Polarized Dendritic Cells for Tumor Immunotherapy

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POLARIZED DENDRITIC CELLS FOR TUMOR IMMUNOTHERAPY

David Allen Hokey, PhD

University of Pittsburgh, 2005

An elusive objective for tumor immunologists has been the development of an effective tumor vaccine capable of inducing potent immune responses to eliminate established tumors and induce long-term protective antitumor immunity. Dendritic cells (DCs) are potent inducers of immunity and represent a promising tool for the purpose of immune-based tumor therapies. However, DC-based vaccines have enjoyed only limited success in clinical trials, probably due to the use of immature/intermediate mature DCs that maintain tolerance during the steady state, or to the use of non-polarized DCs which lack the proper cytokine production that favors cellular immune responses needed to eliminate established tumors. The failure of past tumor vaccines demonstrates a need to examine and enhance immunization strategies on multiple levels. The underlying hypothesis for these studies was that **combining a DC1 polarization signal with an effective antigen-loading strategy will result in enhanced tumor immunotherapy.** Our first aim was to compare cytosolic and membrane-bound antigen presentation of tumor-derived proteins by DCs following three different antigen-loading strategies; coculture of DCs and tumor cells, feeding DCs with tumor lysate, and fusion of DCs and tumor cells. We demonstrated that both DC-tumor coculture and fusion result in a higher level of tumor-derived peptide presentation compared to feeding DCs with tumor lysate. Our second aim was to develop a murine DC1 polarization model to evaluate DC1-based tumor immunotherapy. Herein, we described the synergistic affect of TLR3 and TLR9 ligation on IL-12p70 production by murine DCs, characterizing the timing and exhaustion of IL-12p70 production. Furthermore, we

examined the ability of polarized DCs to stimulate T cell proliferation and cytokine secretion in response to a model antigen *in vitro*. For our third aim we examined the capacity of DC1s to stimulate immune responses to a model antigen as well as native tumor antigens *in vivo* and tested the therapeutic effect of tumor-loaded DC1 vaccines. These studies demonstrate the ability of antigen-loaded polarized DCs to induce strong Th1-mediated anti-tumor immunity characterized by tumor infiltrating $CD4^+$ T cells and macrophages, but not $CD8^+$ T cells, resulting in tumor growth inhibition.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1. CHAPTER ONE

INTRODUCTION

1.1. Dendritic cells and immunity

Dendritic cells (DCs) are "professional" antigen presenting cells (APCs) and are the only APCs known to stimulate naïve T cells [1, 2]. They reside in peripheral tissues in an "immature" state and display high antigen uptake ability. DCs sample antigens from surrounding tissues and migrate to tissue draining lymph nodes, where they can present the antigens in the form of processed peptides in the context of MHC molecules. During steady-state interactions, DCs remain in an immature/semi-mature state and help to maintain peripheral tolerance to self antigens [3, 4]. However, with proper stimulation, DCs become activated and undergo maturation resulting in the up regulation of antigen processing and presenting machinery, co-stimulatory molecules, and cytokine production while down regulating their antigen capturing capabilities. These "mature" DCs are potent inducers of adaptive immune responses through the presentation of specific antigenic peptides on MHC class I and class II molecules for presentation to $CD8^+$ and $CD4^+$ T cells, respectively. In addition, DCs direct the type of adaptive immune response that is generated through the secretion of cytokines [5-7]. Thus, DCs are pivotal in the generation of appropriate adaptive immune responses to a variety of pathogens. DCs also secrete inflammatory cytokines in response to microbial signals that stimulate innate immune responses. In this way, DCs stand at the interface between adaptive and innate immunity, bring both arms of the immune system to effectively eliminate potential threats [8].

Because DCs are so critically important for the initiation of immune responses, they are attractive for use as adjuvants for use in tumor immunotherapy. Elicitation of immune responses to tumor antigens represents a unique set of challenges. Tumor antigens, unlike antigens associated with bacteria and other pathogens, are self antigens, and the immune system is often tolerant of them. Studies have demonstrated the existence of regulatory T cells which hinder the development of immune responses to tumor antigens [9-14]. In addition, tumors acquire mechanisms to evade or inhibit immune responses. For these reasons, much attention has been given to the development of immunization strategies to maximize the immunostimulatory capacity of dendritic cells. Antigen loading strategies designed to elicit $CD4^+$ and $CD8^+$ T cell responses are critical for the development of effective tumor immunotherapeutic approaches as are methods to activate dendritic cells for the induction of potent Th1 skewing.

1.1.1. Antigen capture by dendritic cells

Dendritic cells are charged with the task of sampling antigens in the periphery for presentation to T cells in draining lymph nodes. Antigen can take many forms, including soluble antigens, such as toxins secreted by various pathogens, particulate antigens such as bacteria, viruses, exosomes, and necrotic cellular debris. Dendritic cells must also acquire antigens from surrounding viable cells to ensure detection of virally infected cells and tumors. Because of the diversity of antigen forms, DCs have developed a wide array of mechanisms to acquire antigens. DCs have been demonstrated to readily take up soluble materials from the environment such as fluorescent molecules and proteins through a process known as macropinocytosis [15]. Larger antigens such as particulates and bacteria are acquired through phagocytosis [16-18]. DCs also utilize a variety of receptors to capture and internalize antigens, a process referred to as receptor-mediated

endocytosis. Receptors include Fcγ and Fcε receptors for immune complexes [19, 20], DEC-205 and macrophage mannose receptors for glycosylated proteins [19, 21, 22], CD36 and the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins for presentation of proteins from apoptotic cells [23, 24], and scavenger receptors such as CD91 for internalization of heat shock proteins (HSPs), allowing class I presentation of antigens from necrotic cells [25]. Dendritic cells are also capable of capturing antigens from live cells via class A scavenger receptors in a process known as "nibbling" [26]. Together, these reports suggest a variety of antigen loading strategies that may be utilized for dendritic cell-based tumor immunization strategies.

Approaches utilizing whole tumor cells as a source of antigen for dendritic cells may prove particularly useful for tumor immunotherapy, encompassing the entire repertoire of tumor antigens available for a specific tumor rather than focusing the immune response on one antigen [27, 28]. This would potentially help in preventing tumor immune escape through antigen-loss variants or mutations in critical T cell epitopes [28]. In order for immunotherapy to be effective, the antigen loading method must be able to induce both MHC class I- and class II-mediated presentation of tumor antigens, enabling activation of both $CD8^+$ and $CD4^+$ T cells, respectively. APCs, including DCs, normally process and present exogenous antigens on MHC class II molecules. However, accessing the cross-presentation pathway for the presentation of exogenous antigens for MHC class I presentation appears to me more tightly regulated. Some have proposed tumor/DC fusion (termed "dendritomas") for enhanced MHC class I presentation of tumor antigens, commonly performed using polyethylene glycol-mediated fusion or electrofusion [29-32]. Presumably, the classical MHC class I pathway is utilized through the introduction of tumor antigens directly into the cytosol. Little is known about the resulting effect

on APC function of DCs fused to tumor cells, especially concerning their ability to stimulate T cell polarization. Ultimately, different loading methods must be compared for their relative ability to induce both class I and class II presentation of tumor-derived antigens.

1.1.2. Antigen processing and presentation

The immune system continually surveys cells for the presence of foreign antigenic peptides. In this way, it serves to protect against pathogens, which are composed of foreign proteins, and tumors, which have acquired mutations in proteins that can be perceived as foreign. Intracellular pathogens and tumors present a special obstacle for the immune system since the problem lies within a cell and could potentially hide from phagocytic cells and immunoglobulins. Cytotoxic T cells have evolved to detect and kill infected or mutated cells via recognition of foreign antigenic peptides presented on the surface of nucleated cells in the context of MHC class I molecules. Cellular proteins have a regular turnover during which old proteins are degraded through the ubiquitin-proteasome pathway and replaced by newly synthesized proteins [33, 34]. This pathway also serves to eliminate mistakes made during protein synthesis such as misfolded or truncated proteins. The classical MHC class I presentation pathway takes advantage of this process of protein degradation by shuttling peptides from partially degraded proteins into the endoplasmic reticulum (ER), enabling presentation of peptides by MHC class I molecules [33, 34]. This process can be enhanced through stimulation of cells with IFN-γ, which induces the inclusion of LMP2 and LMP7 subunits in the proteasome to form the immunoproteasome [34, 35]. The immunoproteasome is effective at generating peptides that bind MHC class I molecules, generally 8-13 amino acids in length and ending in a hydrophobic or basic C-terminal amino acid [34]. These peptides are shuttled into the endoplasmic reticulum (ER) by TAP

(transporters associated with antigen processing) molecules, TAP1 and TAP2, for loading into the MHC class I peptide-binding groove. MHC class I molecules are formed from the non-covalent association of an α-chain and a β_2-microglobulin chain. This complex is relatively unstable and requires the presence of chaperone molecules, such as calnexin and calreticulin, until a peptide is bound [34]. The different MHC types can bind peptides according to specific binding motifs. These motifs are based on specific anchor residues generally at the C- and N-termini of the peptide chain [34, 36]. Amino acids that do not play a role in MHC binding can vary greatly in their sequence, allowing for a wide variety of peptides that can be bound by each MHC molecule. Peptides are limited in their size due to the closed nature of the MHC class I binding groove, which is composed solely from the α-chain [34, 36]. Once a peptide is bound to the peptide binding groove, the complex becomes stable and dissociates from chaperone molecules. The peptide/MHC class I complex is then transported through the golgi and to the cell surface where the peptide can be presented to $CD8^+$ T cells (Fig. 1).

DCs serve as sentinels for the immune system. While DCs are found throughout the body, they are in high abundance at the interface between the host and the environment where they are most likely to interact with pathogens, such as mucosal surfaces and the skin. Dendritic cells and other antigen presenting cells are designed to acquire antigens from peripheral sources and present them to antigen-specific T cells in draining lymph nodes. They have adapted to acquire antigens in a variety of forms for subsequent processing, including soluble antigen, particulate antigen, immune complexes, and even cell-associated antigens. Dendritic cells must then process the antigen for presentation to antigen-specific T cells. This process requires special antigen processing machinery referred to as the MHC class II processing pathway. Unlike MHC

class I presentation, MHC class II presentation is restricted to antigen presenting cells such as B cells, macrophages, and dendritic cells. MHC class II presentation involves endocytosis of exogenous antigen which is then degraded in the endolysosome into peptides. Newly formed MHC class II molecules in the ER are associated with invariant chain which acts as a chaperone molecule as well as to prevent class I peptides from binding in the class II peptide binding groove. The MHC class II/invariant chain complexes are shuttled from the ER through the golgi and then merge with endolysosomes to form the MIIC compartment. There, the invariant chain is degraded, leaving the CLIP fragment (class II-associated invariant chain peptide) bound. With the aid of HLA-DM, CLIP is removed and an antigenic peptide is allowed to bind, forming a stable MHC class II/peptide complex [36-38]. The length of peptides found in MHC class II molecules is slightly longer than those found in class I molecules. Unlike the MHC class I molecule, the binding pocket for MHC class II is composed of both the α-chain and the β-chain, resulting in a binding cleft with open ends that can bind longer peptides, generally 13-18 amino acids long [36, 38]. The peptide/MHC class II complex is then transported to the cell surface for presentation to $CD4^+$ T cells (Fig. 1).

Cytotoxic T cells are capable of recognizing the presentation of foreign antigens on all nucleated cells via their T cell receptors (TCRs). However, they must first be activated by professional APCs presenting the antigenic peptides in the context of MHC class I molecules. This poses a significant problem if the infected or mutated cell is not an APC. The immune system has developed a pathway in which APCs can present exogenous antigens on MHC class I molecules, termed cross-presentation [39-43]. While the exact mechanism for cross-presentation is still being investigated, it appears that the process is TAP-dependent, suggesting exogenous antigens

gain access to the cytosol [40, 44, 45]. However, some suggest antigen loading of class I molecules occurs in endocytic compartments that contain all the necessary machinery for antigen processing [46]. In addition, studies have reported the involvement of SEC61 in cross-presentation. SEC61 is normally involved in transport of proteins from the ER to the cytosol for proteasomal degradation, allowing for class I presentation of membrane-bound and ER-targeted proteins. It has been suggested that SEC61 is likely responsible for transport of exogenous antigens from endocytic vesicles to the cytosol [47, 48]. Confusion may be the result of multiple pathways for cross-presentation. What is certain is that the pathway exists and is critical for the activation of $CD8^+$ T cells to fight infections or mutations in non-APC cell lineages. Therefore, tumor vaccination strategies utilizing whole proteins or using cells as a source of antigen must access the cross-presentation pathway in order to elicit effective $CD8^+$ T-cell responses

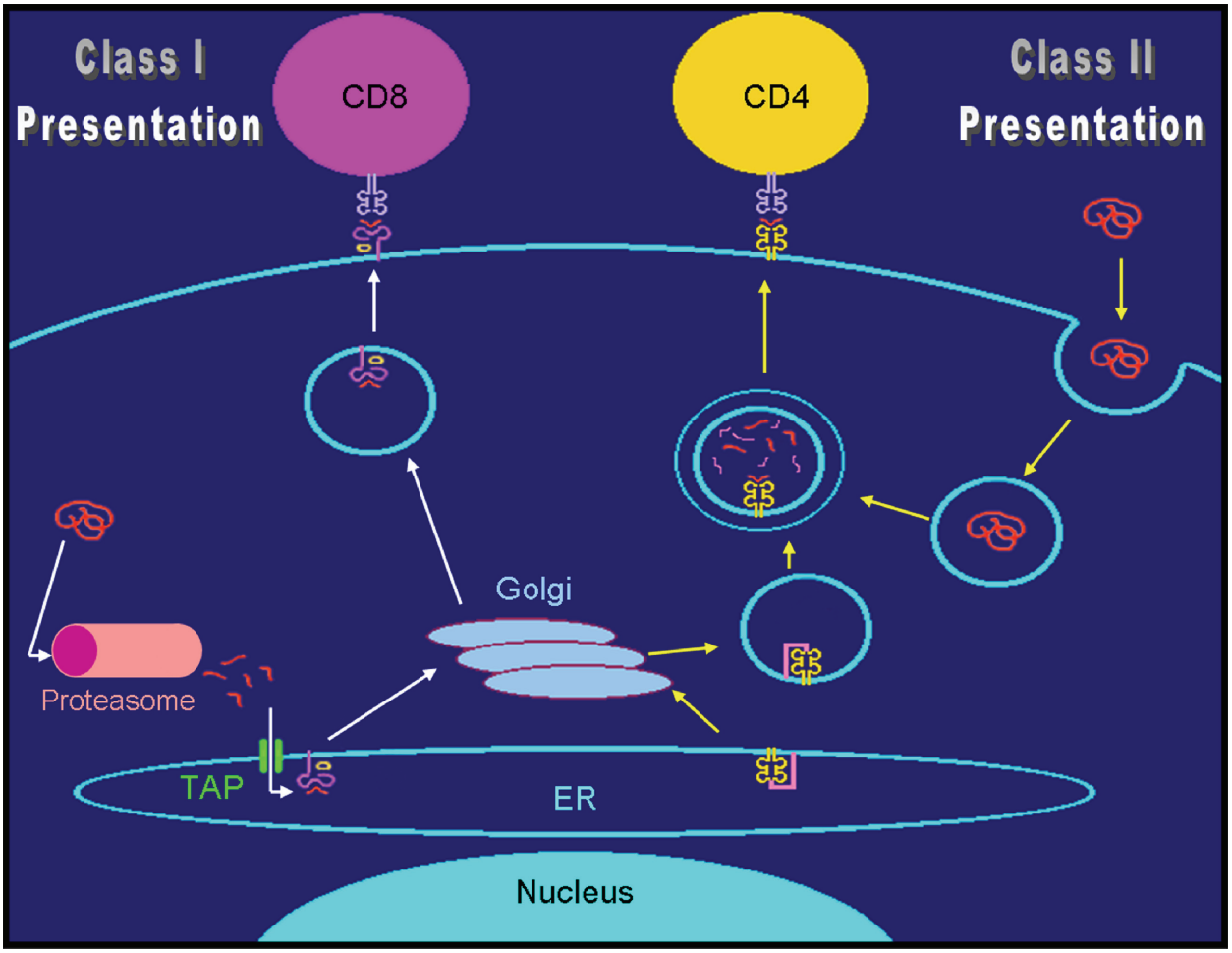

Figure 1. Antigen processing and presentation

Antigen (in red) is processed through two primary pathways. Class I presentation generally involves proteasomal degradation of cytosolic antigen into peptides which are loaded onto MHC class I molecules in a TAP-dependent manner and then transported to the cell surface for presentation to $CD8^+$ T cells. Class II presentation involves endocytosis of exogenous antigen which is degraded into peptides in the endolysosome. These peptides are then loaded onto MHC class II molecules in the MIIC compartment with the aid of HLA-DM and transported to the cell surface for presentation to $CD4^+$ T cells.

1.1.3. DC activation

DCs in the periphery normally exist in an immature state and migrate to draining lymph nodes as "semi-mature" APCs bearing normal self antigens. While in the immature/semi-mature state, DCs help to maintain peripheral self tolerance through the induction of T cell anergy or apoptosis. However, DCs induce potent immune responses when they become fully activated, expressing high levels of 1) important costimulatory molecules such as CD40, CD80, and CD86, 2) antigen presentation molecules such as MHC class I and II, and 3) adhesion molecules such as CD54 (ICAM-1). In addition, activated DCs secrete a number of cytokines for the induction of innate immune responses, such as interleukin-12 (IL-12) and the type-1 interferons IFN-α and IFN-β [8, 49, 50], as well as for the generation of polarized antigen-specific T-cell responses (adaptive immunity), also known as "signal 3" [5-7].

DCs are capable of sensing the presence of microbes in the periphery through pathogen-associated molecular patterns (PAMPs) [51], as predicted by the Infectious-Nonself (INS) model [52]. Stimulation of DCs through PAMPs (referred to here as "stranger signals") results in a series of changes designed to alert the immune system of a threat. Similarly, DCs are also capable of recognizing endogenous signals indicating tissue damage or cellular stress, termed "danger signals" [53-58]. Together, stranger and danger signals, along with cytokines and other factors, instruct DCs in the periphery at the site of an infection about the nature of the pathogen. This environmental instruction results in the polarization of DCs and subsequently determines the type of immune response that is generated [6, 59-61]. Understanding the molecular code that determines particular types of immune responses will allow vaccines to be tailored to specific

pathogens. Importantly for cancer vaccine development, we can utilize this information to elicit robust immune effector responses to eliminate tumor cells.

