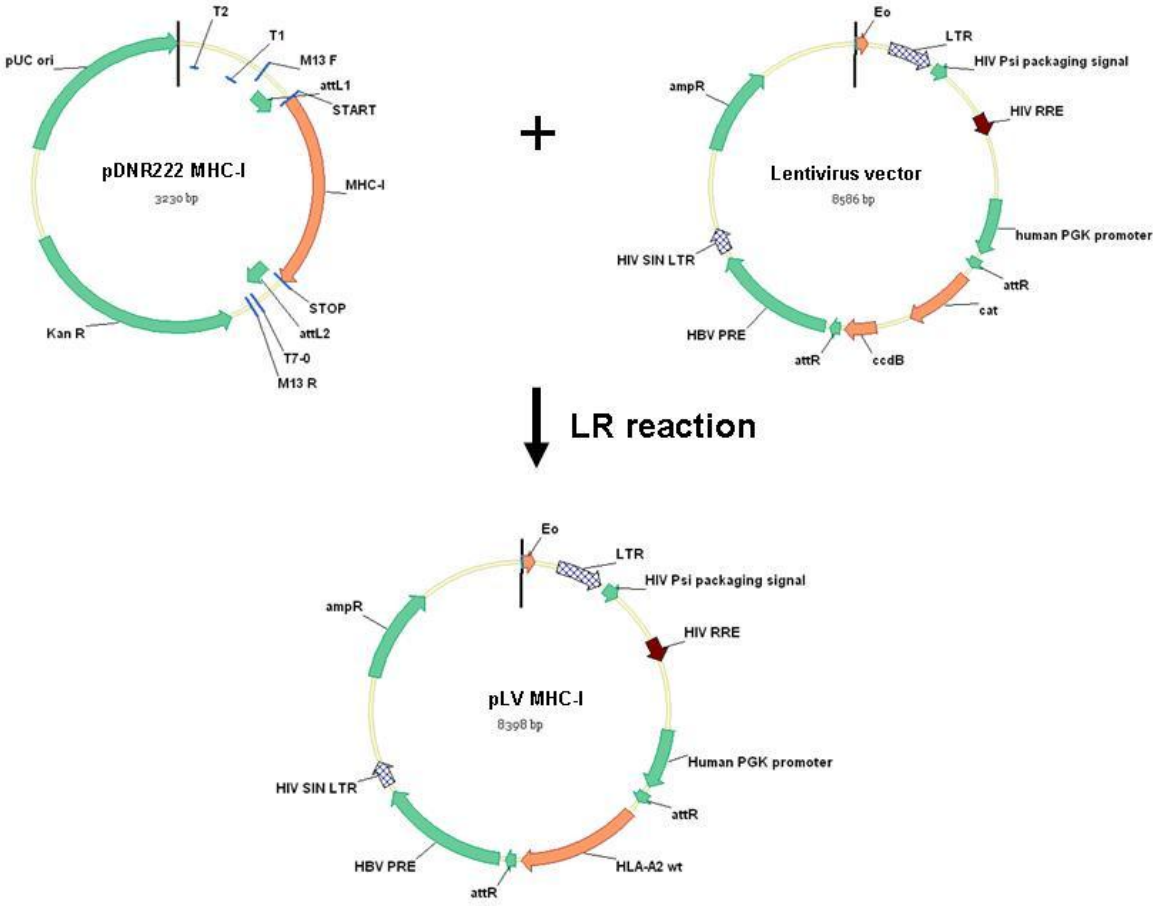

start
B1
kozac
attB1 p
frame
MHC-I
stop
B2
frame
attB2 p
+
T2
T1
pUC ori
M13 F
attL
B1
attR1
pDONR222
4718 bp
ccdB
Kan R
M13 R
T7.0
attL
B2
attR
CmR
BP reaction
T2
T1
pUC ori
M13 F
attL1
START
MHC-I
3230 bp
STOP
attL2
Kan R
T7-0
M13 R

Figure 16: Cloning different MHC class I molecules into Gateway-adapted entry vectors. (A) MHC class I molecules (orange) were amplified using primers containing the recombination sites attp1 and attp2 (patterned arrows). The PCR products were then cloned between the B1 and B2 sites of the gateway-adapted entry vector, pDONR 222 via BP reactions. The resulting vector was selected by kanamicyn (KanR) resistance and used for transferring the gene into gateway-adapted lentiviral expression vectors. **(B)** Recombination of pDONR 222 MHC class I (left) into a lentivirus expression vector containing a mammalian promoter (i.e. human PGK or MSCV promoter) was performed using LR reactions.

B)


T2
T1
pUC ori
M13 F
attL1
START
pDNR222 MHC-I
3230 bp
MHC-I
STOP
attL2
Kan R
T7-0
M13 R
+
Eo
LTR
HIV Psi packaging signal
ampR
HIV RRE
Lentivirus vector
8586 bp
human PGK promoter
HIV SIN LTR
attR
cat
HBV PRE
attR
ccdB
LR reaction
Eo
LTR
HIV Psi packaging signal
ampR
HIV RRE
pLV MHC-I
8398 bp
Human PGK promoter
HIV SIN LTR
attR
HBV PRE
HLA-A2 wt
attR

4.4 Generation of Δ7 MHC class I molecules

Mutagenesis in the cytoplasmic tail of MHC class I molecules was performed using a PCR-based site directed mutagenesis (Stratagene). To generate Δ7 MHC class I molecules**,** we performed site-directed mutagenesis PCR on pDONOR222 WT-A2 and pDONOR222 H-2D^{b} using mutagenic primers designed to specifically delete exon 7 in the cytoplasmic tail of both H-2D^{b} and HLA-A2 molecules (see primer design and primer sequences below). For example, the forward primer (1) was designed to anneal several base pairs located at the end of exon 6 and several base pairs from the start end of exon 8. A second primer (2) was designed to be the reverse complement of the forward primer. Sequences in exon 7 were not included in these primers to allow the deletion of exon 7 during the PCR. PCR products were analyzed by gel electrophoresis and used to transform bacteria. DNA from different bacterial clones was purified and sent out for sequencing analysis (MD Anderson, DNA core). Validation of clones was performed by using the alignment program of the Vector NTI software (Invitrogen). Validated clones were then transferred into a HIV-based lentivirus expression vector containing the human PGK promoter via gateway recombination LR reactions (Gateway technology from Invitrogen). The products of the LR reactions were then used for bacterial transformations to amplify the expression vector followed by generation of lentiviral virions for transductions.

