Promoting Type-1 CD4$^+$ T Cell Immune Responses Against Tumor-Associated Antigen MAGE-A6

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Abstract

One of the main challenges facing tumor immunologists is to develop strategies that would effectively stimulate Type-1 anti-tumor T cell responses, which have been correlated with better clinical outcome and prolonged survival of cancer patients. As $CD4^+$ T cells were shown to play a critical role in mediating these responses, it was of interest to examine novel ways of effectively stimulating and enhancing Type-1 $CD4^+$ T cell responses. For these studies I used MAGE-A6, a tumor associated antigen (TAA) expressed by a broad range of human cancer types. Two novel MAGE-A6 T-helper epitopes were identified and were shown to be recognized by $CD4^+$ T cells isolated from the majority of normal donors or patients with melanoma, regardless of their HLA genotype (i.e. poly-DR presented epitopes). Furthermore, peptide-specific T cells also recognized autologous monocytes pulsed with recombinant MAGE-A6 protein, supporting the natural processing and MHC presentation of these epitopes. Interestingly, one of the novel MAGE-A6 epitopes possesses a high-degree of homology with a microbial peptide. $CD4^+$ T cells stimulated *in vitro* with this microbial peptide cross-reacted against the MAGE-A6 homologue peptide, and could recognize naturally-processed MAGE-A6 epitopes more effectively than T cells stimulated with MAGE-A6 peptides. This study showed that it is possible to stimulate, and even enhance tumor-specific T cell responses using microbial epitopes that are homologous to TAA-derived peptides. In the final study, human dendritic cells (DC) were engineered to secrete high levels of IFN-γ-inducing cytokines IL-12p70 and IL-18 via

recombinant adenoviral infection to generate an *in vitro* stimulus capable of promoting previously deficient patient Th1-type responses. DC engineered to secrete both of these cytokines simultaneously (DC.IL-12/18) were highly effective at stimulating MAGE-A6-specific Th1-type $CD4^+$ T cell responses from patients with melanoma, particularly when loaded with MAGE-A6 protein. Poly-DR presented epitopes and MAGE-A6 protein defined in this thesis, if loaded onto DC.IL-12/18, could prove clinically useful as a vaccine modality capable of promoting the recovery and/or enhancement of tumor antigen-specific, Th1-type $CD4^+$ T cell responses in the majority of patients harboring MAGE-$A6^+$ cancers.

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PREFACE

A Japanese proverb states, “Fall down seven times. Get up eight.” Getting up is particularly easy when you are surrounded by caring and supportive people that will stand by you regardless of adversities. I have been blessed to be surrounded by a number of such individuals. The first of these people I would like to thank is my mentor, Dr. Walter J. Storkus. I have met Dr. Storkus years before I joined his lab, and once I came to the University of Pittsburgh he was the clear choice for my advisor. I feel extremely fortunate and honored to have spent my graduate school years under the tutelage of such an exceptional scientist and human being. I would also like to thank all the people who have been a part of the Storkus Lab during my years here, and who have always provided support, insight, help, and friendship that made my tenure more gratifying. I would next like to thank my Thesis Committee for their guidance and patience throughout my time here. Furthermore, I would like to thank all of my friends who gave me regular reality checks.

Finally, I would like to extend a special thank you to my wonderful family, without whom I would have not made it this far. Throughout my life, my mother Svetlana, sister Dušica, grandmother Nadežda, and father Nikola have provided me with love, wisdom and encouragement that gave me strength in moments of doubt. Your guidance and support has never been more valuable than during my time in graduate school, and for that I dedicate this thesis to you. A Serbian proverb states, “Be humble for you are made of earth. Be noble for you are made of stars.” One always needs to remember his roots.

1. INTRODUCTION

Previously it has been observed that some human tumors, especially melanoma and renal cell carcinoma (RCC), can occasionally undergo spontaneous regression (1, 2). These findings have inspired the imagination of clinicians and scientists, and have created hope that the immune system can specifically recognize and eliminate cancers. Since the first description of a molecularly-defined human tumor-associated antigen (TAA) recognized by cytotoxic T cells 15 years ago (3), advances in understanding the nature of tumor-specific immune responses and mechanisms of tolerance induction have encouraged researchers and clinicians alike to develop a more refined approach to immune-mediated therapies. Studies utilizing expression cloning of TAA cDNAs have been integrated with novel strategies such as reverse immunology, biochemical methods, genetic approaches, and serological analysis of recombination expression libraries (SEREX) to identify a number of TAAs. Reverse immunology refers to a strategy where epitopes are predicted on the basis of known HLA-binding motifs from an already identified TAA. Biochemical methods involve eluting and fractionating TAA peptides naturally expressed on tumor cells in the context of HLA molecules by reverse-phase high-performance liquid chromatography (HPLC) and mass spectrometry. Genetic approaches are used to identify tumor genes coding for the epitopes recognized by isolated patient cytotoxic T cell clones reactive against autologous tumors. SEREX is based on the recognition of tumor antigens by cancer patient's autologous sera. All of these strategies have successfully been utilized to identify a number of TAA that can be presented by tumor cells or by antigen presenting cells (APCs) in the context of major histocompatibility complex (MHC) molecules on their cell surfaces (4-7).

1.1. Tumor Associated Antigen Classification

According to the pattern of expression in neoplastic and normal tissues, TAAs can be classified into four major categories (**Table 1**). The first category is cancer-testis antigens. These are proteins encoded by genes expressed in various tumors but not in normal tissues, except for testis and placenta. Antigens that belong to this group are MAGE, GAGE, and BAGE families, as well as NY-ESO-1 and its alternative ORF products LAGE and CAMEL. The second group represents differentiation antigens that are shared between tumors and the normal tissue from which the tumor arose. Of the ones discovered so far, most are expressed in melanoma and normal melanocytes, such as tyrosinase, Melan-A/MART-1, gp100, TRP-1, and TRP-2. The third category is tumor-specific antigens. These antigens are generated by point mutations (e.g. p53, Ras, CDK4, β-catenin) (5, 6) or tumor-specific splicing aberrations in genes that are ubiquitously expressed (e.g. TRP-2/INT2) (8), and are expressed only in tumors where they were identified (unlike cancer-testis antigens). These molecular changes are associated with neoplastic transformation and/or progression. The fourth group of antigens is widely occurring, over-expressed TAA. These are proteins that have been detected in histologically different types of tumors (often with no preferential expression on a certain type of cancer) as well as in many normal tissues, generally with lower expression levels. Some of the antigens belonging to this group include survivin, MUC1/2 and EphA2, among others.

Table 1. Summary of tumor antigen categories

TAA Categories	Antigen Characteristic	Genes
Cancer-Testis	Expressed in various tumors but not normal tissues except in testis and placenta	MAGE, GAGE, BAGE, NY-ESO-1
Differentiation	Antigens shared between tumors and normal tissues from which they arose	Tyrosinase, Melan-A/MART-1, gp100, TRP-1, and TRP-2
Tumor-Specific	Antigens generated by point mutations or splicing aberrations in ubiquitous genes	p53, Ras, CDK4, β-catenin, TRP-2/INT2
Widely Occurring Over-Expressed	Proteins over-expressed in histologically different types of tumors	Survivin, MUC1/2 and EphA2

1.2. Melanoma Antigen Gene (MAGE) Family

MAGE proteins are a family of closely-related molecules that were initially identified as TAA capable of being recognized by cytolytic T lymphocytes (CTL) isolated from the peripheral blood of cancer patients (3). MAGE genes are classified as either type I (MAGE-A, MAGE-B, and MAGE-C genes located on the X chromosome) or type II (those that are located outside of the type I MAGE genomic cluster) (9, 10). Normally, type I MAGE proteins are selectively expressed in testicular cells among somatic tissues (11, 12), and in some instances placenta (MAGE-A3, MAGE-A4, and MAGE-A8 through –A11; ref. (13)). In fact, MAGE antigen expression has been demonstrated in spermatogonia as well as spermatocytes, as the only cell types regularly expressing MAGE antigens among normal tissues (12). However, MAGE proteins can also be expressed in both pre-malignant and malignant lesions in concert with DNA

hypomethylation (14, 15). As both testis and placenta are considered to represent immunologically privileged regions due to their lack of/deficiency in MHC class I expression, any potential vaccination strategies using these antigens would be expected to have only limited pathologic effects in patients, making these antigens acceptable targets for cancer vaccines.

1.2.1. MAGE Function

While most of the members of the MAGE family have been molecularly characterized, their cellular function remains a major mystery. This is particularly true for type I MAGE proteins. Most of the functional analyses reported have thus far been performed on necdin and MAGE-D1 (also known as NRAGE), growth suppressors expressed predominantly in post-mitotic neurons that have been implicated in their terminal differentiation (reviewed in ref. (16)). Necdin is a cell cycle regulator necessary for the terminal differentiation and survival of primary dorsal root ganglion neurons. It serves as a growth suppressor that is functionally similar to the retinoblastoma (RB) tumor suppressor protein. Necdin is involved in the terminal differentiation and survival of nerve growth factor (NGF)-dependent dorsal root ganglion neurons. Suppression of necdin expression in neurons leads to caspase-3-dependent apoptosis (17). Necdin appears to interact with cell cycle promoting proteins such as simian virus 40 large T antigen, adenovirus E1A, and transcription factor E2F1. It represents a growth suppressor that targets and modulates the biological functions of p53 in post-mitotic neurons (18). Necdin markedly suppresses p53-dependent activation of the p21/WAF promoter, and in doing so, inhibits p53-induced apoptosis of tumor cells. Furthermore, necdin and p53 inhibit cell growth in an additive manner. MAGE-D1 was identified as a binding partner for the p75 neurotrophin receptor, the apoptosis inhibitory

protein XIAP, and the Dlx/MSX homeodomain proteins. It appears to block cell cycle progression, and unlike necdin, is involved in cellular pro-apoptotic pathways (16, 19).

Limited data has been accumulated regarding the function of type I MAGE genes, particularly MAGE-A1 and MAGE-A4. Stable (enforced) expression of MAGE-A1 reduces the susceptibility of tumor cell lines to TNF-α-mediated cytotoxicity (20), suggesting that MAGE-A1 is cyto-protective. Contrary to this paradigm, MAGE-A4 has been reported to bind to, and suppress, the oncoprotein gankyrin in hepatocellular carcinoma (21). MAGE-A4 also partially suppresses both anchorage-independent growth *in vitro* and tumor formation in athymic mice (21). MAGE-A4 appears to promote cellular apoptosis in both p53-dependent and p53-independent manners. It stabilizes p53 protein levels, but decreases cellular expression of p21 by binding to Miz-1, in concert with down-regulating Bcl-xL expression during the process of apoptosis (22). These conflicting reports suggest that, while highly-homologous, MAGE family proteins may mediate disparate functions associated with cell cycling and death.

1.2.2. MAGE-A Subfamily

MAGE-A1 was one of the first TAA reported based on modern molecular cloning approaches (3). Subsequently, new members of this family have been isolated, largely based on homology searches predicated on a MAGE-A1 template. The MAGE-A gene family is currently composed of 12 members (i.e. MAGE-A1 through -A12), that are in aggregate expressed by more than half of all human cancers. The MAGE-A gene cluster is located on chromosome Xq28, and all open reading frames are contained within a single exon (23). These genes encode intracellular proteins that have most commonly been observed in the cytoplasm (24, 25), but in

some cases they have also been observed in the nuclei of well-differentiated tumors (25). MAGE-A expression is frequently observed in melanoma specimens (26, 27), but not in naevi (including Spitz, dysplastic naevi, junctional and compound naevi) (28). In fact, greater immunohistochemical staining of tumor cells with anti-MAGE antibody has been associated with an invasive phenotype and a decreased in the overall survival rate of cancer patients (29, 30).

MAGE-A antigens have been evaluated as targets for immunoreactivity in a number of published studies. A summary of MAGE-A-derived MHC class I- and class II-restricted epitopes that have been previously reported is provided in **Tables 2** and **3** (7). What is unique about these proteins is that they are highly homologous, and immunogenic peptides identified within one MAGE-A protein are often shared or highly-homologous with epitopes encoded by other members of the MAGE-A family. *In vivo* vaccination studies utilizing MAGE-A peptides/cDNAs showed that epitope-specific $CD4^+$ (31) and $CD8^+$ (32) T cell responses can be primed in immunized patients. In one particular study, vaccination of metastatic melanoma patients with cutaneous injections of a recombinant canary pox virus carrying a mini-gene coding for two HLA-A1-restricted peptides encoded by MAGE-A1 and MAGE-A3, resulted in the enhancement of anti-tumor CTL responses. Anti-tumor CTLs (i.e. specific for TAA other than MAGE-A1 and MAGE-A3 epitopes) were 10,000 times more frequent among tumor infiltrating lymphocytes (TILs) than vaccine-specific T cells, suggesting that treatment-induced CTLs were not likely to represent the effectors associated with therapeutic benefit in these patients. It suggests instead that through the process of epitope spreading, vaccine-associated T cells may enable large numbers of anti-tumor (although not necessarily MAGE-specific) CTLs to be effectively cross-primed *in* vivo, yielding a clinically-effective, tumoricidal T cell repertoire

(33, 34). These studies suggest that MAGE-A antigens likely possess potential therapeutic value as targets of vaccine intervention strategies.

Table 2. Class I HLA-Restricted MAGE-A Peptides

Immunogenic MAGE-A-derived, class I HLA-restricted peptides defined in the literature are listed (7). Homologous MAGE-A epitope sequence is written in red.

Gene	Restricting HLA Allele	Peptide epitope
MAGE-A1	A1	EADPTGHSY
	A3	SLFRAVITK
	A24	NYKHCFPEI
	A28	EVYDGREHSA
	B37	REPVTKAEML
	B53	DPARYEFLW
	Cw2	SAFPTTINF
	Cw3	SAYGEPRKL
	Cw16	SAYGEPRKL
MAGE-A2	A2	KMVELVHFL
	A2	YLQLVFGIEV
	A24	EYLQLVFGI
	B37	REPVTKAEML
MAGE-A3	A1	EADPIGHLY
	A2	FLWGPRALV
	A24	TFPDLESEF
	A24	IMPKAGLLI
	B44	MEVDPIGHLY
	B52	WQYFFPVIF
	B37	REPVTKAEML
	B*3501	EVDPIGHLY
MAGE-A4	A2	GVYDGREHTV
MAGE-A6	A34	MVKISGGPR
	B37	REPVTKAEML
	B*3501	EVDPIGHVY
MAGE-A10	A2	GLYDGMEHL
MAGE-A12	Cw7	VRIGHLYIL

Table 3. Class II HLA-Restricted MAGE-A Peptides

Immunogenic MAGE-A-derived, class II HLA-restricted peptides defined in the literature are listed (7). Homologous MAGE-A epitope sequences are written in red.

Gene	HLA Allele	Peptide epitope
MAGE-A1	DRB1*1301, DRB1*1302	LLKYRAREPVTKAE
MAGE-A2	DRB1*1301, DRB1*1302	LLKYRAREPVTKAE
MAGE-A3	DRB1*1301, DRB1*1302	LLKYRAREPVTKAE
	DRB1*1301, DRB1*1302	AELVHFLLLKYRAR
	DR1, DR4, DR7, DR11	RKVAELVHFLLLKYR
		GDNQIMPKAGLLIIV
		TSYVKVLHHMVKISG
	DR1, DR4, DR7, DR11	FFPVIFSKASSSLQL
MAGE-A6	DRB1*1301, DRB1*1302	LLKYRAREPVTKAE
	DRB1*0401	ESEFQAALSRKVAKL
		LLKYRAREPVTKAEMLGSVVGNWQ
		VGNWQYFFPVIFSKASDSLQLVFGIELMEVD
		IFSKASDSLQLVFGIE
		LTQYFVQENYLEYRQVPG

1.2.3. MAGE-A6

MAGE-A6 was originally isolated from the human melanoma cell line DM150, and was shown to be 98% homologous at the protein level with MAGE-A3 (35). Like MAGE-A3, it is expressed in testis, but unlike some other members of the MAGE family, is not expressed in the placenta (13). MAGE-A6 has been reported to be expressed in more than 60% of melanomas (27), 30% of renal cell carcinomas (36), and by many other cancer types, such as breast, esophageal, head and neck, bladder, and lung carcinomas (27, 37-40). CTL responses against

MAGE-A6 have been reported to occur naturally in the setting of spontaneously regressing human melanoma (41), suggesting that the immune targeting of this antigen may be linked with tumor regression *in situ*, bolstering its potential therapeutic value. It has been shown to react with sera extracted from breast cancer patients but not normal donors (42). The clinical relevance of the MAGE-A6 antigen has been further substantiated in a clinical study where MAGE-A6-specific $CD8^+$ T cell clones were detected in a metastatic melanoma patient that had complete tumor regression following adoptive transfer of autologous tumor-specific tumor-infiltrating lymphocytes (TILs) (43). This is compounded by its wide range of expression among cancer types, and its lack of expression by normal tissues, which theoretically limits concerns over autoimmune pathology resulting from MAGE-A6-based cancer vaccines and immunotherapies.

1.3. Tumor Antigen Processing

Endogenous and exogenous (usually internalized by APCs) TAAs are processed principally via the cytosolic and endo/lysosomal pathways, respectively. They are presented as short protein fragments by MHC class I (endogenous peptides 8-9 amino acids long) and class II (exogenous peptides up to 35 amino acids in length) (44, 45). Tumor peptides associate with MHC molecules in intracellular compartments [endoplasmic reticulum (ER) for MHC class I, and endolysosome for MHC class II], and once forming stable complexes, they are transported to the cell surface where they become accessible to T cell scrutiny (45, 46). Most TAAs contain a number of sequences that have been predicted and/or documented to bind to MHC molecules (47). Typically, only a few of the potential epitopes elicit a strong (immunodominant epitopes)

cytotoxic T cell responses, while the majority elicit weak or no responses (sub-dominant or cryptic epitopes) (48, 49).

1.3.1. Classical MHC Class I/Peptide Presentation

Classical MHC class I antigen presentation (**Figure 1**) starts with the degradation of endogenous (intracellularly synthesized) proteins by the proteasome. Peptides of the correct length and sequence (possessing the correct anchor residues) bind to class I with the slowest off-rate (50, 51). Peptides that are longer, or do not have appropriate anchor residues bind with faster off-rates (52, 53). A small fraction of the peptide fragments that result from this degradation survive complete destruction and are transported into the ER and loaded onto the MHC by the peptide loading complex composed of one TAP1/TAP2 (transporter associated with antigen presentation) heterodimer associated with 4 tapasin, 4 calreticulin and 4 MHC class I heavy chain/beta-2 microglobulin (β_2m) dimers (54). In the ER, the peptides are loaded onto newly synthesized MHC class I molecules, forming ternary complexes, each composed of MHC class I heavy chain, β_2m and peptide. These stable complexes are then transported to the cell surface (55) where they are exposed to $CD8^+$ T cell surveillance.

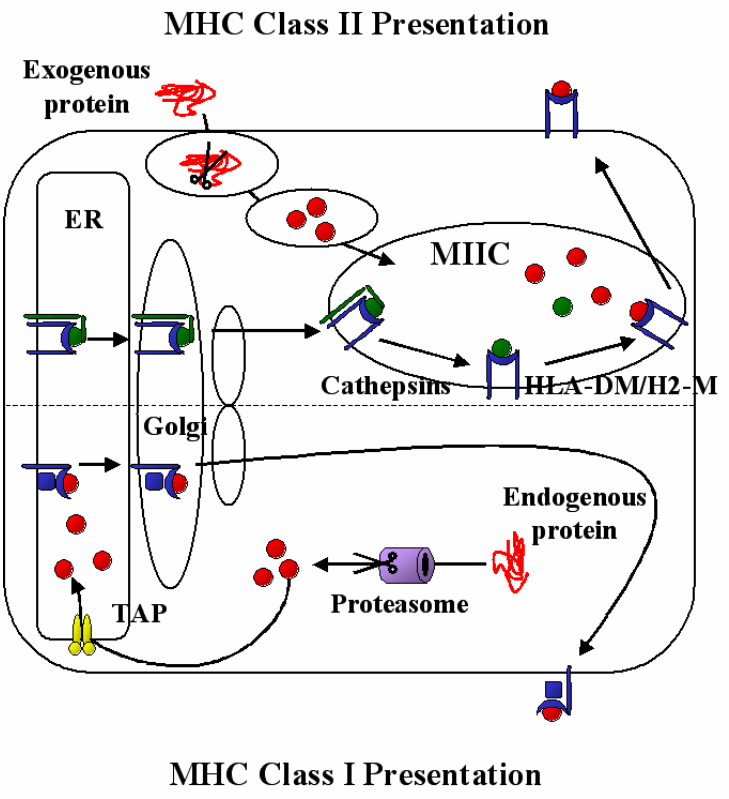


Figure 1. Conventional MHC class I and class II antigen processing and presentation pathways.

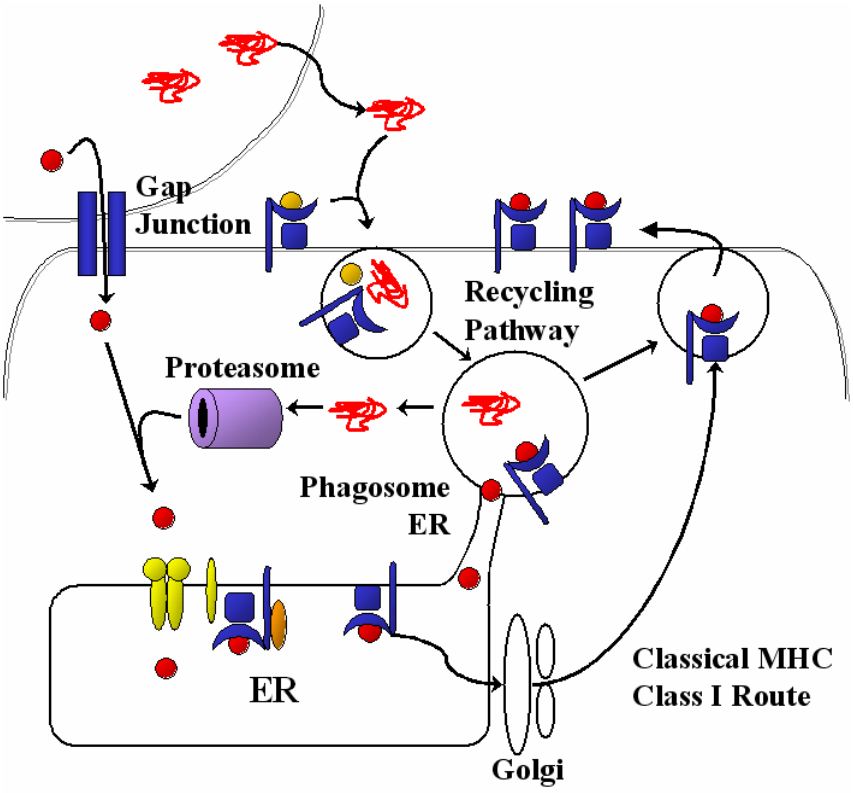
Endogenous antigens are degraded by the proteasome into short peptides that are transported into the ER by TAP. Here they form a complex with a newly synthesized MHC class I/β2m that is transported to the cell surface for presentation to $CD8^+$ T cells. MHC class II α- and β-chains assemble in the ER with Ii. This complex is transported to the MIIC, where Ii is degraded by cathepsins until only CLIP is left in the MHC class II binding groove. HLA-DM (or its mouse homologue H2-M) catalyzes the replacement of CLIP with peptides derived from exogenous antigens taken up by APCs.

1.3.2. Cross-Presentation

In vivo, APCs acquire exogenous antigens (extracellular but often derived from an intracellular source; e.g. tumor cells) in the periphery, and then migrate to the lymph nodes where they display antigenic peptides in association with MHC class I molecules and stimulate epitope-specific $CD8^+$ T cells. In this case, the source of antigen(s) is distinct from that of classical MHC class I antigen presentation pathway, and therefore the mechanism of antigen degradation and delivery of the peptide to MHC class I molecules is also likely to be different. The mechanism by which cross-presentation occurs is still not fully understood (55-57). There are multiple theories as to how this process occurs (**Figure 2**).

Figure 2. Various models of cross-presentation.

There are multiple theories as to how extracellular antigens enter MHC class I antigen-processing pathway. One possibility is that gap junctions allow for direct transfer of peptides from infected cells into the cytosol of APCs. Proteins taken up through the endocytic pathway can enter the cytoplasm, from where they enter the classical MHC class I pathway. Another option is that MHC class I molecules from the cell surface are recycled along the endocytic pathway where they can exchange peptides. One of the more recent models for cross-presentation suggests that there may be direct fusion events involving phagosomes and ER membranes. Therefore, there would be a direct access for exogenous proteins to the enzymatic machinery required for MHC class I presentation.



In one model, intracellular peptides may be "swapped" through gap junctions, small channels that connect the cytosolic compartments of adjacent cells (58). This theory, however, does not explain how extracellular antigens, such as those used in vaccination studies, become cross-presented. As TAP and the proteasome complex have been shown to be crucial to cross-presentation (59), another possibility is that antigens somehow enter the cytosol of the APC, making them accessible to proteasomal degradation, transport into the ER, and presentation in MHC class I molecular complexes via the "classical pathway". It is also possible that MHC class I molecules can be recycled from the cell surface along the endocytic MHC class II pathway and exchange endogenous for exogenous peptides while there (60). One of the more recent models

for cross-presentation suggests that there may be direct fusion events involving phagosomes and ER membranes. Therefore, there would be a direct access to the enzymatic machinery required for MHC class I presentation (59). However, this model is rather controversial and has recently been refuted (61). More studies are clearly needed to further dissect this phenomenon, for without this knowledge, it will be difficult to completely rationalize optimal vaccine development predicated on cross-presentation *in vivo*.