Perhaps the most well-known PAMP receptors are the Toll-like receptors (TLRs). Toll was first recognized in *Drosophila*, which lacks an adaptive immune system [62, 63]. In *Drosophila*, Toll plays a critical role in the insect's innate resistance to fungal infections by inducing the secretion of an antifungal peptide, drosomycin [63]. Subsequently, homologues of Toll (TLRs) have been found in plants [62, 64] and mammals, including 11 family members in humans [51, 65-76] that are known bind both microbial and endogenous compounds, making TLRs important for both stranger and danger signals (Table 1). This family of receptors is characterized by a leucine-rich repeat in the extracellular domain and a cytoplasmic Toll/IL-1R homology (TIR) domain, named for the homology seen with the IL-1R family [65, 77]. Several TLR ligands have been described and are known to polarize DCs toward a DC1 or DC2 phenotype (Table 1) [78-82]. Differences in TLR expression between BALB/c and C57BL/6 mice have been suggested to be responsible for their susceptibly or resistance to *Listeria monocytogenes*, respectively [83], further indicating the important role this family of receptors plays in the induction of appropriate immune responses to pathogens.

TLRs are known to signal through two primary pathways, the MyD88 pathway and the TRIF pathway [77] and all TLRs tested result in an increase in the expression of costimulatory molecules in DCs, dependent on the nuclear translocation of the transcription factor NF-κB. While multiple receptors are able to stimulate through each of these pathways, they are still

unique in their downstream effect, suggesting greater complexity in their signaling pathways. For example, TLR ligands have been demonstrated to enhance CTL development when used as adjuvants with virus-like particles encoding a model antigen in mice. This CTL-inducing adjuvant effect was seen most prominently with ligation of TLR9, moderately so with TLR3, TLR5, and TLR7, and was absent with TLR2 and TLR4 [84]. The signaling mechanisms that allow for the differences in TLR responses are beginning to be understood. Adaptor proteins are able to interact with TLRs through a homotypic interaction between TIR domains. Individual TLRs are capable of recruiting different combinations of adaptor molecules, resulting in the generation of distinctive cellular responses. One adaptor, myeloid differentiation primary-response protein 88 (MyD88), was once thought to be critical for all TLR signaling. However, MyD88 knockout mice revealed the existence of a MyD88-independent pathway that is dependent upon the adaptor molecule TRIF. While TLR3 is capable of signaling through the MyD88-dependent pathway, it is now believed that TLR3 primarily signals through the MyD88-independent/TRIF-dependent pathway. TLR3 is also known to induce NF-κB translocation to the nucleus via the adaptor protein RIP1 [85]. Interferon regulatory factors (IRFs) are also differentially activated between TLRs. IRF3 regulates the production of IFN-β and is activated by stimulation through TLR3 and TLR4 through the MyD88-independent/TRIF-dependent pathway. Similarly, MyD88 has been demonstrated to interact with the IFN-α regulator IRF7, but not with IRF3. Stimulation of TLR7, TLR8, or TLR9 has been shown to result in the activation of IRF7, dependent on an interaction with the adaptor molecule tumor necrosis factor receptor-associated factor 6 (TRAF6). Other TIR-domain-containing molecules are believed to exist to further account for differences seen following ligation of different TLRs, but have yet to be found. Interestingly, synergy has been described between TLR3 and TLR9 signaling

pathways, and even between TLR and cytokine signaling pathways [81, 86, 87]. This synergism has likely evolved to help the immune system discern one pathogen from another, affecting immune effector mechanisms as well as immune skewing through polarization of dendritic cells. The understanding of Toll-like receptors and their signaling pathways provides a molecular code by which we can exploit for the purpose of immunomodulation. Decoding and understanding these molecular mechanisms will support the development of more effective vaccines, providing tools by which we may manipulate immune responses to fight cancer and various pathogens. TLR signaling molecules may also prove useful targets for drug development for the inhibition of immune responses for the treatment of autoimmune disorders.

Toll-like receptors have also been shown to enhance antigen capture and cross presentation by dendritic cells. West et al. demonstrated a transient enhancement of macropinocytosis following stimulation of murine DCs with ligands for TLR2, TLR3, TLR4, and TLR9 [88]. This effect was MyD88-dependent for TLRs 2 and 9, but MyD88-independent in the case of TLR3 and TLR4, consistent with the use of the TRIF-dependent signaling pathway by TLR3 and TLR4 [77, 88]. Together the data suggest that direct signaling though TLRs is required for the enhancement of antigen uptake. In addition, two Toll-like receptors have been implicated in the enhancement of cross-presentation of exogenous antigen [89-91]. These data indicate a potential role for the use of specific TLRs as adjuvants for the enhancement of $CD8^+$ T cell responses.

The creation of murine models for human Toll-like receptor-based DC polarization has not been straightforward. Species-specific differences in TLRs and their expression prevents the generation of polarization cocktails that are equally effective in both mouse and human systems.

For example TLR11, which recognizes a yet unidentified component of uropathogenic bacteria, is believed to be truncated and non-functional in humans, but is critical in protecting mice from urinary tract infections [76]. One of the most extensively studied TLRs is TLR9. In humans, TLR9 expression is restricted to plasmacytoid DCs (pDCs), while in mice, TLR9 expression is promiscuous (Table 1) [80]. Consequently, pDCs are the primary cells responding to CpGs in human systems, secreting large amounts of IFN-α and IFN-β that can then act on other DC subsets. Importantly, CpG-stimulated human pDCs have been implicated in the generation of regulatory T cells, suggesting the role of pDCs in tolerance induction [92]. In murine systems, CpGs are able to act directly on non-plasmacytoid DCs and act in synergy with other TLRs in these cells [81, 86, 87, 93]. Furthermore, murine and human TLR9 preferentially respond to different CpG motifs [72, 93]. Moreover, LPS has been demonstrated to increase the expression of TLR3 in murine cells, but inhibits the expression of TLR3 in human systems [94]. Together these data indicate that it is unrealistic to expect TLR polarization methods to be identical in murine and human systems. These problems are indicative of the issues surrounding the development of a murine model to test DC1 polarization methods currently used in clinical trials. In order to create a murine DC1 model capable of mimicking the effects of human DC1s, polarized murine DCs must be functionally evaluated using standards currently used for evaluating their functional capacity in human systems.

Table 1. Toll-like receptors: skewing properties, ligands, and expression

Receptor	Polarization	Ligand*	Expression[a]				
			Mouse			Human	
			$CD8^+DC$	$CD8^-DC$	pDC	mDC	pDC
TLR1[†,‡]	DC2[§]	Lipoprotein Triacyl lipopeptides	+	+	+	+	+
TLR2	DC2	Diacyl lipopeptides Glycoinositolphospholipids Glycolipids HSP70[¶] Lipoarabinomannan Lipopeptides Lipoprotein Lipoteichoic acids LPS[‖] (atypical/cylindrical lipid A) Peptidoglycan Phenol-soluble modulin Porins Triacyl lipopeptides Zymosan	+	+	+	+	-
TLR3	DC1[#]	dsRNA[**] Poly[I:C][††] mRNA	+	+	-	+	-
TLR4	DC1	Fibronectin Fibrinogen HSP60 HSP70 Hyaluronic acid Lipoteichoic acids LPS (conical lipid A) MMTV[‡‡] envelope protein RSV[§§] fusion protein Taxol	+	+	+/-	+	-
TLR5	DC1	Flagellin	+/-	+	+	+	-
TLR6[‡]	DC2	Diacyl lipopeptides Lipoteichoic acid Peptidoglycan Zymosan	+	+	+	+	+
TLR7	DC1	Bropirimine Imidazoquinoline Loxoribine ssRNA[¶¶]	-	+	+	+	+
TLR8	DC1	Imidazoquinoline ssRNA	+	+	+	+	-
TLR9	DC1	Unmethylated CpG[‖‖]	+	+	+	-	+

*Colors indicate source of ligand--bacterium, virus, fungus, plant, host, synthetic; [a]mDC—myeloid DC, pDC—plasmacytoid DC; [†]TLR—Toll-like receptor; [‡]Appears to require dimerization with TLR2; [§]DC2—type-2 polarized dendritic cell; [¶]HSP—heat shock protein; [‖]LPS—lipopolysaccharide; [#]type-1 polarized dendritic cell; [**]dsRNA—double-stranded RNA; [††]Poly[I:C]—polycytidylic-polyinosinic acid – mimics dsRNA; [‡‡]MMTV—mouse mammary-tumor virus; [§§]RSV—respiratory syncytial virus; [¶¶]ssRNA—single-stranded RNA; [‖‖]CpG—deoxycytidylate-phosphate-deoxyguanylate oligodeoxynucleotides; modified from [77, 80, 95, 96].

1.1.4. T cell activation

Activated DCs play a critical role in the activation of adaptive immunity through the activation of $CD4^+$ and $CD8^+$ T cells. DCs activate T cells through three signals. Signal 1 occurs with T cell recognition, via the T-cell receptor complex, of antigen/peptide complexes on the surface of dendritic cells. This interaction is mediated by T cell CD4 molecules through the interaction with MHC class II or by T cell CD8 through the interaction with MHC class I. Signal 2 is via DC costimulatory molecules such as CD40, and CD80/CD86, which interact with T cell CD40L and CD28, respectively. The interaction of the T cell and the DC results in the formation of an immunological synapse. In the immunological synapse, cell surface molecules involved in antigen recognition, T cell stimulation, and cell adhesion along with signaling molecules are segregated into distinct domains known as supramolecular activation clusters (SMACs) at the site of the DC/T cell interaction. The central SMAC (cSMAC) is composed molecules involved in T cell recognition and activation such as TCR/CD3 complex, MHC/peptide complex, CD80 and its ligand CD28, and associated signaling molecules such as PKC-θ, *lck*, and *fyn*. The peripheral SMAC (pSMAC) contain adhesion molecules such as ICAM-1 and its ligand LFA-1 along with talin, which serves to anchor the adhesion molecules to the cytoskeleton [97, 98]. The immunological synapse has been reported to play a role in the determination of T cell polarization through the recruitment of IFN-γR to the cSMAC, which results in Th1 polarization signals. The recruitment of this receptor to the cSMAC is inhibited by IL-4 and suggests a mechanism by which IL-4 can inhibit Th1 polarization [99].

The third signal for T cell activation is mediated through cytokines and plays a critical role in determining T cell polarization [5-7]. Activated DCs are classified as either DC1 or DC2,

depending on their ability to induce Th1 or Th2 polarization. This ability is believed to be mediated largely due to the secretion of the polarizing cytokines IL-12p70 and IL-4 by DCs which induce Th1 and Th2 immune responses, respectively [1]. However, MacDonald et al. demonstrated that IL-4 or IL-12 secretion by injected DCs was not strictly necessary for the development of Th2 or Th1 immunity in recipient mice, respectively, but rather was dependent on the ability of recipients to secrete IL-4 and IL-12 [100]. Furthermore, the authors were unable to determine what cell types were necessary in the recipient mice for the induction of the polarized responses. The authors concluded that recipient DCs may be responsible for the responses seen, suggesting that antigen loaded donor DCs were able to influence the immune skewing through IL-4- and IL-12-independent mechanisms [100]. Th1 cells secrete IFN-γ, resulting in the activation of macrophages and promoting the development of cytotoxic T cells, while Th2 cells secrete IL-4 and IL-5. IFN-γ has long been accepted to be a critical factor in mediating the effects of IL-12-based tumor therapy and for mediating IL-12-driven immune responses [101, 102]. T cell polarization has also been demonstrated to revolve around two transcription factors, T-bet and GATA-3. T-bet is known to drive Th1 machinery and inhibits the activity of the Th2-promoting transcription factor GATA-3 [103, 104]. The ability of dendritic cells to induce Th1-mediated immune responses appears to involve a variety of factors. A very recent study demonstrates the inability of IL-12p70-secreting DCs to induce Th1 responses and suggests that DCs secreting IFN-γ in addition to IL-12p70 are better able to drive Th1 immunity [105]. These DCs were not stimulated with polarization signals that may be necessary to induce the full Th1-stimulating capacity of these cells. However, IL-12p70 is recognized as a critical factor in contributing to Th1 polarization [106].

While IL-12p70 is thought to be the most potent inducer of Th1 immunity, other factors can also influence Th skewing. Human monocyte-derived DCs have been shown to induce IFN-γ secretion by T cells through the secretion of β-chemokines [107]. Other cytokines such as the IL-12 family members, IL-23 and IL-27, are known to influence Th1 skewing as well [108]. In human cells, Th1 development has been regulated through the IFN-β-mediated regulation of IL-23, IL-27, and IL-18 [109]. In addition, NK cells have been shown to aid in the stimulation of Th1 immunity through the secretion of IFN-γ [110]. Activation of DCs through CD40/CD40L interaction has been shown to enhance the Th1-stimulating capacity of Langerhans cells in a manner that is independent of IL-12 [111]. Importantly, the interaction between T cells and dendritic cells via the immunological synapse also appears to have great influence on Th1 polarization, driven by ICAM1 and high levels of MHC/peptide complex on the DC surface and inhibited by molecules such as OX40L and MCP1 [6, 60, 112, 113]. Together the data demonstrate numerous factors can contribute to the ultimate polarization of T cells. Dendritic cells must take information learned at the peripheral site and subsequently manage the complex molecular interactions during DC/T cell contact that ultimately determine T cell response to pathogens.

Th1-mediated immunity is believed to be critical for the generation of effective anti-tumor immune responses. $CD4^+$ T cell help promotes the development of $CD8^+$ cytotoxic T cell (CTL) activity and is necessary for the generation of memory T cell populations. However, the elicitation of CTL immune responses does not strictly require the presence of $CD4^+$ T cell help. $CD8^+$ T cells have a variety of mechanisms to deal with intracellular pathogens and tumors. The primary function of $CD8^+$ T cells is believed to be through cytotoxic mechanisms, giving them

the ability to eliminate virally infected cells and tumor cells. $CD8^+$ T cells have two primary methods in their arsenal by which they can kill cells. The first method involves the use of perforin and granzymes, which are secreted into the synapse between the T cell and an infected or mutated cell. $CD8^+$ T cells increase expression of cathepsin B upon triggering if their TCR, giving them resistance to perforin-mediated destruction and preventing their own death while killing target cells [114]. The second pathway involves the ligation of Fas on target cells by Fas ligand (FasL) on $CD8^+$ T cells. While the perforin/granzyme pathway is thought to be the primary mechanism by which killing occurs, some tumors appear to require killing through the Fas/FasL pathway for tumor rejection [115]. While many viruses can be eliminated through the destruction of infected cells, some viruses, such as herpes viruses, infect neurons. This represents a challenge for the immune system since neurons cannot be replaced if they are killed. To combat such viruses, $CD8^+$ T cells surround infected nerves and help maintain viral latency through the secretion of IFN-γ [116-118]. The ability of $CD8^+$ T cells to mount effective responses against a variety of intracellular pathogens as well as tumors makes their induction critical for the success of tumor vaccines. Understanding the mechanisms governing $CD8^+$ T cell elicitation will assist in the development of new immunotherapeutic approaches for the treatment of cancer and other diseases.

1.1.5. Interleukin-12 and Th1 immunity

Perhaps the most important factor in the generation of Th1-mediated immunity is IL-12. IL-12 is a heterodimer composed of two subunits, p35 and p40, which together result in bioactive IL-12p70. IL-12 is a member of a family of heterodimeric cytokines that includes IL-23 and IL-27. IL-23 is composed of the IL-12p40 subunit and a p19 subunit [119]. Similarly, IL-27 is

composed of an IL-12p40 homologue, Epstein-Barr virus (EBV)-induced molecule 3 (EBI3), and the IL-12p35 homologue, p28 [120]. The IL-12 family of cytokines is secreted in response to Toll-like receptor ligands as well as in response to interferons. IL-12, IL-23, and IL-27 are all able to stimulate IFN-γ secretion in T cells *in vitro*, and are believed to be important for the regulation of different aspects of the development of Th1 immunity [108].

Due to the role IL-12p70 plays in the development of Th1-mediated immunity, much effort has gone into the development of methods to induce IL-12p70 production by DCs. Interestingly, the Th2-inducing cytokine IL-4, either alone or in synergy with IFN-γ, has been implicated in the enhancement of IL-12 secretion, and has been shown to inhibit the formation of the inhibitory IL-12p40 homodimer [121, 122]. The influence of cytokines on the production of IL-12p70 is well-known. The traditional method used to induce IL-12p70-secreting polarized DC1s involved stimulation of immature DCs with a polarizing cocktails composed of tumor necrosis factor α (TNF-α), IL-1β, IL-6, and prostaglandin E_2 (PGE_2) [123] or TNF-α, IL-1β, and IFN-γ [61]. Recent evidence suggests the maturation of DCs using only inflammatory mediators such as these are insufficient for the full activation of dendritic cells [124]. The addition of TLR ligands to these cytokine cocktails improves the IL-12p70-secreting capacity of DCs as well as their T cell stimulatory capabilities [125, 126], suggesting a synergistic effect between certain cytokines and TLR ligands and highlighting the critical nature of TLR signaling for full immune activation. In addition, TLR ligands are known to synergize with each other, giving rise to polarized type-1 dendritic cells capable of secreting high levels of bioactive IL-12p70 and having an enhanced ability to stimulate anti-tumor immunity [81, 86, 87]. Yet another inducer of IL-12p70 secretion by DCs is CD40 ligand (CD40L). Stimulation of DCs with CD40L has been demonstrated to

stimulate high levels of bioactive IL-12p70 following a primary stimulation with TLRLs [82, 127-129] or in synergy with IFN-γ [129]. The involvement of CD40L in the stimulation of IL-12p70 production suggests a mechanism by which T cells can stimulate properly stimulated DCs to secrete IL-12p70 for the induction of Th1-mediated immunity.

DCs are known to make IL-12p70 over a narrow window of time, followed by a period of exhaustion [130]. Langenkamp et al. suggest that IL-12p70-secreting DCs induce Th1 immunity, while exhausted DCs will induce Th2 responses [130]. However, Mailliard et al. have developed a system in which DCs are stimulated with a polarization cocktail containing TLR3L. In this model, DCs are stimulated for 2 days with polarization signals and results in DCs that secrete high levels of IL-12p70 in response to CD40L [125]. According to previous data in the field, DCs stop making this potent cytokine 8-12 hours following the initial stimulation [130]. Together these data suggest DCs may be able to secrete IL-12p70 in two phases. The first phase would occur in the periphery at the site of infection, where inflammatory cytokines would attract innate immune responders such as macrophages and neutrophils to the site. The second phase would occur in the lymph node, where DCs can interact with antigen-specific T cells and secrete IL-12p70 in response to CD40L stimulation to drive Th1 responses. However, experiments need to be performed to examine this possibility.

1.2. The challenge of tumor vaccines

The development of tumor vaccines represents a distinct set of challenges for immunologists. Traditional vaccines have been directed against foreign pathogens that have a defined set of

antigens. Indeed, some tumors are known to arise from infection with pathogens such as papillomavirus. Vaccines against these tumors are currently in their final phase of clinical trials and appear to be very effective. However, antigens from the majority of tumors are not easily defined, arising from the accrual of genetic mutations from a wide variety of sources that leads to a malignant growth. While certain types of tumors are likely to share some antigens, the majority of antigens in a tumor are likely to be unique to an individual tumor. These antigens are mutated forms of self antigens and have the added benefit of self tolerance mechanisms, adding to the difficulty of mounting immune responses against these antigens. Tumors also utilize a variety of mechanisms to evade immune responses, ranging from altering or inhibiting immune responses through the secretions of various cytokines and growth factors to the down regulation of MHC molecules, allowing the tumors to hide from $CD4^+$ and $CD8^+$ T cells. Finally, tumor vaccines must, by necessity, break self tolerance to tumor antigens while maintaining tolerance to normal self antigens. The potential benefit of tumor-specific immunotherapy capable of eliminating tumors while preserving healthy tissue has inspired a variety of approaches designed to stimulate antitumor immunity.