Mutagenic primers for human and mouse MHC class I molecules :

1. HLA-A*0201

5’ CTCTCAGGCTGCAGTGTGAATCGATG

3’ CATCGATTCACACTGCAGCCTGAGAG

2. H-2D^{b}

5’ GCTCTGGCTCCAGCGTGAGACCCAGC

3’ GCTGGGTCTCACGCTGGAGCCAGAGC

4.5 Generation of different HLA-A2/GFP fusion proteins

To generate different HLA-A2/GFP fusion proteins, the WT and Δ7 variants of HLA-A*0201 generated in the section above were amplified by PCR. The primers used for PCR were designed to contain unique EcoR1 and ClaI sites at the 5’ end and 3’ end, respectively. The Cla-I site at the 3’ end was used to substitute the stop codon of the different HLA-A2 variants in order to allow the translation of the fusion protein. The PCR products were then confirmed by gel electrophoresis and purified using a Qiagen PCR purification kit (Invitrogen). The purified products were then cut using the EcoR1 and ClaI restriction enzymes and transferred into pENTR1A/GFP (a kind gift from Brian Rabinovich, MD Anderson) by ligations. We choose to use the pENTR1A/GFP vector for generating the fusion proteins because it has been characterized to contain a death cassette flanked by unique EcoR1 and ClaI sites at the 5’ end and 3’ end, respectively. Also because it has a linker that allows the proper folding of the fusion protein, and the green fluorescent protein (GFP), both being downstream of the death cassette. The different HLA-A2

molecules (minus stop codon) were ligated between the EcoR1 and Cla I sites of the death cassette. Following ligations, the different HLA-A2/GFP constructs were then used for bacterial transformations and the extracted DNAs were confirmed by DNA sequencing Validated plasmids were then cloned into a gateway-adapted lentivirus expression vector containing the human PGK promoter by using highly specific recombination reactions (Gateway system, Invitrogen). Primers used for PCR are listed below.

Primers:

5' EcoR1---HLA-A2 (Fwd)

GAATTCGCCACCATGGCCGTCA

3' ClaI----HLA-A2 (Rev)

ATCGATCACTTTACAAGCTGTG

4.6 Generation of lentiviral vectors

Lentiviruses were generated by transient transfection of 293METR cells using Lipofectamine 2000 (Invitrogen). Briefly, 293METR cells were transfected with plasmids encoding either WT-HLA-A2, Δ7-HLA-A2, WT-HLA-A2/GFP, Δ7-HLA-A2/GFP, WT-H-2D^b or Δ7-H-2D^b (2.3μg each), along with the packaging plasmids, pΔR8.91 (4.7μg) and CMV-pVSVG (2μg), using Lipofectamine 2000 (1.6μg/ml). pΔR8.91 encodes the structural *gag* and *pol*, and the *tat* and *rev*, which regulate viral gene expression [162]. The CMV-pVSVG encodes the VSV-G envelope protein, which enables viral entry into cells. Viral supernatants were collected at 48 and 72 hrs following transient transfection and filtered using a 0.45μm membrane (Millipore). Viral supernatants were then concentrated

by ultracentrifugation at 25,000rmp for 2 hours. Viral pellets were re-suspended at a 400X concentrate in X-vivo15 serum-free medium and stored at -80°C.

4.7 Titration of Lentiviruses

2.5 x10^4 293 METR cells were resuspended in 1 ml of DMEM medium, plated in 24-well plate and cultured overnight at 37°C. Media was then removed and 200µl of fresh DMEM was added to the cells for virus titrations. Serial dilutions (3 fold) of lentiviral vector stocks were then added to the cells along with 8 µg/ml of polybrene (Sigma), which enhances viral entry efficiency. Cells were then collected to measure transgene expression using specific antibodies and flow cytometry. An anti-HLA-A2 monoclonal antibody conjugated to alexa 488 (Serotec) was used to measure titrations of lentivirus encoding different HLA-A2 cytoplasmic tail isoforms. An anti-$H2D^b$ monoclonal antibody conjugated to alexa 647 (BD Biosciences) was used to measure titrations of lentiviruses encoding different $H2\text{-}D^b$ cytoplasmic tail isoforms. Viral vector titrations were calculated as follows: Transducing units (TU) per ml = % transgene positive cells x 50,000 cells / volume of viral vector used (in ml).

4.8 Generation of primary dendritic cells

Mouse: Bone marrow-derived murine DC progenitors were isolated from the tibia and femur of DBA/2 mice. The bone marrow was triturated using an 18 gauge needle and passed through a 70µm nylon mesh cell strainer (Becton Dickinson) to make single cell suspension in PBS. Bone marrow cells were then counted using a hemacytometer and resuspended in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and GM-CSF (50ng/ml) at a starting concentration of 5×10^5

cells/ml. Cells were incubated at 37°C for 7 days to induce the differentiation of cells into immature dendritic cells. To generate mature dendritic cells, cells were incubated for 2 additional days in the presence of LPS.

Human: $CD34^+$ cells were purified from HLA-A*0201 negative donor stem cells (a kind gift from Nina Shah, M.D. Anderson) using $CD34^+$ selection beads (Miltenyi) following the manufactures instructions. Enriched $CD34^+$ cells were incubated at 37°C for 2 weeks in complete media containing RPMI + 5% human AB serum, Flt3L (100ng/ml), TPO (100ng/ml) and stem cell factor (100ng/ml). Cells were then washed and incubated at 37°C in RPMI + 5% human AB serum. Human recombinant GM-CSF (100ng/ml) and IL-4 (100ng/ml) were also added to the media and cells were incubated for 6 additional days in order to induce the differentiation of immature DCs. To mature DCs, a combination of poly:IC (5ug/ml), IFN-α (100ng/ml) and CD40L (200ng/ml) were added to the culture for 2 additional days. All cytokines were obtained from BD Biosciences.

4.9 Transduction of dendritic cells using lentiviruses

BM-DCs were transduced on day 3 using lentiviral vectors encoding WT-D^b-IRES-GFP, Δ7-D^b-IRES-GFP, or empty-IRES-GFP. Cells were transduced by spinfection using a multiplicity of infection (MOI) of 5 in RPMI supplemented with GM-CSF (50ng/ml), and polybrene (4μg/ml). Transduced cells were then incubated for 7 additional days at 37°C in complete media supplemented with GM-CSF. Cell viability and transduction efficiencies were then analyzed by FacScan (BD Biosciences). The human HLA-A*0201 negative KG-1 cells [163], and $CD34^+$-derived DCs were transduced with lentiviral vectors encoding either WT-HLA-A*0201, Δ7-HLA-A*0201, WT-HLA-

A*0201/GFP fusion protein or the Δ7-HLA-A*0201/GFP fusion protein in XVivo-15 serum free media (Cambrex). A MOI of 5 was used to transduce human cells. Polybrene was also added to transduction conditions. Cells were then incubated for 5 days in the appropriate culture medium, and transduction efficiencies were measured by flow cytometry. KG-1 cells expressing comparable levels of cell surface WT-A2 and Δ7-A2 were purified by cell sorting. To ensure that the same number and percentage of WT- and Δ7-MHC-I expressing cells were used for all functional experiments, we first normalized transduced primary $CD34^+$-derived DCs for HLA-A2 expression.

4.10 T-cell cytokine production: ELISA, Luminex and Intracellular staining

Mouse T-cell cytokines*:* Mouse BM-DCs expressing either WT or Δ7 variants of $H\text{-}2D^b$ were pulsed with titrated concentrations of hgp100 (25-33) peptide, and co-cultured with Pmel-1 T cells at a 1:10 ratio at 37°C for 18hrs. Culture supernatants were collected and T-cell cytokine production was measured by mouse ELISA (Endogen) and Luminex (Millipore). The IFN-γ ELISA method was performed according to the manufacturers instructions after a 1:10 dilution of the culture supernatants. Seven standard curve points were used: 2,500, 1000, 400, 160, 64, 25.6, and 0pg/ml. The samples were added in triplicates of 50ul to each well. The plates were developed using TMB substrate for 15 minutes and the reactions stopped by adding 100µl of Stop Solution to each well. The plates were read in a 96-well ELISA microplate reader (Tecan Group Ltd) using Megallan V.400 software with a 450nm filter. The standard curve was generated by plotting the average absorbance obtained for each standard concentration on the Y axis versus the corresponding IFN-γ concentration (pg/ml) on the X axis. This standard curve was then

used to determine the amount of IFN-γ in an unknown sample. The concentration of IFN-γ in each sample was determined by multiplying the interpolated value obtained from the standard curve by the dilution factor. The Luminex method was performed similarly to the ELISA method but according to the manufacture's instructions.