1.3.3. MHC Class II/Peptide Presentation

The MHC class II processing pathway processes and presents exogenous, as well as self/intrinsic-antigens that are degraded in the endocytic pathway (**Figure 1**). MHC class II αβ dimers assemble in the ER with the chaperone invariant chain (Ii) and its class II-associated Ii peptide (CLIP) portion inserted within the MHC peptide-binding cleft, which stabilizes and protects this site from interacting with other peptides in the ER microenvironment. MHC–Ii complexes are transported to early endosomes, and then via late endosomes into lysosomal compartments, during which time, they may encounter antigenic peptides resulting from the degradation of endocytosed proteins (62). Endocytosed antigens may be unfolded by thiol-reductases and then efficiently degraded by cathepsins, with peptides formed as intermediates during late endosomal protein degradation loaded into MHC class II complexes in a reaction catalyzed by the chaperone protein HLA-DM in the MHC class II compartment (MIIC), before transport of mature class II/peptide complexes to the plasma membrane (63). In order for these peptides to bind within the MHC class II groove, this pocket must be vacated by the Ii-derived CLIP peptide. Displacement of CLIP is facilitated by acidic pH in endosomes which favors an

open conformation in the MHC class II molecule and hence peptide exchange, the activity of the HLA-DM which stabilizes the open conformation, and by proteolytic elimination of the regions of Ii that flank the CLIP peptide. The peptide-MHC class II complexes are then transported to the cell surface, where they may be surveyed by $CD4^+$ T cells (62). A study done by Lazarski et al. has suggested that immunodominance of a given peptides is determined by the comparative stability of MHC class II:peptide complexes (64). In other words, immunodominant peptides typically possess long half-lives in class II complexes, while cryptic or poorly immunogenic peptides display significantly shorter half-lives in these complexes.

1.4. General Overview of T Cell Selection

The T cell repertoire is provided via a broad array of clonotypic T cells exhibiting heterogeneous usage of TCR Vα and Vβ chains. These T cells are capable of distinguishing foreign from self-antigens, and are normally capable of responding uniquely and appropriately to each of these stimuli. Thymic selection of T cells involves both positive (able to be "restricted" by self MHC) and negative selective (not pathologically reactive against self MHC) mechanisms based on the avidity of T cell interaction with antigen-MHC complexes. Apoptosis, or programmed cell death, plays a critical role in selecting the thymocyte pool, deleting cells expressing an unproductive T cell receptor (TCR), or exhibiting hyper-responsiveness upon encountering self MHC/self-peptide complexes. Thymocytes progress through well-defined steps during their maturation, exhibiting characteristic phenotypes at each stage. Immature thymocytes will survive if signals generated by TCR-MHC/peptide engagement are interpreted as appropriate (positive selection), but will be deleted by apoptosis if these generated signals are interpreted as either inappropriately weak (death by neglect/glucocorticoid-induced cell death) or

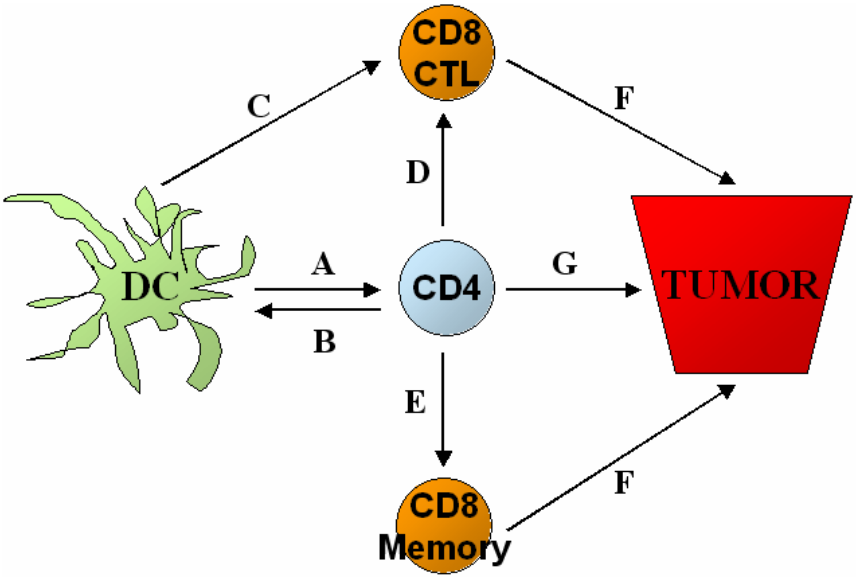
inappropriately strong (negative selection, therefore posing an autoimmune risk) (65, 66). A fraction of all T cells escapes thymic selection and ends up in the circulation, and these cells are subjected to additional peripheral selection criteria during systemic immune responses to antigenic challenge. It has been shown in mouse models that autoimmune T cells bearing high affinity TCR that escape thymic selection may be deleted as a result of immunizing animals with strongly immunogenic epitopes. However, if the animals were immunized with a weakly-immunogenic analogue, high-affinity T cells expand (67). As TAAs are considered to be "self" antigens, stimulating and sustaining an immune response to these antigens is a difficult proposition. An antigen used for vaccination needs to be preferentially expressed on tumor cells, therefore limiting any damage to healthy tissues, and must be capable of inducing as high avidity T cell responders as possible, without consequently promoting their (apoptotic) deletion.

1.5. $CD4^+$ T Cell-Mediated Immunity

Mature $CD4^+$ are typically known as T-helper (Th) cells. $CD4^+$ lymphocytes are believed to polarize the adaptive immune response by secreting a dominant panel of cytokines in response to specific antigen recognition. Based on these cytokine profiles, Th cells can be generally segregated into three major subsets: Th1, Th2, and Th3/T-regulatory (Treg) subsets (68). Th1 cells provide help for cellular immunity and perform several major functions (**Figure 3**).

Figure 3. The role of CD4+ Th1 cells in modulating immune responses against cancer.

DC present antigen to $CD4^+$ T cells and induce the induction of Th1 cells via the production of IL-12 (A). Activated $CD4^+$ T cells activate DCs via CD40/CD40L interaction (B). This provides help for the priming of $CD8^+$ T cells (C). Th1 cells also provide help for the maintenance of the CTL function and their proliferation (D), as well as generation and maintenance of a functional $CD8^+$ T cell memory (E). Activated $CD8^+$ T cells can then recognize and kill tumor (F). Th1 cells can also directly inhibit tumor growth via IFN-γ and even kill MHC class II-presenting tumors via TNF family ligands (G).



It is now clear that anti-tumor $CD4^+$ T cells regulate the quality, magnitude and durability of $CD8^+$ CTL immunity *in vivo* (69-71), and that the Th1-type cytokine, IFN-γ, plays an essential role in this response. Th1-type $CD4^+$ T cells secrete IFN-γ and IL-2, and may mediate delayed type hypersensitivity (DTH) responses that can lead to enhanced cross-presentation of tumor antigens by host APCs (72), and consequent epitope spreading in the evolving anti-tumor T cell repertoire (73, 74). Th1 cells play a major role in the initiation of a primary immune response by providing help to CTLs. DC presenting antigen to $CD4^+$ T cells induce the induction of Th1 cells via the production of IL-12. Activated Th1 cells upregulate CD40 ligand (CD40L) on their cell surface, which engages CD40 expressed on DC and in turn activates DCs, enabling them to effectively prime $CD8^+$ T cells. IL-2 secreted by Th1 cells provides help for the maintenance of CTL function and their proliferation, as well as generation and maintenance of a functional $CD8^+$ T cell memory. Once activated, TAA-specific $CD8^+$ T cells can recognize and kill tumor cells

(75). Furthermore, Th1-type $CD4^+$ T cells may mediate direct tumoricidal activity via TNF family ligand members and can inhibit tumor angiogenesis via locoregional production of IFN-γ (76-79). Th2 cells and their associated cytokines are often linked to strong antibody (humoral) responses, and they tend to inhibit Th1 responses.

The typical Th2-type cytokine profile includes the production of IL-4, IL-5 and IL-13 (68). These cytokines are best known for supporting B cell growth and differentiation, leading to the enhanced ability of B/plasma cells to secrete antibodies. Furthermore, they have been shown to protect tumor cells *in vivo* by suppressing Th1-type anti-tumor immune responses, and their presence in serum is usually correlated with poor prognosis and the reduced overall survival of cancer patients (80). Th3/Treg cells generally produce IL-10 and/or TGF-β, with both cytokines capable of strongly suppressing the proliferative and effector functions of Th1- and Th2-type $CD4^+$ T cells. As a consequence, these Th3/Treg cells are also known as T suppressor cells (68, 81, 82).

1.6. Immunoregulatory Function of IFN-γ

Anti-tumor $CD4^+$ T cells regulate the quality, magnitude and durability of $CD8^+$ CTL immunity *in vivo* (69-71), and IFN-γ, a Type-1 cytokine, plays an essential role in this response. The production and secretion of IFN-γ is promoted by IL-12 family members (i.e, IL-12p70, IL-23 and IL-27), and by IL-18 (83-87). Type-1 $CD4^+$ T cells (Th1), $CD8^+$ T cells (Tc1) and natural killer (NK) cells secrete IFN-γ and may mediate DTH responses, leading to cross-presentation of tumor antigens by host APCs (72), and resulting in anti-tumor epitope spreading (73, 74).

Furthermore, $CD4^+$ T cells were shown to inhibit tumor angiogenesis via locoregional production of IFN-γ (76-79).

IFN-γ plays an important role in regulating key components involved in the MHC class I and II processing and presentation machinery. Previous studies have shown that IFN-γ induces MHC class I and II synthesis and expression (88-90), regulates peptide processing, compartmentalization, MHC loading and MHC/peptide complex delivery to the cell surface (91-93), and qualitatively influences presentation of cryptic MHC class I T cell epitopes (94). IFN-γ induces the exchange of the three catalytic subunits (LMP2, LMP7, and MECL-1) of the proteasome complex, thus forming the so-called "immunoproteasome" (95), which allows for processing and presentation of otherwise cryptic epitopes. Furthermore, IFN-γ induces the expression of PA-28, a proteasome activator, that is able to increase the proteolytic efficiency of the 20S proteasome subunit (96).

Anti-tumor Th1-type $CD4^+$ T cells, however, appear inhibited in many cancer patients (71, 97, 98), as reflected by decreased proliferation and T cell receptor (TCR) signaling (99), as well as, by increased frequencies and activity of regulatory T cells (100, 101). While Th1-type responses have been associated with spontaneous or therapy-induced regression of tumor lesions (98, 102), tumor infiltrating lymphocytes isolated from patients with progressive lesions have been generally reported to exhibit dominant Th2-type (secreting IL-4, IL-5) or regulatory (Th3)-type (secreting IL-10, TGF-β1) responses (97, 98, 102).

1.7. $CD8^+$ T Cell-Mediated Immunity

Past studies have shown that in order for tumors to be rejected by the immune system, a tumor-specific $CD8^+$ T lymphocyte response must be stimulated and sustained in cancer patients. Since tumor cells are considered to be poor APCs due to their inhibitory properties and lack of co-stimulatory molecules such as B7.1 and B7.2 [27], naïve $CD8^+$ T lymphocytes need to be activated by mature DCs presenting TAA-derived epitopes. Upon recognition of their specific peptide, anti-tumor $CD8^+$ T cells undergo a proliferative burst and consequently differentiate into effector/memory cells. Naïve $CD8^+$ T cells are efficient producers of IFN-γ and TNF-α at early time-points after their initial priming. Furthermore, they efficiently synergize with CD40L-expressing naïve Th cells in the optimal activation of DCs in association with enhanced APC secretion of IL-12p70, the key Th1-inducing cytokine (103). Following the interaction with DCs, responding T cells undergo a developmental transformation to become effector cytotoxic T lymphocytes (CTL) and acquire the ability to kill their target cells after specific antigen recognition (104). Once activated, CTLs become "serial" killers (i.e. able to kill multiple targets; ref. (105)). Perforin, granulysin, and granzymes stored in pre-formed lytic granules within CTLs are secreted within the T cell/target cell interface, with perforin and granulysin forming pores in the target cell membrane, resulting in the sensitization of target cells to granzymes (106-108). Granzyme B, that is also secreted, induces apoptosis by directly activating target cell caspase-3 (109) and/or by destabilizing the mitochondrial membrane (110), while Granzyme A causes single-strand DNA breaks and apoptosis via a slower lytic pathway (111).

Another way CTLs induce cell death is by engaging tumor necrosis factor receptors (TNFR) on target cells. While TNFR family members vary in their primary sequence, all of them

contain a homologous intracellular death domain (112). The best-known receptor/ligand pair in this family is Fas and FasL (CD95/CD178). When the CTL TCR are engaged and activated by MHC class I complexes, T cells upregulate FasL expression (113). Just like the rest of the TNF family members, FasL is a homotrimeric protein that binds to 3 Fas receptors on CTL target cells (114). Once bound, the death domains of the 3 Fas receptors are clustered, allowing for the recruitment of pro-apoptotic adaptor proteins (e.g. FADD) via interactions with the death domains on the adaptor proteins. The secondary adaptor proteins then induce apoptosis in a caspase-8 dependent manner (115).

1.8. Regulatory T Cells

In humans, Treg cells represent approximately 1-3% of circulating CD4+ T cells (116), and are concentrated within the $CD4^{+}CD25^{hi}$ (CD25: IL-2 receptor α-chain) subset of $CD4^{+}$ T lymphocytes (117) that express *FoxP3*, a gene that encodes a transcription factor required for Treg development and function (118). These cells were initially described as a subpopulation of suppressor T cells that mediate immune tolerance by suppressing autoreactive T cells (119). Their physiological role in healthy individuals is to protect the host against the development of autoimmunity by regulating immune responses against antigens expressed in normal tissues. Indeed, Treg cells have been shown to recognize self-antigens more efficiently than other T cell subsets (120). This has been further substantiated by observations that animals deficient in Treg cells developed severe autoimmune diseases (116, 121). $CD4^{+}$ Treg can be grouped into two major subsets: 1) naturally-occurring Treg (nTreg) produced in the thymus and that exert

immunosuppressive effect by cell-to-cell contact, and 2) Th3 or Tr1 cells which are induced peripherally and suppress immune responses via secretion of IL-10 and TGFβ (122).

nTreg cells serve the important role of maintaining peripheral immune tolerance, and are largely composed of $CD25^{+}CD62L^{+}$ T cells and natural killer T (NKT) cells. Cell-to-cell contacts mediated through membrane-associated receptors, such as CTLA4, appear critical for their suppressive capacity. Expression of this receptor is increased on Treg, and CTLA4-specific antibody was shown to inhibit the Treg-induced immunosuppression in autoimmunity animal models (123). Another possible receptor involved in this process is glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR; TNFRSF18). However, there is still insufficient evidence to support a causal linkage of this receptor with nTreg function (123).

Previous studies have shown that elevated numbers of Treg cells can be found in the tissues of advanced cancer patients (124) and that high Treg frequencies are associated with reduced overall patient survival (125). Treg cells require TCR ligation and IL-2 to become activated, after which, they can mediate immune suppression in an antigen-independent manner (126, 127). Normally, Treg cells are anergic (i.e. incapable of proliferation and cytokine production in response to conventional T cell stimulation) *in vitro*, however this anergy can be broken by the addition of high doses of exogenous IL-2. Recombinant IL-2 (rIL-2), which is commonly used as an immunotherapeutic agent in cancer patients, has been implicated as playing a major role in the generation and maintenance of Treg cells. Patients with pediatric sarcoma that had been treated with cyclophosphamide (CY)-based chemotherapy followed by a peptide-based tumor vaccine in conjunction with systemically-administered rIL-2 had a marked increase in the number of Treg in their circulation as compared to patients that had not been treated with rIL-2. These cells were not regenerated in the thymus, but were enriched by amplification of circulating $CD4^{+}CD25^{hi}$ T

lymphocytes that survived chemotherapy-induced lymphopenia (128). A number of murine studies have also shown that depletion of Treg cells using anti-CD25 antibodies leads to a more effective anti-tumor immune response, culminating in the prolonged survival of tumor-bearing animals (129-131). Furthermore, deletion of $CD4^{+}CD25^{hi}$ circulating T lymphocytes using a rIL-2 diphtheria toxin conjugate DAB_{389}IL-2 (also known as ONTAK) allowed for the significantly improved stimulation of tumor-specific T cell responses in renal cell carcinoma (RCC) patients following immunization with RNA-transfected DCs, when compared with vaccination alone (132).

In human and mouse neoplasia, Treg cells accumulate in tumors, draining lymph nodes, and the blood stream (133, 134). The mechanisms that lead to Treg cell accumulation in tumor-bearing hosts are still largely unknown. Most current evidence suggests that during tumor progression, DC exposed to the tumor microenvironment acquire the capacity to secrete TGF-β and to stimulate the expansion of nTreg cells through signals mediated through the TGF-β-receptor II (135). These DCs appear to be of an immature, myeloid phenotype that lack expression of co-stimulatory molecules that are needed for promoting antigen-specific T cell responses.

Antigen presentation by immature DCs *in vivo* is considered to be an important pathway by which tolerance to "self" antigens is maintained. This occurs by inhibition of T cell proliferation, the induction of anergy within a cohort of antigen-specific T cells, as well as, the induction of immunosuppressive Treg cells (136). Immature DC have been shown to induce both $CD4^{+}$ and $CD8^{+}$ IL-10-producing Treg (123, 137). Interestingly, CD40 expression by DC has been implicated as a key factor in Treg induction, since antigen-loaded DCs which lack CD40

prevent T cell priming, suppress previously primed immune responses and induce IL-10-secreting $CD4^+$ Treg cells (138).

1.9. Dendritic Cells (DCs)

TAAs have been utilized as active immunogens in numerous anti-tumor vaccine studies (6, 7). Various vaccine strategies have been developed to maximize the therapeutic effect of these antigens. One of the most effective methods utilized so far involves the use of DCs pulsed with tumor peptides or proteins, or transfected with TAA cDNA to induce anti-tumor immunity (139-143). DCs are considered to be the most effective APCs for the priming and maintenance of anti-tumor immunity (144-146) and are considered to be the only APCs capable of productively activating naïve T cells (147). They take up antigens within their microenvironment in the periphery and process them through the endogenous and/or exogenous pathways (45, 49, 146). Soluble or particulate antigens are typically captured by "immature" DCs through phagocytosis, pinocytosis and receptor-mediated endocytosis (e.g. Fc receptors, integrins, C-type lectins, and "scavenger receptors" LOX-1 and CD91) (137). Immature DC also express low surface levels of HLA molecules, CD80, CD86, and CD40 (137), and commonly express the chemokine receptor CCR6 (148). Once they take up antigen (and receive maturational or environmental "danger" signals), DCs migrate to draining lymph nodes where they may efficiently prime and expand anti-tumor T cells. During this time, DCs decrease their ability to uptake antigen and increase their capacity to (cross)present antigens to T cells via their MHC class I and II complexes. These DCs are typically "mature" DCs. Such DCs express increased levels of MHC class I and II complexes as well as co-stimulatory molecules such as CD80, CD86, and CD40 (49, 137), upregulated levels of CD83 (149), downregulated levels of

CCR6 and upregulated levels of CCR7 (150). These phenotypic changes allow mature DC, as compared to immature cells, to not only productively activate naïve T lymphocytes, but to form long-lasting "synaptic" interactions with these responder T cells, even in the immediate absence of antigens (151). This allows the interacting lymphocytes sufficient time to "scan" DC-presented antigens, allowing for consequent cognate signaling into the specific T cell.

1.9.1. Lymphocyte Polarization Depends on the Subtype of Stimulating DC

The fate of naïve T cells upon exposure to Ag is determined by three signals that are provided by DCs: 1) ligation of TCRs by DC-expressed MHC-peptide complexes, 2) engagement of DC-expressed co-stimulatory molecules, without which lymphocytes may become anergic, and 3) DC secretion of polarizing cytokines. The secreted cytokine profile of the stimulating DC determines the type of responder T lymphocyte functional polarization. IL-12, IL-18, IL-23 and IL-27 polarize toward Type-1 responses, while chemokine ligands CCL2, CCL17, CCL22 or the absence of IL-12p70 skews the response towards a Type-2 result (137, 145). The DC cytokine secretion profile depends on many factors including: the DC subtype, the local environment and anatomic location of the DC and the type of maturation stimulus received by DCs (152).

Conditions under which DCs are primed are important for their cytokine profiles, and therefore the resulting class of immune responses resulting from their stimulation. DC1 (myeloid) and DC2 (plasmacytoid) subtypes stimulate Type-1 and Type-2 cells, respectively (153). DC1 are commonly associated with monocyte-derived DCs that typically promote Th1 differentiation, in part due to their secretion of IL-12p70 (153). DC2 are represented by

$CD4^+CD3^-CD11c^-$ plasmacytoid cells that induce Th2 differentiation of $CD4^+$ T cells via mechanisms that do not appear to involve IL-4 or IL-12 (153, 154). DC2 precursors are natural IFN-producing (NIP) cells that are the primary producers of IFN-α and IFN-β *in vivo* (155). Furthermore, a TGF-β-secreting subset of DCs (DC3) has been defined in the tumor microenvironment. DCs that are exposed to tumor cells can acquire the capacity to secrete TGF-β and to stimulate the expansion of nTreg cells through TGF-β-receptor II (135).

Toll-like receptors (TLRs) have been implicated as playing important roles in the process of DC polarization. An original belief held that TLR triggering always resulted in the development of DC1, however it has now been shown that ligation of TLRs may also promote the development of non-Type-1 DCs. In particular, signals mediated through Toll-like receptor 2 (TLR2), which is expressed on most $CD11c^+$ (myeloid) DCs, may induce DC secretion of either IL-23 or IL-10 depending on the specific TLR ligands evaluated. TLR2 forms heterodimers with TLR1 when triggered by bacterial lipoproteins, but when engaged by mycoplasma-derived lipoproteins, they form heterodimers with TLR6 (145). Unlike activation via TLR4 (by LPS) leading to DC production of IL-12, TLR2 ligation by bacterial lipoproteins induces DC expression of messenger RNA encoding the p40 and p19 subunits of the Type-1-polarizing cytokine IL-23, but not the p35 subunit of IL-12 (156). On the other hand, mycoplasma-derived lipopeptide 2 induces DC production of IL-10, but not IL-12, and these resulting DCs, induce unpolarized T-cell (i.e. Th0-type) responses (157). These observations suggest that TLR2 signaling may dictate distinct cytokine profiles secreted by DCs, resulting in differential polarizing effects on T cells primed by these APCs.

1.9.1.1. Role of Interleukin-12 in Promoting Therapeutic Immunity

Interleukin-12 (IL-12) is one of five heterodimeric cytokines that belong to the IL-12 family (others include IL-23, IL-27, CLC-sCNTFR, and CLC-CLF-1). It was originally identified as cytotoxic lymphocyte maturation factor, and is composed of two covalently linked protein chains, p35 and p40, that form the p70 heterodimer that is produced in a restricted manner by antigen presenting cells (DCs, monocytes, macrophages, neutrophils). It binds to the IL-12 receptor (IL-12R) complex that is composed of two chains, IL-12Rβ1 and IL-12Rβ2. IL-12Rβ1 binds IL-12 p40 and is associated with Tyk2, while IL-12Rβ2 recognizes the p70 heterodimer or the p35 and is associated with Jak2 (158). Most of the biological responses to IL-12 are mediated through the STAT4 signaling pathway, and optimal Th1 polarization is only achieved in the continuous presence of IL-12 (158, 159).

IL-12 p70 effectively stimulates IFN-γ production by T, NK, and other cell types, and is a potent inducer of Th1 cell differentiation (158). It is also capable of irreversibly repolarizing Th2 $CD4^+$ T cells towards the Th0/Th1 phenotype, and this change is accompanied by suppression of GATA-3 (Th2-specific transcription factor) and induction of T-bet (Th1-specific transcription factor) (160). While effective at inducing expansion and optimal activation of Th1 $CD4^+$ T cells, its role in $CD8^+$ T cell generation is somewhat less studied. *In vitro* priming of T cells in the presence of IL-12p70 increases the generation and improved survival of memory $CD8^+$ T cells in mice after adoptive transfer of activated cells (161). However, IL-12 p40- and IL-12Rβ1-deficient mice showed similar levels of primary and memory $CD8^+$ T cell responses, when compared to wild-type mice, implying that endogenous IL-12p70 is not critical for the generation

of IFN-γ-secreting, $CD8^+$ cytotoxic T lymphocytes *in vivo* (161, 162). Together, these results suggest that IL-12p70 can serve as an important, but non-essential regulatory factor for the development of $CD8^+$ T cells.