1.2.1. Tumor immune evasion

Elicitation of effective antitumor immunity is inherently difficult. The majority of tumor antigens remain undefined and are likely unique to any given tumor. In addition, tumors enjoy several advantages and employ numerous mechanisms by which they can evade immune responses. Perhaps the most significant benefit to a tumor cell is that, in most cases, the antigens in the tumor cell are self antigens. The immune system has evolved to be tolerant of such antigens, making the elicitation of antitumor responses difficult. Self-reactive T cells are either

depleted or anergized through interactions with DCs during steady-state conditions. In addition, tumors appear to have mechanisms by which they can establish tolerance that resemble mechanisms of normal peripheral tolerance [131, 132].

Responses to self antigens are also believed to be inhibited by $CD4^+$ $CD25^+$ regulatory T cells (Tregs). Tregs can be identified by their expression of Foxp3 and glucocorticoid-induced TNF receptor (GITR) and play a vital role in the inhibition of autoimmune responses [133, 134]. These cells have been shown to inhibit T cell immunity through a variety of mechanisms, including the suppression of T cell proliferation and function [135]. Tregs are also known to inhibit DC maturation and function [136] and have been shown to increase expression of indoleamine 2,3-dioxygenase (IDO) on dendritic cells which has been shown to inhibit immune responses and promote immune tolerance [137]. This inhibitory effect is only partially mediated through the immunosuppressive effects of IL-10 and TGF-β since blocking antibodies to these cytokines have only minimal effects on Treg-mediated immunosuppression [135, 136]. Importantly for our work, Tregs have been implicated in the maintenance of $CD8^+$ T-cell immune tolerance in the face of DC-based immunizations [9, 14, 138]. Several studies have indicated that depletion of Tregs may be necessary for the enhancement of vaccines and for the elicitation of effective antitumor immune responses [9, 10, 14, 139].

Tumors are also known to secrete a variety of factors to hinder antitumor immunity. Tumors in both mice and humans have been described to produce IL-10 [140-145], which is known to inhibit Th1-mediated immunity through a variety of mechanisms. IL-10 can inhibit T cell proliferation by blocking synthesis of IL-2 [146]. IL-10 is also implicated in the generation of

tolerogenic DCs [147, 148] and the induction of $CD4^+$ $CD25^+$ T cells [149]. IL-10 has also been shown to inhibit the production of the Th1-skewing cytokine IL-12p70 and promotes Th2 skewing of immune responses [86]. Subsequently, secretion of IL-10 represents a potent method by which tumors can modulate immune responses.

Tumor cells are also known to secrete vascular endothelial growth factor (VEGF) which has been demonstrated to inhibit DC function and T cell immunity [150]. More damaging, tumors have been shown to specifically recruit DCs to the tumor site where the secretion of VEGF induces the differentiation of dendritic cells into endothelial cells for the purpose of tumor vascularization [151]. Tumors have also been shown to produce factors to inhibit T cells responses that may be generated against tumor antigens. For example, tumor cells have been shown to express FasL, rendering them capable of killing activated antitumor T cells [152-154]. In addition, the expression of galectin-1 has been demonstrated to inhibit Th1 polarization of T cells and even induce apoptosis in activated T cells [155].

Finally, tumor cells can also escape immune detection through the down regulation of MHC class I molecules. The highly aggressive murine melanoma, B16, has been shown to be deficient in MHC class I presentation ability due to a lack of expression of antigen processing machinery, including TAP molecules and immunoproteasome subunits LMP2 and LMP7 [156]. Importantly, class I presentation capability can be restored upon exposure to IFN-γ [156]. This suggests a role by which tumor-specific Th1-polarized $CD4^+$ T cells can up regulate the expression of class I molecules on tumor cells, rendering them susceptible to CTL killing. It is clear that tumors employ many mechanisms by which they can evade or hinder antitumor

immunity. These mechanisms must be considered when developing immunotherapeutic approaches for the treatment of cancer.

1.2.2. Dendritic cells and tumor immunotherapy

Dendritic cells are known to stimulate both innate and adaptive immune responses and are the only APCs known to stimulate Naïve $CD4^+$ T cells. This ability to stimulate a broad array of potent immune responses makes them attractive targets and adjuvants for tumor immunotherapy and vaccine development. Strategies have involved *in vitro* culturing of DCs as well as the *in vivo* targeting of DCs to induce antigen-specific antitumor immunity [157]. In addition, a wide variety of antigen loading strategies have been utilized and tested for their effectiveness at inducing effective tumor-specific immunity [158, 159].

Many strategies have relied on the use of identified tumor antigens. Several tumor antigens have been identified, the majority being melanoma antigens such as MART [160] and MAGE [161-163] families of antigens as well as gp100 [164], tyrosinase [165, 166], and tyrosinase-related proteins (TRPs) [167, 168]. Another important identified tumor antigen is MUC1, which is known to be expressed in a variety of tumors including breast, pancreatitic, lung, colon, ovarian, and prostate tumors [169]. The identification of tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) has led to the development of vaccination strategies designed to target those antigens. Some approaches have involved the deliver of antigens to DCs *in vivo* through the injection of particulate antigens [170] or through genetic immunizations encoding specific antigens [171, 172]. Pulsing DCs with whole proteins or peptides encoding class I-restricted epitopes as enjoyed some success. This approach has been demonstrated to induce potent anti-

tumor immunity mediated by $CD8^+$ T lymphocytes capable of eliciting protective and therapeutic anti-tumor immunity to model tumor antigens as well as native tumor antigens [173-176]. Lentiviral vectors encoding tumor antigen appear to be quite effective at eliciting potent $CD4^+$- and $CD8^+$-mediated antitumor immunity, resulting in effective tumor rejection following [177]. While these approaches are very effective, there are some rather severe limitations. First, these vaccines are dependent on specific antigen expression and presentation by tumor cells. Second, peptide-based approaches require specific MHC alleles to be expressed by recipients, greatly limiting the patient population that would benefit from such vaccines. Third, many peptide-pulsed DC-based vaccines focus on the induction of only $CD8^+$ T cell responses. However, $CD4^+$ T cells have been shown to be required for the induction of a broad array of immune responses and memory [178, 179]. Finally, these strategies tend to focus the immune response on only one antigen. Tumors contain a variety of antigens, most of which have not been defined [180]. Vaccine strategies may be more successful if they encompass a broad array of tumor antigens [181].

Several approaches to this problem revolve around the use of whole tumor cells as a source of antigen. By using whole tumor cells, you can deliver the entirety of antigens within a tumor to dendritic cells. Moreover, allowing the DCs to process and present the antigens naturally results in the presentation of peptides unique to the tumor antigen/MHC allelic combination. However, because the antigen is exogenous, each strategy requires the verification of class I-restricted presentation for the induction of $CD8^+$ T-cell immunity. Strategies include feeding DCs with tumor lysate [182-185], apoptotic tumor cells [27, 186, 187], coculture of DCs with live tumor cells [188-190], and fusion of dendritic cells and tumor cells [29-32]. Several discrepancies have

come about through the comparison of some of these methods, particularly when comparing the immunostimulatory ability of tumor lysate vs. apoptotic tumor cells to deliver antigen to DCs. Some state that both methods are equipotent at stimulating antitumor responses [191] while others claim that only necrotic cells are able to stimulate immune responses [192]. One study examining the injection of necrotic vs. apoptotic tumor cells *in vivo* to target DCs resulted in only apoptotic cells being effective at eliciting immune responses [193]. Differences seen between these studies may reflect the use of different tumor models or minute differences in the preparation of apoptotic or necrotic tumor cells. Further, the maturation and polarization stimuli present in the preparation may also have profound effects on antigen presentation and the induction of antitumor immunity. Clearly, more research must be conducted to conclusively determine which methods are superior at loading dendritic cells for the presentation of antigenic tumor peptides.

1.2.3. Summary

Dendritic cells are the controllers of immunity, making them ideal targets for vaccine development for the treatment of tumors. Several goals must be achieved for the development of effective antitumor immune responses including 1) identification and optimization of antigen loading techniques capable of delivering antigens to both class I and class II antigen presentation pathways, 2) determination of the appropriate adjuvants to drive potent polarized adaptive and innate immune responses, 3) strategies able to overcome tumor immune evasion and tumor-induced immune suppression, and 4) vaccines capable of breaking tolerance to tumor antigens while maintaining tolerance to self antigens. Of critical importance is the understanding of the biological code by which DCs determine what type of immune response to initiate. TLRs have the capability to break tolerance to tumor and non-tumor antigens [138, 194]. Understanding these mechanisms will aid in the development of more effective vaccines for enhancing immunity to combat tumors, viruses, bacteria, and fungi as well as for regulating immune responses for the treatment of autoimmune diseases.

SCOPE OF THIS THESIS

Type 1-polarized dendritic cells are capable of inducing potent Th1-mediated immunity capable of driving $CD4^+$- and $CD8^+$-mediated antitumor immunity. Current vaccine strategies in preclinical trials utilize DC polarization signals for the development of type 1-polarized dendritic cells [125, 126]. However, a murine model is not currently available for testing the efficacy of immunotherapeutic approaches using these specialized cells. In these studies, we observe synergy between TLR3, TLR9, and IFN-γ that results in high-level secretion of bioactive IL-12p70 with a decreased ability to secrete the inhibitory cytokine IL-10. Using IL-12p70 as a surrogate marker for the Th1-stimulating capability of these cells, we characterize the DC response to simultaneous stimulation of DCs using this DC1-driving cocktail. We compare three antigen loading strategies using whole tumor cells as a source of antigen; coculture of DCs with live tumor cells, pulsing DCs with tumor cell lysate, and fusion of DCs with tumor cells to form dendritomas. We compare the ability of these three antigen loading methods by measuring antigen transfer as well as examining the ability of antigen loaded DCs to stimulate antigen-specific $CD4^+$ and $CD8^+$ T-cell responses. We demonstrate the ability of all three methods to deliver tumor antigens to DCs. However, coculture and fusions appeared to be superior at inducing $CD4^+$ and $CD8^+$ T-cell immunity.

One of the primary goals of this study is to examine the benefit of combining an antigen loading strategy with a DC polarization signal to generate IL-12p70-secreting antigen-loaded DCs. Therefore, we examined whether the loading strategies tested affected the ability of the DCs to respond to the DC1 cocktail. What we determined was that only coculture did not inhibit the ability of DCs to secrete IL-12p70. While fusion appeared to result in the most efficient antigen

presentation of tumor antigens, it severely inhibited the secretion of IL-12p70. We determined that coculture was the best method for loading DCs for the generation of antigen-loaded polarized DCs. We tested the ability of these cells to induce antitumor immune responses. We observed potent induction of Th1-mediated tumor immunity following immunization of tumor-bearing mice, suggesting the vaccine was capable of overcoming $CD4^+$ T-cell tolerance and tumor-induced $CD4^+$ T-cell immune suppression. Antigen-loaded polarized DC-based immunizations resulted in a significant decrease in tumor growth. Unexpectedly, we were unable to detect $CD8^+$ T-cell responses in these mice by conventional ^{51}Cr-release assays or by examination of tumor infiltrate. The response observed appeared to be mediated by $CD4^+$ T cells and activated macrophages, suggesting these cells play important roles in the suppression of tumor growth. We determined that the lack of a $CD8^+$ T-cell response was not due to a lack of class I presentation by our vaccine and conclude that inhibition was due to the presence of regulatory T cells previously shown to inhibit the development of $CD8^+$ T cell-mediated immunity [9, 14, 138].

2. CHAPTER TWO

GENERATION OF TYPE-1 POLARIZED DENDRITIC CELLS USING SYNERGISTIC TLR LIGATION

2.1. Introduction

Dendritic cells (DCs) are professional antigen presenting cells (APCs) with the unique ability to initiate and control antigen-specific immune responses. In peripheral tissues DCs reside in an "immature" state with high antigen uptake and processing ability. Upon activation, DCs migrate from peripheral tissues to draining lymph nodes, displaying antigenic peptides in the context of MHC-class I or MHC-class II for presentation to $CD8^+$ or $CD4^+$ T cells, respectively. Fully mature DCs influence the type of immune response they stimulate through expression of a combination of cell surface and secreted "polarizing" signals referred to collectively as "signal 3" [5-7, 195]. Although many factors contribute to T-cell polarization, secretion of Th1-driving cytokines such as IL-12, IL-18, IL-23, and IL-27 by DCs favors the induction of a Th1-biased response. DCs secreting Th1-driving cytokines have been referred to as type-1 polarized DCs or DC1s [6, 79, 125, 196-198]. Recent evidence suggests Th1-type immune responses have the potential to mediate tumor therapy through multiple effector mechanisms. $CD8^+$ cytotoxic T-lymphocytes (CTLs) are well-established as important effector cells for tumor immunity, and Th1-skewed $CD4^+$ T-helper cells are known to support effective and durable $CD8^+$ T-cell immunity [199-201].

Although T-cell polarization is a result of the convergence of several factors, secretion of IL-12p70 has been a useful surrogate marker for DC1-type function. DCs in peripheral tissues can be stimulated to secrete Th1-driving cytokines, including IL-12p70, by "danger signals"

including ligands that stimulate Toll-like receptors (TLRs) [52, 56, 202]. Depending on the specific DC TLRs ligated, DCs are polarized toward DC1 or DC2 function and stimulate Th1- or Th2-biased T-cell immunity respectively. Importantly for the purpose of tumor immunotherapy, TLR ligation also stimulates DC maturation, making TLR agonists attractive adjuvants for the generation of fully functional DC1s [203]. In preclinical studies, various cocktails of cytokines, commonly including IL-1β, TNF-α, IL-6, PGE2, and/or IFN-γ have been developed to generate DCs with type-1 function from peripheral blood derived precursors [123, 126, 203]. Most recently, it has been shown that the addition of poly[I:C], a TLR3 agonist, to IL-1β, TNF-α, and IFN-γ generates a potent type-1 polarized DC characterized by higher levels of production of IL-12p70 and improved functional capacity for T-cell activation [125, 126, 204]. As yet, a murine equivalent of the human monocyte derived DC1s being evaluated in clinical trials has not been developed, limiting efforts to define the mechanisms and limitations of DC1-induced tumor immunity *in vivo*.

We sought to investigate the ability of *in vitro* TLR ligation to induce type-1 polarized function in murine bone marrow derived DCs. We show that stimulation of bone marrow derived murine DC populations with poly[I:C] and CpGs results in phenotypic maturation of DCs and synergistic induction of durable, high level IL-12p70 secretion characteristic of human type-1 polarized DCs. Functionally, these DCs induce antigen-specific Th1-type $CD4^+$ T-cell activation *in vitro* and *in vivo* characterized by secretion of IFN-γ. The data demonstrate important synergistic effects between TLRs that have implications for the development of more effective vaccines.

2.2. Results

2.2.1. Poly[I:C] and CpGs activate DCs synergistically to induce high level IL-12p70 secretion

First we evaluated the effects of DC1-driving stimuli on the activation and polarization of murine bone marrow derived DCs. CD11c purified DCs were cultured in DC medium in the presence or absence of IFN-γ, poly[I:C], and/or CpGs. We determined the levels of IL-10 and IL-12p70 in 18h culture supernatants (Fig. 2A). Individually, IFN-γ and poly[I:C] were unable to induce significant levels of spontaneously secreted IL-12p70, and CpGs alone stimulated low/intermediate levels of IL-12p70 secretion. However, simultaneous stimulation of DCs with a combination of poly[I:C], CpGs, and IFN-γ, or poly[I:C] and CpGs without IFN-γ, induced very high levels of IL-12p70 secretion. While none of the stimulants tested induced high level secretion of IL-10, the addition if IFN-γ to poly[IC] and CpGs suppressed IL-10 production compared to poly[I:C] and CpG alone.

We next addressed the effects of ligand dose, either individually or in combination, on IL-12p70 secretion. Higher doses of either CpGs or poly[I:C] alone did not result in an increase of IL-12p70 secretion compared to that observed with low dose CpG stimulation (Fig. 2B). However, the combination of CpGs and poly[I:C] resulted in over a two-fold increase in IL-12p70 secretion compared to any single ligand dosing, demonstrating a potent synergistic effect of simultaneous TLR3 and TLR9 stimulation (Fig. 2B). Importantly, stimulation of DCs with this combination also resulted in phenotypic DC maturation/activation as demonstrated by increased levels of cell surface expression of important antigen presentation molecules MHC-class II, CD86, and CD40 similar to that seen with LPS stimulation, a gold standard for murine bone

marrow derived DC activation (Fig. 2C) [205]. Based on these results we utilized a combination of poly[I:C] (20 μg/ml), CpGs (1 μM), and IFN-γ (20 ng/ml) in subsequent experiments, and refer to this as a "DC1 cocktail".