Human T-cell cytokines: Transduced KG-1 or $CD34^+$-derived DCs were pulsed with titrated amounts of MART-1 or FluM1 peptides and co-cultured with MART-1 or Flu-specific T cells at a 1:10 ratio for 18h at 37°C. IFN-γ production was measured in culture supernatants collected 18hrs following stimulation of antigen-specific T cells by peptide-pulsed dendritic cells expressing either WT/HLA-A2 or Δ7/HLA-A2 molecules using human IFN-γ ELISA kit (Endogen) and Luminex. The human ELISA and Luminex methods were performed as described above.

Intracellular cytokine staining: Antigen-specific T cells were co-cultured with transduced DCs for 4h in the presence of GolgiStop (BD Biosciences), washed, fixed, permeabilized, and stained using anti-mouse or anti-human IFN-γ conjugated to FITC (BD Biosciences). Mouse T cells were also stained with a fluorescently labeled CD107a specific antibody (BD Biosciences) to measure degranulation. Human antigen-specific T cells were also stained with MART-1 or FluM1 tetramers (Baylor College of Medicine) and a fluorescently labeled anti-human CD8 antibody (BD Biosciences). Antigen-specific intracellular IFN-γ production by $CD8^+$ T cells was then determined by flow cytometric analysis.

4.11 CFSE labeling

1μM of CFSE (Invitrogen) was used for labeling T cells to track their proliferation. CFSE has a fluorescence exiting/emission profile similar to FITC. The proliferating cells

can dilute CFSE and generate proliferation curves following stimulation experiments. T cells were first washed in PBS and resuspended in PBS containing CFSE. Labeling was performed in room temperature for 5 minutes followed by three times of wash using culture medium. Stimulation was next set up as described earlier and CFSE dilution was measured by flow cytometry using the FL-1 channel.

4.12 T-cell proliferation

Mouse studies: Naïve T cells isolated from the spleen of Pmel-1 mice were labeled with CFSE (Sigma) and co-cultured *invitro* with D^b-transduced DCs that had been pulsed with titrated amounts of hgp100 peptide (0.1, 1 and 10 nM) at 37°C for 48, 72 and 96 hours. Cells were collected and stained using a fluorescently labeled anti-Thy.1.1 antibody (eBioscience) and anti-mouse CD8 antibody conjugated to alexa 647 to label Pmel-1 CD8+T cells. Proliferation of gated Thy.1.1 CD8+ Pmel T cells was assessed by flow cytometry over time. At least 10,000 cells were analyzed in each sample.

Human studies: HLA-A2-positive PBMCs from normal donors were obtained from Gulf Coast Blood Center (Houston, TX). Transduced KG-1 cells were pulsed with different amounts of peptides for 2hr at room temperature. The HLA-A*0201-restricted peptides used in the experiments were MART-1 (26-35,27L: ELAGIGILTV), influenza matrix 1 protein (58-66: GILGFVFTL), or CMVpp65 (495-503: NLVPMVATV). All peptides were obtained from Beckman Coulter. Following peptide pulsing, DCs were irradiated with 20,000 rads, and co-cultured with antigen-specific human T cells at a ratio of 1:10 at 37°C for 8 days. Cells were then collected and analyzed by flow cytometry to determine the number of tetramer positive cells in the $CD8^+$ T-cell population. The

function of the expanded antigen-specific T cells was analyzed by measuring intracellular IFN-γ staining and CD107a in response to antigen presentation by T2 cells.

4.13 Adoptive T-cell transfer, DC vaccination and Tumor treatment

To induce tumor growth, 5×10^5 B16 melanoma cells were injected subcutaneously into C57BL/6 mice (8-10 weeks old). On day 7 after tumor injection, tumor-bearing mice were subjected to irradiation (350 rad). Following irradiation, 5×10^6 T-cells from Pmel-1 mice were mixed with 1×10^6 transduced DCs pulsed with 300 nM of $hgp100_{25–33}$ peptide and adoptively transferred into irradiated tumor bearing mice for tumor treatment. IL-2 (6×10^5 units twice daily) was injected intraperitoneally for 3 consecutive days to support T-cell proliferation. Tumor size was measured every day following treatment using calipers, and the products of perpendicular diameters were recorded. On day 7 and 21 following DC vaccination, the percentages of peripheral blood Thy1.1+ Pmel-1 cells were analyzed by flow cytometry. Mice were sacrificed when tumors exceeded 15 mm in diameter or became ulcerated or mice became moribund. All *invivo* experiments were carried out in a blinded, randomized fashion and performed three times.

4.14 Internalization of cell surface HLA-A2 in human dendritic cells

KG-1 cells induced to express either WT or Δ7 variants of HLA-A2 were stained on ice for 30 minutes by using a monoclonal HLA-A2 specific antibody conjugated to alexa 888 (Serotec). Transduced KG-1 were washed with ice-cold PBS and incubated at 37°C or 4°C for 90 minutes. Cells were then fixed with 2% parafomaldehyde (PFA) and

plated onto poly-L-lysine coated coverslips. The remaining fluorescence at the cell surface following incubation at 37°C was analyzed by confocal microscopy (Leica).

4.15 APC/T-cell conjugates

KG-1 cells induced to express WT-A2/GFP or Δ7-A2/GFP fusion proteins were pulsed with MART-1 peptide (20nM) for 2 hrs at room temperature. Cells were then co-incubated with MART-1 specific T cells at a 1:1 ratio, plated in 48 well plates for 2hrs in order to visualize KG-1/T-cell cluster formation using light microscopy. The mixture of KG-1 cells and MART-1 specific T cells was also plated onto poly-L-lysine-coated coverslips and incubated at 37°C for up to 2hrs. The mixture of cells were then fixed using 2% PFA (Fisher) and stained with anti-CD3-ζ (6B10.2, BD Biosciences) mAb conjugated to alexa 647 on ice in order to label MART-1 specific T cells. KG-1/MART-1 T-cell conjugates were evaluated by confocal microscopy using a 60X oil objective. Z-stacks of with 20 planes and 2μM spacing between each plane were collected for each image. KG-1/MART-1 T-cell conjugates were also quantified by visually counting numbers of MART-1 specific T cells conjugated to a random collection of 20 to 30 transduced KG-1 cells. Conjugates were scored only if MART-1 specific T cells were in contact with one KG-1 cell expressing either WT or Δ7 variants of HLA-A2 fused to GFP.

4.16 Statistical analysis

Microsoft Excel was used for graphing and statistical analysis. The Kaplan-Meier test was used to compare mouse survival between treatment and control groups. A Student

T test was used to analyze the statistical significance of the results. A p-value of less or equal to 0.05 was the cut-off to determine significance of the statistics.