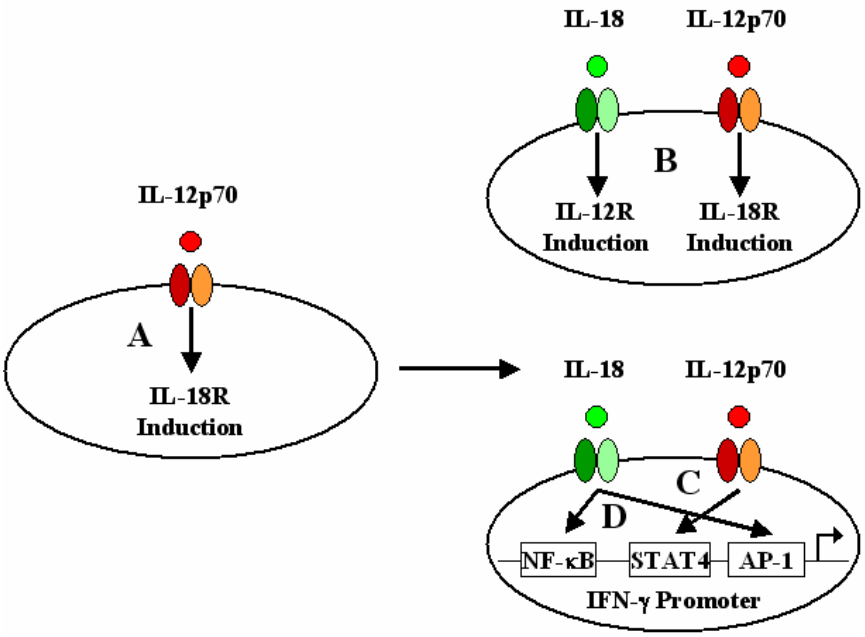
1.9.1.2. Role of Interleukin-18 in Promoting Therapeutic Immunity

Interleukin-18 (IL-18) is a member of the IL-1 cytokine superfamily that plays an important role in regulating immune responses. IL-18 is produced by antigen presenting cells (DCs, monocytes), as well as Kupffer cells (phagocytes lining the hepatic sinusoids), keratinocytes, osteoblasts, pituitary gland, adrenal cortical cells, and intestinal epithelial cells (163). It is initially synthesized as a biologically inactive precursor, pro-IL-18. This precursor is then cleaved by caspase-1 (IL-1β-coverting enzyme) to form the biologically active mature cytokine that can be secreted. Furthermore, IL-18 can be cleaved by caspase-3 yielding an inactive product. While these proteases are also involved in apoptosis, there is no apparent relationship between apoptosis and IL-18 production (163).

While it was initially characterized as IFN-γ-inducing factor (83), later studies have shown that IL-18 is a unique cytokine capable of inducing either Th1 or Th2 polarization, depending on the type and context of stimuli, the ambient cytokine priming milieu, and underlying genetic influences (164). IL-18 synergizes with IL-12p70 to promote IFN-γ secretion from, and proliferation of, $CD4^+$ T effector and NK cells (165, 166). IL-12p70 induces T cell surface expression of the IL-18 receptor (IL-18R) by naïve T cells (167); while IL-18 potentiates the differentiation of Th1 cells (**Figure 4**) instigated by IL-12 (168).

Figure 4. Synergistic action of IL-12 and IL-18 in IFN-γ production from Th1 cells.

IL-12 stimulation induces IL-18R expression by naïve T cells. Once IL-18R is induced, IL-12 and IL-18 induce the reciprocal upregulation of their receptors. Upon binding to its receptor, IL-12 induces activation of STAT4, which translocates to the nucleus and binds to the IFN-γ promoter. IL-18 directly activates NF-kB and AP-1, which bind to and activate the IFN-γ promoter. For IL-12-dependent INF-γ promoter activation, the AP-1 site is also required. Synergistic action of IL-12 and IL-18 may occur via simultaneous activation of STAT4, NF-kB and AP-1(transcription factors).



Paradoxically, when cultured alone or in combination with IL-2 or IL-4, IL-18 induces IgE expression and Th2 differentiation (169). Indeed, Th2 polarization of $CD4^+$ T cells after IL-18 administration appears to involve the activation of NK T cells, that have been previously shown to be a major source of IL-4 (170). Furthermore, IL-18 has been shown to be a chemoattractant for both myeloid DC1 and plasmacytoid DC2 (171, 172).

The IL-18R is a heterodimeric cytokine composed of an IL-18-binding α chain, and a non-binding, signal transducing β chain. Once IL-18 binds to the IL-18Rα chain, the IL-18Rβ chain is recruited and induces one of several possible intracellular signaling pathways. Most commonly, IL-18R-mediated signaling induces the nuclear factor κB (NF-κB) activation cascade in a manner similar to that mediated by the IL-1R. However, studies in specific mouse knockout strains have also shown that like IL-12, IL-18 can signal via STAT-4. In fact, IL-12-induced

STAT-4 signaling is synergistically enhanced in combination with IL-18 (**Figure 4**) via NF-κB and AP-1 transcription factors (164).

1.10. Cancer Vaccines and Therapies

The three traditional therapies for the clinical management of cancer are surgery, radiation, and chemotherapy. Surgery, the process of physically removing the existing tumor from the body, is usually the first step in treating the disease. If lesions are not easily accessible for surgical resection, tumors are typically treated with locoregional radiation therapy. Radiotherapy involves exposing cancerous tissues and its supportive vascular bed to various forms of radiation in order to cause DNA damage, forcing these tissues to undergo differential apoptosis. If there is a possibility that the disease has metastasized (spread to other tissues) or if the disease affects leukocytes, chemotherapy is commonly applied as a systemic therapy. This generally involves the administration of chemicals that inhibit the ability of cancer cells to survive and replicate. While these three methods have showed some degree of clinical success, their long-term benefits, particularly in the cases of radiotherapy and chemotherapy, are generally perceived as limited. These treatments are often very destructive not only to tumor cells, but to normal tissues as well. Furthermore, recurrence of disease is very common and is frequently found to be resistant to the original treatment modality. For these reasons, it is necessary to establish novel therapy methods for tumors that will provide more specific treatment, and long-term protection from recurrence. Various immunotherapy strategies have the potential to provide these benefits.

Immunotherapeutic strategies utilize various components of the immune system to promote immune responses against a specific disease, such as cancer. There are three lines of evidence

that suggest that cancer immunotherapy can be beneficial in humans: 1) immunosuppressed transplant recipients display higher incidences of non-viral tumors, such as melanomas, colon, lung, pancreas, bladder, kidney, and endocrine system cancers than immunocompetent control populations (173); 2) the presence of lymphocytes within the tumor is often a positive prognostic indicator of patient survival (174), and 3) a minority of cancer patients (< 5%) are able to develop spontaneous innate and acquired immune responses to the tumors they bear (7, 175, 176). One of the first pieces of clinical evidence suggesting that the manipulation of the immune system could be beneficial as a cancer therapy involved the administration of interleukin-2 (IL-2, which is a lymphocyte proliferation-inducing cytokine produced by T cells that has the ability to induce proliferation of T cells that have recognized their specific antigen) (177). In that study, IL-2 treatment of patients with metastatic renal carcinoma or metastatic melanoma induced tumor regression in 15-20% of treated patients. Since then, great progress has been made in understanding the immune response to tumors, and based on this knowledge a number of different immunization strategies designed to further augment the tumor-specific T cell immune response in patients have been developed and tested.

1.10.1. Pre-clinical experience of DC-based cancer vaccines and therapies

Over the past several decades, tumor immunology has increasingly focused on approaches to define, accentuate and sustain T cell-mediated immunity as a means to effectively prevent or regulate tumor development and progression. With the discovery of TAAs and their derivative MHC-presented epitopes, the molecular targets of immune reactivity have begun to be resolved. Multiple active specific immunotherapy (i.e. immunization with specific TAA) strategies have

been employed, and utilizing the innate adjuvant properties (antigen uptaking, processing, and presenting ability) of autologous (i.e. derived from the same donor) DCs emerged as the most effective one for priming and maintenance of TAA-specific responses (144-146).

A number of DC-based approaches tested *in vitro* and in animal models have been evaluated as the basis of understanding the potential clinical value of DCs. These strategies differ in the type of tumor, source of DCs (directly sorted out of blood or solid tumor; monocyte-derived, bone-marrow-derived, $CD34^+$ hematopoietic precursor-derived) type of TAA used, method of loading DCs with antigen (TAA-derived peptide, whole protein, TAA gene expression, tumor cell lysate, tumor apoptotic bodies, DC-tumor cell fusion hybrid), method of gene introduction (recombinant retroviral or adenoviral vectors, plasmid transfection, gene gun) and/or DC maturation stimuli (cytokines, CpG motifs, microbial membrane motifs). These strategies have shown promise for treating or preventing cancer, and several important conclusions have been reached as a consequence of these studies.

Tumors are not homogenous tissues that can be treated with a single vaccination tactic. They vary in TAA repertoire, as well as immuno-evasive properties. These variations are observed between patients, tissues affected and at different time points in the malignant process (178, 179). Such differences require strategies that are "tailor"-made for the specific tumor and a specific patient. Since the expression of TAA is not uniform between tumors, it seems preferable to co-administer several antigens rather than only one, to avoid the possibility that the single TAA will prove non-immunogenic or that its epitopes may have been downregulated on the tumor cell membrane *in situ*.

There are three criteria that are believed to be required in an effective anti-tumor therapy: 1) the ability to promote a sufficient number of high-avidity effector T cells that are capable of

recognizing tumor cells; 2) the ability to support the effective trafficking and penetrance of immune cells into tumor lesions; and 3) the ability to maintain anti-tumor effector cells in a functional manner within the tumor lesion for an extended period of time. As Th1-type responses have been associated with spontaneous or therapy-induced regression of tumor lesions (98, 102), DC-based therapies should stimulate high-avidity, Th1-type T cell responders capable of penetrating the tumor microenvironment and appropriately responding to the disease. To achieve this, DCs need to be of a mature phenotype, and they should secrete a dominant balance of Th1-polarizing cytokines in order to override the inhibitory effects of the tumor microenvironment (137, 145).

Another important factor in inducing TAA-specific immunity is the format of antigen used. The antigen format used in vaccination impacts which T cell subset may be preferentially stimulated. Synthetic peptides can be used to stimulate either $CD4^+$ or $CD8^+$ T cell populations. If DCs are loaded with whole TAA protein, the antigen is introduced to the endosomal, MHC class II processing pathway and peptides derived from it will primarily stimulate $CD4^+$T cell responses. On the other hand, if DCs are infected/transfected with TAA cDNA, the protein may be preferentially expressed in the cytosol where it enters the classical MHC class I antigen processing pathway, and peptides derived from it may prompt primarily $CD8^+$ T cell responses (59, 62, 146).

1.10.2. IL-12-based therapy of cancer: recombinant protein vs. engineered DC

One strategy tested to enhance Th1-type responses *in vitro* and *in vivo* has been the administration of various forms of IL-12p70. Furthermore, IL-12 combined with other

immunotherapy approaches, particularly co-administration of IL-18, has been shown to achieve even better immuno-stimulatory results. While IL-12 has demonstrated significant efficacy in inducing effective anti-tumor T cell responses in experimental models, clinical trials with systemic recombinant IL-12 (rIL-12) have displayed unacceptable toxicities. Common toxicities included fever/chills, fatigue, nausea, vomiting, headache, anemia, neutropenia, lymphopenia, hyperglycemia, thrombocytopenia, hypoalbuminemia, and even death in humans (180-185). Mouse studies have shown that these toxicities are largely mediated by IFN-γ overproduction by NK cells (186). IL-12-mediated toxicity was particularly exacerbated with co-administration of recombinant IL-18 (rIL-18). Mouse studies have also shown that simultaneous administration of rIL-12 and rIL-18 causes, in a STAT4-dependent manner, severe systemic inflammation due to NK cell-secreted IFN-γ and 100% mortality (187).

One possible way to eliminate these toxic effects is to utilize gene transfer methods to confine IL-12 production within the tumor environment, thereby preventing systemic toxicity. Tumor cells, dendritic cells, or autologous fibroblasts have been transfected with recombinant adenoviruses or retroviruses encoding IL-12 cDNA, the injected intratumorally/perilesionally in order to focus cytokine production. These approaches have demonstrated increased efficacy and acceptable safety profiles (188-190). Indeed, my group has previously shown in murine models that DC engineered to secrete both IL-12p70 and IL-18 *ex vivo*, and subsequently injected intratumorally, promote acute tumor rejection in concert with enhanced Th1-type immunity and determinant spreading in the curative anti-tumor CTL repertoire (191).

1.10.3. Enhancement of TAA-Specific T Cell Responses Using Epitope Analogues

In the past it was believed that individual T cell clones were capable of distinguishing and responding to a unique epitope sequence presented in the context of an autologous MHC complex. Recent studies have instead suggested that a fair degree of T cell cross-reactivity exists and is in fact necessary to maintain an immune system with sufficient flexibility to adapt to a continuously changing antigenic environment. Indeed, T cell clones thought to be specific for an antigen have been shown to recognize peptides differing considerably in their amino acid sequences (192-194). "Analogue" or "heteroclitic" peptides refer to those peptides that share a high degree of homology with naturally-occurring, wild-type tumor epitopes, and induce cross-reactive T cells to their homologues. Most of these studies examined $CD8^+$ T cell responses against 9-mer peptides. While the anchor residues at positions 2 and 9 of HLA class I-presented epitopes have been shown to be highly restricted, the other amino acids of reactive peptides differed at as many as six or seven of the remaining positions. Importantly, a large proportion (one-third to one-half) of analogue peptides stimulated T cells to produce IFN-γ at concentrations far lower than that of the naturally-occurring peptide, suggesting the higher functional avidity of clonal TCR for analogue peptides presented by MHC class I molecules. In fact, analogue epitopes have been shown to be more effective at breaking immunological tolerance than cognate wild-type epitopes (195). *In vivo* studies further substantiated these observations. One study showed that immunization with a $gp100_{209\text{-}217}$ (210M) heteroclitic melanoma antigen peptide promoted the development of circulating effector-memory T cells that were reactive against the wild type $gp100_{209\text{-}217}$ epitope (196). Individual amino acid substitutions have also been associated with differential cytokine responses by MART-$1_{26\text{-}35}$-specific $CD8^+$ T

lymphocytes. Substitution of the N-terminal amino acid of this 10-mer dictated whether the T cell response would be Type-1 or Type-0 (both Type-1 and Type-2 cytokines secreted) (197).

Analogue peptides have also been observed in nature, and these cross-reactive epitopes have been coined "epitope mimics". Epitope mimicry has been described as a potential mechanism underlying the induction of autoimmune diseases due to pathologic T cells primed against infectious microorganisms that cross-react against host proteins in susceptible individuals. Diseases such as viral myocarditis, lyme disease, rheumatoid arthritis (198), multiple sclerosis (199), and virus-induced autoimmune diabetes (200, 201) have long been considered to be initiated or exacerbated by microbial pathogens. From this observation came an idea that immunotolerance to TAA could be broken by employing mimicking epitopes to stimulate TAA-specific lymphocytes. A study performed by Loftus, et al. showed that the HLA-A2-presented MART-1_{27-35} epitope bears sufficient sequence or conformational homology to peptides derived from microbial proteins to which many individuals may have become naturally primed against, allowing for functional T cell-mediated cross-reactivity (202). Therefore, one may hypothesize that there is a limited flexibility of TCR antigenic specificity that could potentially be exploited in order to stimulate TAA-specific lymphocyte responses in patients who may have become functionally tolerant to their progressive TAA.

1.10.4. Poor clinical results for DC-based vaccines: limited by lack of Type-1 Th responses?

In 2004, Rosenberg and colleagues published a "watershed" article on the state of active specific immunotherapy cancer trials (203). They analyzed 9 years worth of their own data, as

well as data from 35 reports of vaccine trials performed outside of NCI. Overall, they reviewed 1,306 solid tumor patients using the modified Response Evaluation Criteria in Solid Tumors (RECIST; clinical response is defined as at least 50% reduction in the sum of the products of the perpendicular diameters of all lesions without the 25% growth of any lesion or the appearance of new lesions). The picture they portrayed was very grim, with an overall therapy-induced tumor regression rate of only 3.3% (in patients vaccinated with either synthetic peptides, "naked" DNA, peptide-pulsed dendritic cells, recombinant vaccinia viruses, recombinant fowlpox viruses and recombinant adenoviruses expressing various TAAs). Of these immunization methods, peptide-pulsed DCs seemed to be the most effective strategy, with 7.1% of treated patients exhibiting tumor regression. While this frequency of response was higher than those frequencies found for other vaccination strategies, the response was still very low. Furthermore, the overall vaccine treatments of metastatic melanoma patients, when successful, were predominantly effective in patients with disease at cutaneous or lymphatic sites, but not those with disseminated, visceral disease.

Unlike chemotherapy, immunological vaccines do not follow linear dose-effect kinetics. Instead, these strategies depend on the complex interplay of a number of variables, including the administration method, minimum effective dose, vaccination schedule, type of immunological adjuvant and the existing state of host immunological competence. The slightest discrepancy in any of these variables can affect the patient outcome following therapeutic immunization. The majority of patients treated in theses studies were late-stage metastatic patients that were heavily pre-treated with conventional chemotherapeutic reagents prior to immunizations. Not only do such tumors have potent immuno-inhibitory functions, but the implemented chemotherapies have also been shown to non-specifically decrease the number of leukocytes in recipients, making

metastatic patients severely immuno-compromised. It was reported that pediatric sarcoma patients treated with chemotherapy followed by a peptide-based tumor vaccine in conjunction with recombinant IL-2 had a marked increase in the number of Treg in the circulation, as compared to patients that were treated without IL-2 (128). These observations suggest that the conditions under which patients were immunized was most likely sub-optimal for the priming of clinically-meaningful tumor-specific Th1-type T cell responses.

There are several other possibilities to explain why the clinical response to these vaccines was so poor. The immune system, while potentially effective, is limited by the frequency of responders that can be stimulated by vaccinations. Even if TAA-specific responses were stimulated by immunization, it is possible that the bulk tumor mass was too large at the time of the treatment for the available effector T cell population to eliminate it efficiently. It is also possible that while the vaccine-targeted antigens are expressed by the tumors, their derivative peptides are not presenting on the cell surface in the context of MHC class I molecules, making the tumor cells effectively invisible to $CD8^+$ T cell recognition. Another possibility is that TAA used for vaccinations were not expressed by targeted tumors and/or that TAA-derived peptides used were not effective at eliciting high avidity T cell responders. Therefore, instead of tailoring the vaccine to the individual patient's TAA repertoire, these individuals may have been treated with arguably irrelevant or weakly-immunogenic antigens that yield a clinically-meaningless immune response. Due to the potential limitations under which these clinical trials have been performed to date, novel vaccine strategies need to be developed that have the potential to improve therapeutic outcome.

1.11. Basis for This Project

The breaking of immunological tolerance to TAA epitopes has been a central interest of tumor immunologists in the last decade. As previously discussed, there are two main problems when it comes to breaking immunological tolerance. Firstly, most TAA are considered to be "self"-antigens that are commonly found in many healthy tissues, and the immune system regards these as potential autoimmune targets. Most of the lymphocytes specific for these antigens either undergo thymic and peripheral deletion or acquire a regulatory phenotype. That means that in most cases only low-avidity and/or regulatory T lymphocytes specific for these antigens remain in the circulation, limiting the sensitivity, magnitude, and appropriate functional polarization of T cell immune responses to tumors. Secondly, tumors exhibit immunosuppressive capacity that may protect them from immunosurveillance. Lymphocytes that are recruited into the tumor site may be neutralized, ablated or acquire a regulatory phenotype. Multiple strategies used to rescue and enhance tumor-specific T lymphocyte responses have been contemplated over the past decade, with a number of these predicated on the antigen uptaking, processing, and presenting capabilities of DCs. Two DC-based strategies that we evaluated were founded on previous observations that antigen-specific responses can be enhanced by utilizing T cell epitope analogues, and that the IFN-γ-inducing cytokines, IL-12 and IL-18, can synergize in the enhancement of Type-1 epitope-specific responses.

1.12. Summary

Due to its wide range of expression among cancer types, its innate immunogenicity, and its restricted expression in normal tissues, MAGE-A6 is an attractive target on which to base cancer vaccines and immunotherapies. While MHC class II-restricted peptide epitopes have been previously reported for MAGE-A6, their clinical monitoring and immunotherapeutic value is limited due to the fact that the relevant HLA-DR alleles that present these epitopes to T cells are expressed by only a minority of patients. Given these limitations, we sought to define MAGE-A6 T-helper epitopes that would be immunogenic to a high frequency of responders regardless of their HLA-DR phenotype. I used MAGE-A6 peptide-pulsed DC as *in vitro* stimulators to ascertain to whether these novel MAGE-A6 epitopes could elicit specific T cell responses. To test for the natural-processing and MHC-presentation of these epitopes, I used autologous donor monocytes loaded with newly constructed recombinant MAGE-A6 (rMAGE). Two of the peptides investigated elicited epitope-specific, physiologically relevant $CD4^+$ T cell responses in a large cohort of randomly-selected melanoma patient and normal blood donors. Given particularly strong primary *in vitro* sensitization of normal donor $CD4^+$ T cells by these epitopes, and theorizing that this could represent cross-reactivity against environmental stimuli, I identified homologous peptides of microbial origin for each of these immunogenic peptides. $CD4^+$ T cells stimulated *in vitro* with one of these microbial peptides cross-reacted against autologous monocytes pulsed with the MAGE-A6 homologue peptide or rMAGE, as well as, HLA-matched MAGE-$A6^+$ melanoma cell lines. These $CD4^+$ T cells responses appeared heteroclitic in nature and at a higher functional avidity than those primed with the MAGE-A6

peptide itself. This is a significant discovery because these results suggest that tolerance to tumor self-antigens can potentially be broken using mimicking microbial epitopes.

The observation that anti-tumor Th1-type $CD4^+$ T cells are inhibited in many cancer patients strongly suggests that the future immune-based therapies must overcome existing deficiencies in Type-1 anti-tumor $CD4^+$ T cell responses in cancer patients with active disease in order to be clinically effective. In my second study, I generated and applied novel recombinant adenoviral vectors encoding Th1-polarizing human IL-12p70 (Ad.IL-12) and the mature form of human IL-18 (Ad.IL-18) to engineer human DC *in vitro*, and subsequently evaluated the ability of these gene-modified antigen presenting cells to promote Th1-type $CD4^+$ T cell responses against the MAGE-A6 tumor antigen in HLA-$DR4^+$ normal donors and patients with melanoma. My results indicate that DC co-infected with both Ad.IL-12 and Ad.IL-18 that are consequently loaded with tumor peptides or recombinant tumor antigen effectively promoted *in vitro* epitope-specific Type-1 $CD4^+$ T cell immunity in patients with cancer who may display existing immune dysfunction.

My studies are innovative for the following reasons: firstly, two novel Th epitopes derived from a common tumor antigen have been defined that will dramatically expand the range of cancer patients and types of cancer that may be treated with peptide-based vaccines; secondly, this is the first study that shows that tumor-specific $CD4^+$ T cells can be stimulated using heteroclitic microbial peptides, that may circumvent limitations associated with tolerance mechanisms linked to self tumor antigens. Thirdly, we are the first group to describe an adenoviral vector encoding the mature form of human IL-18, which in conjunction with IL-12, appears to define a combinational cytokine gene therapy that overcomes tumor-associated inhibition of Type-1 T cell responses in patients. These results can serve as the basis for the

development of novel prospective immunotherapy protocols designed to elicit, enhance and sustain the in vivo efficacy of therapeutic MAGE-A6-specific T cells.

Scope of This Thesis

MAGE-A6 was previously characterized as a TAA in the setting of melanomas (Mel), as well as renal cell carcinomas (RCC). I sought to define MAGE-A6 T-helper (Th) epitopes that would be immunogenic to a high frequency of responders regardless of their HLA-DR phenotype. Based on a computer algorithm analysis designed to identify peptides likely to have "promiscuous" HLA-DR-binding tendencies (poly-DR), I defined two novel MHC class II-restricted MAGE-A6 epitopes and confirmed that a previously-defined peptide was immunogenic in a high frequency of normal donors and patients with melanoma that were evaluated. Each of these epitopes was naturally processed and cross-presented by monocytes after these APCs were pulsed with recombinant, full-length MAGE-A6 protein. The most immunogenic peptides tested shared significant homologies with multiple microbial sequences according to GenBank homology search. $CD4^+$ T cells stimulated *in vitro* with one of the microbial homologues exhibited a higher functional avidity for target cells presenting the MAGE-A6 epitope than T cells evoked against the MAGE-A6 peptide itself. Furthermore, they recognized MAGE-A6 protein-loaded, autologous monocytes as well as MAGE-$A6^+$, HLA-DR-matched melanoma cell lines. I then sought to enhance Th1-stimulating ability of DCs by engineering them to secrete high levels of the IFN-γ-inducing cytokines IL-12p70 and IL-18 via recombinant adenoviral infection to generate an *in vitro* stimulus capable of promoting previously-deficient patient Th1-type responses. DC co-infected with IL-12 and IL-18 vectors were more effective at stimulating MAGE-A6-specific Th1-type $CD4^+$ T cell responses than DC infected with either of the cytokine vectors alone, control empty virus or uninfected DC. Furthermore, I show that IL-12 and IL-18 co-infected DCs loaded with recombinant MAGE-A6 protein (rMAGE) and used as *in vitro* stimulators, promote Th1-type immunity that is frequently directed against multiple MAGE-A6-

derived epitopes. Based on these results, I believe that the MAGE-A6 and microbial Th epitopes described here may prove useful in the development of cancer vaccines or immunomonitoring strategies for patients harboring MAGE-A6^{+} tumor lesions, without limiting patient accrual due to a requirement for expression of specific HLA haplotypes. Furthermore, I believe this Th1-enhancing modality may prove clinically useful as a vaccine platform to promote the recovery of tumor antigen-specific, Th1-type CD4^{+} T cell responses in patients with cancer.