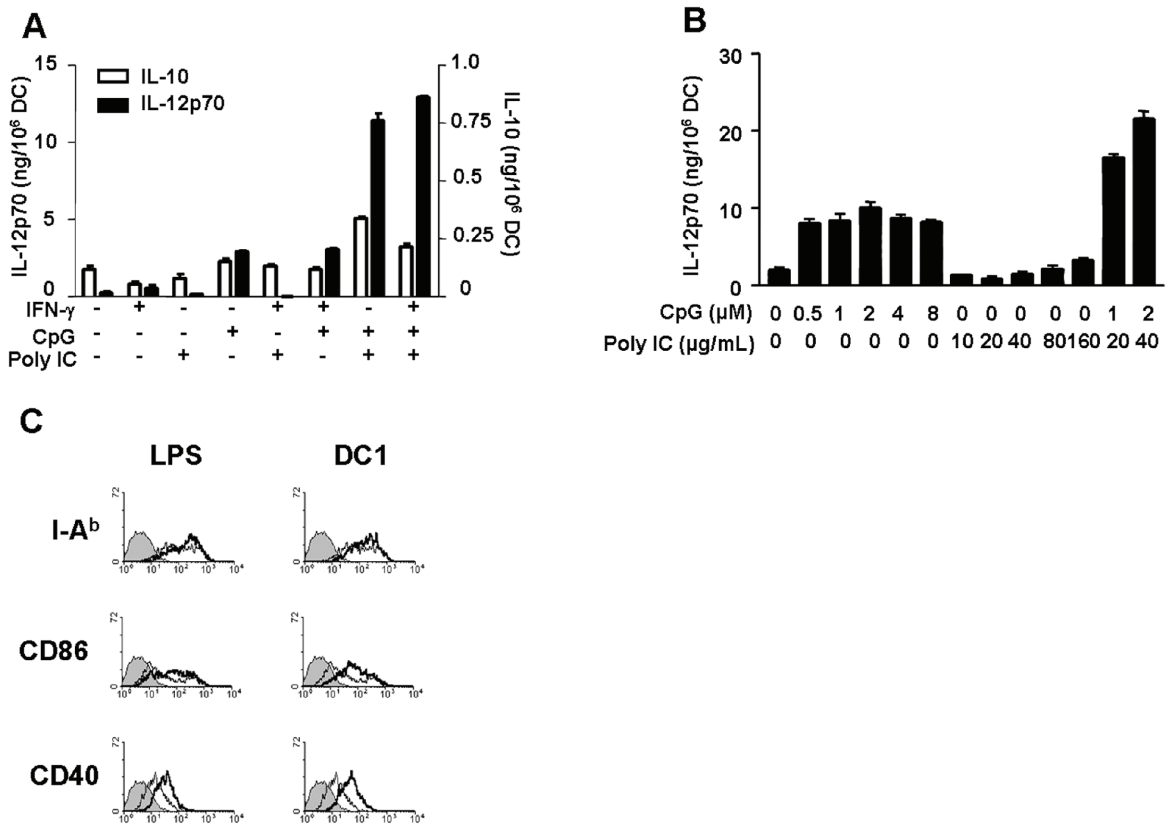

Figure 2. CpGs and poly[I:C] act synergistically to induce DC maturation and high levels of IL-12p70 secretion

A. Purified DCs were stimulated by culture with IFN-γ (20 ng/mL), poly[I:C] (20 μg/mL), and/or CpGs (1 μM) for 18h at 37°C as indicated after which IL-10 (open bars) and IL-12p70 (solid bars) secretion was determined by ELISA. B. Purified DCs were suspended in DC media supplemented with IFN-γ (20 ng/mL) and stimulated with varying concentrations of CpGs alone, poly[I:C] alone, or both as indicated and IL-12p70 concentration was determined in 18h supernatants. C. Purified DCs were suspended in DC medium and stimulated with LPS (1μg/mL) (heavy line), DC1 cocktail (heavy line), or left unstimulated (thin line) for 18h and expression of the indicated maturation markers on the $CD11c^+$ gated cell population was determined by flow cytometry. Results are representative of at least 3 similar experiments. Error bars indicate mean ± SEM.

2.2.2. Characterization of IL-12p70 secretion by DCs stimulated with DC1 cocktail

Previous studies with human DCs demonstrate that potential for DCs to produce IL-12p70 is limited to a narrow window of time, a phenomenon referred to as DC "exhaustion" [130]. Similarly, under our experimental conditions, secretion of IL-12p70 by DC1s occurred in a narrow window of time. High IL-12p70 secretion was observed during the first 4h of stimulation, was increased between 4h and 8h of stimulation, and then significantly diminished after 8h (Fig. 3A).

To evaluate IL-12p70 secretion in relationship to the length of stimulation with DC1 cocktail, DCs were cultured in DC1 cocktail for 2, 3, or 4h and then washed and recultured in normal DC medium. To directly compare IL-12 secretion over the 4-8h time period, all groups of DCs were again washed at the 4h time point and then recultured in DC media or DC1 media for an additional 4h before collecting supernatants for cytokine ELISAs. In general, longer incubation with DC1 cocktail correlated with higher secretion of IL-12p70, reaching a maximum with 8h of stimulation (Fig. 3B). DCs stimulated for 8h secreted nearly the same level of IL-12p70 as that secreted by DCs for 18h with ligand continuously present. Previous studies suggest that DCs are able to stimulate Th1 immune responses while they secrete IL-12p70 but promote Th2 responses following exhaustion [130]. Based on previous human studies demonstrating significant IL-12p70 secretion by DC1s following CD40 ligation, we hypothesized that IL-12p70 secretion by murine DC1s could be sustained through DC-T-cell contact [61]. To address this we determined the duration of IL-12p70 secretion by DC1s following CD40 ligation. DC1 cultures were prepared as described except that CD40L-expressing J558 cells were added 5h or 24h after the addition of DC1 driving cocktail. Signaling DC1s through CD40 5h after initial TLR ligation

resulted in sustained high-level secretion of IL-12p70 through the initial 24h time period and significant but lower level expression from 24-48h (Fig. 3C). In contrast, CD40 ligation 24h after TLR ligation neither rescued nor sustained high level IL-12p70 secretion (Fig. 3D). Both DC1s and DCs that had not been exposed to TLR ligands secreted similar, smaller amounts of IL-12p70 following CD40 ligation at 24h. Together, the data suggest a benefit for Th1 skewing if polarized DCs interact with CD40L expressing T cells within several hours of initial TLR ligation. In subsequent experiments TLR ligated DC1s were used for *in vitro* and *in vivo* analysis 4h after initial TLR ligation.

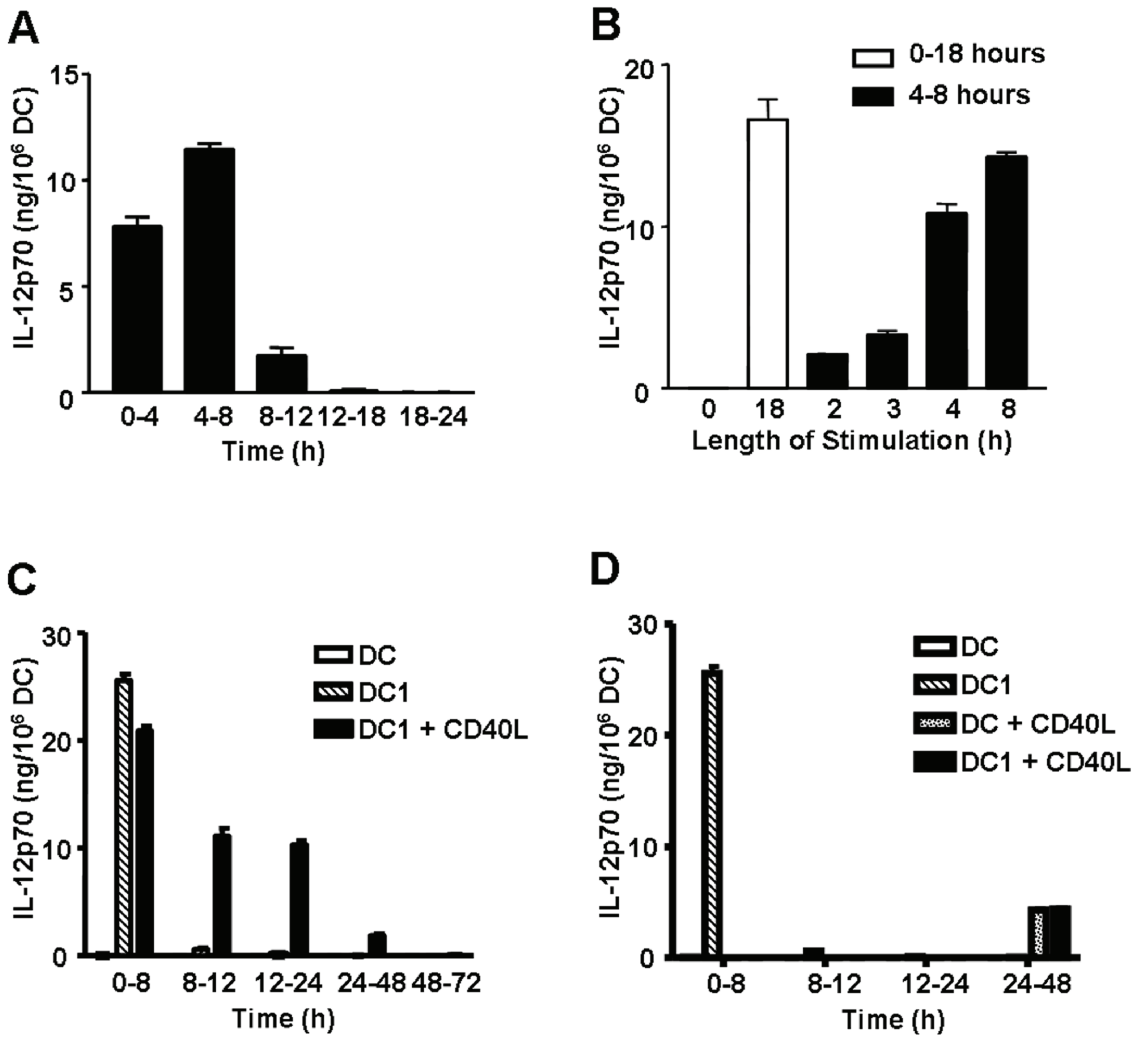

Figure 3. Characterization of IL-12p70 secretion by murine DCs stimulated with DC1 cocktail in the presence or absence of CD40 ligation

A. DCs were stimulated with DC1 cocktail and IL-12p70 concentration in the supernatants was determined at the indicated time points by ELISA. For each time point, supernatants were collected and frozen for future analysis. DCs were then recultured in fresh DC medium containing DC1 cocktail and incubated for the next time point. B. DCs were stimulated with DC1 cocktail for 2, 3, or 4h then supernatants were discarded and DCs recultured in DC medium without stimulation. At 4h, supernatants from all groups were discarded and DC were recultured in DC medium alone (2, 3, or 4h groups) or in DC medium with DC1 cocktail (8h group) for an additional 4h at which time supernatants were collected and analyzed for IL-12p70 concentration by ELISA (solid bars). For comparison, cells were maintained in DC medium or DC medium + DC1 cocktail for 18h before collecting supernatants for ELISA (open bars). Error bars represent mean $\pm$ SEM. C, D. DC cultures were prepared as in A except that CD40L-expressing J558 cells were added 5h (C) or 24h (D) after the addition of DC1 cocktail. At each time point, supernatants were collected and DCs were recultured in DC medium supplemented with DC1 cocktail. The concentration of IL-12p70 in culture supernatants was later assessed by ELISA. Results are representative of at least 3 similar experiments. Error bars indicate mean $\pm$ SEM.

2.2.3. Polarized DC1s induce CD4$^+$ T-cell proliferation and Th1 differentiation

While IL-12p70 secretion is frequently used as a surrogate marker of the ability of DCs to induce Th1-skewed T-cell immunity, the ultimate definition of DC1s is functional. We sought to determine the ability of these murine DC1s to stimulate Th1-skewed antigen-specific T-cell immunity *in vitro* and *in vivo*. OVA-pulsed DC1s were used to stimulate naïve OT-II T cells in *in vitro* cultures. OT-II T cells recognize OVA peptide 323–339 (ISQAVHAAHAEINEAGR) when presented by I-A^b MHC-class II molecules [206]. Determining the OT-II T-cell response to antigen-pulsed DCs enables an evaluation of the ability of DCs to process and present exogenous antigen, as well as their ability to stimulate and skew naïve T cells in an antigen-dependent manner. Specifically, we compared OT-II T-cell responses stimulated by DC1s or non-polarized DCs that were antigen loaded or not by pulsing with soluble OVA. As expected, stimulation of OT-II T cells with OVA-pulsed DCs (■ and □) or DC1s (○ and ●) resulted in significant and comparable proliferation of OT-II T cells (□ and ○) compared to unpulsed DC/DC1 stimulators (■ and ●) (Fig. 4A). However, OVA-pulsed DC1s induced significantly greater IFN-γ secretion by responder OT-II T cells compared to OVA-pulsed non-polarized DCs, indicating a strong Th1 bias in responders stimulated by the polarized DC1s (Fig. 4B). In these experiments IFN-γ secreted by DCs alone, DC1s alone, or OT-II T cells alone was negligible (data not shown). Under the same experimental conditions we were unable to detect secretion of IL-5 by ELISA (data not shown).

We next evaluated the ability of DC1s to stimulate a CD4$^+$ Th1-polarized OVA-specific immune response in vivo. Mice were immunized intradermally with DCs or DC1s pulsed with soluble OVA. CD4$^+$ T cells isolated from the splenocytes of immunized mice were incubated with

OVA-loaded splenic target cells and antigen-specific IFN-γ and IL-5 secretion was determined using ELISPOT (Fig. 4C) and ELISA (Fig. 4D). Polarized DC1s induced a clear Th1-biased immune response, as indicated by pronounced IFN-γ production. Non-polarized DCs induced a mixed Th1/Th2 response characterized by significantly less IFN-γ and more IL-5 production by antigen-specific $CD4^+$ T cells. The number of IFN-γ-secreting T cells observed in ELISPOT was consistent with the amount of secretion of cytokines determined by ELISA. Together, these data indicate that DCs stimulated with DC1 cocktail drive Th1-biased $CD4^+$ T-cell immune responses *in vitro* and *in vivo*.

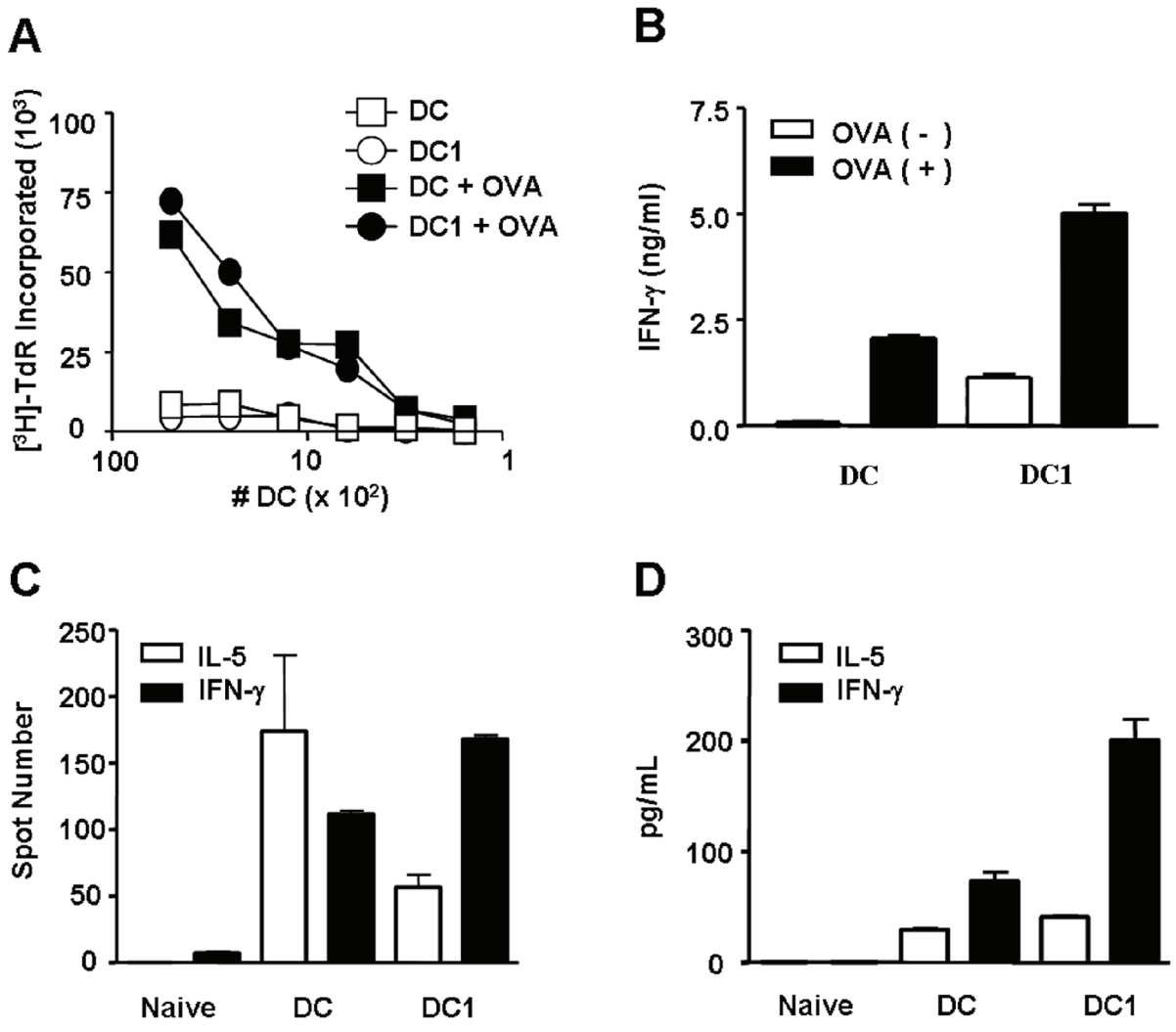

Figure 4. Polarized DC1s promote Th1 responses *in vitro* and *in vivo*

A. DCs were cultured in DC medium in the presence or absence of OVA (1 mg/ml) for 6 hours. At 2h, DC1 cocktail was added to some cultures as indicated. At 6h, DCs were washed 3x in PBS and cultured with freshly isolated OT-II T cells. T-cell proliferation was measured by ^{3}H thymidine incorporation. B. DCs were prepared as in A and cultured with freshly isolated OT-II T cells. On 3 day microculture supernatants were collected and IFN-γ concentration was measured by ELISA. C, D. DCs were loaded with OVA and polarized as in A and then used to immunize naïve mice via footpad/haunch injections on days 1, 7, and 14 with a total of $3x10^{6}$ DC delivered per immunization. Spleens were harvested on day 21 and $CD4^{+}$ T cells purified with magnetic beads. Splenic APC from naïve C57BL/6 were loaded with OVA (1 mg/mL) for 2 hours, washed 3x in PBS, and used as target cells for ELISPOT and ELISA assays to detect concentrations of IL-5 (open bars) and IFN-γ (solid bars). Results are representative of at least 3 similar experiments. Error bars indicate mean $\pm$ SEM.

2.3. Summary

Here we have verified previous reports describing TLR synergy. Stimulation of DCs using a cocktail of TLR3 and TLR9 ligands resulted in enhanced secretion of the Th1-driving cytokine IL-12p70 and DC maturation (Fig. 2). The addition of IFN-γ to our cocktail resulted in the suppression of IL-10 secretion by DCs (Fig. 2A). We examined the duration of IL-12p70 secretion and found our DCs exhaust 12 hours after the addition of DC1 cocktail (Fig. 3A) and determined that DCs require at least 4 hours of stimulation with the cocktail to achieve the maximal secretion of IL-12p70 (Fig. 3B). Our primary goal in this study was to evaluate the ability of our polarized DCs to induce Th1-mediated immunity. Because IL-12p70 plays an important role in the generation of Th1 immune responses, we examined the ability of DCs to secrete IL-12p70 following stimulation using CD40L, mimicking the interaction of DCs with T cells. We found that DC1 cocktail-stimulated DCs will sustain their secretion of IL-12p70 providing they encounter T cells prior to exhaustion, but fail to secrete IL-12p70 over that seen by non-polarized DCs if this interaction takes place after exhaustion (Fig. 3C and D). Together, the data suggest the Th1-skewing potential of DC1-based vaccines in our system may be more effective if the DCs reach draining lymph nodes prior to exhaustion. We next evaluated the ability of OVA-loaded DCs or DC1s to stimulate antigen-specific proliferation and skewing in and OT-II model *in vitro* and found that while both DC groups stimulate T cells equally well, but the DC1s are able to stimulate more potent Th1 responses compared to non-polarized DCs (Fig. 4A and B). Finally, we examined the ability of OVA-loaded DCs or DC1s to stimulate polarized antigen-specific T cell responses *in vivo*. Finally, we demonstrate antigen-loaded DC1s generated using our DC1 cocktail are more potent inducers of Th1 immunity *in vivo* than antigen-loaded non-polarized DCs (Fig. 4C and D), verifying the effectiveness of our cocktail.