CHAPTER 5-BIOBIOLGRAFY

[1] B. Korber, M. Muldoon, J. Theiler, F. Gao, R. Gupta, A. Lapedes, B.H. Hahn, S. Wolinsky, and T. Bhattacharya, Timing the ancestor of the HIV-1 pandemic strains. Science 288 (2000) 1789-96.

[2] H. Chapel, R. Geha, and F. Rosen, Primary immunodeficiency diseases: an update. Clin Exp Immunol 132 (2003) 9-15.

[3] G.P. Dunn, A.T. Bruce, H. Ikeda, L.J. Old, and R.D. Schreiber, Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 3 (2002) 991-8.

[4] L. Zitvogel, A. Tesniere, and G. Kroemer, Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 6 (2006) 715-27.

[5] J.E. Pease, Asthma, allergy and chemokines. Curr Drug Targets 7 (2006) 3-12.

[6] J.E. Murray, Human organ transplantation: background and consequences. Science 256 (1992) 1411-6.

[7] W. Cookson, The immunogenetics of asthma and eczema: a new focus on the epithelium. Nat Rev Immunol 4 (2004) 978-88.

[8] E.A. Green, and R.A. Flavell, The initiation of autoimmune diabetes. Curr Opin Immunol 11 (1999) 663-9.

[9] P.J. Delves, and I.M. Roitt, The immune system. First of two parts. N Engl J Med 343 (2000) 37-49.

[10] P.J. Delves, and I.M. Roitt, The immune system. Second of two parts. N Engl J Med 343 (2000) 108-17.

[11] R.N. Germain, MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 76 (1994) 287-99.

[12] S.Y. Tseng, and M.L. Dustin, T-cell activation: a multidimensional signaling network. Curr Opin Cell Biol 14 (2002) 575-80.

[13] T.K. Starr, S.C. Jameson, and K.A. Hogquist, Positive and negative selection of T cells. Annu Rev Immunol 21 (2003) 139-76.

[14] J.D. Stone, A.S. Chervin, and D.M. Kranz, T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. Immunology 126 (2009) 165-76.

[15] Y. Yin, Y. Li, M.C. Kerzic, R. Martin, and R.A. Mariuzza, Structure of a TCR with high affinity for self-antigen reveals basis for escape from negative selection. EMBO J 30 1137-48.

[16] S.O. Andreasen, J.E. Christensen, O. Marker, and A.R. Thomsen, Role of CD40 ligand and CD28 in induction and maintenance of antiviral CD8+ effector T cell responses. J Immunol 164 (2000) 3689-97.

[17] Y. Sykulev, M. Joo, I. Vturina, T.J. Tsomides, and H.N. Eisen, Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. Immunity 4 (1996) 565-71.

[18] S. Valitutti, and A. Lanzavecchia, Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. Immunol Today 18 (1997) 299-304.

[19] S. Valitutti, S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia, Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature 375 (1995) 148-51.

[20] T.W. McKeithan, Kinetic proofreading in T-cell receptor signal transduction. Proc Natl Acad Sci U S A 92 (1995) 5042-6.

[21] A.M. Kalergis, N. Boucheron, M.A. Doucey, E. Palmieri, E.C. Goyarts, Z. Vegh, I.F. Luescher, and S.G. Nathenson, Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. Nat Immunol 2 (2001) 229-34.

[22] F. Denizot, A. Wilson, F. Battye, G. Berke, and K. Shortman, Clonal expansion of T cells: a cytotoxic T-cell response in vivo that involves precursor cell proliferation. Proc Natl Acad Sci U S A 83 (1986) 6089-92.

[23] C.K. Baumgartner, and L.P. Malherbe, Antigen-driven T-cell repertoire selection during adaptive immune responses. Immunol Cell Biol 89 54-9.

[24] V. Shankaran, H. Ikeda, A.T. Bruce, J.M. White, P.E. Swanson, L.J. Old, and R.D. Schreiber, IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 410 (2001) 1107-11.

[25] C. Prezzi, M.A. Casciaro, V. Francavilla, E. Schiaffella, L. Finocchi, L.V. Chircu, G. Bruno, A. Sette, S. Abrignani, and V. Barnaba, Virus-specific CD8(+) T cells with type 1 or type 2 cytokine profile are related to different disease activity in chronic hepatitis C virus infection. Eur J Immunol 31 (2001) 894-906.

[26] C.M. Trambas, and G.M. Griffiths, Delivering the kiss of death. Nat Immunol 4 (2003) 399-403.

[27] M.E. Pipkin, and J. Lieberman, Delivering the kiss of death: progress on understanding how perforin works. Curr Opin Immunol 19 (2007) 301-8.

[28] L.E. Broker, F.A. Kruyt, and G. Giaccone, Cell death independent of caspases: a review. Clin Cancer Res 11 (2005) 3155-62.

[29] R. Greil, G. Anether, K. Johrer, and I. Tinhofer, Tracking death dealing by Fas and TRAIL in lymphatic neoplastic disorders: pathways, targets, and therapeutic tools. J Leukoc Biol 74 (2003) 311-30.

[30] R.M. Steinman, and I. Mellman, Immunotherapy: bewitched, bothered, and bewildered no more. Science 305 (2004) 197-200.

[31] S.A. Rosenberg, and M.E. Dudley, Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol 21 (2009) 233-40.

[32] S.A. Rosenberg, N.P. Restifo, J.C. Yang, R.A. Morgan, and M.E. Dudley, Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer 8 (2008) 299-308.

[33] J. Kuball, B. Hauptrock, V. Malina, E. Antunes, R.H. Voss, M. Wolfl, R. Strong, M. Theobald, and P.D. Greenberg, Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. J Exp Med 206 (2009) 463-75.

[34] C. Wrzesinski, C.M. Paulos, A. Kaiser, P. Muranski, D.C. Palmer, L. Gattinoni, Z. Yu, S.A. Rosenberg, and N.P. Restifo, Increased intensity lymphodepletion enhances tumor treatment efficacy of adoptively transferred tumor-specific T cells. J Immunother 33 1-7.

[35] K. Palucka, J. Banchereau, and I. Mellman, Designing vaccines based on biology of human dendritic cell subsets. Immunity 33 464-78.

[36] Y. Lou, G. Wang, G. Lizee, G.J. Kim, S.E. Finkelstein, C. Feng, N.P. Restifo, and P. Hwu, Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells in vivo. Cancer Res 64 (2004) 6783-90.

[37] D.M. Andrews, E. Maraskovsky, and M.J. Smyth, Cancer vaccines for established cancer: how to make them better? Immunol Rev 222 (2008) 242-55.

[38] R.M. Steinman, Dendritic cells and the control of immunity: enhancing the efficiency of antigen presentation. Mt Sinai J Med 68 (2001) 160-6.

[39] Y.J. Liu, Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 106 (2001) 259-62.

[40] T. De Smedt, E. Butz, J. Smith, R. Maldonado-Lopez, B. Pajak, M. Moser, and C. Maliszewski, CD8alpha(-) and CD8alpha(+) subclasses of dendritic cells undergo phenotypic and functional maturation in vitro and in vivo. J Leukoc Biol 69 (2001) 951-8.

[41] J.M. den Haan, and M.J. Bevan, Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(-) dendritic cells in vivo. J Exp Med 196 (2002) 817-27.

[42] M. Gilliet, W. Cao, and Y.J. Liu, Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 8 (2008) 594-606.