Preface Chapter 2

Using DC1-based *in vitro* vaccinations as a model system, my goal was to define MAGE-A6-derived epitopes recognized by $CD4^+$ T-lymphocytes. I analyzed T cell responses to 4 putative epitopes that were hypothesized to be immunogenic to a high frequency of responders regardless of their HLA-DR phenotype. Two novel (MAGE-$A6_{172\text{-}187}$ and MAGE-$A6_{280\text{-}302}$) epitopes and a previously-defined (MAGE-$6_{140\text{-}170}$) epitope were recognized by $CD4^+$ T cells isolated from most normal donors and patients with melanoma that were evaluated. Peptide-specific $CD4^+$ T cells also recognized autologous monocytes pulsed with full length MAGE-A6 protein, supporting the natural-processing and MHC-presentation of these epitopes. Interestingly, I identified a peptide within the *Mycoplasma penetrans* HF-2 permease protein (MPHF2) sequence that is highly-homologous to the MAGE-$A6_{172\text{-}187}$ epitope. $CD4^+$ T cells primed with the MPHF2 peptide cross-reacted against autologous monocytes pulsed with the MAGE-$A6_{172\text{-}187}$ peptide or MAGE-A6 protein, and recognized HLA-matched MAGE-$A6^+$ melanoma cell lines. These responses appeared heteroclitic in nature since the functional avidity of MPHF2 peptide-primed $CD4^+$ T cells for the MAGE-$A6_{172\text{-}189}$ peptide was approximately 1000 times greater than for $CD4^+$ T cells primed with the MAGE-A6 peptide itself.

The studies in **Chapter 2** support the immunogenicity of three poly-DR MAGE-A6 epitopes and that these may have broad clinical utility in cancer vaccines or immunomonitoring strategies, without having to limit patient accrual due to a requirement for expression of specific HLA haplotypes. Furthermore, this study shows that it could be possible to effectively stimulate TAA-specific T cell responses using microbial peptide homologues.

2. MAGE-A6 Encodes Multiple Naturally-Processed, Promiscuous Th Epitopes, One of Which is Immunologically-Related to a Mycoplasma Penetrans HF-2 Permease-Derived Peptide

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All the results reported in this study were obtained by Lazar Vujanovic. Functional avidity assays were repeated by Maja Mandic, M.D.. A portion of ELISPOT plate analyses were performed by Walter C. Olson, Ph.D.

2.1. ABSTRACT

While T-helper (Th) epitopes have been previously reported for tumor antigen MAGE-A6, the relevant HLA-DR alleles that present these peptides are expressed by only a minority of patients, serving to limit their potential clinical utility. Given these limitations, I sought to define poly-DR presented MAGE-A6 Th epitopes that would be immunogenic in a high frequency of responders. I identified two novel (MAGE-A6$_{172\text{-}187}$ and MAGE-A6$_{280\text{-}302}$) epitopes and confirmed that a previously-defined (MAGE-6$_{140\text{-}170}$) epitope can be recognized by CD4^{+} T cells isolated from most normal donors and patients with melanoma that were evaluated. Peptide-specific CD4+ T cells also recognized autologous monocytes pulsed with recombinant MAGE-A6 (rMAGE) protein, supporting the natural-processing and MHC-presentation of these epitopes. Given strong primary *in vitro* sensitization of normal donor CD4^{+} T cells by the MAGE-A6$_{172\text{-}187}$ epitope, and theorizing that this could represent cross-reactivity against an environmental stimulus, I identified a highly-homologous peptide within the *Mycoplasma penetrans* HF-2 permease protein (MPHF2) sequence. MPHF2 peptide-primed CD4^{+} T cells cross-reacted against autologous monocytes pulsed with the MAGE-A6$_{172\text{-}187}$ peptide or rMAGE protein, and recognized HLA-matched MAGE-A6^{+} melanoma cell lines. These responses appeared heteroclitic in nature since the functional avidity of MPHF2 peptide-primed CD4^{+} T cells for the MAGE-A6$_{172\text{-}189}$ peptide was approximately 1000 times greater than for CD4^{+} T cells primed with the MAGE-A6 peptide itself. I believe that these epitopes may have broad clinical utility in cancer vaccines or immunomonitoring strategies, without having to limit patient accrual due to a requirement for expression of specific HLA haplotypes. (246 words)

2.2. INTRODUCTION

Melanoma antigen gene (MAGE) proteins are a family of closely-related molecules that were initially identified as tumor associated antigens (TAA) capable of being recognized by cytolytic T lymphocytes (CTL) isolated from the peripheral blood of cancer patients (204). MAGE genes are classified as either type I (MAGE-A, MAGE-B, and MAGE-C genes located on the X chromosome) or type II (those that are located outside of the type I MAGE genomic cluster) (9, 10). Normally, type I MAGE proteins are selectively expressed in testicular cells among somatic tissues (11). However, they can also be expressed in both pre-malignant and malignant lesions in concert with DNA hypomethylation (205). The MAGE-A proteins, composed of 12 members (i.e. MAGE-A1 through -A12), are expressed by more than half of all human cancers (26). For instance, MAGE-A6 is expressed in more than 60% of melanomas (27), 30% of renal cell carcinomas (36), and by many other cancer types, such as breast, esophageal, head and neck, bladder, and lung carcinomas (27, 37-40). This wide range of expression among cancer types, as well as the limited/lack of expression by normal tissues, has made MAGE family members (including MAGE-A6) attractive targets on which to base cancer vaccines and immunotherapies.

Previous studies have shown that melanoma is among the most responsive cancers to immunotherapy (98, 206), making it a prototype for the development of anti-tumor vaccine models. While most vaccine studies have focused on the effector $CD8^+$ T cell compartment of the anti-melanoma immune response as being most important for objective clinical responses, it is clear that anti-tumor $CD4^+$ T cell responses regulate the quality, magnitude and durability of $CD8^+$ CTL immunity *in vivo* (70, 207). $CD4^+$ T cells have been shown to play a crucial role in

the induction of effective cellular anti-tumor immune responses (69, 207). They mediate IFN-γ-mediated delayed type hypersensitivity (DTH)-like responses that can lead to enhanced cross-presentation of tumor antigens by host APCs, and consequent epitope-spreading in the anti-tumor T cell repertoire (74). Furthermore, $CD4^+$ T cells may mediate direct tumoricidal activity and inhibit tumor angiogenesis (76-79).

In the current study I analyzed three novel (MAGE-$A6_{172-187}$, MAGE-$A6_{192-214}$, and MAGE-$A6_{280-302}$), and one previously described (i.e. MAGE-$A6_{140-170}$; ref. (36)), MAGE-A6-derived peptide(s) as candidate poly-HLA-DR presented epitopes *in vitro*. I observed that the MAGE-$A6_{172-187}$ and MAGE-$A6_{280-302}$ epitopes appeared to be the most effective at eliciting Th1-type (i.e. IFN-γ) responses in both normal donors and patients with melanoma. Each of these epitopes was naturally-processed and cross-presented by monocytes after these APCs were pulsed with recombinant, full-length MAGE-A6 protein.

I also discerned that the MAGE-$A6_{172-187}$ epitope was highly homologous to, and immunologically cross-reactive with, a peptide derived from the *Mycoplasma penetrans* HF-2 permease protein (MPHF2). Exposure to this ubiquitous environmental pathogen could explain the common $CD4^+$ T cell responsiveness against the MAGE-A6 peptide I detected among normal donors after primary *in vitro* sensitization. Notably, $CD4^+$ T cells stimulated *in vitro* with this microbial homologue recognized MAGE-A6 protein-loaded, autologous monocytes as well as MAGE-$A6^+$, HLA-DR-matched melanoma cell lines. Indeed, MPHF2 peptide-based stimulation yielded $CD4^+$ T cells exhibiting a higher functional avidity for target cells presenting the MAGE-$A6_{172-187}$ epitope than T cells evoked against the MAGE-$A6_{172-187}$ itself. I believe that the MAGE-A6 and MPHF2 Th epitopes described here may prove useful in the development of cancer vaccines or immunomonitoring strategies for patients harboring MAGE-$A6^+$ tumor

lesions, without limiting patient accrual due to a requirement for expression of specific HLA haplotypes.

2.3. MATERIALS AND METHODS

2.3.1. Cell lines

Cell lines used included the melanoma cell lines Mel526 and SLM2, the SLR20 renal carcinoma cell line (208), and T2.DR4, a human B x T cell hybrid cell line expressing HLA-DR4 class II molecules (8). Cell lines were cultured in T75 culture flasks (COSTAR, Cambridge, MA), in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% HEPES, 1% L-glutamate and 1% non-essential amino acids (all reagents from Invitrogen, Carlsbad, CA), in a humidified 37^{o}C incubator under 5% CO_2 tension.

2.3.2. Isolation of Patient and Normal Donor PBMC

Peripheral blood was obtained from normal donors or melanoma patients by venipuncture with written consent, under an IRB-approved protocol. Blood was diluted 1:2 with PBS, applied to ficoll-hypaque gradients (Cellgro; Mediatech, Inc., Herndon, VA), and centrifuged at 550 x g for 25 min at room temperature. Peripheral blood mononuclear cells (PBMC) were recovered from the buoyant interface and washed three times with PBS in order to remove residual platelets and ficoll-hypaque.

2.3.3. HLA-DR Typing

To test for donor HLA-DR alleles, genotyping was performed. DNA was extracted from PBMC using the DNeasy Tissue Kit (Qiagen), according to the manufacturer's protocol, with consequent HLA-DR genotyping performed using the Dynal Allset+ SSP DR "low resolution" Kit (Dynal Inc., Lake Success, NY) with extracted DNA samples. HLA-DR4^{+} phenotype of

PBMC or tumor cell lines was also corroborated by flow cytometric analysis using the 359-F10 mAb (36, 208).

2.3.4. DC1 Preparations

Type-1 polarized dendritic cells (DC1) were generated from $CD14^+$ MACs (MACS; Miltenyi Biotech, Auburn, CA)-isolated human monocyte precursors, as previously described (24). Additional $CD14^+$ monocytes were cryopreserved at -80^{o}C and used as antigen presenting cells in ELISPOT assays.

2.3.5. $CD4^+$ T cell isolation from PBMC and in vitro stimulation (IVS)

Following monocyte separation, $CD4^+$ T cells were isolated from $CD14^-$ PBMC by magnetic cell sorting (MACS; Miltenyi Biotech), according to manufacturer's protocol, and then cryopreserved until needed. To establish DC-T cell cultures, $CD4^+$ T cells were thawed at 37^{o}C and washed in AIM-V medium (GIBCO-Invitrogen, Carlsbad, CA), then resuspended in T cell media [AIM-V supplemented with 5% human serum (GIBCO)]. DC1s were incubated for 1-3 hours in 1ml of T cell media with or without MAGE-A6 peptides (10 μg/ml) at 37^{o}C. Afterwards, DC1s were co-cultured with autologous $CD4^+$ lymphocytes at a 1:10 DC1:T cell ratio in T cell media for 11 days.

2.3.6. ELISPOT

On day 11 of IVS, the frequencies of peptide-specific $CD4^+$ T cell responders were measured using anti-human IFN-γ ELISPOT assays, as previously described (36, 208). Tumor cells used in ELISPOT assays were pre-treated with IFN-γ (1000 U/ml) for 24 hours in order to upregulate

MHC class II expression and then irradiated (100 Gy) to prevent their proliferation. $CD4^+$ T cells, along with autologous $CD14^+$ cells or HLA-DR-matched tumor cell lines were added to ELISPOT wells at a 5:1 T cell:antigen presenting cell ratio. In antibody blocking tests, antigen-presenting cells were pre-incubated with 20 μg/ml of L243 HLA-DR blocking antibody (ATCC, Gaithersburg, MD) for 1h at 37°C prior to loading in ELISPOT wells. Peptides or rMAGE-A6 were added at 10 μg/ml, except in titration experiments where peptide concentrations were varied between 0 and 30 μM. ELISPOT plates were incubated at 37°C for 24h (peptide and tumor recognition), or 48h (protein responses), developed and evaluated using an ImmunoSpot automatic plate reader (Cellular Technology Ltd., Cleveland, OH), as previously reported (36, 208). The number of peptide-specific $CD4^+$ T cell responders was always statistically compared to the background number of IFN-γ spots produced by T cells in response to APCs pulsed with the malarial circumsporozooite $CS_{326-345}$ peptide (for peptide-based assays) or with the TOP10 processed bacterial lysate (for protein-based assays). Positive control wells contained T cells and 10 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich).

2.3.7. Peptides

The MAGE-A6 (GenBank accession no. AAA68875) and *Mycoplasma penetrans* HF-2 permease protein (GenBank accession no. NP_757962) proteins were analyzed using the ProPred HLA-DR peptide-binding algorithm (http://www.imtech.res.in/raghava/propred/index.html). MAGE-A6 peptide sequences were selected based on their predicted ability to bind the broadest HLA-DR repertoire (**Table 4**). Subsequently, microbial peptides were selected based on a homology search of GenBank sequences similar to that performed for the MAGE-A6 peptides (**Table 6**). All peptides were synthesized using 9-fluorenylmethoxycarbonyl (FMOC) chemistry

by the University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Facility (Shared Resource). Peptides were >95% pure based on high-performance liquid chromatography (HPLC) and tandem mass spectrometry analyses performed by the UPCI Protein Sequencing Facility (Shared Resource).

Table 4. Predicted and synthesized poly-DR binding epitopes derived from MAGE-A6.

The MAGE-A6 protein (GenBank accession no. AAA68875) was analyzed using the ProPred HLA-DR peptide-binding algorithm. Peptides were defined/selected according to their ability to theoretically bind the broadest HLA-DR repertoire. The most "promiscuous" HLA-DR binding peptide sequences predicted by the algorithm are underlined, and the predicted anchor residues for HLA-DR binding bolded. In the rightmost column, the HLA-DR alleles to which the peptides are predicted to bind are indicated.

Peptide Position	Peptide Sequence	HLA-DR Alleles Predicted to Bind
MAGE-A6$_{140-170}$	VGN**W**QYFFPVIFSKASDS**L**QLVFGIELMEVD	DRB*01, 03, 04, 07, 13, 15, and DRB5*01
MAGE-A6$_{172-187}$	IGH**VY**IFATCLGLSYD	DRB1*01, 04, 07, 08, 11, 13, 15, and DRB5*01
MAGE-A6$_{192-214}$	DNQ**I**MPKTGF**L**IIILAIIAKEGD	DRB1*01, 03, 04, 07, 08, 11, 13, 15, and DRB5*01
MAGE-A6$_{280-302}$	ETS**Y**VKVLHHM**V**KISGGPRISYP	DRB1*01, 03, 07, 08, 11, 13, 15, and DRB5*01

2.3.8. PCR

Cell lines were screened for MAGE-A6 expression by reverse transcription (RT)-PCR, while *Mycoplasma penetrans* HF-2 contamination was tested by PCR. For MAGE-A6 analysis, RNA was isolated from the cell lines using the RNeasy Tissue Kit (Qiagen, Valencia, CA) and cDNA prepared using the GeneAmp^{R} RNA PCR Kit (Applied Biosystems, Foster City, CA). MAGE-A6 transcripts were analyzed, as previously described (36), using the following primer set; forward: TGGAGGACCAGAGGCCCCC; reverse: CAGGATGATTATCAGGAAGCCTGT. *Mycoplasma penetrans* HF-2 DNA contamination of cell lines was tested by PCR, as previously described (209) using the primers; forward: CATGCAAGTCGGAC; reverse: AGCATTTCCTCTTC. *Mycoplasma penetrans* HF-2 bacteria (ATCC# 55252; Gaithersberg, MD) were used as positive DNA control, as was assessment for β-actin DNA using the primer set; forward: GGCATCGTGATGGACTCCG; reverse: GCTGGAAGGTGGACAGCGA. The PCR reaction parameters consisted of an initial 3 min denaturation step at 94°C followed by 32 amplification cycles that consisted of denaturation at 94°C for 45 sec, annealing at 68°C for 45 sec, and extension at 72°C for 1 min. The final cycle was followed by an additional extension step at 72°C for 10 min.

2.3.9. rMAGE-A6 generation and Western Blot analysis

Full-length MAGE-A6 cDNA was generated by RT-PCR using the primer set; forward: TGGAGGACCAGAGGCCCCC; reverse: AGGATGATTATCAGGAAGCCTGTC. cDNA was isolated from the MAGE-A6^{+} SLR20 renal carcinoma cell line (208) and inserted into the pBAD TOPO TA (Invitrogen) cloning vector, then amplified in TOP10 (Invitrogen) bacteria, according

to the manufacturer's protocol. The sequence was confirmed using the sequencing primers provided in the pBAD TOPO TA Cloning Kit. Bacterial extracted poly-His-tagged recombinant MAGE-A6 (rMAGE) was purified using the BD Talon Purification Kit (BD Biosciences, San Jose, CA), according to the manufacturer's protocol. Non-transformed TOP10 bacteria were grown and processed in an identical manner as for rMAGE purification, with the processed elution fractions (TOP10) used as a negative control in ELISPOT readouts for immune response to rMAGE. LPS levels for rMAGE and TOP10 control protein were tested using the QCL-1000 Kit (BioWhittaker, Walkersville, MD), and determined to be < 3 ng/ml (data not shown). For Western Blotting, proteins were transferred onto Immobilon-P transfer membranes (polyvinylidene fluoride microporous membrane; Millipore, Billerica, MA) and stained using the anti-MAGE antibody, 57B (kindly provided by Dr. G. C. Spagnoli, University Hospital of Basel, Basel, Switzerland; ref. 26) or isotype matched control Ab (Sigma-Aldrich, St. Louis, MO). Goat anti-mouse HRP conjugated antibody (Sigma-Aldrich) was used as a detection antibody. After extensive washing with PBS supplemented with 0.05% Tween, Western Lightning Chemoluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) was added and the blot developed by ECL chemiluminescence radiography (Kodak, Rochester, NY).

2.3.10. Statistical Analysis

Statistical comparisons were made using a two-tailed Student's T-test, with a p value ≤ 0.05 considered significant.

2.4. RESULTS

2.4.1. Selection and testing of poly-DR binding peptides derived from MAGE-A6.

I subjected the MAGE-A6 protein sequence to a computer algorithm screen designed to identify peptides likely to have "promiscuous" HLA-DR-binding tendencies. Four such peptides were identified (**Table 4**). While three of these peptides represent novel sequences (MAGE-A6$_{172-187}$, MAGE-A6$_{192-214}$, and MAGE-A6$_{280-302}$), I have previously defined the MAGE-A6$_{140-170}$ peptide as containing an HLA-DR4-restricted epitope (208).

These 4 peptides were evaluated for their immunogenicity in vitro using normal donor and melanoma patient $CD4^+$ T cells as responders and autologous Type-1 polarized DCs (i.e. DC1; ref. (210, 211) as stimulator cells. A single round of in vitro stimulation was employed in order to amplify recall Th responses to these epitopes and to limit the priming of naïve $CD4^+$ T cells, with IFN-γ ELISPOT assays performed to enumerate the resultant peptide-specific $CD4^+$ T cell responses in an initial assessment of 14 melanoma patients and 7 normal donors. A summary of donor characteristics and their T cell responses to MAGE-A6 peptides are provided in **Table 5** and **Fig. 5**, respectively.

Table 5. Normal Donor and Patient Characteristics.

Peripheral blood was obtained from normal donors or melanoma patients with written consent, under an IRB-approved protocol. DNA was isolated from PBMC and tested for HLA-DR genotype as described in Materials and Methods. Abbreviations: C, chemotherapy; I, immunotherapy; Mel, melanoma patient; Met, metastatic disease; N.D., normal donor; NED, no evidence of disease at time of blood draw; N.T., not tested; R, radiotherapy; S, surgery.

Donor	Age	Sex	HLA-DR Genotype	Stage	Status	Treatment Received
N.D.01	49	M	07, 13	-	-	-
N.D.02	64	M	07, 16	-	-	-
N.D.03	35	M	11, 13	-	-	-
N.D.04	17	F	03, 15	-	-	-
N.D.05	30	F	13	-	-	-
N.D.06	44	M	07, 13	-	-	-
N.D.07	41	F	07, 15	-	-	-
N.D.08	42	F	15, 16	-	-	-
N.D.09	48	M	07	-	-	-
Mel01	42	M	01, 11	II	Met	S, I
Mel02	62	M	03, 07	IV	Met	S, C, I
Mel03	80	M	01, 04	I	NED	S
Mel04	69	M	03, 13	III	Met	S, I
Mel05	37	F	01, 07	III	Met	S, R, I
Mel06	75	M	13, 14	II	NED	S
Mel07	69	M	03, 07	II	NED	S
Mel08	75	M	01, 07	I	NED	S
Mel09	75	F	04, 15	IV	NED	S, R
Mel10	58	F	07	III	NED	S
Mel11	30	M	N.T.	IV	Met	S, C
Mel12	34	F	13, 15	IV	Met	S, I, R
Mel13	63	M	07, 13	IV	Met	S, I, C
Mel14	32	F	01, 11	IV	Met	S
Mel15	67	F	03, 09	IV	Met	S
Mel16	45	M	15, 17	IV	Met	S
Mel17	54	F	01, 103	IV	NED	S
Mel18	31	M	01, 04	IV	Met	S
Mel19	52	F	11, 13	II	Met	S, C
Mel20	38	F	11, 13	IV	Met	S, R
Mel21	45	F	04	I	NED	S

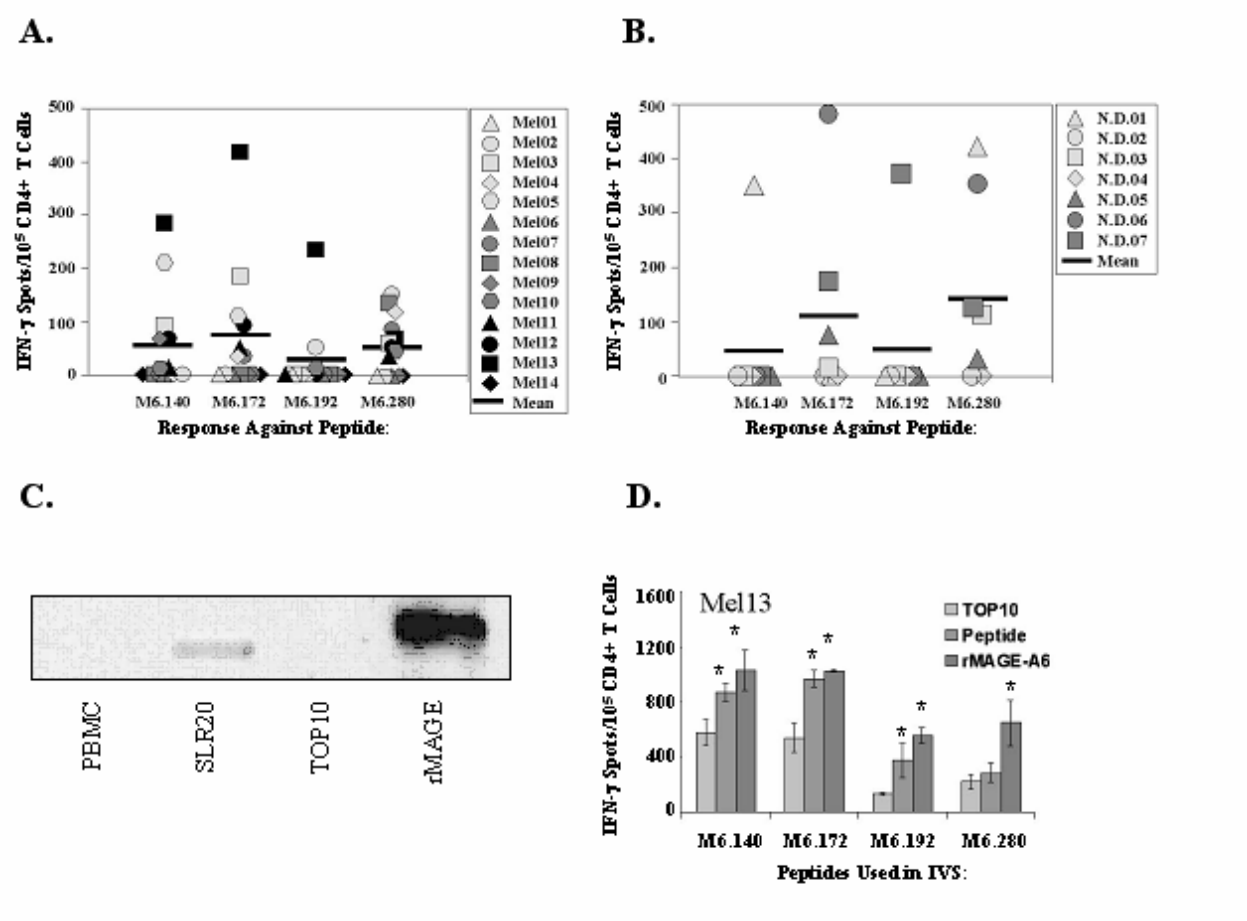


Figure 5. Type-1 CD4$^+$ T cell responses against MAGE-A6-derived poly-DR peptides and naturally-processed rMAGE epitopes.