3. CHAPTER THREE

COMPARATIVE ANALYSIS OF STRATEGIES DESIGNED TO DELIVER TUMOR-DERIVED PROTEINS TO DENDRITIC CELLS

3.1. Introduction

Many DC-based tumor immunization strategies have focused on the identification of tumor-specific and tumor-associated antigens [159, 207-209]. An obvious benefit to this approach is the ability to target only one antigen or peptide, decreasing the likelihood of activating self-reactive T cells and inducing subsequent autoimmunity. However, there are disadvantages to using identified proteins or peptides for use in immunotherapy. First, due to the nature of tumors, the vast majority of tumor antigens are unknown having derived from random mutations within the genomic DNA of the cell [180]. The end result is that every tumor expresses a unique set of antigens that may be used as targets for the immune response. Focusing the immunotherapeutic approach on only one antigen may result in an ineffective immune response if the tumor does not express that antigen. Second, peptide-based vaccines require that the recipients express a particular MHC allele to present the tumor-derived peptide, excluding a large number of patients from the vaccine approach. Third, identification of tumor antigens is time consuming, tedious, and expensive and ultimately impractical in a clinical setting on a patient-to-patient basis. Finally, focusing the immune response to only one protein or one epitope increases the probability that the tumor will generate an escape variant that does not express the particular antigen, rendering the vaccine ineffective [159, 180, 181].

It has been suggested that polyvalent immunization strategies have greater potential to induce tumor-specific immune responses than those that employ only single proteins or epitopes [181]. One approach to address this issue is the use of whole autologous tumor cells as a source of

antigen, allowing for potential immune recognition of a broad range of antigens in the tumor. DCs have the capacity to internalize, process, and present exogenous antigens on both MHC class I and II molecules [1]. While it has been demonstrated that different methods of inducing antigen transfer from tumor cells to DCs can result in internalization and presentation of tumor-derived peptides by dendritic cells [207, 209], little is known about the relative efficacy of antigen delivery using these methods. Here we compare three methods used to deliver tumor antigens to DCs using whole tumor cells as a source of antigen; 1) coculture of tumor cells with DCs (coculture), 2) feeding tumor lysate to DCs (lysate), and 3) fusion of tumor cells and DCs (fusion). Antigen transfer to DCs is evaluated using flow cytometry and fluorescence microscopy, demonstrating transfer in both murine and human systems and using multiple tumor lines. We verify that antigen transfer to DCs in cocultures is due to transfer from live tumor cells, rather than dead tumor cells that may be in the cultures, through the use of live-cell imaging. Functional presentation of both cytosolic and membrane-bound antigens following the three methods of antigen transfer to DCs is evaluated using OVA-expressing tumor clones.

3.2. Results

3.2.1. Measurement of antigen transfer from tumor cells to DCs using flow cytometry

Antigen transfer from tumor cells to DCs following coculture, lysate, and fusion was assessed using flow cytometry. Tumor cells were stained using CFSE, which binds free amine groups in the cytosol, effectively labeling the protein content of the tumor cells with the fluorescent dye. Since this attachment is through a covalent bond, any transfer of CFSE from the tumor cells to a DC can be assumed to be attached to a tumor-derived protein and, therefore, an indication that antigens have been passed to the DC. DCs were cultured with CFSE-labeled tumor cells or

tumor cell lysate at a ratio of 3:1 overnight. Samples were stained with CD11c-PE and fixed for analysis using a flow cytometer. The data demonstrate that both coculture and lysate effectively transfers tumor antigens to DCs in a murine (Fig. 5A) system. The ability of these methods to transfer antigens to DCs is also effective for human DCs (Fig. 5C). We also tested the ability of coculture and lysate to transfer antigens to DCs with a variety of tumors and found that the method was effective in every case (Table 2). While both methods are effective, lysate appears to be more efficient at delivering antigens to DCs (Fig. 5 and Table 2). Polyethylene glycol was used to fuse CFSE-labeled B16 tumor cells with murine BMDCs using a method previously described [188]. After fusion, samples were stained with CD11c-PE and analyzed by flow cytometry. Fusion alters the size of cells in an unpredictable manner. Therefore, gating by forward/side scatter was not performed in order to obtain all fusion events. The data demonstrate substantial antigen transfer to DCs following fusion (Fig. 5B). Together, the data suggest that all three methods of antigen transfer to DCs are effective, with lysate providing the highest level of transfer.

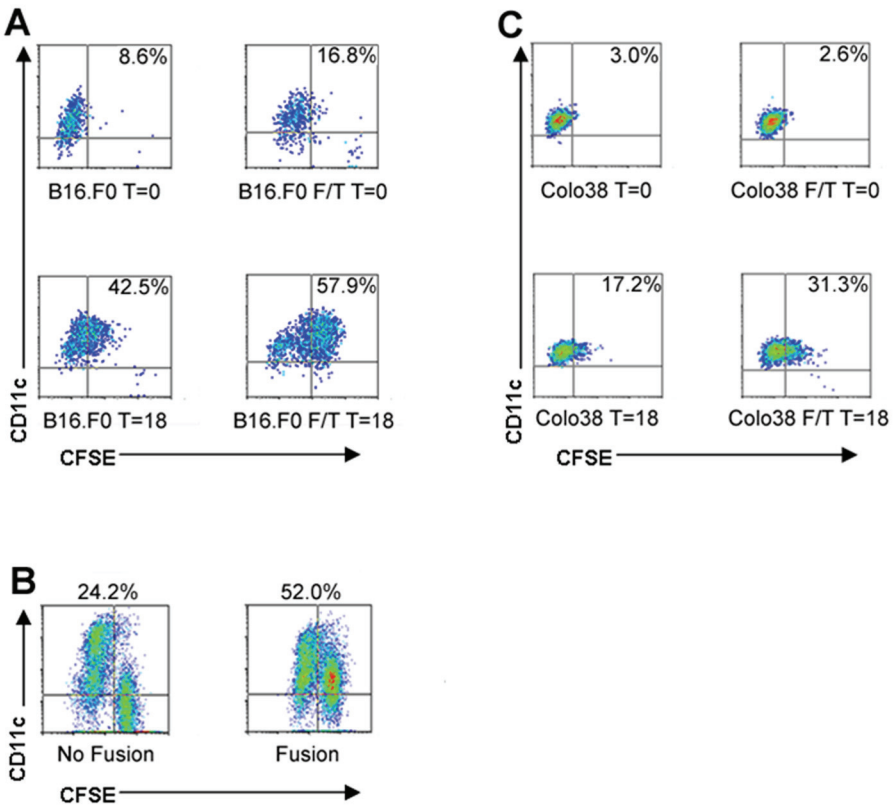

Figure 5. Comparative analysis of antigen transfer to DCs using flow cytometry

Tumor cells were labeled with CFSE and used either alive or used to generate tumor lysate by undergoing 3 rounds of freeze/thaw (F/T). Live tumor cells or lysate was cultured with murine (A) or human (C) DCs for 18 hours at 37º. In some experiments, murine DCs were fused with CFSE-labeled B16 tumor cells (B). Following coculture, lysate, or fusion, samples were stained with CD11c-PE and analyzed by flow cytometry. Coculture and lysate experiments were gated on the DC population using forward/side scatter. Numbers shown represent the percent of the $CD11c^+$ cells that are $CFSE^+$.

Table 2. Transfer of antigens in murine and human systems using coculture and lysate

Tumor	Species	Coculture		Lysate	
		% CFSE* Positive DCs at T=0	% CFSE Positive DCs at T=18	% CFSE Positive DCs at T=0	% CFSE Positive DCs at T=18
B16	Mouse	8.6	42.5	16.8	57.9
MethA	Mouse	15.1	60.9	7.8	82.5
3LL	Mouse	0.9	25.9	4.6	42.0
Mel526	Human	2.9	13.7	3.3	28.6
Colo38	Human	3.0	17.2	2.6	31.3

*Tumor cells were labeled with CFSE prior to coculture or generation of lysate, effectively labeling tumor proteins with the fluorescent dye. $CFSE^+$ DCs indicate the dendritic cells have acquired tumor antigens from the antigen loading method.

3.2.2. Evaluation of antigen transfer using fluorescence microscopy

We evaluated antigen transfer to DCs using fluorescence microscopy. As before, tumor cells were labeled with CFSE to tag cytosolic proteins with the fluorescent dye. Coculture and lysate was performed as described above using both murine tumors (B16 and MethA) and a human tumor (Colo38). Samples were fixed after 18 hours of culture. Fusions were performed using only B16 tumors and fixed immediately after fusion. Samples were stained with CD11c-PE to identify the dendritic cells and images acquired using a fluorescence microscope. Consistent with flow cytometric data, the data demonstrate effective antigen transfer to DCs using all three methods and with all tumors imaged (Fig. 6).

Because of the nature of flow cytometry and fluorescence microscopy, it is possible that what is perceived as antigen transfer could be the result of tumor-derived proteins adhering to the outside of the DCs. Similarly, fusions could actually be the result of a close association of tumor cells and DCs. Therefore, we used confocal microscopy to address this issue. In addition, we used LysoTracker dye to determine whether antigen was accessing the lysosomal compartments of the cell for degradation. Using CFSE-labeled B16 cells, we performed coculture, lysate, and fusion as before. Samples were stained with LysoTracker dye and then stained with CD11c-Cy5 and fixed for imaging. Images were acquired using confocal fluorescence microscopy. The data demonstrate both cytoplasmic localization of tumor antigens in DCs and colocalization of some tumor antigens with lysosomal compartments (arrows) following coculture and lysate (Fig. 7A). In addition, we verify the presence of tumor/DC fusions demonstrated by single-plane confocal microscopy (Fig. 7B). The data confirm that antigens are transferred to DCs using all three

methods and suggest that antigens are reaching compartments designed for antigen degradation (lysosomes).

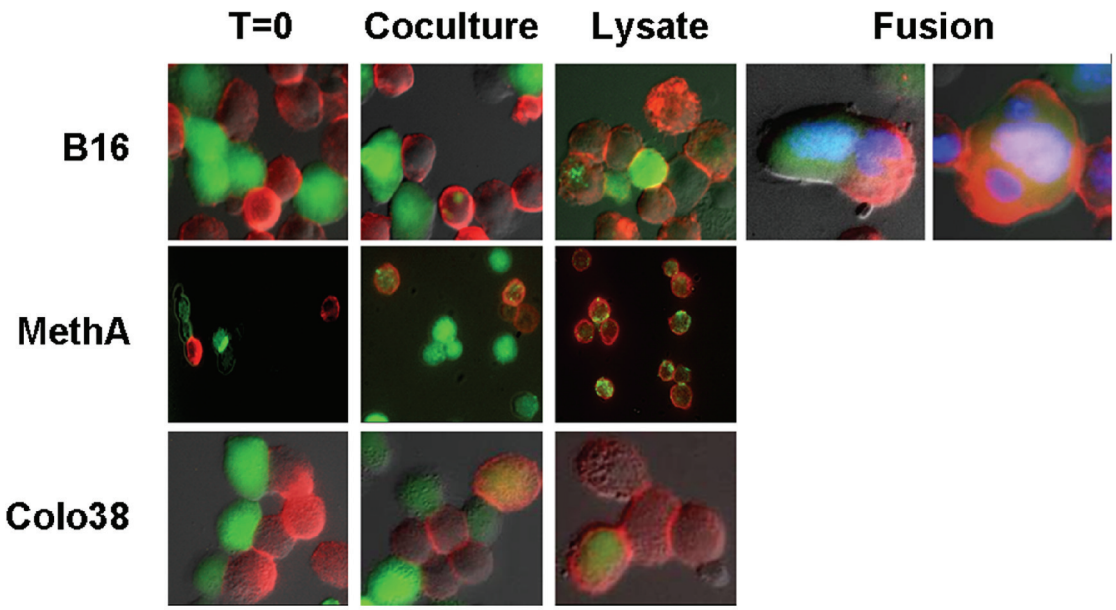

Figure 6. Evaluation of antigen transfer using fluorescence microscopy

The ability of coculture, lysate, and fusion to deliver tumor-derived proteins to dendritic cells was evaluated using fluorescence microscopy. Briefly, tumor cells were labeled with CFSE prior to antigen transfer. Samples were then stained using CD11c-PE to label dendritic cells and then fixed in 2% paraformaldehyde for imaging. As a control, tumor cells and dendritic cells were mixed immediately prior to staining and fixation (T=0). Antigen transfer is verified by visualization of tumor antigen (green) within dendritic cells (red).

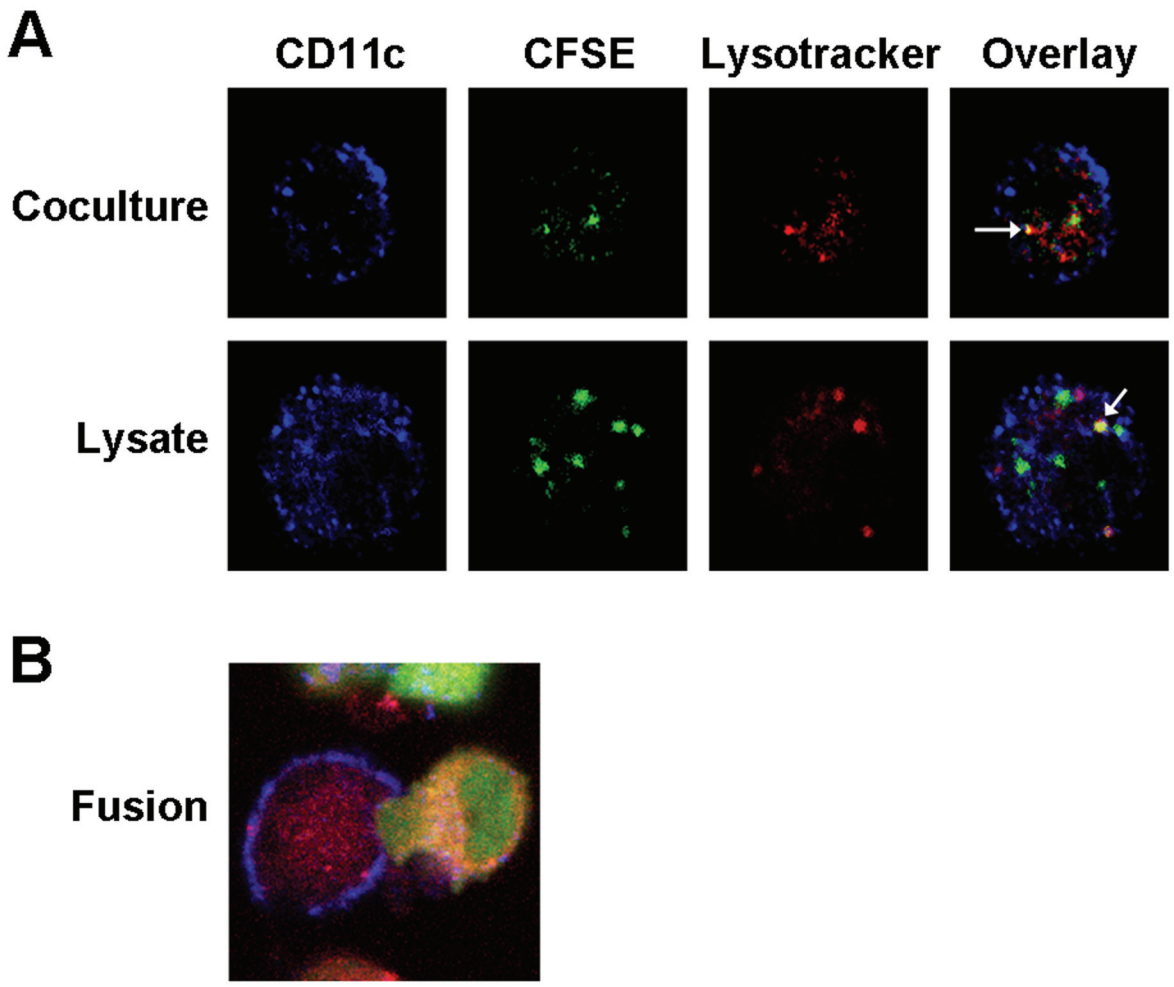

Figure 7. Verification of antigen transfer by confocal microscopy

Confocal microscopy was used to verify cytosolic localization of antigens following coculture and lysate (A) and to verify the presence of tumor/DC fusions (B). B16 tumor cells were labeled with CFSE (green) and used in coculture, lysate, and fusion with day 5 dendritic cells. Just prior to fixation, samples were labeled with LysoTracker (red) and stained using CD11c-Cy7 [210]. Arrows indicate tumor antigen access to lysosomes following coculture and lysate (A).

3.2.3. Dendritic cells acquire antigen from live tumor cells during coculture

DCs are known to sample antigens from peripheral tissues *in vivo* for presentation to antigen-specific T cells in draining lymph nodes [1]. We hypothesized that DCs acquire antigens from live cells in a similar manner during coculture with tumor cells. However, there was a distinct possibility that antigen transfer seen with flow cytometry and microscopy was due to uptake of antigens from apoptotic or necrotic tumor cells. To address this issue, we performed fluorescence live cell imaging to generate movies depicting antigen uptake by DCs during coculture. B16 tumor cells were stained using the cytosolic dye, CFSE, and allowed to adhere to a coverglass. The tumor cell coated coverglass was placed in the imaging chamber and day 5 CMTMR-labeled DCs injected and allowed to coculture with the tumor cells over a period of two hours. Images were taken of random fields every 2 minutes and then compiled to generate live cell movies. Still images from a live cell movie demonstrate dendritic cells acquire tumor antigens from live tumor cells during coculture (Fig. 8).