[43] K. Palucka, H. Ueno, J. Fay, and J. Banchereau, Harnessing dendritic cells to generate cancer vaccines. Ann N Y Acad Sci 1174 (2009) 88-98.

[44] J.W. Fay, A.K. Palucka, S. Paczesny, M. Dhodapkar, D.A. Johnston, S. Burkeholder, H. Ueno, and J. Banchereau, Long-term outcomes in patients with metastatic melanoma vaccinated with melanoma peptide-pulsed CD34(+) progenitor-derived dendritic cells. Cancer Immunol Immunother 55 (2006) 1209-18.

[45] J. Banchereau, and R.M. Steinman, Dendritic cells and the control of immunity. Nature 392 (1998) 245-52.

[46] R. Medzhitov, Toll-like receptors and innate immunity. Nat Rev Immunol 1 (2001) 135-45.

[47] M. Montoya, G. Schiavoni, F. Mattei, I. Gresser, F. Belardelli, P. Borrow, and D.F. Tough, Type I interferons produced by dendritic cells promote their phenotypic and functional activation. Blood 99 (2002) 3263-71.

[48] P. Guermonprez, J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena, Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol 20 (2002) 621-67.

[49] P. Cresswell, A.L. Ackerman, A. Giodini, D.R. Peaper, and P.A. Wearsch, Mechanisms of MHC class I-restricted antigen processing and cross-presentation. Immunol Rev 207 (2005) 145-57.

[50] J. Albring, J.O. Koopmann, G.J. Hammerling, and F. Momburg, Retrotranslocation of MHC class I heavy chain from the endoplasmic reticulum to the cytosol is dependent on ATP supply to the ER lumen. Mol Immunol 40 (2004) 733-41.

[51] U.M. Abdel Motal, C.L. Sentman, X. Zhou, P.J. Robinson, J. Dahmen, and M. Jondal, Glycosylphosphatidylinositol-linked Db does not induce an influenza-specific cytotoxic T lymphocyte response or recycle membrane-bound peptides. Eur J Immunol 25 (1995) 1121-4.

[52] A.L. Ackerman, C. Kyritsis, R. Tampe, and P. Cresswell, Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. Proc Natl Acad Sci U S A 100 (2003) 12889-94.

[53] G. Lizee, G. Basha, and W.A. Jefferies, Tails of wonder: endocytic-sorting motifs key for exogenous antigen presentation. Trends Immunol 26 (2005) 141-9.

[54] A. Hall, and C.D. Nobes, Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos Trans R Soc Lond B Biol Sci 355 (2000) 965-70.

[55] T. Di Pucchio, B. Chatterjee, A. Smed-Sorensen, S. Clayton, A. Palazzo, M. Montes, Y. Xue, I. Mellman, J. Banchereau, and J.E. Connolly, Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. Nat Immunol 9 (2008) 551-7.

[56] S. Burgdorf, C. Scholz, A. Kautz, R. Tampe, and C. Kurts, Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. Nat Immunol 9 (2008) 558-66.

[57] J.D. Dasgupta, S. Watkins, H. Slayter, and E.J. Yunis, Receptor-like nature of class I HLA: endocytosis via coated pits. J Immunol 141 (1988) 2577-80.

[58] H. Radhakrishna, and J.G. Donaldson, ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. J Cell Biol 139 (1997) 49-61.

[59] M.A. Vega, and J.L. Strominger, Constitutive endocytosis of HLA class I antigens requires a specific portion of the intracytoplasmic tail that shares structural features with other endocytosed molecules. Proc Natl Acad Sci U S A 86 (1989) 2688-92.

[60] S.G. Santos, A.N. Antoniou, P. Sampaio, S.J. Powis, and F.A. Arosa, Lack of tyrosine 320 impairs spontaneous endocytosis and enhances release of HLA-B27 molecules. J Immunol 176 (2006) 2942-9.

[61] D.J. Owen, and P.R. Evans, A structural explanation for the recognition of tyrosine-based endocytotic signals. Science 282 (1998) 1327-32.

[62] A. Sorkin, Cargo recognition during clathrin-mediated endocytosis: a team effort. Curr Opin Cell Biol 16 (2004) 392-9.

[63] G.J. Doherty, and H.T. McMahon, Mechanisms of endocytosis. Annu Rev Biochem 78 (2009) 857-902.

[64] G.J. Praefcke, and H.T. McMahon, The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol 5 (2004) 133-47.

[65] A. Simonsen, R. Lippe, S. Christoforidis, J.M. Gaullier, A. Brech, J. Callaghan, B.H. Toh, C. Murphy, M. Zerial, and H. Stenmark, EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature 394 (1998) 494-8.

[66] B.D. Grant, and J.G. Donaldson, Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol 10 (2009) 597-608.

[67] M. Deneka, M. Neeft, I. Popa, M. van Oort, H. Sprong, V. Oorschot, J. Klumperman, P. Schu, and P. van der Sluijs, Rabaptin-5alpha/rabaptin-4 serves as a linker between rab4 and gamma(1)-adaptin in membrane recycling from endosomes. EMBO J 22 (2003) 2645-57.

[68] A.M. Powelka, J. Sun, J. Li, M. Gao, L.M. Shaw, A. Sonnenberg, and V.W. Hsu, Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. Traffic 5 (2004) 20-36.

[69] H. Radhakrishna, R.D. Klausner, and J.G. Donaldson, Aluminum fluoride stimulates surface protrusions in cells overexpressing the ARF6 GTPase. J Cell Biol 134 (1996) 935-47.

[70] R. Weigert, A.C. Yeung, J. Li, and J.G. Donaldson, Rab22a regulates the recycling of membrane proteins internalized independently of clathrin. Mol Biol Cell 15 (2004) 3758-70.

[71] J.G. Donaldson, Arf6 and its role in cytoskeletal modulation. Methods Mol Biol 189 (2002) 191-8.

[72] J.G. Donaldson, and H. Radhakrishna, Expression and properties of ADP-ribosylation factor (ARF6) in endocytic pathways. Methods Enzymol 329 (2001) 247-56.

[73] J. Luo, J. Li, R.L. Chen, L. Nie, J. Huang, Z.W. Liu, L. Luo, and X.J. Yan, Autologus dendritic cell vaccine for chronic hepatitis B carriers: a pilot, open label, clinical trial in human volunteers. Vaccine 28 2497-504.

[74] S.G. Marsh, Nomenclature for factors of the HLA system, update July 2005. Hum Immunol 66 (2005) 1183-6.

[75] K. Kuroki, and K. Maenaka, Immune modulation of HLA-G dimer in maternal-fetal interface. Eur J Immunol 37 (2007) 1727-9.

[76] P.J. Bjorkman, M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley, Structure of the human class I histocompatibility antigen, HLA-A2. Nature 329 (1987) 506-12.

[77] L.C. Wu, D.S. Tuot, D.S. Lyons, K.C. Garcia, and M.M. Davis, Two-step binding mechanism for T-cell receptor recognition of peptide MHC. Nature 418 (2002) 552-6.

[78] J.S. Haurum, L. Tan, G. Arsequell, P. Frodsham, A.C. Lellouch, P.A. Moss, R.A. Dwek, A.J. McMichael, and T. Elliott, Peptide anchor residue glycosylation: effect

on class I major histocompatibility complex binding and cytotoxic T lymphocyte recognition. Eur J Immunol 25 (1995) 3270-6.