CD4$^+$ T cells were isolated from the indicated (A) 14 melanoma patients and (B) 7 normal donors and tested for their ability to be stimulated by, and react against, the indicated MAGE-A6 peptides. CD4$^+$ T cells were stimulated once *in vitro* using autologous DC1 pulsed with 10 μg/ml MAGE-A6 peptide for 11 days. Responder T cells were assessed for their functional reactivity in IFN-γ ELISPOT assays against autologous monocytes pulsed with 10 μg/ml of control ($CS_{326-345}$) or relevant (M6.140, MAGE-$6_{140-170}$; M6.172, MAGE-$A6_{172-187}$; M6.192, MAGE-$A6_{192-214}$, M6.280, MAGE-$A6_{280-302}$) peptides. (C) Western Blotting was performed using the anti-MAGE mAb 57B against rMAGE, or the following controls: normal donor PBMC (MAGE-A6-) lysate; SLR20 renal carcinoma cell line (MAGE-A6+) lysate and TOP10 (MAGE-A6-) bacterial lysate. (D) IFN-γ ELISPOT assays were performed on IVS (peptide)-primed CD4$^+$ T cells isolated from seven melanoma patients to assess their functional reactivity against rMAGE-pulsed autologous monocytes (a representative experiment for patient Mel13 is shown). Controls included monocytes pulsed with the indicated relevant (MAGE-A6) or irrelevant (CS) peptides, and the TOP10 protein (negative control for rMAGE). (*) indicates significant responses ($p < 0.05$ for rMAGE vs. TOP10 or MAGE-A6 peptide vs. CS peptide, data not shown). Abbreviations used: N.D. = normal donor; Mel = melanoma patient.

Peptide-specific responses were observed in both melanoma patients and, at typically lower frequencies, in normal donors. Melanoma patients displayed variable reactivity against each of the four peptides tested. The MAGE-$A6_{280-302}$ peptide was associated with the most frequent $CD4^+$ T cell responses among the patients evaluated, with 9/14 patients evaluated reacting against this sequence (mean response [MR] = 50 specific spots/10^5 $CD4^+$ T cell evaluated). MAGE-$A6_{140-170}$ (5/13 patients reactive; MR = 58.4 specific spots/10^5 $CD4^+$ T cell evaluated) and MAGE-$A6_{172-187}$ (7/13 patients reactive; MR = 67 specific spots/10^5 $CD4^+$ T cell evaluated) were also commonly immunostimulatory, while the MAGE-$A6_{192-214}$ (2/14 patients reactive; MR = 22 specific spots/10^5 $CD4^+$ T cell evaluated) epitope exhibited the poorest overall immunogenicity (**Fig. 5A**). Similarly, in normal donors (**Fig. 5B**), the MAGE-$A6_{280-302}$ peptide yielded the strongest and most frequent responses (5/7 normal donors reactive; MR = 147 specific spots/10^5 $CD4^+$ T cell evaluated), while the MAGE-$A6_{172-187}$ peptide was the second most stimulatory peptide (3/7 normal donors reactive; MR = 108 specific spots/10^5 $CD4^+$ T cell evaluated) among the donors evaluated. The MAGE-$A6_{140-170}$ (1/7 normal donors reactive; MR = 50 specific spots/10^5 $CD4^+$ T cell evaluated) and MAGE-$A6_{192-214}$ (1/7 normal donors reactive; MR = 53 specific spots/10^5 $CD4^+$ T cell evaluated) epitopes were less effective in promoting specific immune responses. Overall, 11/14 melanoma patients (i.e. with the exception of patients Mel01, Mel06, and Mel14) and 5/7 normal donors (except for N.D.02 and N.D.04) evaluated were responsive against at least one of these epitopes following a single round of IVS.

2.4.2. Recognition of naturally-processed MAGE-A6 epitopes by peptide-stimulated $CD4^+$ T cells.

While my preliminary data suggest that the selected MAGE-A6 epitopes can stimulate specific $CD4^+$ T cell responses in vitro from the majority of randomly-selected donors evaluated, this does not prove that these peptides are naturally-processed and HLA-presented. Hence, to provide support for the physiological relevance for these epitopes, I analyzed the capacity of peptide-stimulated T effector cells to react against autologous monocytes pulsed with recombinant MAGE-A6 protein in vitro. I first constructed, produced and purified recombinant MAGE-A6 as outlined in the Materials and Methods (rMAGE; **Fig. 5C**). I then analyzed whether MAGE-A6 peptide-stimulated T cells could recognize autologous $CD14^+$ monocytes loaded in vitro with rMAGE using IFN-γ ELISPOT assays as a readout system (**Fig. 5D**). As controls, T cells were assessed for reactivity against monocytes loaded with either the relevant MAGE-A6 or irrelevant CS peptides, or the TOP10 processed bacterial lysate as negative control for rMAGE protein. An evaluation of peptide-primed $CD4^+$ T cells generated from melanoma patients supported the conclusion that each of the peptide epitopes tested was naturally-processed and -presented by autologous monocytes. A representative experiment is shown in **Fig. 5D**, where after one round of stimulation with individual peptides, $CD4^+$ responder T cells isolated from patient Mel13 recognized the relevant MAGE-A6 peptide, as well as, rMAGE-pulsed autologous monocytes.

2.4.3. Recognition of poly-DR presented MAGE-A6 epitopes by normal donors and potential cross-reactivity against environmental pathogens.

It was interesting to note that several normal donors were able to mount detectable Th1-type responses against the MAGE-A6$_{172-187}$ and MAGE-A6$_{280-302}$ peptides after a single round of IVS. One possible explanation for this finding is that some normal donors harbor pre-malignant MAGE-A6^{+} lesions, since MAGE antigens can be expressed in such tissues (212, 213). However, I believe it highly unlikely that such a large frequency of donors would be impacted in this manner. An alternate possibility is that the MAGE-A6 peptides evaluated bear sufficient sequence or conformational homologies to environmentally expressed proteins to which many individuals may have become naturally primed against, allowing for functional cross-reactivity to be detected in my assays. This type of phenomenon has been previously suggested for the HLA-A2-presented MART-1$_{27-35}$ epitope (202).

While conformational epitope mimics are not easily evaluated, I was able to perform sequence homology searches of the GenBank database for potential sources of cross-reactive linear epitopes. These screens suggested that the MAGE-A6$_{172-187}$ and MAGE-A6$_{280-302}$ (but not the MAGE-A6$_{140-170}$ or MAGE-A6$_{192-214}$) epitopes exhibited significant homologies to known microbial sequences (**Table 6**). The MAGE-A6$_{172-187}$ VYIFATCL octamer was similar to a peptide (i.e. the HF-2$_{219-226}$; MPHF2) derived from the HF-2 permease protein of *Mycoplasma penetrans* [a common opportunistic human pathogen (214)]. The MAGE-A6 peptide contains conservative V1I and A5T positional amino acid substitutions when compared to the pathogen homologue. Similarly, the MAGE-A6$_{280-302}$ VLHHMVKI octamer was highly homologous to a Chlamydia muridarum Nigg [a strain of rodent origin (215)] encoded, conserved hypothetical

protein TC0097 (CHP). When these pathogen-derived core peptides were expanded to include three native (from the protein of origin) amino-acids on each flank (**Table 6**), and then analyzed using the ProPred HLA-DR peptide-binding algorithm, they were predicted to bind a wide range of HLA-DR alleles, similar to their MAGE-A6 homologues. Subsequently, both peptides were synthesized. Due to its high hydrophobicity index, the MPHF2 peptide flanking regions were additionally modified to include diaminopropionic acid (Z) groups (that are very similar to Lysine but less bulky), in order to improve peptide solubility.

Table 6. Sequence homology of MAGE-A6 epitopes with pathogen-associated proteins.

The MAGE-A6 Th epitopes were analyzed using a GeneBank homology program and microbial homologues identified. Underlined sequences represent areas of homology between matched sequences. Diaminopropionic acid (Z) groups were added to MPHF2 flanking regions in order to enhance its solubility. Sequences were analyzed as described in the Materials and Methods section. Symbols: +, Conserved amino-acid family; -, Non-conserved amino-acids.

Protein	Homologous sequences	Conserved sequence
MAGE-A6$_{172-187}$	IGHVYIFATCLGLSYD	
		+**Y**IFA-CL
Permease	(ZZZ)AIYIYIFAACLLLI(ZZZ)	
[*Mycoplasma penetrans* HF-2]		
MAGE-A6$_{280-302}$	ETSYVKVLHHMVKISGGPRISYP	
		VLH-M**V**KI
Conserved hypothetical protein TC0097		
[*Chlamydia muridarum Nigg*]	KRRVLHEMVKIYSL	

2.4.4. $CD4^+$ T cell responses to the MAGE-$A6_{172-187}$ and the MPHF2 homologue peptide are immunologically-related.

I next evaluated the ability of these 2 pathogenic sequences to be recognized by MAGE-A6 peptide-primed $CD4^+$ T cells, as well as, to serve as immunogens capable of priming $CD4^+$ T cells capable of cross-reacting against the homologous MAGE-A6 epitopes in vitro (**Fig. 6**). The resulting IVS responder T cells were assessed for their reactivity against autologous monocytes pulsed with the priming peptide, its homologue peptide or rMAGE in IFN-γ ELISPOT assays. MAGE-$A6_{172-187}$ peptide-primed $CD4^+$ T cells derived from 2/6 patients recognized the stimulating peptide and also cross-reacted against the MPHF2 homologue peptide in a statistically-significant manner (**Fig. 6A**). Similarly, $CD4^+$ T cells generated from 4/6 patients after stimulation with the MPHF2 peptide recognized the stimulating peptide, with half of these responders cross-reacting against the MAGE-$A6_{172-187}$ homologue peptide. Interestingly, while $CD4^+$ T cells generated from patients stimulated with the MAGE-$A6_{172-187}$ epitope only modestly recognized naturally-processed rMAGE protein (**Fig. 6B**), MPFH2-primed $CD4^+$ T cells from all 6 patients recognized autologous monocytes pulsed with rMAGE. In contrast, in the MAGE-$A6_{280-302}$/CHP peptide analyses that were performed in parallel, I observed no evidence for peptide cross-recognition by $CD4^+$ T cells after peptide-based in vitro stimulation (**Fig. 6A**).

Similar results were observed for $CD4^+$ T cells isolated from three normal donors, with data from a representative experiment depicted in **Fig. 6C**. In this donor (N.D.08), MAGE-$A6_{172-187}$ peptide priming did not promote a specific $CD4^+$ T cell response in vitro, while MPHF2-stimulated $CD4^+$ T cells cross-reacted against both target peptides and naturally-processed and presented

rMAGE-derived epitope(s) in the ELISPOT readout assay. This donor also responded to the MAGE-A6$_{280-302}$, but not the CHP peptide (**Fig. 6C**).

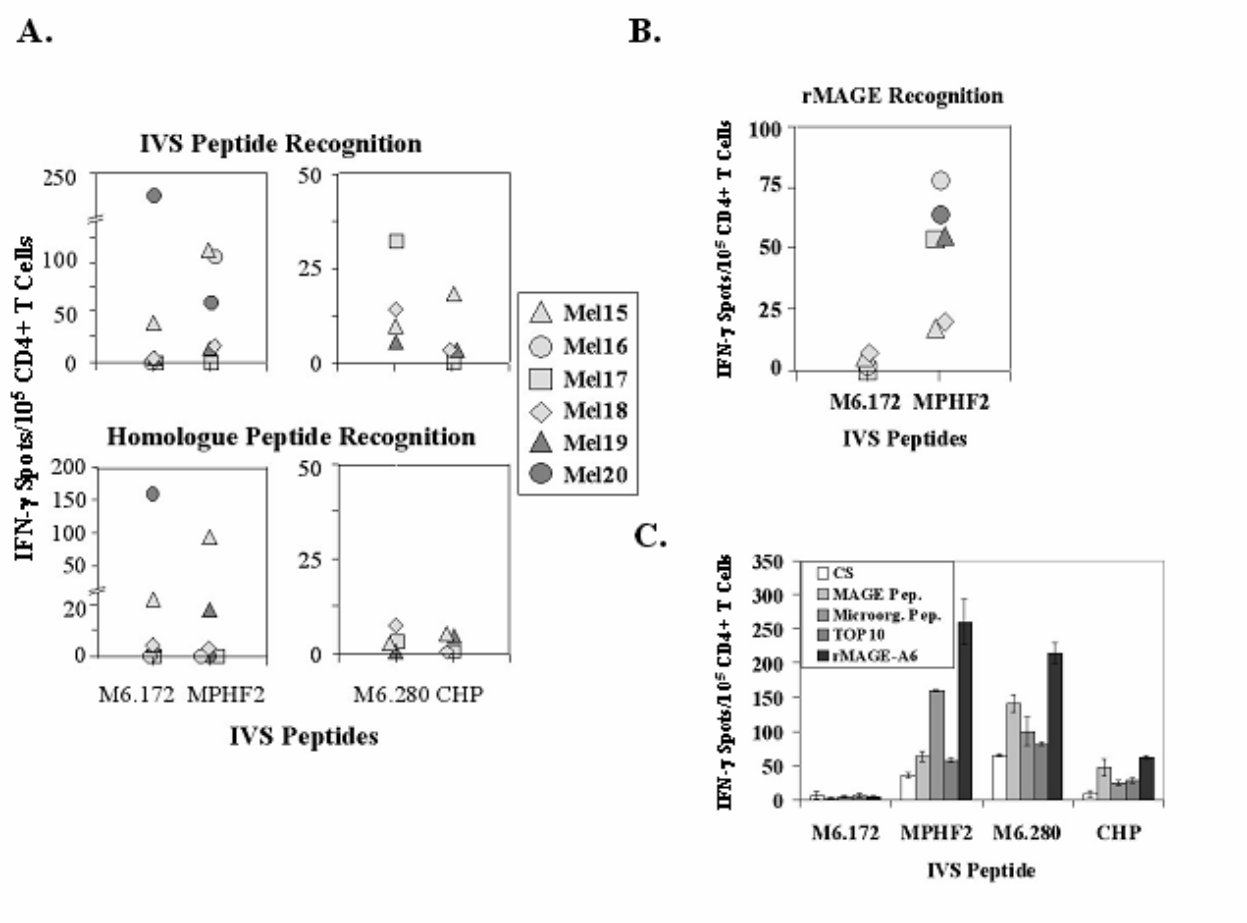


Figure 6. Normal donor and melanoma patient CD4$^+$ T cell responses against MPHF2 and CHP peptides: cross-reactivity against MAGE-A6 epitopes?

(A) CD4$^+$ T cells isolated from melanoma patients were tested for their ability to be stimulated by, and react against, MAGE-A6$_{172-187}$ and MAGE-A6$_{280-302}$, and their homologues MPHF2 and CHP, respectively. CD4$^+$ T cells were stimulated as described in the **Fig. 5** legend, with responder T cells analyzed in IFN-γ ELISPOT assays for reactivity against autologous monocytes pulsed with 10 μg/ml of stimulating peptide (A, top panel), the corresponding homologue peptide (A, bottom panel) or rMAGE (B). Negative controls included the CS$_{326-345}$ peptide and TOP10 bacterial proteins. (C). CD4$^+$ T cells isolated from normal donors were stimulated as above using the individual MAGE-A6, MPHF2 and CHP peptides, then analyzed for their reactivity against autologous monocytes pulsed with the indicated peptides or proteins (insert) in IFN-γ ELISPOT assays. The data displayed in panel C are representative of 3 normal donors analyzed in this manner.

2.4.5. MPHF2-stimulated CD4$^+$ T cells recognize HLA-DR matched, MAGE-A6$^+$ melanoma cell lines in vitro.

To further evaluate the potential physiological relevance of CD4$^+$ T cell priming against the MPH2 epitope, I compared MAGE-A6$_{172\text{-}187}$ and MPHF2-stimulated CD4$^+$ T cells for their ability to recognize HLA-DR-matched, MAGE-A6$^+$ melanoma cell lines in vitro. In these experiments, CD4$^+$ T cells isolated from an HLA-DR4$^+$ melanoma patient blood were stimulated twice at weekly intervals with autologous DC1 pulsed with either the MAGE-A6$_{172\text{-}187}$ or MPHF2 peptides. Two HLA-DR4$^+$ (**Fig. 7A**)/MAGE-A6$^+$ (**Fig 7B**) melanoma cell lines, SLM2 and Mel526.DR4, were then used as targets for responder T cells in IFN-γ ELISPOT assays (**Fig. 7C**). Pan-DR mAb (L243) was also added to replicate wells, as indicated, to demonstrate the MHC class II-restricted nature of T cell responses. I observed that both populations of peptide-primed CD4$^+$ T cells recognized the two tumor cell lines in a manner that was partially blocked by addition of mAb L243, with the MPHF2-stimulated CD4$^+$ T cells exhibiting a greater magnitude of response to tumor cell lines than T cells primed with the MAGE-A6$_{172\text{-}187}$ epitope (**Fig. 7C**). To rule out the possibility that T cell recognition was due to specific mycoplasma infection of the target cell lines, both melanoma cell lines were shown to be negative for *Mycoplasma penetrans* HF-2 contamination using a sensitive PCR method (**Fig. 7D**).

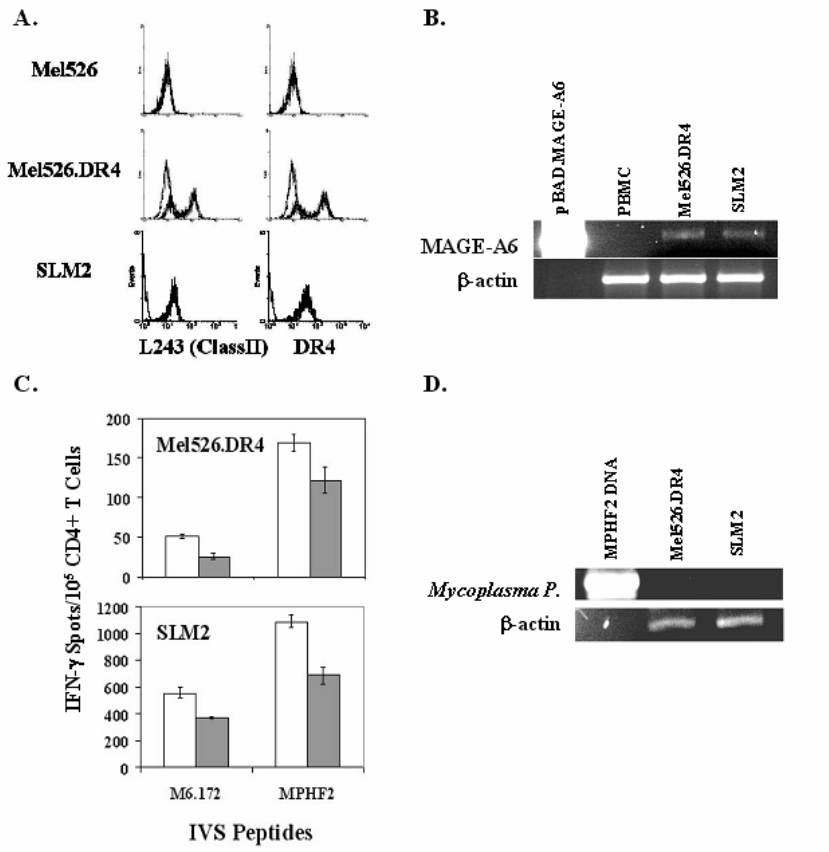


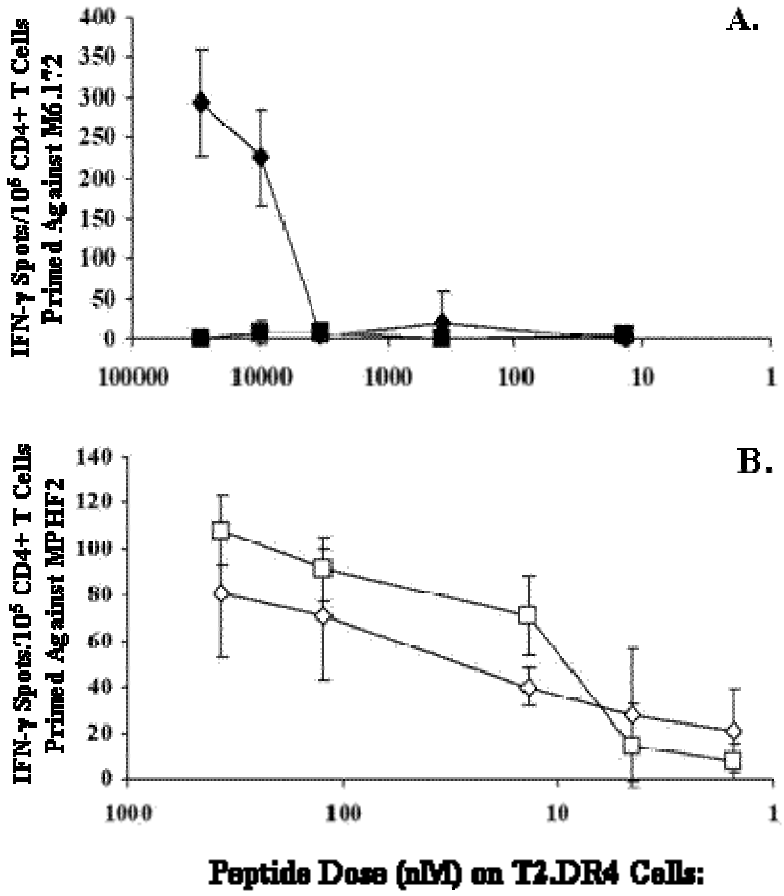
Figure 7. MPHF2-primed CD4$^+$ T cells more effectively recognize HLA-matched, MAGE-A6$^+$ tumor cells than T cells primed in vitro against the homologous MAGE-A6$_{172\text{-}187}$ peptide.

In (A), flow cytometry was used to assess the target cell lines used in these experiments (Mel526.DR4, SLM2), as well as the MHC class II negative Mel526 cells, for expression of pan-MHC class II (L243) and HLA-DR4 molecules [thin line: IgG control; thick line: MHC class II or HLA-DR4 antibody]. While SLM2 cells spontaneously expressed HLA-DR4, the Mel526.DR4 cell line is HLA-DR4$^+$ as a consequence of infection with a retrovirus encoding the HLA-DRα/-DRβ1*0401 cDNAs (33). Tumor cell lines were also evaluated by: (B) RT-PCR for expression of MAGE-A6 mRNA expression and (D) by PCR for contamination by *Mycoplasma penetrans* HF-2. In (C), CD4$^+$ T cells were isolated from an HLA-DR4$^+$ melanoma patient (Mel21) and stimulated with autologous DC1 pulsed with the MAGE-A6$_{172\text{-}187}$ or MPHF2 peptides as described in the **Fig. 5** legend, with responder T cells were evaluated for functional reactivity against the Mel526.DR4 (panel C, top) and SLM2 (panel C, bottom) tumor cell lines in IFN-γ ELISPOT assays. L243 (anti-pan-class II mAb; 20 μg/ml; gray filled histograms)) or no mAb (open histograms) were added to wells to confirm the class II-restricted nature of IFN-γ secretion by responder CD4$^+$ T cells. All data for L243 vs. no mAb are significant, with $p < 0.05$. Data are representative of 3 independent assays performed.

2.4.6. MPHF2-stimulated CD4$^+$ T cells exhibit a higher functional avidity for MAGE-A6$_{172-187}$ loaded target cells than T cells primed against the MAGE-A6 peptide itself.

To determine whether the superior capacity of MPHF2 (vs. MAGE-A6$_{172-187}$) peptide-primed CD4$^+$ T cells to recognize autologous APC pulsed with rMAGE-A6 protein as well as HLA-matched, MAGE-A6$^+$ tumor cells could be attributed to differences in T cell functional avidity, I compared the abilities of peptide-primed CD4$^+$ T cells generated from an HLA-DR4$^+$ normal donor to recognize titrated doses of the MAGE-A6 peptide pulsed onto T2.DR4 presenting cells in IFN-γ ELISPOT assays. CD4$^+$ T cells generated using the MAGE-A6 peptide recognized T2.DR4 cells only when pulsed with relatively high concentrations peptide (with a half-maximal response associated with a peptide dose of 3-10 μM; **Fig. 8A**), while MPHF2-stimulated cells recognized target cells pulsed with far (approximately 1000-fold) lower concentrations of peptide (**Fig. 8B**).

Figure 8. MPHF2-primed CD4$^+$ T cells exhibit a higher functional avidity for the MAGE-A6$_{172-187}$ epitope than T cell primed against the MAGE-A6 peptide itself.



To test the functional avidity of (A) MAGE-A6$_{172-187}$-primed and (B) MPHF2-primed CD4$^+$ T cells (as outlined in the **Fig. 7** legend), lymphocytes were evaluated for their ability to recognize titrated doses of MAGE-A6$_{172-187}$ (diamonds) or MPHF2 (squares) peptide pulsed onto the HLA-DR4+ T2.DR4 cell line in IFN-γ ELISPOT assays, as described in Materials and Methods. All data are reflective of 3 independent assays performed. MAGE-A6$_{172-187}$ MW = 1728 g/mol [e.g. 10 μM = 17.3 μg/ml]; MPHF2 MW = 2115 g/mol [e.g. 10 μM = 21.2 μg/ml].

2.5. DISCUSSION

Previous studies of peptide-specific, $CD4^+$ T cell responses against tumor antigens, including MAGE-A6 have been traditionally skewed towards an analysis of a single (i.e. HLA-DR4) or very limited set of HLA-DR restriction elements (208, 216, 217). Given the extreme polymorphism among HLA-DR alleles, the translational utility of the epitopes defined to date would be limited to a modest cohort of 20-30% of patients (23). In the current study I have attempted to circumvent this limitation by identifying peptides that are likely to bind to, and be presented by, as broad a range of HLA-DR alleles (i.e. poly-DR) as possible, thereby expanding the range of patients to which MAGE-A6-based therapies might be applied clinically.