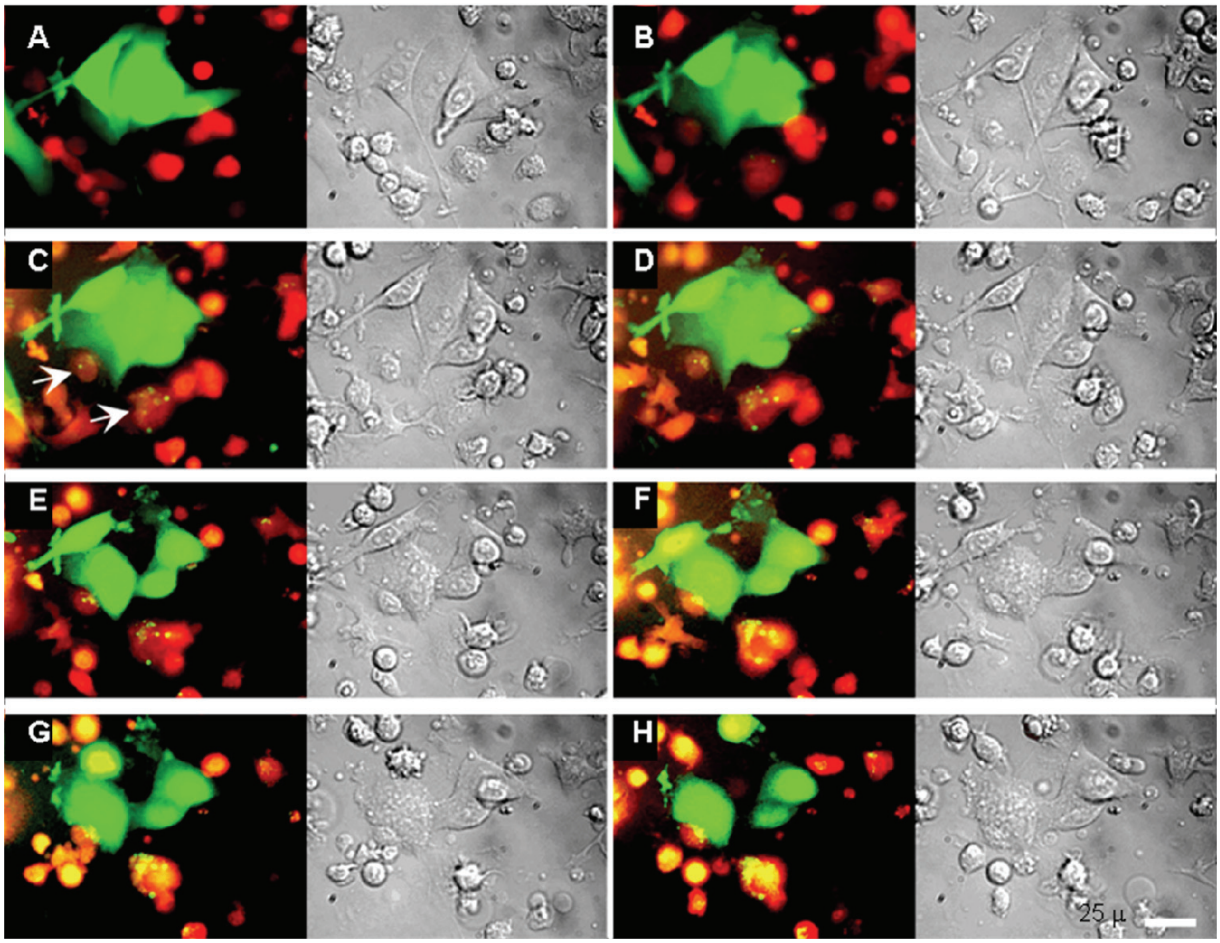

Figure 8. DCs acquire antigen from live tumor cells during coculture

Day 5 BMDC were purified as before and stained with CMTMR (Red). B16 tumor cells were labeled with CFSE (green) and allowed to adhere to a coverslip and then placed in a chamber for live fluorescence microscopy. DCs were pumped into the chamber to co-culture with tumor cells, with images collected at 2-minute intervals. The panels represent intervals taken at 0-minutes (A), 15-minutes (B), 30-minutes (C), 45-minutes (D), 60-minutes (E), 75-minutes (F), 90-minutes (G), and 105-minutes (H). Both the fluorescence images (left side of each panel) and the DIC images (right side of each panel) are shown. Tumor-derived protein uptake by DC (yellow) is evident after 30 minutes of incubation as indicated by the arrows in panel C. The data demonstrate co-culture as a viable method for antigen transfer to DC for possible use in tumor immunotherapy.

3.2.4. Evaluation of functional presentation of tumor-derived antigens following coculture, lysate, and fusion

Our data thus far has focused only on transfer of tumor antigens to DCs. For tumor vaccine development, it is important that tumor antigens be degraded and processed in a manner that induces presentation of tumor-derived peptides on MHC class I and class II molecules. To facilitate evaluation of functional tumor antigen presentation, we generated B16 clones expressing either membrane-bound or cytosolic variants of chicken egg ovalbumin. We used two previously described constructs of OVA, OVA-TR489 (membrane-bound) and OVA-138 (cytosolic), a version of OVA-489 modified to include a start codon on the 5' end of the sequence [37, 177, 211].

We began by verifying the cellular localization of the constructs. 293T cells were transiently transfected overnight using OVA-TR489 and OVA-489. Cells were fixed and stained for OVA using a FITC-conjugated polyclonal antibody with or without permeabilization. OVA-TR489 is generated from the fusion of the cytosolic variant of OVA fused to the transferrin receptor, resulting in the OVA protein existing as an extracellular domain with a transmembrane anchor [37, 211]. Cells transfected with OVA-TR489 will, therefore, stain positive with and without permeabilization. In a similar manner, cells transfected with OVA-489 will only stain positive for OVA when permeabilized since the localization of OVA is restricted to the cytoplasm. The results demonstrate that the constructs tested have the appropriate cellular localization, with OVA-489-transfected cells staining positive only when permeabilized and OVA-TR489 staining positive with and without permeabilization (Fig. 9).

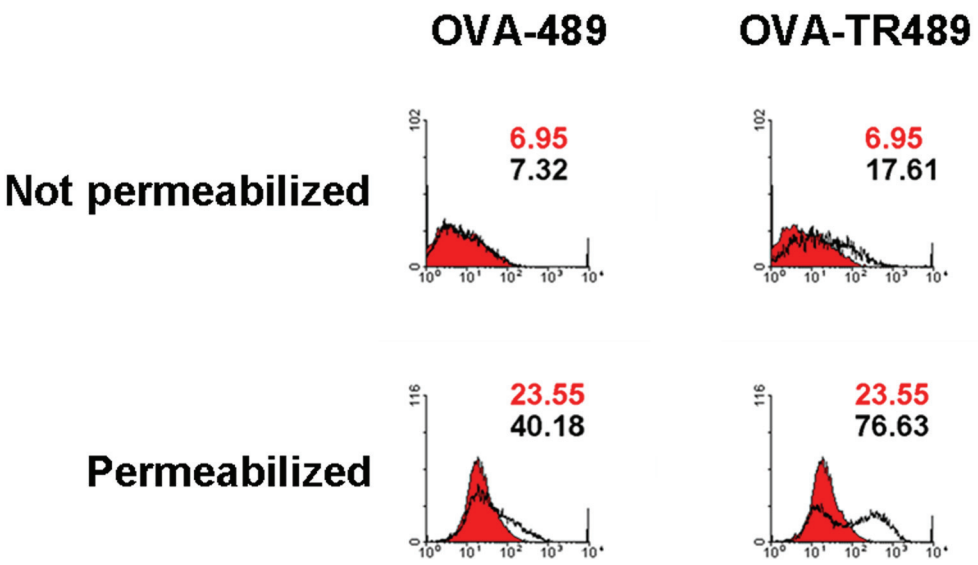

Figure 9. Cellular localization of OVA constructs

293T cells were transfected with OVA-489 and OVA-TR489. Cells were fixed and stained with FITC-conjugated anti-OVA antibodies with or without permeabilization to determine the cellular localization of the antigens (black). Isotype staining is shown in red. Numbers indicate the MFI of the respective samples.

Transient transfections often yield clones that express a varying amount of antigen that is short-lived. Therefore, we chose to utilize lentiviral vectors capable of generating clones that express a desired antigen over long periods of time. To accomplish this, OVA-TR489 and OVA-138 were amplified from existing plasmids using PCR and purified by gel electrophoresis. The size of purified bands was consistent with the desired fragments (Fig. 10A). These fragments were cloned into the lentiviral plasmid and used to transform bacteria. Colonies were selected and PCR ran on 4 colonies to verify the presence of the desired constructs (Fig. 10B). One colony was chosen for each construct and expanded for DNA purification. Plasmid DNA was purified using a Qiagen miniprep kit and then used to generate lentiviral vectors. Lentiviral vectors were tested for accuracy by transforming 293T cells. Cells were selected in blasticidin and then solubilized for gel electrophoresis. Western blots confirmed the presence of OVA-TR489 and OVA-138 (Fig. 10C). Confident with our lentiviral vectors, we transformed B16 tumor cells and isolated the OVA-expressing clones B16-TR489 and B16-138. OVA expression was verified by measurement of mRNA using reverse transcription PCR (RT-PCR) using primers internal to the OVA portion of the constructs (Fig. 10D). These clones were subsequently used for functional analysis of membrane-bound and cytosolic antigen presentation of tumor-derived proteins. Coculture, lysate, and fusion were performed as before using B16, B16-138, and B16-TR489. DCs were purified from coculture, lysate, and fusion groups using CD11c magnetic beads, stimulated with DC1 cocktail for 4 hours, and used to stimulate OT-I and OT-II T cells. Following three days of stimulation, supernatants were collected and analyzed by ELISA for IL-2 and IFN-γ as indicators of functional antigen presentation. Both coculture and fusion were able to induce class I presentation of both cytosolic and membrane-bound tumor antigens as demonstrated by OT-I T-cell production of IL-2 (Fig. 11A) and IFN-γ (Fig. 11B). Fusion was

able to induce high levels of IL-2 production, suggesting a greater level of function antigen presentation. (Fig. 11A). No IL-2 and low levels of IFN-γ were detected in OT-I culture supernatants following stimulation with lysate-fed DCs (Fig. 11A and B) suggesting minimal class I presentation. In a similar manner, coculture and fusion induced high levels of class II presentation of cytosolic and membrane-bound antigens with fusion inducing higher levels of IL-2 production by OT-II T cells (Fig. 11C). Both methods induced high levels of IFN-γ secretion by OT-II T cells (Fig. 11D). While lysate was able to induce greater class II presentation of tumor-derived OVA than class I presentation, the levels were still comparatively low to that seen in coculture and fusion groups and lysate was still unable to induce detectable IL-2 in culture supernatants (Fig. 11C and D). Detection of IFN-γ and not IL-2 in supernatants is probably due to the usage of IL-2 by activated OT-I and OT-II T cells. Together, the data demonstrate all three methods of antigen loading are capable of inducing functional class I and class II presentation of tumor-derived antigens. While lysate appears far more efficient at delivering tumor antigens to DCs (Fig. 5 and Table 1), it is unable to induce the functional presentation at levels seen by coculture and lysate (Fig. 11).

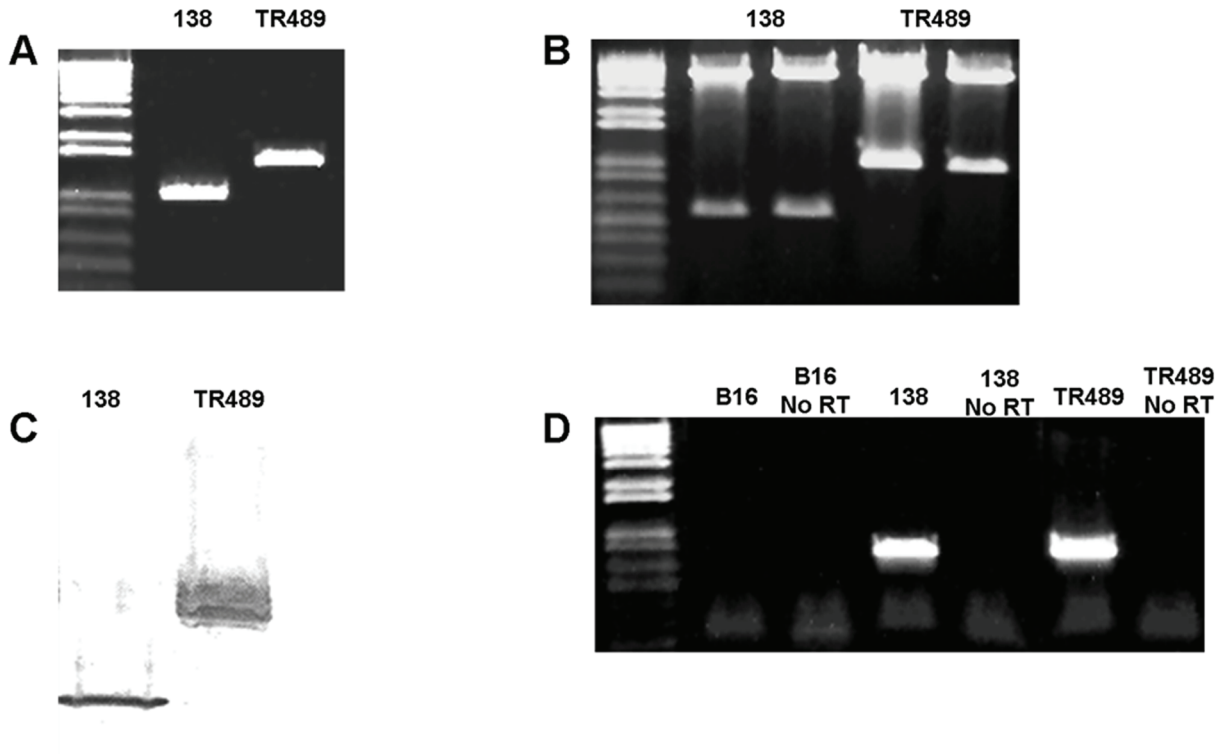

Figure 10. Generation of OVA-expressing B16 clones

DNA fragments encoding cytosolic (138) and membrane-bound (TR489) forms of OVA were amplified from plasmids using PCR and purified by gel electrophoresis. (A). Fragments were cloned into a pLenti vector and the resulting plasmids used to transform bacteria. Colonies were screened (B) and plasmid DNA used to generate viral vectors. 293T cells were infected with Lentivirus encoding OVA-138 and OVA-TR489. Protein expression by transduced cells was verified by OVA western blot (C). Once verified, OVA-138 and OVA-TR489 lentiviral vectors were used to generate stable OVA-expressing B16 clones (B16-138 and B16-TR489) as determined by RT-PCR (D). In the absence of reverse transcriptase, no bands are seen, indicating a lack of genomic DNA contamination (D).

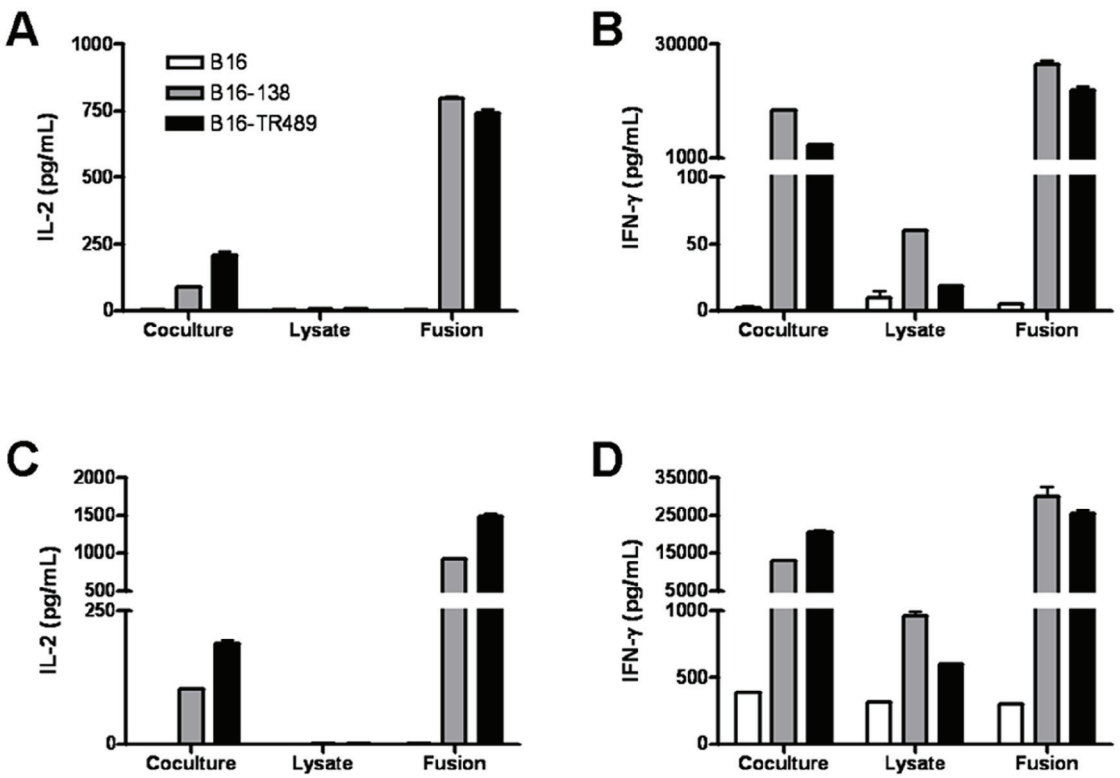

Figure 11. Functional presentation of tumor-derived cytosolic and membrane-bound antigens following coculture, lysate, and fusion

Coculture, lysate, and fusion were performed as before using Day 5 BMDCs and B16 (negative control) (open bars), B16-138 (gray bars), and B16-TR489 (black bars). DCs were purified using CD11c magnetic beads, stimulated with DC1 cocktail for 4 hours, and used to stimulate OT-I (A, B) and OT-II (C, D) T cells to measure class I and class II presentation, respectively. Following 3 days of stimulation, culture supernatants were collected and frozen for ELISA. Antigen presentation is determined by the detection of IL-2 (A, C) and IFN-γ (B, D) in supernatants. Error bars represent mean $\pm$ SEM.

3.3. Summary

Here we have compared the relative ability of coculture, lysate, and fusion to deliver tumor antigens to DCs using flow cytometry (Fig. 5), fluorescence microscopy (Fig. 6), and confocal microscopy (Fig. 7). Our results suggested that all three methods were efficient at delivering the antigens to DCs, and also suggested coculture and lysate methods resulted in tumor-derived antigens access the lysosomal compartments, allowing for potential degradation and presentation of these antigens. We verified the ability of DCs to acquire antigens from live tumor cells via live cell microscopy (Fig. 8). Finally, we generated stable B16 clones expressing cytosolic or membrane-bound OVA (Fig. 9 and 10) and used those clones to examine functional class I and class I presentation of tumor-derived antigens by DCs. Our data demonstrate that while all three methods were similar in their capacity to deliver antigens to DCs, fusion resulted in the highest level of functional presentation, coculture resulted in a high/intermediate level of presentation, and lysates provided only low levels of antigen presentation as determined by *in vitro* OT-I and OT-II assays (Fig. 11).

4. CHAPTER FOUR

TUMOR IMMUNOTHERAPY USING ANTIGEN-LOADED POLARIZED DENDRITIC CELLS

4.1. Introduction

DCs initiate antigen-specific immune responses through the presentation of MHC class I and class II antigenic peptides in the context of appropriate costimulation to antigen-specific T cells. Cytokines produced by activated DCs not only control the type of specific immune responses induced, but also activate innate immunity. The ability of DCs to orchestrate both arms of the immune system makes them attractive candidates for preventative and therapeutic vaccine development. Considerable effort is being made to develop strategies to use DCs to induce tumor-specific immunity, including nearly 100 clinical trials designed to evaluate safety or efficacy in humans [157, 158, 207].