[79] R. Gavioli, Q.J. Zhang, M. Marastoni, R. Guerrini, E. Reali, R. Tomatis, M.G. Masucci, and S. Traniello, Effect of anchor residue modifications on the stability of HLA-A11/peptide complexes. Biochem Biophys Res Commun 206 (1995) 8-14.

[80] G. Lizee, G. Basha, J. Tiong, J.P. Julien, M. Tian, K.E. Biron, and W.A. Jefferies, Control of dendritic cell cross-presentation by the major histocompatibility complex class I cytoplasmic domain. Nat Immunol 4 (2003) 1065-73.

[81] M. Gromme, F.G. Uytdehaag, H. Janssen, J. Calafat, R.S. van Binnendijk, M.J. Kenter, A. Tulp, D. Verwoerd, and J. Neefjes, Recycling MHC class I molecules and endosomal peptide loading. Proc Natl Acad Sci U S A 96 (1999) 10326-31.

[82] T.G. Rodriguez-Cruz, S. Liu, J.S. Khalili, M. Whittington, M. Zhang, W. Overwijk, and G. Lizee, Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity. PLoS One 6 e22939.

[83] C. Murre, C.S. Reiss, C. Bernabeu, L.B. Chen, S.J. Burakoff, and J.G. Seidman, Construction, expression and recognition of an H-2 molecule lacking its carboxyl terminus. Nature 307 (1984) 432-6.

[84] S.A. Ellis, K.A. Braem, and W.I. Morrison, Transmembrane and cytoplasmic domain sequences demonstrate at least two expressed bovine MHC class I loci. Immunogenetics 37 (1992) 49-56.

[85] J. McCluskey, L.F. Boyd, W.L. Maloy, J.E. Coligan, and D.H. Margulies, Alternative processing of H-2Dd pre-mRNAs results in membrane expression of differentially phosphorylated protein products. EMBO J 5 (1986) 2477-83.

[86] B.C. Guild, and J.L. Strominger, Human and murine class I MHC antigens share conserved serine 335, the site of HLA phosphorylation in vivo. J Biol Chem 259 (1984) 9235-40.

[87] J.S. Pober, B.C. Guild, and J.L. Strominger, Phosphorylation in vivo and in vitro of human histocompatibility antigens (HLA-A and HLA-B) in the carboxy-terminal intracellular domain. Proc Natl Acad Sci U S A 75 (1978) 6002-6.

[88] G. Basha, G. Lizee, A.T. Reinicke, R.P. Seipp, K.D. Omilusik, and W.A. Jefferies, MHC class I endosomal and lysosomal trafficking coincides with exogenous antigen loading in dendritic cells. PLoS One 3 (2008) e3247.

[89] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, and D. Forman, Global cancer statistics. CA Cancer J Clin 61 69-90.

[90] R. Taby, and J.P. Issa, Cancer epigenetics. CA Cancer J Clin 60 376-92.

[91] K.G. Lewis, and M.A. Weinstock, Trends in nonmelanoma skin cancer mortality rates in the United States, 1969 through 2000. J Invest Dermatol 127 (2007) 2323-7.

[92] P.M. Howell, Jr., S. Liu, S. Ren, C. Behlen, O. Fodstad, and A.I. Riker, Epigenetics in human melanoma. Cancer Control 16 (2009) 200-18.

[93] J.M. Thomas, D.C. Strauss, and A.J. Hayes, Some of the 2009 American Joint Committee on Cancer melanoma staging and classification is not evidence based. J Clin Oncol 28 e479; author reply e480-1.

[94] N. Agar, and A.R. Young, Melanogenesis: a photoprotective response to DNA damage? Mutat Res 571 (2005) 121-32.

[95] K. Kido, H. Sumimoto, S. Asada, S.M. Okada, T. Yaguchi, N. Kawamura, M. Miyagishi, T. Saida, and Y. Kawakami, Simultaneous suppression of MITF and BRAF V600E enhanced inhibition of melanoma cell proliferation. Cancer Sci 100 (2009) 1863-9.

[96] S.S. Legha, Treating metastatic melanoma: further considerations. Oncology (Williston Park) 23 (2009) 500, 508.

[97] P. Arenberger, M. Arenbergerova, and T. Vedral, [Malignant melanoma: standards and innovation in diagnostics and therapy]. Cas Lek Cesk 148 (2009) 151-7.

[98] H.A. Tawbi, and S.C. Buch, Chemotherapy resistance abrogation in metastatic melanoma. Clin Adv Hematol Oncol 8 259-66.

[99] W.B. Coley, The Treatment of Inoperable Sarcoma by Bacterial Toxins (the Mixed Toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). Proc R Soc Med 3 (1910) 1-48.

[100] S.A. Rosenberg, J.C. Yang, and N.P. Restifo, Cancer immunotherapy: moving beyond current vaccines. Nat Med 10 (2004) 909-15.

[101] G. Lizee, L.G. Radvanyi, W.W. Overwijk, and P. Hwu, Improving antitumor immune responses by circumventing immunoregulatory cells and mechanisms. Clin Cancer Res 12 (2006) 4794-803.

[102] G. Lizee, M.A. Cantu, and P. Hwu, Less yin, more yang: confronting the barriers to cancer immunotherapy. Clin Cancer Res 13 (2007) 5250-5.

[103] C. Blank, I. Brown, A.C. Peterson, M. Spiotto, Y. Iwai, T. Honjo, and T.F. Gajewski, PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. Cancer Res 64 (2004) 1140-5.

[104] G.J. Freeman, A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, and T. Honjo, Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192 (2000) 1027-34.

[105] Y. Iwai, M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, and N. Minato, Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci U S A 99 (2002) 12293-7.

[106] I. Melero, S. Hervas-Stubbs, M. Glennie, D.M. Pardoll, and L. Chen, Immunostimulatory monoclonal antibodies for cancer therapy. Nat Rev Cancer 7 (2007) 95-106.

[107] D.G. Maloney, A.J. Grillo-Lopez, C.A. White, D. Bodkin, R.J. Schilder, J.A. Neidhart, N. Janakiraman, K.A. Foon, T.M. Liles, B.K. Dallaire, K. Wey, I. Royston, T. Davis, and R. Levy, IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. Blood 90 (1997) 2188-95.

[108] D. Harari, and Y. Yarden, Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. Oncogene 19 (2000) 6102-14.

[109] C.A. Hudis, Trastuzumab--mechanism of action and use in clinical practice. N Engl J Med 357 (2007) 39-51.

[110] M.E. Dudley, J.R. Wunderlich, J.C. Yang, R.M. Sherry, S.L. Topalian, N.P. Restifo, R.E. Royal, U. Kammula, D.E. White, S.A. Mavroukakis, L.J. Rogers, G.J. Gracia, S.A. Jones, D.P. Mangiameli, M.M. Pelletier, J. Gea-Banacloche, M.R. Robinson, D.M. Berman, A.C. Filie, A. Abati, and S.A. Rosenberg, Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol 23 (2005) 2346-57.

[111] E. Jager, D. Jager, and A. Knuth, Clinical cancer vaccine trials. Curr Opin Immunol 14 (2002) 178-82.

[112] K.R. Jordan, R.H. McMahan, C.B. Kemmler, J.W. Kappler, and J.E. Slansky, Peptide vaccines prevent tumor growth by activating T cells that respond to native tumor antigens. Proc Natl Acad Sci U S A 107 4652-7.