Peripheral blood $CD4^+$ T cells were harvested from normal donors and patients with melanoma, stimulated with a single round of peptide-based IVS, and evaluated for their ability to recognize MAGE-A6 peptides (selected to be promiscuously presented by HLA class II) in IFN-γ ELISPOT assays. My data support the identification of 3 novel MAGE-A6 peptide epitopes recognized by Type-1 Th cells and substantiate the ability of these epitopes, along with the previously-defined MAGE-$A6_{140\text{-}170}$ peptide, to be recognized by a large proportion of individuals (who overall exhibited a diverse array of HLA-DR haplotypes). Of the four peptides analyzed, the MAGE-$A6_{172\text{-}187}$ and MAGE-$A6_{280\text{-}302}$ peptides were recognized at the highest frequencies in peptide ELISPOT readouts by $CD4^+$ T cells. In contrast, the MAGE-$A6_{192\text{-}214}$ appeared comparatively non-immunogenic, and the MAGE-$A6_{140\text{-}170}$ peptide was moderately effective in this capacity. Based on the ability of peptide-primed $CD4^+$ T cells to recognize autologous monocytes pulsed with rMAGE protein, each of the peptides evaluated appear to contain naturally-processed and -presented Th epitopes.

These MAGE-A6 poly-DR epitopes displayed a high degree of homology with sequences contained in other MAGE-A family members, especially MAGE-A3. MAGE-$A6_{140-170,}$ MAGE-$A6_{172-187,}$ and MAGE-$A6_{280-302}$ differed from their MAGE-A3 counterparts by only a single amino acid in each case. For MAGE-$A6_{140-170}$ this difference is at position 156, where a D > S substitution occurs in the MAGE-A3 protein. The MAGE-$A6_{172-187}$ peptide differs from its MAGE-A3 counterpart based on a conservative V175L substitution, and the MAGE-$A6_{280-302}$ to MAGE-A3 difference reflects an R298H substitution. Hence, while it remains to be formally evaluated, I believe that in many cases, the selected MAGE-A6 epitopes will likely elicit $CD4^+$ T cell responses in a high frequency of patients that are capable of cross-reacting against their MAGE-A3 homologues when presented by autologous APCs. As a result, these peptides could represent promising candidates for inclusion in peptide-based vaccines designed to treat the majority of patients harboring tumors that exhibit MAGE-$A6^+$ and/or MAGE-$A3^+$ phenotypes *in situ*.

It was also noted in this study that $CD4^+$ T cells isolated from several normal donors were able to effectively recognize the MAGE-$A6_{172-187}$ and MAGE-$A6_{280-302}$ epitopes after a single round of IVS. This observation could be attributed to epitope mimicry. Epitope mimicry has been described as a potential mechanism underlying the induction of autoimmune diseases due to pathologic T cells primed against infectious microorganisms that cross-react against host proteins in susceptible individuals. Diseases such as viral myocarditis, lyme disease, rheumatoid arthritis (198), multiple sclerosis (199), and virus-induced autoimmune diabetes (200, 201) have long been considered to be initiated or exacerbated by microbial pathogens. As was previously noted for the HLA-A2-presented, melanoma-associated MART-1_{27-35} epitope (202), I hypothesized that the high degree of normal donor response against the MAGE-A6 Th peptides

might be due to the cross-reactivity of T cells initially primed *in vivo* against highly-homologous peptides within environmentally-encountered proteins. After performing a homology search of the GenBank database, I selected the MPHF2 and CHP peptides as two likely candidate homologues of the MAGE-A6$_{172\text{-}187}$ and MAGE-A6$_{280\text{-}302}$ peptides, respectively. The MPHF2 peptide derives from *Mycoplasma penetrans* HF-2, a ubiquitous species of *Mycoplasmataceae*, which infects humans in the urogenital and respiratory tracts. A typical feature of this microorganism is penetration into human cells and long-term intracellular replication and persistence. In human disease, *M. penetrans* is clinically observed in cases of HIV-1 infection, but has also been suggested to represent a primary cause of non-HIV-related urethritis and respiratory disease (214). Importantly, while many strains of mycoplasma can commonly infect laboratory cultures and affect derivative assay results, *M. penetrans* has not been reported to do so, and I did not detected this agent in my cell lines. The CHP peptide derives from *Chlamydia muridarum* strain Nigg, a mouse-tropic strain capable of causing respiratory disorders in mice. This strain of *Chlamydia* diverges significantly from human-tropic strains (215). Given the low degree of likelihood that humans would encounter this microbe, it was perhaps not surprising that I did not observe any evidence for the cross-reactivity of these peptides by T cells in my studies. This does not rule out a pathogenic homologue for the MAGE-A6$_{280\text{-}302}$ peptide being responsible for my common observation of IVS responses against this MAGE-A6 peptide among normal donors, but suggests that additional studies will need to be performed to illuminate its identity.

My results suggest that the MPHF2 peptide is immunogenic and capable of promoting Type-1 effector CD4$^+$ T cells in a majority of melanoma patients and normal donors. Responder T cells were typically able to cross-react against the MPHF2 and MAGE-A6$_{172\text{-}187}$ peptides and

to recognize the naturally-processed rMAGE epitope when presented by autologous monocytes. Notably, anti-MPHF2 $CD4^+$ T cells also appeared preferentially able (in contrast to MAGE-A6 peptide induced T cells) to recognize HLA-DR matched melanoma cell lines that constitutively express the MAGE-A6 gene product. The ability of the MPHF2 peptide to promote "heteroclitic" immunity against the MAGE-A6 (and presumably MAGE-A3) protein(s) may make this epitope extremely attractive as a vaccine candidate in patients bearing tumor histotypes in which MAGE-A3/MAGE-A6 expression is commonly observed (such as melanoma, renal cell carcinoma and others).

Preface Chapter 3

Given that patients with cancer exhibit dysfunctional Th1-type responses against epitopes derived from tumor antigens, such as MAGE-A6, I next investigated whether human DCs engineered to secrete high levels of the IFN-γ-inducing cytokines IL-12p70 and IL-18 via recombinant adenoviral infection could effectively generate Th1-type responses *in vitro*. I showed that DC co-infected with Ad.IL-12 and Ad.IL-18 (DC.IL-12/18) and pulsed with HLA-DR4-restricted MAGE-A6 peptides were more effective at stimulating MAGE-A6-specific Th1-type $CD4^+$ T cell responses than DC infected with either of the cytokine vectors alone, control Ad.Ψ5 virus or uninfected DC. Furthermore, DC.IL-12/18 loaded with recombinant MAGE-A6 protein effectively promote Th1-type immunity that is frequently directed against multiple MAGE-A6-derived epitopes.

The studies in **Chapter 3** demonstrate the immuno-stimulatory properties of DC.IL-12/18, particularly when loaded with the whole TAA. Based on these results, I believe this modality may prove clinically useful as a vaccine platform to promote the recovery of tumor antigen-specific, Th1-type $CD4^+$ T cell responses in patients with cancer. This data has just been reported in *Cancer Gene Therapy* in 2006.

3. IL-12p70 and IL-18 Gene-Modified Dendritic Cells Loaded with Tumor Antigen-Derived Peptides or Recombinant Protein Effectively Stimulate Specific Type-1 CD4$^+$ T cell Responses From Normal Donors and Melanoma Patients *In Vitro*

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This data has just been reported in *Cancer Gene Therapy* in 2006. All the results reported in this study were obtained by Lazar Vujanovic. Evaluation of recombinant adenoviruses was repeated by Elena Ranieri, Ph.D. ELISPOT plate analyses were performed by Walter C. Olson, Ph.D.

3.1. Abstract

While $CD4^+$ Type-1 T helper (Th1) cells secreting interferon-γ (IFN-γ) appear to play an essential role in promoting durable anti-tumor immunity, I have previously shown that patients with cancer exhibit dysfunctional Th1-type responses against epitopes derived from tumor antigens, such as MAGE-A6. Here, I engineered human dendritic cells (DC) to secrete high levels of the IFN-γ-inducing cytokines IL-12p70 and IL-18 via recombinant adenoviral infection to generate an *in vitro* stimulus capable of promoting previously-deficient patient Th1-type responses. DC co-infected with Ad.IL-12 and Ad.IL-18 (DC.IL-12/18) were more effective at stimulating MAGE-A6-specific Th1-type $CD4^+$ T cell responses than DC infected with either of the cytokine vectors alone, control Ad.Ψ5 virus or uninfected DC. Furthermore, I show that DC.IL-12/18 loaded with recombinant MAGE-A6 protein (rMAGE) and used as *in vitro* stimulators, promote Th1-type immunity that is frequently directed against multiple MAGE-A6-derived epitopes. The superiority of DC.IL-12/18-based stimulations in melanoma patients was independent of disease stage or current disease-status. Based on these results, I believe this modality may prove clinically useful as a vaccine platform to promote the recovery of tumor antigen-specific, Th1-type $CD4^+$ T cell responses in patients with cancer.

3.2. Introduction

Previous studies have shown that melanoma represents a prototype for neoplasia responsive to immunotherapy, and serves as a suitable model system for the development of anti-tumor vaccines (206, 218). While most vaccine studies have focused solely on effector $CD8^+$ T cell responses as surrogates for clinical cancer responses, it is now clear that anti-tumor $CD4^+$ T cells regulate the quality, magnitude and durability of $CD8^+$ CTL immunity *in vivo* (69-71), and that the Th1-type cytokine, IFN-γ, plays an essential role in this response. Th1-type $CD4^+$ T cells secrete IFN-γ and may mediate delayed type hypersensitivity (DTH) responses that can lead to enhanced cross-presentation of tumor antigens by host APCs (72), and consequent epitope spreading in the evolving anti-tumor T cell repertoire (73, 74). Furthermore, $CD4^+$ T cells may mediate direct tumoricidal activity via TNF family ligand members and can inhibit tumor angiogenesis via locoregional production of IFN-γ (76-79).

Anti-tumor Th1-type $CD4^+$ T cells, however, appear inhibited in many cancer patients (71, 97, 98), as reflected by decreased proliferation and T cell receptor (TCR) signaling (99), as well as, by increased frequencies and activity of regulatory T cells (100, 101). While Th1-type responses have been associated with spontaneous or therapy-induced regression of tumor lesions (98, 102), tumor infiltrating lymphocytes isolated from patients with progressive lesions have been generally reported to exhibit dominant Th2-type (secreting IL-4, IL-5) or regulatory (Th3)-type (secreting IL-10, TGF-β1) responses (97, 98, 102). Our group has previously shown that peripheral blood $CD4^+$ T cells isolated from melanoma or renal cell carcinoma (RCC) patients with active disease exhibit highly-skewed, non-Type-1 $CD4^+$ T cell reactivity against epitopes derived from tumor antigens, such as MAGE-A6 (36). These findings contrast noticeably with

normal donors and cancer patients who exhibit no current evidence of disease as a consequence of therapeutic intervention, in whom either mixed Th1/Th2-type or strongly Th1-polarized responses to MAGE-A6 peptides occur, respectively (208). These results strongly suggest that the future immune-based therapies must overcome existing deficiencies in Type-1 anti-tumor $CD4^+$ T cell responses in cancer patients with active disease in order to be clinically effective.

IL-12p70 and IL-18 are crucial Th1-type cytokines that synergize in promoting IFN-γ secretion from, and proliferation of, $CD4^+$ T effector cells (165, 166). IL-12p70 induces T cell surface expression of the IL-18 receptor (IL-18R) by naïve T cells (167); while IL-18, an IL-1 family member, potentiates the differentiation of Th1 cells instigated by IL-12p70 (168). I hypothesized that dysfunctional anti-tumor Th1-type responses in cancer patients with active disease might be recovered/enhanced by *in vitro* stimulation of patient $CD4^+$ T cells using vaccines containing autologous dendritic cells (DC) engineered to secrete IL-12p70 and/or IL-18. This gene therapy approach could not only prove capable of supporting Type-1 immunity, but would have the potential to obviate toxicities previously observed for systemic application IL-12p70 alone (184, 186, 190) or IL-12p70 combined with IL-18 (187). Indeed, our group has previously shown in murine models that DC engineered to secrete both IL-12p70 and IL-18 *ex vivo*, and subsequently injected intratumorally, promote acute tumor rejection in concert with enhanced Th1-type immunity and determinant spreading in the curative anti-tumor CTL repertoire (191).

In the current study, I generated and applied novel recombinant adenoviral vectors encoding human IL-12p70 (Ad.IL-12) and the mature form of human IL-18 (Ad.IL-18) to engineer human DC *in vitro*, and subsequently evaluated the ability of these gene-modified antigen presenting cells to promote Th1-type $CD4^+$ T cell responses against the MAGE-A6

tumor antigen (36, 208) in HLA-DR4$^+$ normal donors and patients with melanoma. To my knowledge, this is the first study to describe a recombinant adenoviral vector encoding for a mature, physiologically active form of human IL-18. Furthermore, I am the first to describe the effects of Ad.IL-12 and Ad.IL-18 co-infection of DC on the induction of effective polarization and enhancement of Th1-type CD4$^+$ T cell responses in cancer patients. My results show that DC.IL-12/18 loaded with tumor peptides or recombinant tumor antigens may represent an effective vaccine capable of selectively promoting Type-1 specific immunity in patients with cancer who may display existing immune dysfunction.

3.3. Materials and Methods

3.3.1. Recombinant Adenoviral Vectors

The Ad.ψ5, Ad.IL-12 and Ad.IL-18. E1- and E3-deleted adenoviral vector encoding the human interleukin-12 (hIL-12) p70 and interleukin-18 (hIL-18) cDNAs were constructed through Cre-lox recombination with reagents generously provided by Dr. S. Hardy (Somatix, Alameda, CA). The prepro leader sequence of human GM-CSF was fused to the 5' end of the mature human IL-18 cDNA in order to facilitate secretion of bioactive IL-18 by infected cells. Sal I-Not I fragments containing p40-IRES-p35 or modified mature form of hIL18 cDNAs were inserted into the shuttle vector, pAdlox. Recombinant adenoviruses were generated by co-transfection of Sfi I-digested pAdlox-hIL-12 or pAdlox-hIL-18 and ψ5 helper virus DNA into the adenoviral packaging cell line CRE8 that expresses Cre recombinase, as previously described (219). Recombinant adenoviruses were propagated on CRE8 cells, purified by cesium chloride density gradient centrifugation and subsequent dialysis, prior to storage in 3% threalose at –80^o C.

3.3.2. Cytokine ELISAs

Adenoviral function and cytokine bioactivity were evaluated using ELISA assays for hIL-12p70 (Pharmingen), hIL-18 (R&D Systems) and hIFN-γ (antibody pairs from Mabtech; Mariemont, OH), per the manufacturer's protocols. The lower limits of detection for these assays were 4.0 pg/ml, 12.5 pg/ml and 4.0 pg/ml, respectively.

3.3.3. MAGE-A6 Protein and Peptides

Recombinant MAGE-A6 protein (rMAGE)[1] and MAGE-A6 peptides (MAGE-$A6_{121-144}$ (36), MAGE-$A6_{140-170}$ (36), MAGE-$A6_{246-263}$ (36), and MAGE-$A6_{280-302}$[1] were pulsed onto DC to generate stimulator cells for *in vitro* sensitization of T cells. Non-transformed TOP10 (Invitrogen, Carlsbad, CA) bacteria were grown and processed in an identical manner as for rMAGE purification, with the processed/eluted material (TOP10)[1] later used as a negative control in ELISPOT readouts.

3.3.4. Isolation of Patient and Normal Donor PBMC

Peripheral blood was obtained by venipuncture from normal donors or melanoma patients a single time with consent, under an IRB-approved protocol. Peripheral blood was diluted 1:2 with PBS, applied to Ficoll-Hypaque gradients (Cellgro; Mediatech, Inc., Herndon, VA), and centrifuged at 550 x g for 25 min at RT. Peripheral blood mononuclear cells (PBMC) were recovered from the buoyant interface and washed at least three times with PBS in order to remove residual platelets as well as Ficoll-Hypaque. Donor HLA-DR4 status was determined using a specific anti-HLA-DR4 mAb 359-F10 and FACS analysis, as previously described (36,

208). Only PBMC determined to be HLA-DR4$^+$ were used in these experiments. The 8 melanoma patients analyzed were between 31 and 83 years of age. Six of the eight patients (except Mel4 and Mel 6) tested were male, with all but Mel4 and Mel5 having active disease status at the time of blood draw. Blood was obtained at least 1 month after patient treatment with surgery alone (all), surgery + chemotherapy (Mel1, Mel2) or surgery + radiotherapy (Mel 1). Disease stage at the time of initial diagnosis for each patient is listed in **Table 7**.

3.3.5. DC Generation

Following Ficoll density separation, PBMC were plated in T75 culture flasks (COSTAR, Cambridge, MA) flasks in AIM-V (GIBCO-BRL, Gaithersburg, MD) medium for 60 minutes at 37^o C. Following incubation, non-adherent (NA) cells were removed from flasks by gentle washing with PBS, and cryo-preserved in freezing media [10% DMSO (Sigma-Aldrich), 90% fetal bovine serum (FBS; Invitrogen)] using controlled-rate freezing technique in a -80^o C freezer. Adherent monocytes were cultured at 37^o C and 5% CO_2 in DC medium [AIM-V supplemented with 500 U/ml GM-CSF (Sargramostim; Amgen, Thousand Oaks, CA) and 1000 U/ml IL-4 (Schering Plough; Kenilworth, NJ)] for 5-7 days, as previously described (36).

3.3.6. CD4$^+$ T cell isolation

On the day of establishing DC-T cell cultures, NA cells were thawed at 37^o C and washed in AIM-V medium. CD4$^+$ T cells were isolated from the NA cells by magnetic cell sorting (MACS; Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol.

3.3.7. In Vitro Stimulation (IVS)

DC were used as stimulators of autologous T cells and were either not infected or infected with recombinant adenoviral vectors (rAd): Ad.Ψ5 (empty vector; MOI 250); Ad.IL-12 (MOI 100); and Ad.IL-18 (MOI 250), using a previously-described protocol (220). The impact of viral infection on DC viability (Annexin-V) and maturation status (upregulation of CD83, MHC class I and II, CD80, CD86) was assessed by FACS analyses, as previously described (220). After infections, DC were incubated for 3 hours in 1 ml of T cell media along with peptides (10 μg/ml) or rMAGE (5 μg/ml). Once pulsed with antigen, DC were co-cultured with autologous $CD4^+$ T lymphocytes at a 1:10 (DC:T cell) ratio in T cell media.

3.3.8. ELISPOT

On day 11 of T cell cultures, the frequencies of peptide-specific $CD4^+$ T cell responders were measured using T2.DR4 cells as antigen-presenting cells in commercial human IFN-γ ELISPOT assays, as previously described (36, 208). The number of peptide-specific $CD4^+$ T cell responders was always compared to the background number of IFN-γ spots produced against APCs pulsed with the malarial circumsporozooite $CS_{326\text{-}345}$ peptide (for peptide-based assays) or with the TOP10 processed bacterial lysate in the analyses of the natural processing and presentation of rMAGE protein-derived peptides. Positive control wells contained T cells cultured in the presence of 10 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich).

3.3.9. Statistical Analyses

The statistical significance of differenced between T cell responses was determined using a Student's T test, with differences with an associated $p < 0.05$ considered significant.

3.4. Results

3.4.1. Recombinant adenoviral (Ad) vectors encoding IL-12p70 and mature IL-18 efficiently transduce DC resulting in the secretion of bioactive cytokines

Adenoviral vectors were initially evaluated for their ability to infect tumor cells, as well as immature DCs. Viral load used for infections was optimized for protein production and cell survival (data not shown). It was determined that a multiplicity of infection (MOI) of 100 for Ad.IL-12, and an MOI of 250 for Ad.IL-18 were optimal for efficient DC gene transduction. As previously described (220), adenoviral infection at these MOI did not induce terminal DC maturation (as determined by alterations in cell surface expression of maturation markers), nor did infections alter the capacity of these cells to subsequently mature in response to a cocktail of stimuli including IL-1, IL-6 and TNF-α (data not shown).

The secretion of transgene-encoded IL-12p70 and IL-18 protein was confirmed using specific ELISAs applied to supernatant samples harvested from cells infected *in vitro*. After infection of 10^6 DC with Ad.IL-12 or Ad.IL-18, and subsequent incubation for 48h at 37^o C, cell supernatants contained approximately 10-20 ng/ml of IL-12p70 and 200-300 pg/ml of IL-18, on average. Representative data are depicted in **Fig. 9**. Interestingly, although DC and tumor cells displayed comparable infection efficiencies, the amount of IL-18 produced by gene-modified tumor cells was approximately ten-fold higher than that observed for infected DC (despite comparable efficiencies of transduction, data not shown), suggesting differential post-translational regulation of IL-18 protein secretion may occur in these two cell types.

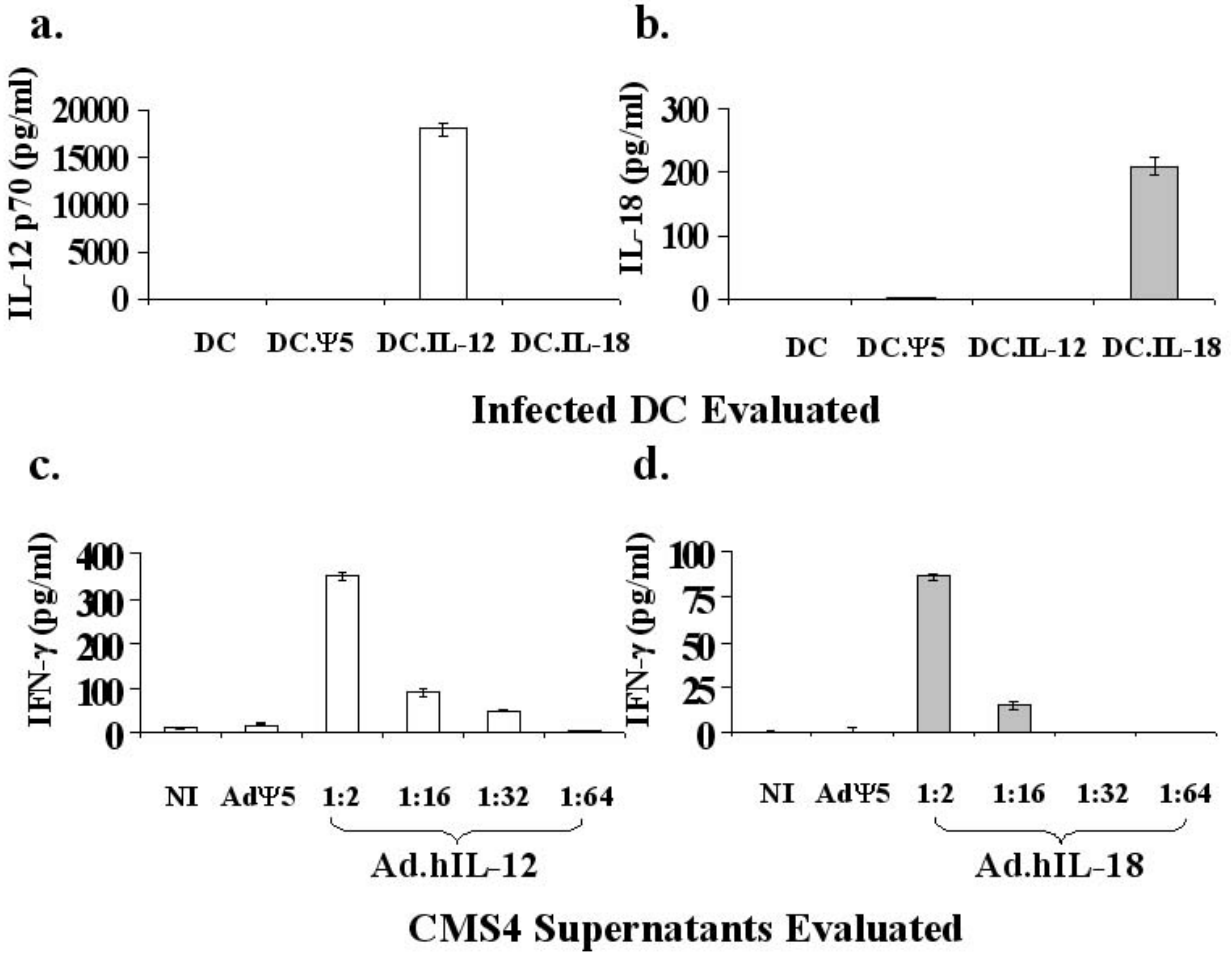


Figure 9. Recombinant adenoviruses (Ad) engineering of human DC to secrete bioactive IL-12p70 and IL-18.