It has long been established that activated $CD4^+$ T-cells can have a direct role in tumor rejection, potentially through a mechanism involving recruitment and activation of macrophages [212-217]. In addition, IFN-γ secreted by Th1 $CD4^+$ T-cells can have direct anti-tumor and anti-angiogenic activities [213, 216, 218, 219]. Very recent studies support the role of $CD4^+$ T-cells in tumor immunity by providing evidence that effector $CD4^+$ T-cells mediate immunity against cervical cancer and myeloma in the apparent absence of tumor-specific $CD8^+$ T cells, and suggest that this effect is mediated at least in part by macrophages activated by T-cell derived IFN-γ [179, 220, 221]. Taken together, these observations provide rationale for the use of type-1 polarized DCs to induce Th1-skewed immune responses for the treatment or prevention of cancer.

We sought to investigate the ability of antigen-loaded DC1s to induce therapeutic responses against the non-immunogenic melanoma B16 following adoptive transfer. The B16 model was chosen because it is a formidable model tumor for the evaluation of immunotherapeutic strategies. B16 has multiple described mechanisms of immune evasion. These include 1) down regulation of MHC-class I molecules and antigen processing machinery [156, 222, 223], 2) a lack of expression of MHC-class II molecules [27], 3) production of vascular endothelial growth factor that inhibits DC function and T-cell immunity [150, 151, 224], and 4) production of galectin-1, a negative regulator of T-cell activation and survival [155, 225]. In addition, recent studies demonstrate that $CD4^+CD25^+$ regulatory T cells can prevent induction of effective anti-B16 $CD8^+$ T-cell responses [9-14]. Taken together, these features suggest that B16 melanoma models the most challenging tumor escape mechanisms thus far described for a variety of human tumors.

To load DCs with tumor antigen we utilized a modified version of an autologous whole cell coculture strategy we described previously [188]. We have shown that cellular vaccines consisting of tumor cells and dendritic cells that have been co-cultured overnight can elicit effective tumor immunity [188]. Using two relatively non-immunogenic murine tumor models, we have shown that this immunization strategy can induce protective tumor immunity *in vivo*, and is capable of causing regression of established tumors, resulting in persistent anti-tumor immunity [188]. Importantly, DC-tumor cell immunization has the potential to simultaneously stimulate $CD4^+$ and $CD8^+$ T cell-mediated immunity against multiple tumor antigens. Since the autologous tumor cell is the source of antigen, immunization does not depend on the prior identification of unique or "shared" tumor antigens, and is not limited to individuals expressing a

particular corresponding MHC allele (as in the case of synthetic tumor peptide epitopes). Furthermore, because the immunization is patient-specific, it has the potential to stimulate immunity against uniquely expressed (polymorphic or mutated) tumor antigens that may be an important component of an effective "regressor" anti-tumor response.

Studies we describe here combine two novel approaches to DC-based tumor immunotherapy – *in vitro* TLR ligation to induce effective DC1 function, and whole tumor cell loading of DCs to enable presentation of a broad range of autologous tumor antigens through multiple processing pathways. We show that DC maturation and polarization are not inhibited by the presence of live tumor cells, and DCs exposed to tumor cells induce DTH responses *in vivo*. Polarized DCs loaded with tumor cells and injected into tumor-bearing mice induce Th1-skewed tumor-specific $CD4^+$ T cells and a significant reduction in tumor growth. Tumor infiltrates in DC1 immunized animals are characterized by the presence of $CD4^+$ T cells and macrophages, and the apparent absence of $CD8^+$ T cells. Finally, we demonstrate the absence of a detectable $CD8^+$ T-cell response is not due to a lack of MHC class I presentation. These results demonstrate a murine model of DC1 function and suggest an important role for $CD4^+$ T cells and macrophages in DC1-induced anti-tumor immune responses. They have implications for the future development of DC1-based immunotherapies and strategies for relevant clinical immune monitoring of their effectiveness.

4.2. Results

4.2.1. Tumor cell loaded DC1s induce Th1-skewed anti-tumor immunity

We sought to determine the capacity of DC1s loaded with autologous tumor cells to induce Th1-skewed tumor-specific immunity. B16 melanoma cells have multiple immune escape mechanisms, including their ability to directly inhibit DC maturation and function [151, 224]. Based on the potent DC activation and polarization we observed with synergistic TLR3 and TLR9 ligation, we hypothesized that the potential inhibitory effects of co-cultured B16 melanoma cells on the DCs in the vaccine could be overcome by TLR ligation. DCs may also be negatively affected by stressful antigen loading strategies, such as fusion. The aim of our study is to evaluate antigen-loaded polarized DCs for tumor immunotherapy. Therefore, it is critical that DCs are capable of secreting the Th1-driving cytokine IL-12p70 in response to DC1 cocktail following antigen loading. To address these issues we compared DC IL-12p70 production in response to DC1 cocktail following coculture, lysate, and fusion. Following antigen loading, DCs were stimulated 18 hours with DC1 cocktail and supernatants collected for IL-12p70 ELISA. The results demonstrate that coculture of DCs with B16 tumor cells does not inhibit IL-12p70 production (Fig. 12A). Interestingly, IL-12p70 secretion by DCs in response to DC1 cocktail is slightly inhibited by feeding DCs with tumor lysate and is nearly absent following fusion (Fig. 12A). Based on these results and previous results comparing antigen presentation, we chose coculture as the optimal antigen loading strategy for use with DC1 polarization for the remainder of this study.

We next compared maturation of DCs cultured in the presence or absence of B16 melanoma cells. DCs were co-cultured with or without B16 tumor cells and stimulated with DC1-driving cocktail or LPS (1μg/ml) as a positive control. Samples were collected after 18h of culture and analyzed for expression of phenotypic markers (Fig. 12B). As determined by comparison of

MHC-class II, CD86, and CD40 expression, stimulation of DCs with TLR3 and TLR9 ligation induces DC activation even in the presence of B16 tumor cells.

Th1-type immune responses support the induction of delayed-type hypersensitivity (DTH) reactions [50, 226]. To initially evaluate the *in vivo* immunogenicity of B16 loaded DC1s, we evaluated the capacity of this DC1/melanoma vaccine to induce DTH reactions. We immunized mice by intradermal/subcutaneous injection of DCs alone, DCs loaded with B16 melanoma by co-culture (as described above) (DC+B16), or DCs loaded with B16 melanoma by co-culture in the presence of DC1-driving cocktail (DC1+B16). Five days later immunized animals were challenged (elicitation) with the vaccines by intradermal injection into the contralateral footpads. Injection of the vaccines alone did not induce increases in footpad swelling at the immunization site (data not shown). DC1+B16 immunizations induced a strong DTH response as demonstrated by a two-fold increase in contralateral footpad thickness at the elicitation site 48hrs after elicitation compared to the response induced by non-polarized DCs (Fig. 12C).

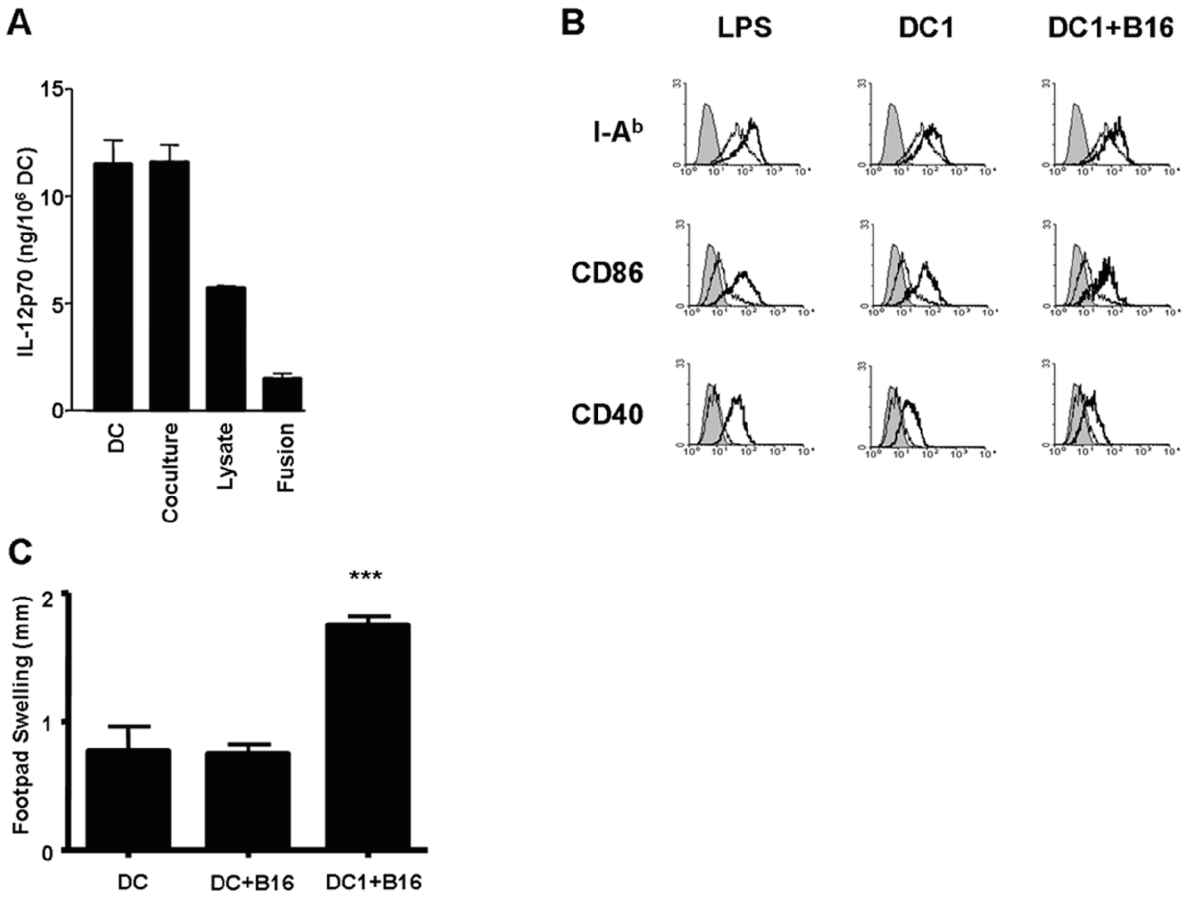

Figure 12. DC1-driving cocktail stimulates DC maturation and type-1 function in the presence of tumor cells

A. Dendritic cells were loaded with B16 tumor antigen by coculture, lysate, or fusion (dendritic cells/tumor cell, 3:1) with the addition of DC1 cocktail after 2 hours of culture. Culture supernatants were collected after 18 hours and tested for IL-12p70 concentration. Representative of at least three similar experiments. B. DCs were cultured in the presence or absence of B16 tumor cells (coculture as in A), stimulated with LPS (1μg/mL) or DC1 cocktail (heavy lines) for 18 hours and $CD11c^+$ cells were phenotypically compared to $CD11c^+$ cells cultured in medium alone (thin line). Shaded regions, isotype control. C. Dendritic cells were cultured for 6 hours with or without tumor cells, with some groups receiving DC1 cocktail after 2 hours of coculture. Naïve mice received 5 x 10^6 DCs per immunization via left footpad/left haunch injections on day 0 (sensitization) and via right footpad injections on day 5 using the same dendritic cells used for sensitization (elicitation). Footpad thickness was measured just before and 48 hours following elicitation injections. Representative of at least two similar experiments. Columns, means; bars, $\pm$ SE. ***, $P < 0.0001$.

To directly evaluate the immunotherapeutic effect of DC1-based immunization, groups of tumor-bearing mice were immunized by intradermal/subcutaneous injection of DCs loaded with B16 tumor cells for 6h, in the presence (DC1+B16) or absence (DC+B16) of poly [I:C], CpGs, and IFN-γ over the final 4h of incubation. We observed a significant delay in tumor growth in mice receiving the DC1+B16 immunization, while immunization with non-polarized DC+B16 resulted in tumor growth similar to that seen in untreated controls (Fig. 13A). $CD4^+$ T cells from splenocytes were isolated and used to evaluate tumor-specific T-cell responses by ELISPOT. B16 tumor cells do not express MHC class II and, therefore, cannot be used directly to stimulate $CD4^+$ T cells *ex vivo*. To overcome this obstacle we isolated splenic APC from un-immunized mice and loaded them with B16 tumor lysates, allowing for the presentation of tumor-derived peptides in the context of MHC class II molecules. Unloaded splenic APC were used as a control. Mice immunized with the DC1+B16 coculture vaccine demonstrated a high level of IFN-γ-secreting tumor-specific $CD4^+$ T-cells (Fig. 13B). DC1+B16 immunized mice demonstrated much lower, but significant, levels of IL-5-secreting tumor-specific $CD4^+$ T cells. In comparison, mice immunized with DC/melanoma demonstrated only low levels of tumor-specific IFN-γ-secreting $CD4^+$ T cells, which were comparable to the levels of IL-5-secreting tumor-specific $CD4^+$ T cells generated. These results are consistent with the induction of a tumor-specific Th1-skewed response by DC1s as demonstrated by a significant and predominant induction of tumor-specific IFN-γ-secreting Th cells.

Interestingly, despite the significant inhibition of tumor growth and the potent Th1-skewed $CD4^+$ T-cell responses observed in DC1+B16 immunized animals, we were unable to detect tumor-specific CTL activity in these animals using standard ^{51}Cr release assays (data not shown). This

was not completely unexpected given the previously reported results of others and the multiple well-described mechanisms of immune evasion observed with the B16 tumor model [10-13, 150, 155, 156, 222, 223]. To further characterize the anti-tumor response in responding animals, we utilized microscopy with hematoxilin and eosin stains (Fig. 13C) and immunofluorescence (Fig. 13D) to characterize tumor infiltrates. Tumors from responding DC1-immunized mice demonstrated a moderate $CD4^+$ T-cell infiltrate and extensive macrophage infiltration compared to naïve mice or mice immunized with non-polarized DC. Tumor-infiltrating macrophages staining positive for iNOS/NOS2 were found only in DC1-immunized mice, demonstrating macrophage activation (Fig. 13D, panel c). This pattern is consistent with an IFN-γ-mediated response similar to that seen in classic DTH responses. As predicted by our inability to detect a tumor-specific CTL response, the infiltrates in tumors of responding animals did not appear to contain significant numbers of $CD8^+$ T cells. At day 15, macrophage infiltration in tumors appeared diminished (Fig. 13E, panel l). In addition, macrophages at this stage of tumor growth appeared small and monocyte-like, suggesting the cells are not activated. Together, the data demonstrate control of tumor growth at day 7, but subsequent loss of control of tumor growth by day 15, suggesting the antitumor immune response was downregulated by immunoregulatory mechanisms or that immunosuppressive mechanisms employed by the tumor were able to shut down antitumor effector responses.

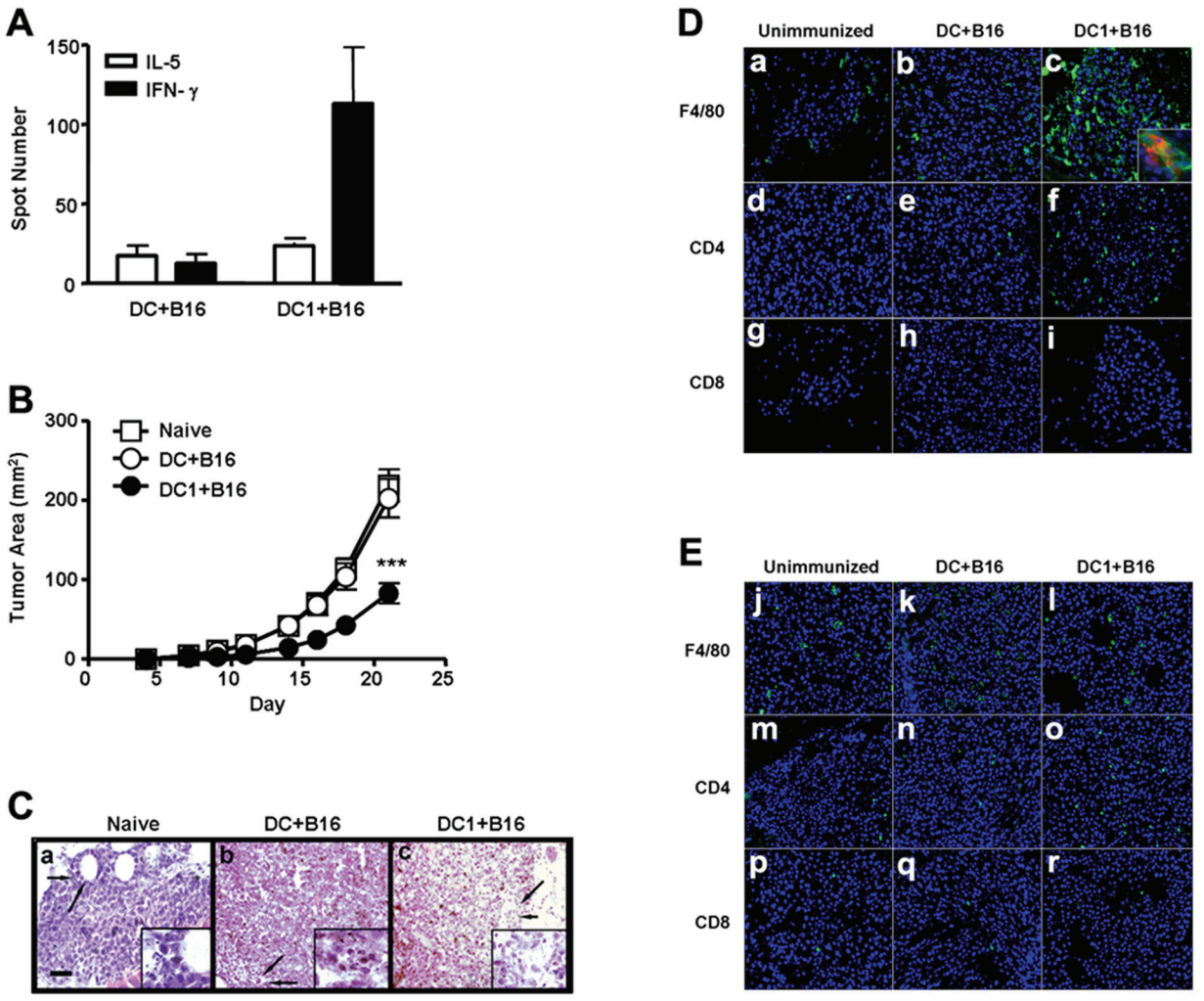

Figure 13. Tumor cell-loaded polarized DC1s induce Th1 tumor-specific immunity *in vivo* that inhibits growing tumors

Dendritic cells (DCs) were cocultured with B16 melanoma cells for 6 hours in the presence or absence of DC1 cocktail added after 2 hours. On day 0, B16 melanoma cells were implanted i.d. into naïve mice. Tumor-bearing mice were then immunized via footpad/haunch injections on days 7 and 14 with tumor-loaded dendritic cells or DC1s. A. On day 21, $CD4^+$ T cells were purified from spleens and used for ELISPOT assays to detect tumor-specific production of IL-5 (open columns) and IFN-γ (solid columns). B. Tumor-bearing mice were immunized or not (control) with tumor-loaded dendritic cells or DC1s via footpad/haunch injections on day 1 and every 7 days thereafter. Tumor area was measured using calipers; tumor growth curves were generated by combining the results from three separate experiments (total of 14 mice per group). Columns, means; bars, $\pm$ SE. ***, $P < 0.0001$. C,D,E. For image analysis, tumors were excised on day 8 (C and D) or on day 15 (E), fixed with 4% paraformaldehyde, and frozen for sectioning. C. H&E. D and E. Immunofluorescence. Nuclei were stained using Hoechst stain [210]. Markers for macrophages (F4/80) and T cells (CD4, CD8) are shown in green. *Inset*, some sections were double-stained for the macrophage marker F4/80 (green) and iNOS/NOS2 (red). Magnification, x 200 with x 1,000 inset (H&E) and x 200 with x 600 inset (immunofluorescence). Representative of two similar experiments.