[113] M. Muraro, O.M. Mereuta, F. Saglio, F. Carraro, C. Cravero, and F. Fagioli, Tumor-associated-antigens or osteosarcoma cell line lysates: two efficient methods for in vitro generation of CTLs with special regard to MHC-I restriction. Cell Immunol 266 123-9.

[114] S. Seiter, V. Monsurro, M.B. Nielsen, E. Wang, M. Provenzano, J.R. Wunderlich, S.A. Rosenberg, and F.M. Marincola, Frequency of MART-1/MelanA and gp100/PMel17-specific T cells in tumor metastases and cultured tumor-infiltrating lymphocytes. J Immunother 25 (2002) 252-63.

[115] M.J. Pittet, A. Zippelius, D. Valmori, D.E. Speiser, J.C. Cerottini, and P. Romero, Melan-A/MART-1-specific CD8 T cells: from thymus to tumor. Trends Immunol 23 (2002) 325-8.

[116] N.N. Hunder, H. Wallen, J. Cao, D.W. Hendricks, J.Z. Reilly, R. Rodmyre, A. Jungbluth, S. Gnjatic, J.A. Thompson, and C. Yee, Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. N Engl J Med 358 (2008) 2698-703.

[117] S. Stager, and P.M. Kaye, CD8+ T-cell priming regulated by cytokines of the innate immune system. Trends Mol Med 10 (2004) 366-71.

[118] S.G. Lee, D.S. Heo, S.J. Yoon, Y.S. Jee, J.O. Kang, K. Kim, C.D. Kim, M.W. Sung, and N.K. Kim, Effect of GM-CSF and IL-2 co-expression on the anti-tumor immune response. Anticancer Res 20 (2000) 2681-6.

[119] K.J. Palmer, M. Harries, M.E. Gore, and M.K. Collins, Interferon-alpha (IFN-alpha) stimulates anti-melanoma cytotoxic T lymphocyte (CTL) generation in mixed lymphocyte tumour cultures (MLTC). Clin Exp Immunol 119 (2000) 412-8.

[120] A.K. Palucka, H. Ueno, J. Fay, and J. Banchereau, Dendritic cells: a critical player in cancer therapy? J Immunother 31 (2008) 793-805.

[121] N. Cartier, S. Hacein-Bey-Abina, C.C. Bartholomae, G. Veres, M. Schmidt, I. Kutschera, M. Vidaud, U. Abel, L. Dal-Cortivo, L. Caccavelli, N. Mahlaoui, V. Kiermer, D. Mittelstaedt, C. Bellesme, N. Lahlou, F. Lefrere, S. Blanche, M. Audit, E. Payen, P. Leboulch, B. l'Homme, P. Bougneres, C. Von Kalle, A. Fischer, M. Cavazzana-Calvo, and P. Aubourg, Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science 326 (2009) 818-23.

[122] A.K. Palucka, H. Ueno, J. Connolly, F. Kerneis-Norvell, J.P. Blanck, D.A. Johnston, J. Fay, and J. Banchereau, Dendritic cells loaded with killed allogeneic melanoma cells can induce objective clinical responses and MART-1 specific CD8+ T-cell immunity. J Immunother 29 (2006) 545-57.

[123] R. Sartorius, C. Bettua, L. D'Apice, A. Caivano, M. Trovato, D. Russo, I. Zanoni, F. Granucci, D. Mascolo, P. Barba, G.D. Pozzo, and P.D. Berardinis, Vaccination with filamentous bacteriophages targeting DEC-205 induces DC maturation and potent anti-tumor T-cell responses in the absence of adjuvants. Eur J Immunol 41 2573-84.

[124] J. Tel, D. Benitez-Ribas, S. Hoosemans, A. Cambi, G.J. Adema, C.G. Figdor, P.J. Tacken, and I.J. de Vries, DEC-205 mediates antigen uptake and presentation by both resting and activated human plasmacytoid dendritic cells. Eur J Immunol 41 1014-23.

[125] T. Tsuji, J. Matsuzaki, M.P. Kelly, V. Ramakrishna, L. Vitale, L.Z. He, T. Keler, K. Odunsi, L.J. Old, G. Ritter, and S. Gnjatic, Antibody-targeted NY-ESO-1 to mannose receptor or DEC-205 in vitro elicits dual human CD8+ and CD4+ T cell responses with broad antigen specificity. J Immunol 186 1218-27.

[126] R.E. Shrimpton, M. Butler, A.S. Morel, E. Eren, S.S. Hue, and M.A. Ritter, CD205 (DEC-205): a recognition receptor for apoptotic and necrotic self. Mol Immunol 46 (2009) 1229-39.

[127] Y.J. Jeong, S.W. Hong, J.H. Kim, D.H. Jin, J.S. Kang, W.J. Lee, and Y.I. Hwang, Vitamin C-treated murine bone marrow-derived dendritic cells preferentially drive naive T cells into Th1 cells by increased IL-12 secretions. Cell Immunol 266 192-9.

[128] P.A. Burch, G.A. Croghan, D.A. Gastineau, L.A. Jones, J.S. Kaur, J.W. Kylstra, R.L. Richardson, F.H. Valone, and S. Vuk-Pavlovic, Immunotherapy (APC8015, Provenge) targeting prostatic acid phosphatase can induce durable remission of metastatic androgen-independent prostate cancer: a Phase 2 trial. Prostate 60 (2004) 197-204.

[129] V.T. DeVita, Jr., The Provenge decision. Nat Clin Pract Oncol 4 (2007) 381.

[130] H. Mahmutefendic, G. Blagojevic, N. Kucic, and P. Lucin, Constitutive internalization of murine MHC class I molecules. J Cell Physiol 210 (2007) 445-55.

[131] J. Stagsted, Journey beyond immunology. Regulation of receptor internalization by major histocompatibility complex class I (MHC-I) and effect of peptides derived from MHC-I. APMIS Suppl 85 (1998) 1-40.

[132] T.G. Rodriguez-Cruz, S. Liu, J.S. Khalili, M. Whittington, M. Zhang, W. Overwijk, and G. Lizee, Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8 T-cell responses and boosts anti-tumor immunity. PLoS One 6 e22939.

[133] W.W. Overwijk, A. Tsung, K.R. Irvine, M.R. Parkhurst, T.J. Goletz, K. Tsung, M.W. Carroll, C. Liu, B. Moss, S.A. Rosenberg, and N.P. Restifo, gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. J Exp Med 188 (1998) 277-86.

[134] A. Serrano, C.S. Brady, P. Jimenez, M.F. Duggan-Keen, R. Mendez, P. Stern, F. Garrido, and F. Ruiz-Cabello, A mutation determining the loss of HLA-A2 antigen expression in a cervical carcinoma reveals novel splicing of human MHC class I

classical transcripts in both tumoral and normal cells. Immunogenetics 51 (2000) 1047-52.

[135] T.S. Dalgaard, L. Vitved, K. Skjodt, B. Thomsen, R. Labouriau, K.H. Jensen, and H.R. Juul-Madsen, Molecular characterization of major histocompatibility complex class I (B-F) mRNA variants from chickens differing in resistance to Marek's disease. Scand J Immunol 62 (2005) 259-70.