One million DC were infected with Ad.Ψ5 (DC.Ψ5; MOI 250), Ad.IL-12 (DC.IL-12; MOI 100) or Ad.IL-18 (DC.IL-18; MOI 250) or were left uninfected (DC). After 48h of incubation, supernatants were collected from cultures and tested for (a) IL-12 p70 or (b) IL-18 protein levels using specific ELISAs. To test for cytokine bioactivity, 1 x 10^6 murine CMS4 cells were infected, as described above, and after 48h incubation, supernatants were collected, measured for transgene expression (231 ng/ml IL-12 p70; 3.5 ng/ml IL-18) and added into PBMC cultures at titrated doses (1:2, 1:16, 1:32, 1:64 dilution ratios of CMS4 supernatant to T cell media). After 48h, supernatants from 1 x 10^7 PBMC stimulated with (c) Ad.IL-12 infected (CMS4.IL-12) or (d) Ad.IL-18 infected (CMS4.IL-18) CMS4 supernatants were collected and analyzed for IFN-γ levels by ELISA. The results represent mean cytokine concentrations (pg/ml) +/- SD. Data are representative of 3 independent experiments performed for each virus. NI, not infected. *asterisks indicate statistically significant differences vs. all other cohorts ($p < 0.05$).

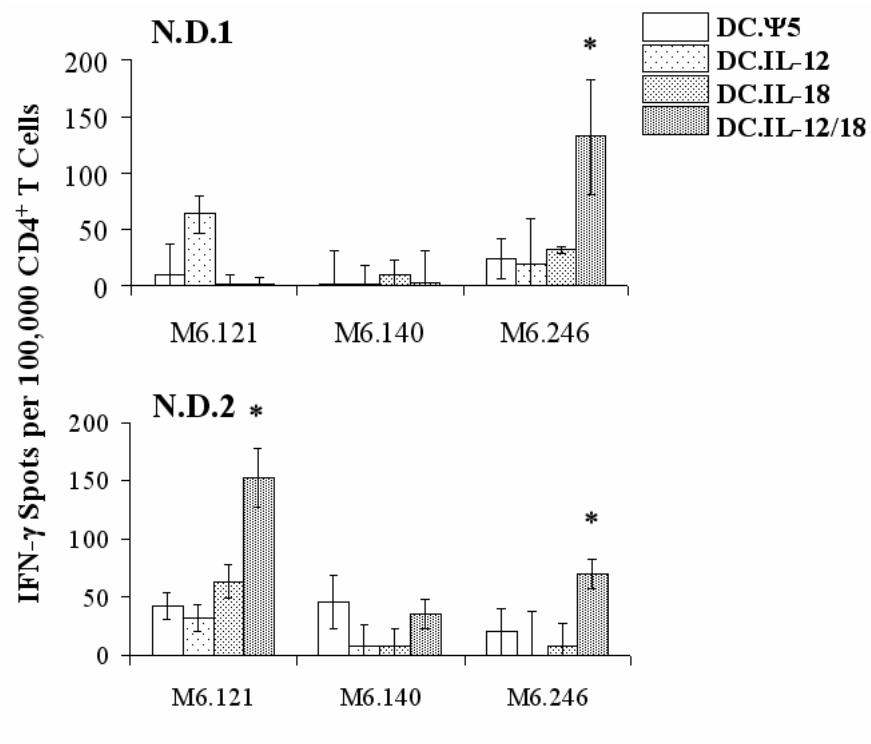
The bioactivity of these transgene products was determined indirectly by assessing the ability of infected cell supernatant samples to subsequently induce IFN-γ secretion from responder human PBMC. To minimize the impact of additional non-virus-induced factors on my assay results (i.e. other than IL-12p70 or IL-18), I analyzed supernatants from infected murine CMS4 tumor cells (191), to stimulate IFN-γ secretion from freshly-isolated PBMC. CMS4 supernatants were titrated on responder cells in order to discern any dose-dependent induction of IFN-γ secretion. Both the IL-12p70 (**Fig. 9c**) and IL-18 (**Fig. 9d**) transgene products were observed to induce IFN-γ secretion from PBMC in a dose-dependent manner, while control Ad-infected CMS4 supernatants did not.

3.4.2. DC co-infected with Ad.IL-12 and Ad.IL-18 exhibit enhanced Th1-type $CD4^+$ T cell immunostimulatory capacity when compared to control DC.

DC derived from two normal $HLA\text{-}DR4^+$ donors (ND) were infected with either Ad.Ψ5 control vector (DC.Ψ5), Ad.IL-12 (DC.IL-12) or Ad.IL-18 (DC.IL-18) alone, or co-infected with both Ad.IL-12 and Ad.IL-18 (DC.IL-12/18) at the previously determined, optimized MOIs. Following infection, DC were pulsed with $MAGE\text{-}A6_{121\text{-}144}$, $MAGE\text{-}A6_{140\text{-}170}$ and $MAGE\text{-}A6_{246\text{-}263}$ peptides for 3h, and then added to culture wells containing autologous $CD4^+$ T cells. A single round of *in vitro* stimulation was employed in order to most directly discern the ability of DC stimulations to amplify Th1-type responses against these epitopes (i.e. by avoiding extended *in vitro* cultures where the impact on existent immunity would be less interpretable). Eleven days after the initiation of the IVS protocol, IFN-γ ELISPOT assays were performed to enumerate peptide-specific Th1-type $CD4^+$ T cell responses (**Fig. 10**). While individual adenoviral infections had little effect on the induction of responses (except for ND1, where Ad.IL-12

infection of DC led to enhanced Th1-type responsiveness to a single MAGE-A6$_{121-144}$ peptide), co-infection of DC with both Ad.IL-12 and Ad.IL-18 led to enhanced stimulation of Type-1 CD4$^+$ T cell reactivity against the MAGE-A6$_{246-263}$ peptide in both donors, as well as against the MAGE-A6$_{121-144}$ peptide in ND2. Based on the reproducible superiority of DC.IL-12/18 vs. all other DC cohorts in promoting specific Type-1 anti-MAGE-A6 CD4$^+$ T cell responses *in vitro* among 6 normal donors analyzed (**Fig. 10** and data not shown), this modality was systematically adopted for all consequent analyses involving patient materials.

Figure 10. DC.IL-12/IL18 elicit superior Type-1 anti-MAGE-A6 CD4+ T cell responses from normal HLA-DR4+ normal donors.



CD4$^+$ T cells isolated from two HLA-DR4$^+$ normal donors were evaluated for their ability to be stimulated by, and react against, MAGE-A6$_{121-144}$ (M6.121), MAGE-A6$_{140-170}$ (M6.140), and MAGE-A6$_{246-263}$ (M6.246) peptides. A single stimulation using autologous DCs pulsed with 10 μg/ml of each of the individual MAGE-A6 peptides was used in each case. Prior to being pulsed with peptides, DCs were infected with Ad.Ψ5 (DC.Ψ5; MOI 250), Ad.IL-12 (DC.IL-12; MOI 100), Ad.IL-18 (DC.IL-18; MOI 250) or both Ad.IL-12 and Ad.IL-18 (DC.IL-12/18; MOI 100 for IL-12, and MOI 250 for IL-18). Responder T cells were assessed in IFN-γ ELISPOT assays for their functional reactivity on day 11 post-initial stimulation. Tests were performed in triplicate wells, using 100,000 CD4$^+$ T cells per well, along with 20,000 T2.DR4 cells and 10 μg/ml of the peptide being evaluated. Specific mean spot numbers per 10^5 CD4$^+$ T cells +/- SD values are reported. *asterisks indicate statistically significant differences vs. Ad.ψ5 controls ($p < 0.05$).

3.4.3. DC.IL-12/18 loaded with MAGE-A6 peptides/protein effectively stimulate epitope-specific Th1-type responses in melanoma patients following IVS

In these experiments, I evaluated $CD4^+$ T cell responses to cytokine gene-engineered DC-based stimulations in 8 HLA-$DR4^+$ patients with melanoma (**Table 7**). DC derived from patient PBMC were either not treated or co-infected with Ad.IL-12 and Ad.IL-18 (DC.IL-12/18). Both groups of DCs were either pulsed with a peptide mix (including MAGE-$A6_{121-144}$, MAGE-$A6_{140-170}$, MAGE-$A6_{246-263}$, and in some indicated cases, MAGE-$A6_{280-302}$) or with recombinant MAGE-A6 (rMAGE), then added to cultures of autologous $CD4^+$ T cells. Eleven days after the initiation of the IVS protocol, IFN-γ ELISPOT assays were performed in order to enumerate the frequencies of peptide-specific Th1-type $CD4^+$ T cell responders. Peptide-pulsed DC.IL-12/18 enhanced the number/frequency of responder T cells as well as the magnitude of mean responses, especially against the MAGE-$A6_{121-144}$ and MAGE-$A6_{280-302}$ epitopes, while peptide stimulations with control, uninfected DC were ineffective in modulating specific $CD4^+$ T responses (**Table 7**). Similarly, rMAGE-pulsed DC.IL-12/18 were more effective than protein-pulsed, uninfected DC in stimulating epitope-specific $CD4^+$ T cell responses *in vitro*. Anti-MAGE-$A6_{121-144,}$ -MAGE-$A6_{280-302}$, as well as -MAGE-$A6_{246-263}$ mean $CD4^+$ T cell responses were significantly enhanced following stimulation with rMAGE-pulsed DC.IL-12/18 (**Table 7**). Indeed, all of the donors evaluated responded to at least one MAGE-A6 epitope in the ELISPOT assay following stimulation with rMAGE protein-loaded DC.IL-12/18. In the majority of cases, protein-pulsed uninfected DC appeared more effective than peptide-pulsed, uninfected DC in promoting Type-1 responses (**Table 7**). Overall, these results suggest the superior immunostimulatory effectiveness of DC.IL-12/18 (vs. control DC) loaded with either synthetic

MAGE-A6 peptides or in particular, rMAGE protein, in stimulating specific Th1-type $CD4^+$ T cell responses from patients with melanoma *in vitro*.

Table 7. $CD4^+$ T cell responses by HLA-DR4+ melanoma patients against MAGE-6 peptide epitopes elicited by DC-based in vitro stimulation.

$CD4^+$ T cells isolated from the peripheral blood of eight melanoma patients were tested for their ability to be stimulated by, and react against, the indicated MAGE-A6 peptide epitopes. Patient disease stage is cited, with (*) indicating patients who had no evidence of disease at the time of blood donation. $CD4^+$ T cells were stimulated a single time *in vitro* using autologous DC pulsed with a mixture of 10 μg/ml of each MAGE-A6 peptide (Peptide Mix), rMAGE protein or control peptide/protein. Prior to being pulsed with antigen, DCs were infected with Ad.Ψ5 (MOI 250) or Ad.IL-12 and Ad.IL-18 (DC.IL-12/18; MOI 100 for IL-12, and MOI 250 for IL-18), or were left uninfected. Responder T cells were assessed in IFN-γ ELISPOT assays as described in the **Fig. 8** legend. Individual patient Th1-type responses against specific MAGE-A6 peptide epitopes and their cumulative responses against all 4 epitopes are listed. In addition, I provide the (**) mean values for all patients' peptide-specific and cumulative responses against all 4 peptides analyzed. Mean IFN-γ spots/10^5 $CD4^+$ T cells are reported; with [A]**bolded** values representing significant ($p < 0.05$) increases in response to the DC.IL-12/18- vs. control DC-based stimulations and [B]underlined values representing significant ($p < 0.05$) increases in rMAGE protein- vs. peptide-based stimulations. Abbreviations: NT, not tested.

IFN-γ Spots/10^5 $CD4^+$ T Cells Evaluated[A,B]:

		Mel1	Mel2	Mel3	Mel4	Mel5	Mel6	Mel7	Mel8	
	Stage	IV	IV	I	IIB*	IIA*	II	III	II	
Stimulation	Readout									Mean**
DC Peptide Mix	M6.121	18	0	6	0	0	28	0	0	7
	M6.140	18	0	10	0	0	0	0	0	4
	M6.246	14	0	4	0	0	0	0	0	2
	M6.280	NT	NT	NT	0	0	2	0	NT	0.5
	Cumulative	50	0	20	0	0	30	0	0	13
DC rMAGE	M6.121	214	0	18	30	0	45	0	0	38
	M6.140	0	0	0	0	0	22	0	4	3
	M6.246	122	0	16	0	0	44	0	0	23
	M6.280	NT	NT	NT	0	0	30	0	NT	8
	Cumulative	336	0	34	30	0	141	0	4	68
DC.IL-12/18 Peptide Mix	M6.121	0	NT	NT	**136**	0	22	0	**88**	**41**
	M6.140	0	NT	NT	0	0	0	0	0	0
	M6.246	0	NT	NT	**74**	0	24	0	0	16
	M6.280	NT	NT	NT	**98**	0	0	0	NT	**25**
	Cumulative	0	NT	NT	**308**	0	46	0	88	**73**
DC.IL-12/18 rMAGE	M6.121	**320**	**268**	20	**124**	0	6	**42**	**58**	**105**
	M6.140	0	22	2	**94**	0	38	0	12	21
	M6.246	**204**	0	0	**136**	**70**	**120**	**94**	**116**	**93**
	M6.280	NT	NT	NT	**32**	**58**	**180**	**62**	NT	**83**
	Cumulative	**524**	**290**	22	**386**	**128**	**344**	**198**	**186**	**260**

3.5. Discussion

In this study, I generated a novel, functional adenoviral vector encoding the mature form of human IL-18 protein, as well as a functional adenoviral vector encoding for human IL-12p70 protein, and analyzed their ability to infect human DC, thereby yielding a superior *in vitro* "vaccine" capable of promoting tumor-specific Type-1 $CD4^+$ T cell responses. Consistent with previous reports by our group and others evaluating adenoviral infection of DC (191, 220, 221), neither vector induced terminal DC maturation following infection (based on phenotypic criteria), resulting in engineered DC that were functionally competent to uptake and process soluble antigens (such as rMAGE).

To directly assess the Th1-stimulating properties associated with Ad.IL-12 and Ad.IL-18 infection on DC, and not alternate stimuli, engineered DC were not concomitantly or consequently matured using cytokine/prostanoid cocktails. Given the desire to interpret the acute effects of DC-based stimulations on a perceived Type-1 dysfunctional T cell repertoire in cancer patients (208, 222), I analyzed peripheral blood $CD4^+$ T cells isolated from HLA-$DR4^+$ normal donors activated after a single-round of *in vitro* sensitization. I evaluated normal donor and patient $CD4^+$ T cells stimulated with uninfected DC or Ad-engineered DC loaded with MAGE-A6 peptides or protein for their ability to secrete IFN-γ when restimulated (in ELISPOT assays) with three known HLA-DR4-restricted MAGE-A6-derived (MAGE-$A6_{121\text{-}144}$, MAGE-$A6_{140\text{-}170}$, MAGE-$A6_{246\text{-}263}$ epitopes (208, 222); and a novel poly-DR-presented MAGE-$A6_{280\text{-}302}$[1]) epitopes. My results suggest that DC.IL-12/18 were the most effective antigen presenting cells for the promotion of Th1-type responses (that may be associated with tumor regression *in situ*; ref. (191)) among those tested in this study. In contrast, DC infected with either Ad.IL-12

(DC.IL-12) or Ad.IL-18 (DC.IL-18) exhibited immunostimulatory capacities that were no greater than those noted for DC infected with Ad.Ψ5 (DC.Ψ5). The improved efficacy associated with DC.IL-12/18 stimulator cells may reflect the knowledge that IL-12p70 is required to induce T cell surface expression of the IL-18 receptor (IL-18R) on naïve T cells (167), while the IL-1 family member, IL-18, potentiates the differentiation of Th1 cells instigated by IL-12p70 (168), and that IL-12 and IL-18 synergize in the activation of T cells and induction of IFN-γ secretion from T cell responders (165, 166).

To evaluate the efficacy of this approach in the cancer setting, monocyte-derived DC were generated from eight HLA-DR4$^+$ melanoma patients and used to stimulate autologous CD4$^+$ T cells *in vitro*. Uninfected DC were compared to DC.IL-12/18 for their ability to stimulate Th1-type responses after being pulsed with synthetic peptides or rMAGE protein. I observed that DC.IL-12/18 were more effective in stimulating peptide-specific Th1-type responses than uninfected DC, especially when loaded with rMAGE protein, in which case, every patient (regardless of clinical staging and the presence/absence of disease at the time of analysis) evaluated responded to at least one of the previously-defined, naturally-processed/presented MAGE-A6-derived epitopes evaluated following *in vitro* sensitization. This suggests that DC.IL-12/18 may be suitable for use in vaccines designed to override the general Type-1 dysfunction in CD4$^+$ T cell responses to tumor antigens observed in many cancer patients with advanced stage disease (100, 101, 208, 222), in support of therapeutic immunity in the clinical setting.

In conjunction with the knowledge that IL-18 is a chemoattractant for myeloid and plasmacytoid DC (171, 172), one could also hypothesize that protein-pulsed DC.IL-12/18, if injected s.c. or intratumorally, might not only induce Th1-type responses, but also attract

additional host DCs into the vaccine/tumor microenvironment. Within this microenvironment, such a paradigm could enhance consequent tumor cell apoptosis, tumor antigen uptake and the cross-priming of anti-tumor T cells *in vivo* (191).

In summary, I have generated a novel Ad.hIL-18, and have shown that when used in conjunction with Ad.hIL-12 to co-infect human DC, a vaccine platform (DC.IL-12/18) is produced that may be loaded with a recombinant tumor antigen or its derivative peptides in order to effectively induce specific Th1-type $CD4^+$ T cell responses *in vitro* from normal donors and patients with melanoma. Further investigation is warranted to determine whether this strategy can be translated into the clinic as a therapeutic vaccine, either as a stand-alone modality, or when administered in combination with agents that ablate or neutralize functional regulatory T cell activity *in situ* (223-225), in order to optimally potentiate therapeutic Type-1 anti-tumor immunity *in vivo*.

GENERAL DISCUSSION

While sustained $CD8^+$ T lymphocyte responses are required for effective clearance of tumors, anti-tumor $CD4^+$ T cells are at least equally important due to their ability to regulate the quality, magnitude and durability of $CD8^+$ CTL-mediated immunity *in vivo*. Previous studies of peptide-specific, $CD4^+$ T cell responses against tumor antigens, including MAGE-A6, have been traditionally focused on an analysis of a single or very limited set of HLA-DR restriction elements. Given the extreme polymorphism among HLA-DR alleles, the potential utility of the previously defined epitopes is limited to only a minority of patients. Optimally, TAA-derived peptides should be promiscuously binding (i.e. presented by multiple HLA-DR alleles; poly-DR), which would increase their clinical applicability by making them immunogenic in a high frequency of responders. Furthermore, any potential clinical modality implementing these peptides needs to circumvent immuno-inhibitory properties of tumors, and stimulate Th1-type responses. In these studies I sought to define poly-DR presented MAGE-A6 Th epitopes that would clinically be broadly applicable. Also, I wanted to know whether I could stimulate Type-1 responses *in vitro* by engineering antigen-presenting DCs to secrete high levels of the IFN-γ-inducing cytokines IL-12p70 and IL-18. I defined two highly-immunogenic poly-DR-binding, MAGE-A6-derived epitopes, one of which shares immunogenicity with a *Mycoplasma penetrans* HF-2 permease-derived epitope (MPHF2). Interestingly, the microbial epitope was more effective at stimulating MAGE-A6-specific responses *in vitro* than its MAGE-A6 counterpart, capable of promoting previously deficient patient Th1-type responses. Furthermore, I showed that human DCs engineered with recombinant adenoviral vectors encoding IL-12p70 and IL-18 represent an efficient *in vitro* stimulus capable of effectively promoting specific Th1-type responses.

First, using DC1-based *in vitro* vaccinations as a model system, I wanted to define poly-DR-binding MAGE-A6-derived epitopes that would be recognized by $CD4^+$ T-lymphocytes in a high frequency of responders regardless of their HLA-DR phenotype. The MAGE-A6 protein sequence was analyzed using the ProPred HLA-DR peptide-binding algorithm, and MAGE-A6 peptide sequences were selected based on their predicted ability to bind the broadest HLA-DR repertoire. I synthesized 4 putative poly-DR peptides, and analyzed Th1 cell responses against them. Two novel (MAGE-$A6_{172\text{-}187}$ and MAGE-$A6_{280\text{-}302}$) epitopes and a previously-defined (MAGE-$6_{140\text{-}170}$) HLA-DR4-restricted epitope were recognized by $CD4^+$ T cells isolated from most normal donors and patients with melanoma that were evaluated, with MAGE-$A6_{172\text{-}187}$ and MAGE-$A6_{280\text{-}302}$ being the most immunogenic. These epitopes appear to be naturally-processed and presented in the context of MHC class II molecules, as peptide-specific $CD4^+$ T cells also recognized autologous monocytes pulsed with full length MAGE-A6 protein.

Interestingly, normal donors mounted detectable Th1-type responses against the MAGE-$A6_{172\text{-}187}$ and MAGE-$A6_{280\text{-}302}$ peptides after only a single-round of IVS. I hypothesized that the MAGE-A6 peptides evaluated shared sufficient sequence homologies to microbial proteins to which many individuals may have become naturally primed against, allowing for functional cross-reactivity to be detected in my assays. I performed sequence homology searches of the GenBank database for potential sources of cross-reactive epitopes. These analyses suggested that the most immunogenic MAGE-$A6_{172\text{-}187}$ and MAGE-$A6_{280\text{-}302}$ epitopes exhibited significant homologies to a number of known microbial sequences, of which the most likely encountered candidates are *Mycoplasma penetrans* HF-2 permease-derived (MPHF2) and Chlamydia muridarum Nigg conserved hypothetical protein TC0097-derived (CHP) epitopes respectively. Unlike MAGE-$A6_{172\text{-}187}$ and MAGE-$A6_{280\text{-}302}$, MAGE-$A6_{140\text{-}170}$ (moderately immunogenic) and

MAGE-A6$_{192-214}$ (poorly immunogenic) epitopes did not share any identified microbial homologies according to the GenBank database search.

CHP homologue peptide did not exhibit any significant cross-stimulatory ability to MAGE-A6$_{280-302}$, which was perhaps not surprising as it is derived from a mouse-tropic strain of Chlamydia and is rarely encountered by humans. Contrary to this, CD4$^+$ T cells primed with the MPHF2 peptide cross-reacted against autologous monocytes pulsed with the MAGE-A6$_{172-187}$ peptide or rMAGE protein, and recognized HLA-matched MAGE-A6$^+$ melanoma cell lines in an MHC class II-restricted fashion. CD4$^+$ T cells generated using the MAGE-A6 peptide recognized APCs only when pulsed with relatively high concentrations of peptide (with a half-maximal response associated with a peptide dose of 3-10 μM), while MPHF2-stimulated cells recognized target cells pulsed with approximately 1000-fold lower concentrations of peptide. Furthermore, stimulations with MPHF2 were capable of promoting previously deficient patient Th1-type responses to MAGE-A6, further supporting the potential clinical relevance for this peptide.

Mycoplasma infections have been shown to stimulate Th1, Th2, and Treg responses. However, in a manner similarly to that of tumors, their effective clearance is associated with Th1-type responses (226). As stimulations with MPHF2 yielded rapid, heteroclitic MAGE-A6 Th1 responses, it will be of interest to examine whether this is prompted from a resident pool of memory CD4$^+$ T cells in patients, or from an as yet antigen-inexperienced population of T cells that could potentially be primed *in vivo* and provide therapeutic benefit to patients with MAGE-A6$^+$ lesions. My preliminary studies show that both Th1 and Th2 responses could be observed in freshly-isolated CD4$^+$ T cells against the MPHF2, but not the CS$_{326-345}$ or MAGE-A6$_{172-187}$ peptides (**Figure 11**). While these results suggest the likelihood of a memory response, this observation requires further investigation of MPHF2 responsiveness (in ELISPOT assays and/or

by HLA-DR-restricted MPHF2 tetramer staining assays) by $CD45RO^+$ $CD4^+$ memory T cells sorted from patient peripheral blood.

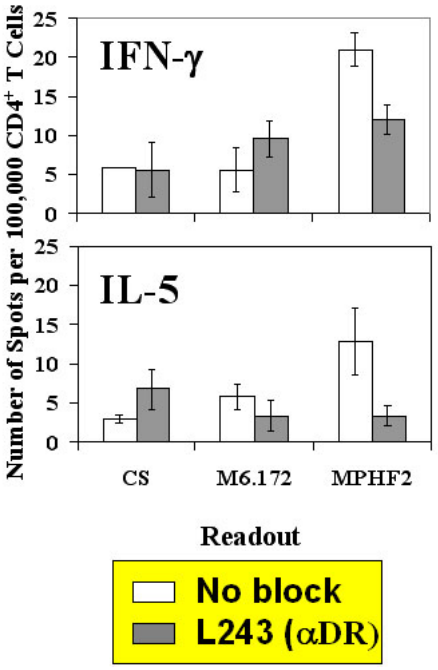


Figure 11. Evaluation of Freshly Isolated $CD4^+$ MPHF2 Recall T Cell Responses in a Normal Donor.

$CD4^+$ T cells isolated from two normal donors were directly plated onto pre-coated IFN-γ and IL-5 ELISPOT plates, along with autologous monocytes at a 1:5 ratio. HLA-DR blocking was performed in appropriate wells for 3 h with L243 antibody (20 μg/ml). 10 μg/ml of $CS_{326-345}$, MAGE-$A6_{172-187}$, and MPHF2 peptides were subsequently added, and cells were incubated for 72 h, when the ELISPOT plates were developed. A representative experiment is shown.