[136] H. Keren, G. Lev-Maor, and G. Ast, Alternative splicing and evolution: diversification, exon definition and function. Nat Rev Genet 11 345-55.

[137] M.J. Cannon, C.K. Osborn, R.A. Nazaruk, V. Grigoriev, and M.D. Crew, Diminished recognition of HLA-A2 proteins lacking a cytoplasmic domain (CY) by A2-restricted, EBV-specific CTLs: possible role of the CY in TAP association. Immunogenetics 49 (1999) 346-50.

[138] G.G. Capps, M. Van Kampen, C.L. Ward, and M.C. Zuniga, Endocytosis of the class I major histocompatibility antigen via a phorbol myristate acetate-inducible pathway is a cell-specific phenomenon and requires the cytoplasmic domain. J Cell Biol 108 (1989) 1317-29.

[139] G.G. Capps, and M.C. Zuniga, Phosphorylation of class I MHC molecules in the absence of phorbol esters is an intracellular event and may be characteristic of trafficking molecules. Mol Immunol 37 (2000) 59-71.

[140] M.M. Al-Alwan, G. Rowden, T.D. Lee, and K.A. West, The dendritic cell cytoskeleton is critical for the formation of the immunological synapse. J Immunol 166 (2001) 1452-6.

[141] D.R. Fooksman, G.K. Gronvall, Q. Tang, and M. Edidin, Clustering class I MHC modulates sensitivity of T cell recognition. J Immunol 176 (2006) 6673-80.

[142] A.B. Vogt, S. Spindeldreher, and H. Kropshofer, Clustering of MHC-peptide complexes prior to their engagement in the immunological synapse: lipid raft and tetraspan microdomains. Immunol Rev 189 (2002) 136-51.

[143] J. Matko, Y. Bushkin, T. Wei, and M. Edidin, Clustering of class I HLA molecules on the surfaces of activated and transformed human cells. J Immunol 152 (1994) 3353-60.

[144] M. Edidin, Class I MHC molecules as probes of membrane patchiness: from biophysical measurements to modulation of immune responses. Immunol Res 47 265-72.

[145] J. Kwik, S. Boyle, D. Fooksman, L. Margolis, M.P. Sheetz, and M. Edidin, Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. Proc Natl Acad Sci U S A 100 (2003) 13964-9.

[146] S. Damjanovich, R. Gaspar, Jr., and C. Pieri, Dynamic receptor superstructures at the plasma membrane. Q Rev Biophys 30 (1997) 67-106.

[147] S. Damjanovich, L. Bene, J. Matko, A. Alileche, C.K. Goldman, S. Sharrow, and T.A. Waldmann, Preassembly of interleukin 2 (IL-2) receptor subunits on resting Kit 225 K6 T cells and their modulation by IL-2, IL-7, and IL-15: a fluorescence resonance energy transfer study. Proc Natl Acad Sci U S A 94 (1997) 13134-9.

[148] M. Edidin, S.C. Kuo, and M.P. Sheetz, Lateral movements of membrane glycoproteins restricted by dynamic cytoplasmic barriers. Science 254 (1991) 1379-82.

[149] M. Edidin, Fluorescence resonance energy transfer: techniques for measuring molecular conformation and molecular proximity. Curr Protoc Immunol Chapter 18 (2003) Unit 18 10.

[150] G.G. Capps, S. Pine, M. Edidin, and M.C. Zuniga, Short class I major histocompatibility complex cytoplasmic tails differing in charge detect arbiters of lateral diffusion in the plasma membrane. Biophys J 86 (2004) 2896-909.

[151] H. Bian, P.E. Harris, A. Mulder, and E.F. Reed, Anti-HLA antibody ligation to HLA class I molecules expressed by endothelial cells stimulates tyrosine phosphorylation, inositol phosphate generation, and proliferation. Hum Immunol 53 (1997) 90-7.

[152] H. Bian, P.E. Harris, and E.F. Reed, Ligation of HLA class I molecules on smooth muscle cells with anti-HLA antibodies induces tyrosine phosphorylation, fibroblast growth factor receptor expression and cell proliferation. Int Immunol 10 (1998) 1315-23.

[153] Y.P. Jin, R.P. Singh, Z.Y. Du, A.K. Rajasekaran, E. Rozengurt, and E.F. Reed, Ligation of HLA class I molecules on endothelial cells induces phosphorylation of Src, paxillin, and focal adhesion kinase in an actin-dependent manner. J Immunol 168 (2002) 5415-23.

[154] S. Skov, S. Bregenholt, and M.H. Claesson, MHC class I ligation of human T cells activates the ZAP70 and p56lck tyrosine kinases, leads to an alternative phenotype of the TCR/CD3 zeta-chain, and induces apoptosis. J Immunol 158 (1997) 3189-96.

[155] K. Narayanan, A. Jaramillo, D.L. Phelan, and T. Mohanakumar, Pre-exposure to sub-saturating concentrations of HLA class I antibodies confers resistance to endothelial cells against antibody complement-mediated lysis by regulating Bad through the phosphatidylinositol 3-kinase/Akt pathway. Eur J Immunol 34 (2004) 2303-12.

[156] Y.P. Jin, M.C. Fishbein, J.W. Said, P.T. Jindra, R. Rajalingam, E. Rozengurt, and E.F. Reed, Anti-HLA class I antibody-mediated activation of the PI3K/Akt signaling pathway and induction of Bcl-2 and Bcl-xL expression in endothelial cells. Hum Immunol 65 (2004) 291-302.

[157] H. Gur, F. el-Zaatari, T.D. Geppert, M.C. Wacholtz, J.D. Taurog, and P.E. Lipsky, Analysis of T cell signaling by class I MHC molecules: the cytoplasmic domain is not required for signal transduction. J Exp Med 172 (1990) 1267-70.

[158] T.S. Ramalingam, A. Chakrabarti, and M. Edidin, Interaction of class I human leukocyte antigen (HLA-I) molecules with insulin receptors and its effect on the insulin-signaling cascade. Mol Biol Cell 8 (1997) 2463-74.

[159] L.M. Wakim, and M.J. Bevan, Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. Nature 471 629-32.

[160] J.W. Yewdell, and B.P. Dolan, Immunology: Cross-dressers turn on T cells. Nature 471 581-2.

[161] E. Bartee, M. Mansouri, B.T. Hovey Nerenberg, K. Gouveia, and K. Fruh, Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. J Virol 78 (2004) 1109-20.

[162] J. Zhao, and A.M. Lever, Lentivirus-mediated gene expression. Methods Mol Biol 366 (2007) 343-55.

[163] A.L. Ackerman, and P. Cresswell, Regulation of MHC class I transport in human dendritic cells and the dendritic-like cell line KG-1. J Immunol 170 (2003) 4178-88.

VITA

Tania Giselle Rodríguez-Cruz was born in Rio Piedras, Puerto Rico on January 1st, 1981 to Ricardo Rodríguez and Carmen M. Cruz. In 1999 she completed her high school studies at Colegio Católico Notre Dame in Caguas, Puerto Rico. The same year she joined the University of Puerto Rico at Mayagüez Campus and earned her Bachelor of Science *Magna Cum Laude* in May, 2004, with a major in Biology. In August 2005, she entered the PhD program at The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. In May 2006 she began her Ph.D. training under the supervision of Dr. Gregory Lizee in the Immunology Program at the University of Texas M.D. Anderson Cancer Center.