If these results confirm the existence of a resident memory $CD4^+$ T cell population that is specific for MPHF2 in a large cohort of cancer patients, this would elicit several important questions regarding the tumor immunity and patient survival. Optimally, the cross-reactive memory cells would provide protective immunity against the TAA. Nevertheless, the available data suggests that tumor immunogenicity can be modified by the selective pressure of the immune system, resulting in the growth of tumors that are poorly immunogenic, and capable of escaping immune detection, and/or to actively inhibit immune effectors (227). Hence, in the MAGE-A6 system, this would suggest that if heteroclitic memory responses exist, the selective pressure of the immune system could potentially delete MAGE-$A6^+$ tumor cells, leaving behind MAGE-$A6^-$ lesions. However, as greater expression of MAGE proteins has been associated with

an invasive phenotype and a decreased survival of cancer patients, it could be hypothesized that in a majority of patients there is not a sufficient stimulus to active MAGE-A6 cross-reactive T cells, and to therefore provide clinical benefit to the affected patients. As a result, one would need to analyze whether potential memory responses to MPHF2 could be exploited in order to effectively stimulate cross-reactive, long-term protective responses to MAGE-A6 *in vivo*. Furthermore, it would be of interest to determine whether an active *Mycoplasma penetrans* HF-2 (*M. penetrans*) infection provides beneficial immunity to tumor patients with MAGE-A6$^+$ lesions.

To study potential tumor-preventive or -therapeutic benefits of MPHF2 peptide immunization, a next step could be to develop an immunization system in animals. Using the "humanized" HLA-DR4 transgenic mouse model (228), one can stimulate MPHF2-specific responses by immunizing mice with DCs conditioned to stimulate Type-1 responses and loaded with MPHF2 peptide or engineered to express full length MPHF2. Subsequent CD4$^+$ T cell memory responses can be tracked by tetramer and/or ELISPOT analyses. Immunized mice can further be challenged with a MAGE-A6$^+$ HLA-DR4$^+$ tumor cell line (possibly a murine tumor cell line engineered to express MAGE-A6 and HLA-DR4), and the therapeutic/preventing relevance of MPHF2 can be measured in terms of animal survival, tumor growth, CD4$^+$ T cell recall responses, as well as the phenotype and function of TILs. If the results were to show that this modality had protective value, this would provide strong support for to the potential clinical utility of this epitope.

To study potential immuno-protective effects of *M. penetrans* infection against MAGE-A6$^+$ lesions, I would have to perform an epidemiological evaluation of cancer patients with a simultaneous *M. penetrans* infection. The most likely candidates for this study would be patients

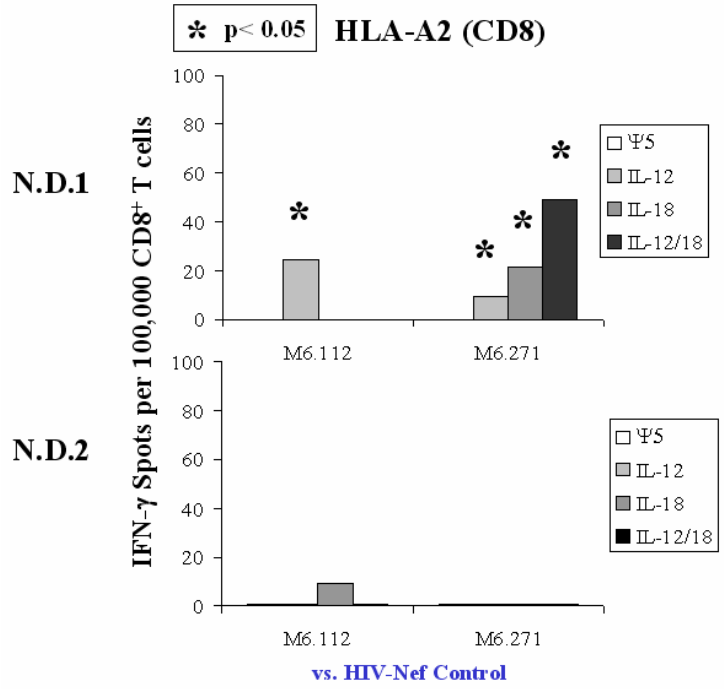
with spontaneously-regressing melanoma lesions since the majority of them are MAGE-A6$^+$ and easily accessible for tumor evaluation. As *M. penetrans* has been suggested to be a primary cause of non-HIV-related urethritis and respiratory disease, I would need to correlate spontaneously-regressing melanomas to an active or recently concluded *M. penetrans* infection. Frequencies of circulating MPHF2-specific CD4$^+$ T cells can be measured by tetramer analysis and, upon surgical removal, melanoma lesions could be analyzed for MAGE-A6 expression by RT-PCR. These results would provide indirect correlation between *M. penetrans* infection and its possible protective properties against MAGE-A6.

As patients with malignant tumors exhibit dysfunctional Th1-type responses against TAA-derived epitopes, it is imperative to re-establish the Th1 functionality in order to enhance their chances of survival. I hypothesized that patient anti-tumor Th1-type responses might be recovered/enhanced by *in vitro* stimulation of CD4$^+$ T cells using vaccines containing autologous DCs engineered to secrete high levels of the IFN-γ-inducing cytokines, IL-12p70 and/or IL-18. In the second study, I investigated whether human DCs engineered to secrete IL-12p70 and IL-18 by recombinant adenoviral infection can promote and enhance Th1-type, MAGE-A6-specific responses *in vitro*. I chose this modality as it could not only prove capable of supporting Type-1 immunity, but also would have the potential to limit toxicities previously observed for systemic application IL-12p70 alone or IL-12p70 combined with IL-18. Initial results showed that DC infected with both Ad.IL-12 and Ad.IL-18 (DC.IL-12/18) were more effective at stimulating MAGE-A6-specific Th1-type CD4$^+$ T cell responses than DCs infected with either of the cytokine vectors alone, Ad.Ψ5 virus (control vector) or uninfected DC. Further tests of this vaccine strategy showed that DC.IL-12/18 loaded with MAGE-A6 peptides or rMAGE effectively stimulated epitope-specific Th1-type CD4$^+$ T cell responses in melanoma patients

following a single round of IVS regardless of their disease stage or current disease status. In particular, DC.IL-12/18 loaded with rMAGE were the most effective stimulators of Th1-type immunity *in vitro,* with responses frequently being directed against multiple MAGE-A6-derived epitopes. My results show that DC.IL-12/18 loaded with tumor peptides or recombinant tumor antigens may represent an effective vaccine capable of selectively promoting Th1-type $CD4^+$ T cell immunity in patients with cancer who may display existing immune dysfunction.

Using the same modality, I compared the ability of DC.IL-12/18 loaded with MAGE-A6-derived HLA-A2 peptides or rMAGE to stimulate Type-1 $CD8^+$ T cell responses. My initial results suggested that DC.IL-12/18 could potentially enhance Type-1 $CD8^+$ T cell responses when compared to DCs infected with either of the cytokine vectors alone, Ad.Ψ5 virus (control vector) or uninfected DC (**Figure 12**).

Figure 12. DC.IL-12/IL18 enhance Type-1 anti-MAGE-A6 CD8+ T cell responses from normal HLA-A2+ normal donors.



$CD8^+$ T cells isolated from two HLA-$A2^+$ normal donors were evaluated for their ability to be stimulated by, and react against, MAGE-$A6_{112\text{-}120}$ (M6.112), and MAGE-$A6_{271\text{-}279}$ (M6.271) peptides. Stimulation was performed as described in **Figure 12**. Responder T cells were assessed in IFN-γ ELISPOT assays for their functional reactivity on day 11 post-initial stimulation. Tests were performed in triplicate wells, using 100,000 $CD8^+$ T cells per well, along with 20,000 T2.DR4 cells and 10 μg/ml of the peptide being evaluated. Specific mean spot numbers per 10^5 CD8+ T cells +/- SD values are reported. *asterisks indicate statistically significant differences vs. Ad.Ψ5 controls ($p < 0.05$).

However, after evaluating the ability of DC.IL-12/18 to stimulate melanoma patient Type-1 $CD8^+$ T cell responses, it was clear that the strategy was not as effective as it was for stimulating Th1 $CD4^+$ T cell responses (**Figure 13**). While DC.IL-12/18-based IVS enhanced the number of $CD8^+$ T cell responders when compared to uninfected DCs, particularly when used in conjunction with rMAGE, actual frequencies of peptide-specific Type-1 $CD8^+$ T cells stimulated were low (**Figure 13**).

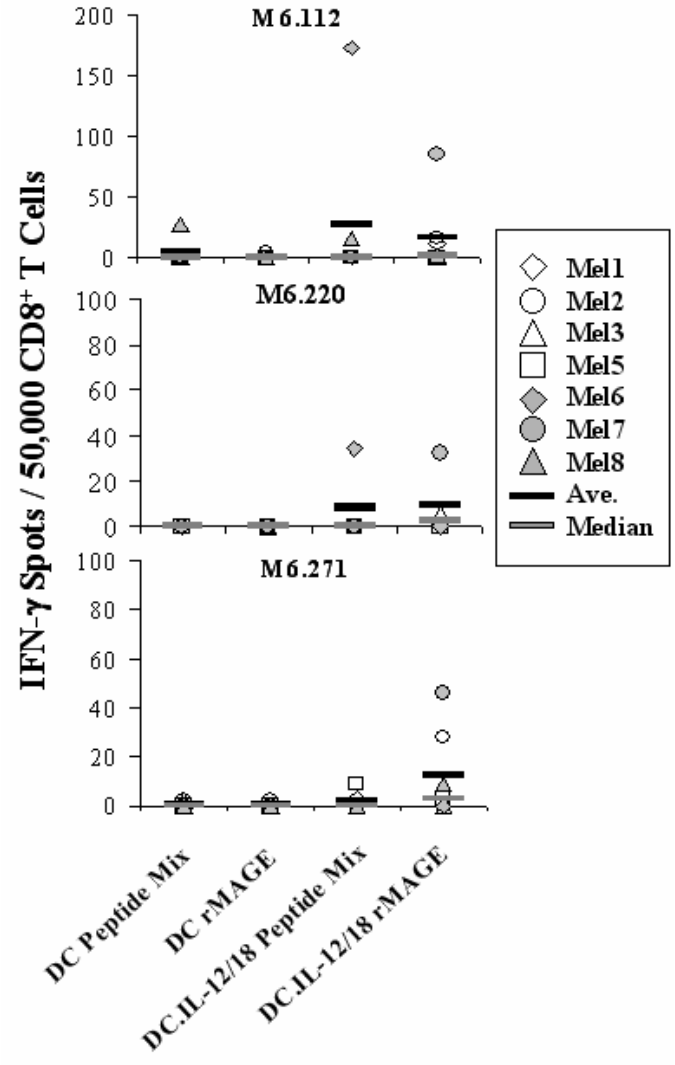


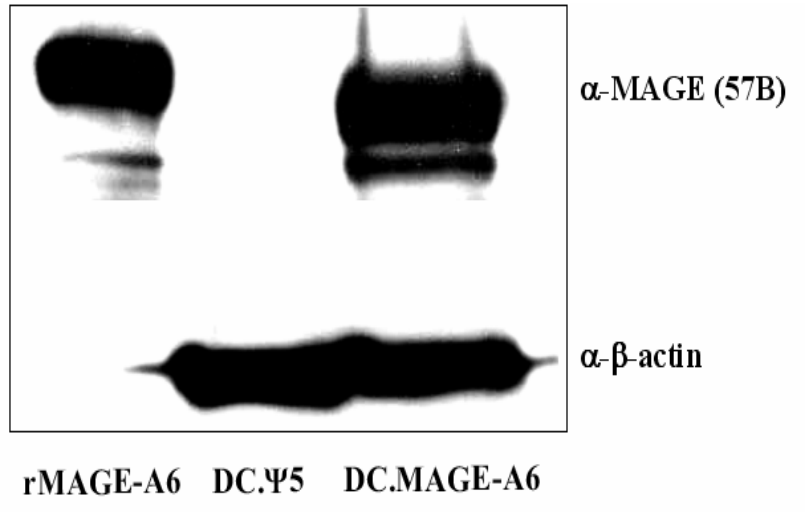
Figure 13. $CD8^+$ T cell responses by HLA-A2+ melanoma patients against MAGE-6 peptide epitopes elicited by DC-based in vitro stimulation.

$CD8^+$ T cells isolated from the peripheral blood of eight melanoma patients were tested for their ability to be stimulated by, and react against, the indicated MAGE-A6 peptide epitopes. $CD8^+$ T cells were stimulated as described in **Table 6**. Responder T cells were assessed in IFN-γ ELISPOT assays as described in the **Fig. 12** legend. Individual patient Type-1 responses against specific MAGE-A6 peptide epitopes and their cumulative responses against 3 HLA-A2 epitopes are listed.

There are several potential reasons for these observations. DCs used in this study were of immature phenotype, therefore they were not efficient at presenting antigens due to their low

expression of co-stimulatory molecules that are needed for optimal activation of $CD8^+$ T cells. While it appears that IL-12 and IL-18 cytokines secreted by DC.IL-12/18 provided enough stimuli for Th1 $CD4^+$ T cells to get activated, this was not the case when it came to stimulating Type-1 $CD8^+$ T cell responses. Another possibility is that the antigen format used for $CD8^+$ T cell stimulation was not adequate for properly stimulating MAGE-A6-specific responses. For that reason, I constructed a recombinant adenoviral vector encoding MAGE-A6 (Ad.MAGE), and capable of efficiently infecting DCs (**Figure 14).**

Figure 14. Recombinant adenovirus engineering of human DCs to express MAGE-A6.



DCs were either infected using the Ad.Ψ5 (empty adenoviral vector; MOI 250) or using the recombinant MAGE-A6 adenoviral virus (Ad.MAGE-A6; MOI 250). Following the 48 h incubation, DCs were collected and lysed using the freeze-thaw method. Western Blotting was performed on the collected lysates, as well as the rMAGE-A6 (positive control), using the anti-MAGE mAb 57B against rMAGE, or the anti-β-actin mAb.

Mature DC1s infected with Ad.MAGE efficiently stimulated Type-1 $CD8^+$ T cell responders but not $CD4^+$ T cells (**Figure 15**). I hypothesize that DC.IL-12/18 infected with Ad.MAGE and subsequently matured would be an efficient APC for stimulating Type-1 $CD8^+$ T cells.

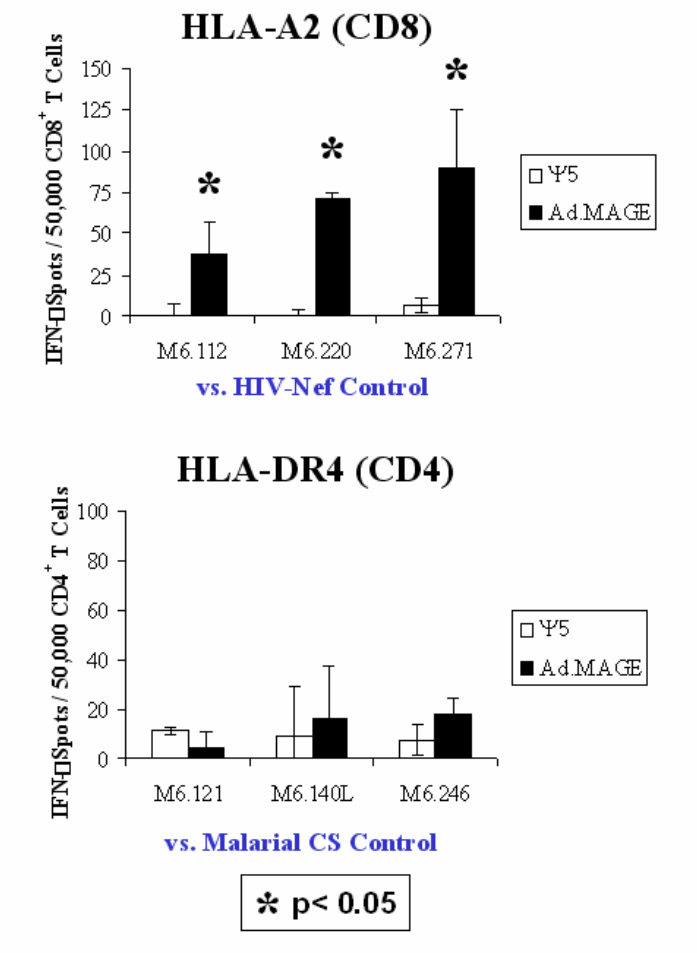


Figure 15. DCs engineered to express MAGE-A6 effectively stimulate Type-1 CD8$^+$ T cell responses to MAGE-A6-derived HLA-A2 epitope.

DC1s (described in **Chapter 2**) infected with the Ad.Ψ5 (empty adenoviral vector; MOI 250) or with the recombinant MAGE-A6 adenoviral virus (Ad.MAGE-A6; MOI 250) were used to stimulate CD8$^+$ T cells *in vitro* for one round of stimulation. Responder T cells were assessed in IFN-γ ELISPOT assays as described in the **Fig. 12** legend. Individual patient Type-1 responses against specific MAGE-A6 peptide epitopes and their cumulative responses against 3 HLA-A2 epitopes are listed.

My analysis of DC.IL-12/18 stimulated and activated T cells did not include the analysis of expression of chemokine receptors (CCR) and cell-adhesion molecules. While resting T cells do not express any CCRs, in activated and memory T lymphocytes chemokines serve to activate T cell integrins and allow them to mediate strong binding to vascular adhesion molecules such as VCAM-1 and ICAM-1. CCRs characteristic for CD4$^+$ Th1 cells are CCR5 and CXCR3, and they have been implicated in their recruitment to inflammatory sites. IL-12p70 was implicated as a central cytokine required for induction of CCR5 on TCR/CD28-stimulated CD4$^+$ and CD8$^+$ T

cells. It was also shown in a mouse tumor model that IL-12p70 induces enhanced intratumoral T cell migration that is dependent on the interaction between LFA-1 and VLA-4 adhesion molecules with their ligands (ICAM-1 and VCAM-1 respectively) on tumor-associated vasculature (229). Therefore it will be of interest to see whether T cells stimulated with DC.IL-12/18 would stimulate expression of CCR5 that would allow for their potential trafficking into tumor sites.

Multiple vaccination strategies utilizing the innate immunostimulatory ability of DCs have been developed, however the ideal vaccination protocol with these cells has not yet been described. Previous clinical trials implementing various DC-based vaccination strategies did not yield completely satisfactory results, but did show potential that supported their further development. Effective immunostimulatory strategies described in these studies could provide potentially novel vaccination modalities that could circumvent immunoinhibitory properties of certain tumors and enhance Type-1 responses in cancer patients. I have described here three different MAGE-A6 antigen formats that in a clinical setting could be used alone or in conjunction with DCs to preferentially stimulate $CD4^+$ or $CD8^+$ T cell responses in patients with MAGE-$A6^+$ lesions.

An optimal immunization strategy would simultaneously stimulate both of these T cell subsets in a Type-1 manner. One clinical strategy would require direct immunization of patients with the rMAGE or MAGE-A6- and/or MPHF2-derived peptides in conjunction with an immunological adjuvant such as SBAS-2 [the mix of the QS21 saponin and of monophosphoryl lipid A (MPL)] (230), relying on patient's immune system to process antigens and stimulate T cell responses. This strategy would not be labor-intensive, however its clinical benefits, based on previous studies, would most likely be less than satisfactory (203).

As DC-based immunizations were shown to be one of the better vaccination strategies, it could be hypothesized that antigen-loaded, Type-1-polarizing DC.IL-12/18 modality tested in these studies has the potential to be implemented in a clinical setting. These cells can be pulsed with MHC class I and II-restricted peptides, engineered to express MAGE-A6 using Ad.MAGE-A6, or loaded with rMAGE. The translational utility of most of the peptides defined is clinically limited to a modest group of patients due to a requirement for expression of specific HLA haplotypes. While poly-DR-binding MAGE-A6 and MPHF2 Th epitopes described here could be applicable in an extended cohort of patients to stimulate MAGE-A6-restricted Th1 responses, previously-described MHC class I-binding peptides were shown to have a greater level of HLA haplotype restriction. rMAGE-loaded and/or Ad.MAGE-A6-infected DC.IL-12/18 could provide a way to stimulate $CD4^+$ and $CD8^+$ T cell responses respectively in cancer patients without HLA haplotype restrictions. Combinational therapy with both rMAGE and Ad.MAGE-A6, that primarily stimulate $CD4^+$ and $CD8^+$ T cell subsets respectively, could provide for an effective immunization strategy. Furthermore, as mature DCs were shown to be superior at inducing T-cell responses than immature DCs in a clinical setting (231), it could be theorized that an optimal DC.IL-12/18-based immunization strategy should utilize matured, antigen-loaded DC.IL-12/18 capable of migrating to a lymph node and effectively stimulating both T cell subsets. Ultimately the goal of these vaccinations would be to not only stimulate MAGE-A6-specific responses, but also promote vaccine-induced epitope spreading, leading to enhanced, clinically-effective T cell repertoire against other TAAs.

The effectiveness of a DC-based vaccine depends not only on the choice of TAA and its format (i.e. peptide, recombinant protein or gene transfection), but on the vaccination schedule in terms of dose (i.e. number of autologous DCs), frequency, number and site of immunization.

Reported doses range from 3-20 x 10^6 DCs per immunization, with an average dose of 10 x 10^6 cells, with multiple vaccination schedules ranging from 1-6 week intervals. Patents are immunized either by intratumoral (i.t.), subcutaneous (s.c.) in the leg or arm closest to intact lymph nodes, or ultrasound-guided intranodal (i.n.) injections (231-233). One thing that is common in these studies is that patients have to be repeatedly vaccinated in order to stimulate high enough frequencies of effective Type-1 T cells. While there are no studies that specifically correlate TAA-specifc T cell numbers to effective tumor rejection, an increase in frequency of TAA-specific lymphocytes following immunizations is generally correlated to a better survival of patients (234). Based on these reports, a potential antigen-loaded DC.IL-12/18 strategy could require a s.c. or i.n. bi-weekly immunizations. Due to high levels of IL-12 and IL-18 being secreted by DC.IL-12/18, the number of cells used for immunization would have to be subsequently determined based on any toxicities that could arise as a result of vaccinations. An additional concern is whether adenovirally-engineered DCs can persist long enough *in situ* to stimulate effective T cell responses as most people have developed immune responses to naturally-encountered adenoviruses that could eliminate adenovirally-infected cells. Due to this concern, multiple strains of recombinant adenoviral vectors have been developed that, if engineered to encode for IL-12p70 and IL-18, could allow for multiple vaccinations.

Any potential DC.IL-12/18-based vaccination should also encompass a strategy to antagonize Treg numbers and function, as it was shown that this subset of cells has a profound ability to suppress anti-tumor immune responses in cancer patients. While there are no agents that would affect Treg specifically, there are strategies that could eliminate these cells at a greater frequency than other T lymphocyte subsets. DC.IL-12/18 could be administered in combination with agents that ablate or neutralize functional regulatory T cell activity *in situ*,

such as cyclophosphamide, anti-CD25 antibody, GITR ligand (235, 236), or rIL-2 diphtheria toxin conjugate DAB_{389}IL-2/ONTAK (132). These potential combinational therapies could prove to be more effective at providing clinical benefit than any of these modalities alone.

As an alternative to maturing antigen-loaded DC *ex vivo*, immature DC.IL-12/18 without the antigen load could be injected directly into a tumor pre-treated with chemotherapy or locally treated with radiotherapy, exploiting the local TAA repertoire to stimulate tumor-specific lymphocyte responses. These conventional treatments could enhance the efficacy of subsequent immunotherapy as they induce apoptosis, and apoptotic cells were shown to be a good source of cross-presented antigens under inflammatory conditions (237, 238). IL-12 and IL-18 generated by DC.IL-12/18, as well as, proinflammatory mediators such as heat shock proteins and IL-6 that are released due to chemotherapy-induced apoptosis (237), would provide enough inflammatory signal for DCs to effectively uptake and cross-present tumor antigens. Similarly, destruction by radiofrequency ablation creates massive tissue apoptosis/necrosis which the immune system is unable to completely clear. Because of the failure to clear apoptotic cells, post-apoptotic, secondary necrosis occurs (238). Necrotic cells are a good source of antigens while simultaneously providing strong maturation signals (self DNA and RNA, uric acid) to DCs. Therefore, immature DC.IL-12/18 exposed to necrotic tumor cells could undergo maturation after antigen uptake, and become effective antigen presenting cells. As the result, DC.IL-12/18 with or without the antigen load could be an effective immunization strategy if used in combination with conventional cancer therapies.

The work performed in this thesis has defined two novel poly-DR-binding, MAGE-A6-derived Th epitopes, as well as a mimicking peptide cross-reactive to a MAGE-A6 Th epitope, that are immunogenic in a high frequency of responders. Furthermore, I have generated rMAGE

and Ad.MAGE-A6 that can be effectively used for stimulating $CD4^+$ and $CD8^+$ T cell subsets respectively. Combined with my findings that DC.IL-12/18 could effectively generate Th1-type responses *in vitro*, this thesis introduces the possibility of a novel DC-based therapeutic approach for the treatment and possibly even prevention of MAGE-$A6^+$ tumors. I hypothesize that DC.IL-12/18 could be an effective platform for stimulating anti-tumor Th1 responses, particularly if used in combination with conventional cancer treatments as well as agents that antagonize Treg numbers and function. They could be used as an immunization modality by themselves, or they could be loaded with a variety of antigen formats, including the ones described in this dissertation. This type of therapy could be effective against a wide range of tumor types, and would not be restricted to a patients HLA types particularly if used in combination with rMAGE and/or Ad.MAGE-A6. Furthermore, if evidence gives further support to a residing MPHF2 memory $CD4^+$ T cells in a large portion of the population, it could be hypothesized that this epitope could be potentially employed as a preventive and/or therapeutic agent.

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