4.2.2. The absence of detectable $CD8^+$ T-cell responses is not due to a lack of class I presentation

Our data demonstrate a lack of $CD8^+$ T-cell responses in DC1-immunized tumor-bearing mice. We have previously demonstrated the ability of coculture to induce class I presentation after 18 hours of coculture (Fig. 11A, B) [188]. However, there is a possibility that the shorter coculture procedure used for immunization of tumor-bearing mice is unable to induce class I-restricted tumor antigen presentation. To address this issue, we performed cocultures with DCs and B16-TR489 for 6 or 18 hours, with DC1 cocktail added for the final 4 hours of coculture. Cells were extensively washed and naïve mice immunized via footpad/haunch injections on day 0 (6 hour coculture) or day 1 (18 hour coculture). As a positive control, a subset of mice was immunized with SIINFEKL-pulsed DC1s. OT-I T cells were stained with CFSE and injected into naïve mice (negative control) and immunized mice on day 2. Polpliteal and inguinal lymph nodes were collected on day 5 and cells stained for the OT-I T cell receptor. As OT-I T cells divide, CFSE fluorescence is halved, generating a series of peaks indicative of proliferation. Class I presentation of tumor-derived antigens is verified if coculture of DCs with OVA-expressing B16 tumor cells results in OT-I proliferation. The data demonstrate no proliferation in non-immunized mice (Fig. 14A) and high level OT-I proliferation in mice immunized with SIINFEKL-pulsed DCs (Fig. 14B). Importantly, an intermediate level of OT-I T-cell proliferation is observed in mice immunized with either 6 hour cocultures (Fig. 14C) or 18 hour cocultures (Fig. 14D). Together, the data demonstrate the lack of $CD8^+$ T-cell responses seen in tumor-bearing DC1-immunized mice is not due to a lack of class I presentation. Other laboratories have reported an inhibition of $CD8^+$ T-cell responses in mice that is overcome

through the depletion of regulatory T cells [9-14]. It is likely that $CD8^+$ T cells are inhibited in a similar manner in our DC1-based tumor immunization model.

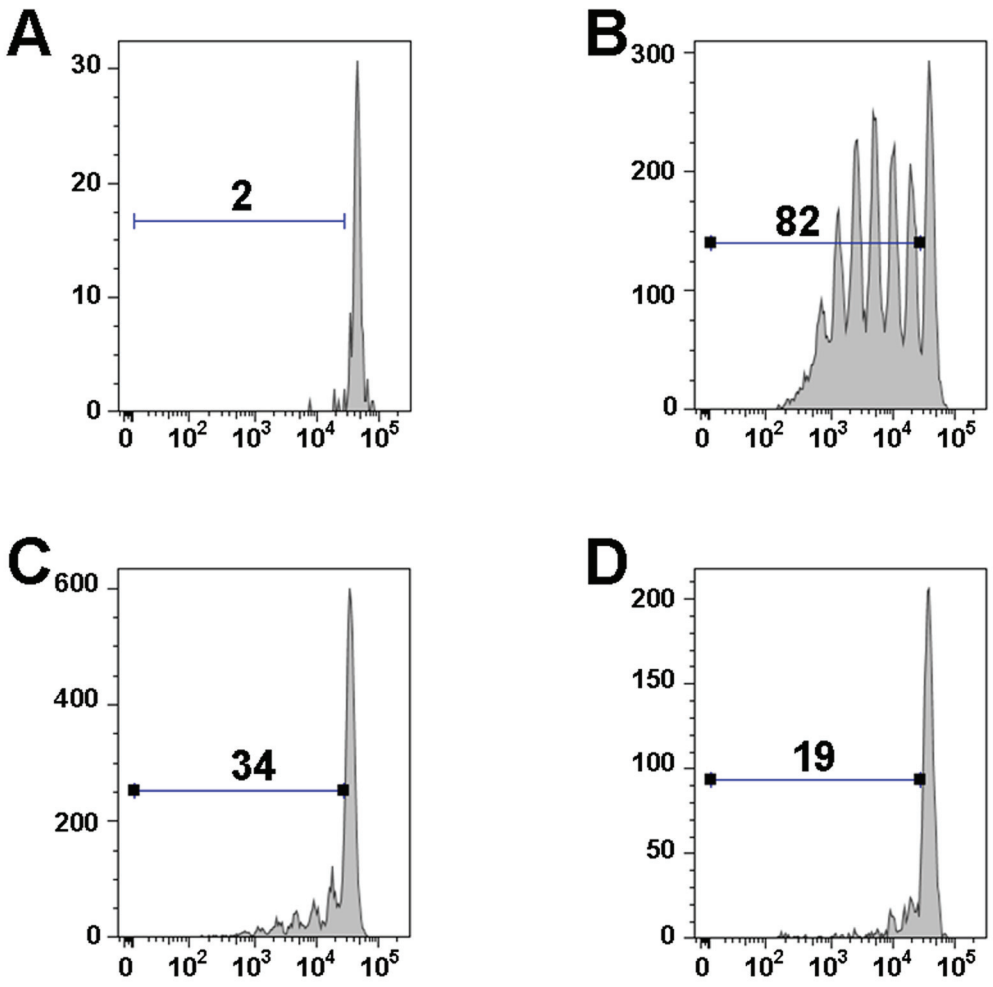

Figure 14. Coculture of dendritic cells and tumor cells for 6 or 18 hours results in class I presentation of tumor-derived antigens

Day 5 DCs were cocultured with B16 tumor cells for 6h or 18h, receiving DC1 cocktail over the final 4h of coculture. Cocultures were injected into naïve mice via footpad/haunch on day 0 or day 1, respectively. On day 2, mice received CFSE-labeled OT-I via tail vein, footpad, and haunch injections. Mice were sacrificed on day 5 and popliteal and inguinal lymph nodes were collected and stained for OT-I T cell receptor expression. Numbers represent percent proliferation of OT-I T cells in naïve (A), SIINFEKL-pulsed DC-immunized (B), 6h coculture-immunized (C) and 18h coculture-immunized (D) mice.

4.3. Summary

Here we combine two novel approaches for tumor immunotherapy. We began by examining the impact of the antigen-loading strategy on the ability of DCs to secrete the Th1-polarizing cytokine, IL-12p70. We found that while fusion resulted in the highest level of antigen presentation (Fig. 11), the method greatly inhibited the ability of our DCs to respond to DC1 cocktail (Fig. 12A). Importantly, coculture did not inhibit this secretion (Fig. 12A). Based on these results, we decided to use coculture to load our dendritic cells prior to maturation with DC1 cocktail. Tumors are known to inhibit DC function. Therefore, we examined whether live tumor cells would inhibit the upregulation of important costimulatory molecules following stimulation with our DC1 cocktail. We found that DCs increased their expression of costimulatory molecules in the presence of live tumor cells equally well compared to DCs matured in the absence of live tumor cells (Fig. 12B). We verified the ability of tumor-loaded DCs to induce Th1 immune responses through measurement of DTH responses (Fig. 12C) and via ELISPOT measuring cytokine secretion *ex vivo* in response to antigen-specific stimulation (Fig. 13A). Finally, we examined the ability of tumor-loaded DC1s to stimulate antitumor immunity compared to tumor-loaded non-polarized DCs. We found that only tumor-loaded DC1s were able to inhibit tumor growth (Fig. 13B). Further, H&E and immunofluorescence microscopy examining the tumor infiltrate indicates the presence of $CD4^+$ T cells and activated macrophages, suggesting a DTH-like mechanism is responsible for tumor growth inhibition. Despite the polarized nature of our DCs, they were unable to stimulate detectable $CD8^+$ T-cell responses. To determine whether this lack of $CD8^+$ T cells was due to a lack of class I presentation, we immunized naïve mice with cocultures using the OVA-expressing clones described earlier and

examined the ability of the vaccine to induce OT-I T cell proliferation. Our results demonstrate the lack of $CD8^+$ T-cell responses was not due to a lack of class I presentation (Fig. 14).

5. CHAPTER FIVE

DISCUSSION

Recent advances in our understanding of DC biology and increasing evidence that DC vaccines can induce tumor-specific immune responses in cancer patients are leading to renewed optimism for the development of therapeutic DC-based cancer vaccines [157, 158, 207]. In particular, DC-based vaccines are attracting much attention due to their increased ability to induce clinical responses in patients with melanoma compared to peptide-, viral vector-, and tumor cell-based vaccines [207]. Nevertheless, DC-based immunotherapies have shown only a limited success in human clinical trials. These negative results might be attributed to the use of immature/intermediate mature DCs able to maintain self tolerance during the steady state, or to the use of non-polarized DCs unable to induce potent cellular immune responses.

Our increasing understanding of the mechanisms underlying immune regulation and deviation in tumor-bearing hosts is providing rationale for a new generation of adoptive transfer and *in vivo* targeted DC vaccines. While immunization strategies that target and manipulate DCs *in vivo* offer considerable theoretical advantages over adoptive transfer therapies, currently, *ex vivo* manipulation of DCs enables better monitoring and control of DC function. *Ex vivo* engineered DC vaccines potentially offer both increasing therapeutic benefit and a unique means to develop a better understanding of human immunoregulatory mechanisms, including those in cancer patients. Information obtained from the clinical application of adoptive transfer DC therapies is uniquely contributing to the development of *in vivo* targeted DC vaccines and other evolving immunotherapies.

As an example of this, *ex vivo* engineered DC vaccines now in clinical trials are testing the hypothesis that more efficacious antitumor immunity may be obtained by inducing Th1-skewed antitumor immunity. Several lines of evidence support this approach. In cancer patients, Th1-skewing has been correlated with improved clinical outcomes [162, 227]. Substantial evidence from murine models and human preclinical studies suggest that Th1-skewed $CD4^+$ T cells support potent $CD8^+$ T-cell responses important for tumor and viral immunity [9, 200, 201]. In addition, both historical evidence and several very recent studies suggest that effector mechanisms other than $CD8^+$ T cells can play an important role in tumor regression [179, 212, 220]. To induce Th1-skewed immunity, human DCs have been engineered *ex vivo* using cytokine cocktails and/or TLR ligands to express high levels of cell surface molecules associated with T-cell activation, and to secrete high levels of IL-12p70, an indicator of Th1-skewing function [6]. These human DC1s have been shown to stimulate both $CD4^+$ T-cell responses dominated by IFN-γ secretion and potent $CD8^+$ T cell inducing activity *in vitro* [61, 125]. Surrogate markers of DC1 function can readily be monitored *ex vivo* before DCs are injected into cancer patients, enabling future correlations with results from immune monitoring and clinical outcomes.

The development of DC1 therapies is currently limited by the lack of a representative murine model. Much of the effort to develop and characterize DC1s to date has focused on *ex vivo* manipulated human DCs. Because of differences between human and murine DCs, such as differences in subset characteristics and TLR expression between subsets, extrapolating DC1-driving protocols designed to polarize human DCs to murine bone marrow derived DCs has not

been straightforward. Here we present a murine model of *ex vivo* engineered DC1s developed by monitoring the same indicators used as surrogates for human DC1 function.

Mechanisms underlying the maturation and polarization of DCs have been the focus of several studies, lending to the development of vaccines capable of inducing polarized immune responses [81, 87, 124-126]. Very recent work has focused on the synergy seen between Toll-like receptors and the ability of that synergy to result in high level IL-12p70 secretion by DCs [81, 86, 87]. Our results confirm the secretion of IL-12p70 at high levels by DCs following synergistic TLR ligation. We found that a DC1-polarizing cocktail that included poly[I:C], CpGs, and IFN-γ induced both phenotypic maturation and IL-12p70 secretion consistent with that observed with polarized human monocyte derived DC1s. Importantly, these polarized DC1s, when loaded with antigens and injected intradermally/subcutaneously, induced potent antigen-specific $CD4^+$ T-cell immunity characterized by predominant IFN-γ secretion, further supporting the functional equivalence of these cells to human DC1s. Although the mechanism of synergy we observe remains unclear, very recent studies show synergy between TRIF-coupled TLRs (TLR3 and TLR4) and endosomal TLRs (TLR7, TLR8, and TLR9) in induction of IL-12p70 in human dendritic cells and murine macrophages and synergistic induction of IL-12p70 by IFNs and TLR ligation in murine dendritic cells [81, 86, 87, 228]. TRIF-coupled TLRs are known to stimulate secretion of IFN-β. However, Napolitani et al. concluded that IFN- β played only a minor role in the synergism seen between TLR3 or TLR4 with TLR8 [81]. IL-12p35 and IL-23p19 mRNA levels were also greatly increased and transcription sustained following synergistic TLR ligation, suggesting TLR synergism can result in enhanced secretion of the Th1-driving cytokine IL-23 as well as IL-12p70 [81]. The authors also demonstrate an increased

expression of Delta-4 and decreased expression of Jagged-1 in response to TLR synergy in human DCs, supporting a shift from a Th2-stimulating capacity to a Th1-stimulating capacity [81]. Consistent with previous data in the field, IFN-γ and CD40L were also able to boost the secretion of IL-12p70 by human DCs. However, the authors concluded that the level of enhancement was ultimately dependent on the combination of TLR signals received by the DCs [81]. The enhanced Th1-polarization capacity of DCs stimulated by synergistic TLR ligation appeared to be due to the enhanced expression of only a few genes and highlights the importance of these genes in the induction of potent Th1 immunity. Consistent with our data, the synergy seen appears to be dependent upon TLRs located in endosomal compartments, suggesting that the generation of DCs secreting high levels of Th1-driving cytokines is restricted to those cells that are likely to have acquired microbial antigens [81].

IFN-α secreted by CpG-stimulated plasmacytoid DCs (pDCs) is known to stimulate IL-12p70 secretion by myeloid DCs in human systems, representing an important mechanism for CpGs to affect myeloid polarization indirectly since TLR9 is restricted to pDCs in humans [127] while it is expressed in both lymphoid and myeloid DCs in the murine system [68]. Additionally, recent literature demonstrates increased secretion of type I interferons (IFNs) and TNF-α by cultured murine macrophages in response to stimulation with CpG and poly[I:C] [87]. While our model is different in that we are using DCs instead of macrophages and pDCs are not detectable in our bone marrow derived DCs (data not shown), we cannot rule out the possibility that these cytokines are mediating the synergy seen in our system. Examination of the expression of these genes and others in murine DCs following synergistic TLR signaling is a future target for investigation.

A very recent paper suggests DCs grown in GM + IL-15 are better inducers of Th1 immunity than DCs grown in GM alone or GM + IL-4 [105]. The authors propose the ability of GM + IL-15 DCs to stimulate Th1 immunity was due to their ability to secrete IFN-γ. However, the authors clearly state that none of the DC populations tested secreted IL-12p70 or IFN-γ unless they were stimulated with TLR3L or TLR4L. Nonetheless, in the absence of TLRL stimulation, GM + IL-15 DCs were capable of inducing strongly polarized Th1 immune responses compared to GM + IL-4 DCs [105]. This is not inconsistent with our data which demonstrate non-polarized GM + IL-4 DCs stimulate primarily a mixed/Th2 response in the absence of strong polarization signals (Fig. 4 and Fig. 13). Importantly, the authors demonstrate that GM + IL-4 DCs are far superior at stimulating T cell proliferation compared to GM or GM + IL-15 DCs [105], suggesting that even though non-polarized GM + IL-15 DCs may be better at stimulating Th1 immunity than non-polarized GM + IL-4 DCs, GM + IL-4 DCs that have been polarized with synergistic TLR ligation may be far better at stimulating robust Th1 immune responses. Importantly, our data demonstrate the ability of IFN-γ to increase the secretion of the Th1-driving cytokine IL-12p70 while suppressing the secretion of IL-10 (Fig. 2). Perhaps the use of GM + IL-15 DCs that have been stimulated with synergistic TLR ligation will secrete IFN-γ, obviating the need to include this cytokine in the DC1 cocktail. It is possible that murine DCs secrete IFN-γ in response to certain TLRs or TLR combinations and contributes to the DC1-inducing properties. However, DCs stimulated with CpG, poly[I:C], and IFN-γ failed to secrete IFN-γ, as demonstrated by the lack of IFN-γ in OT-I assay supernatants when DCs were loaded with B16 cells not expressing OVA (Fig. 11). Our DC1s were able to stimulate robust Th1-mediated immune responses (Fig. 4 and Fig. 13), suggesting the ability of DCs to induce a Th1

phenotype in naïve T cells may be independent of their ability to secrete IFN-γ in our system. There may be multiple mechanisms by which TLR synergy can result in the enhanced Th1-stimulating capacity of DCs. Elucidating the mechanisms by which TLR synergy induces a DC1 phenotype will prove valuable in the further understanding of DC biology and the Th1/Th2 dichotomy.

Much of the concern surrounding polarization of DCs has centered on DC exhaustion, a state in which DCs have been presumed to be unable to secrete IL-12p70. In *ex vivo* studies, human monocyte derived DC1s have been shown to secrete high levels of IL-12p70 over a narrow window of time, peaking 8-12h after stimulation. Secretion then returns to baseline levels, a phenomenon referred to as "exhaustion" [125, 130, 229]. Previous reports suggest that the capacity of DC1s to prime Th1 immunity is lost following exhaustion, resulting in the generation of Th2-skewed immunity [130]. This imposes a theoretical limit on the use of DC1s for immunization, i.e., there is concern that IL-12p70 secretion may exhaust before injected DC1s engage T cells in the relevant lymphatic tissues. Several lines of evidence are in conflict with this theory. First, while IL-12p70 is known to enhance and drive Th1-mediated immune responses, there is clear evidence that DCs incapable of secreting IL-12p70 are able to stimulate Th1-mediated immunity, albeit to a lesser extent to their IL-12p70-secreting counterparts [100]. Second, experiments using human DCs have demonstrated a sustained capacity to secrete high levels of IL-12p70 upon CD40 ligation [125]. These DCs were treated for 48 hours with a cocktail containing the TLR3 ligand, poly[I:C] [125]. Finally, like human DCs, polarized murine DC1s used in this study secret high-levels of IL-12p70 early in response to TLR ligation, with exhaustion occurring after 12h. Interestingly, we found that engagement of CD40 before

exhaustion leads to extended high level IL-12p70 secretion, suggesting that DC1s will continue to secrete a high level of IL-12p70 if they encounter antigen-specific T cells, further promoting Th1 responses. Together, these data challenge the traditional view of DC exhaustion. Rather than switch from Th1-inducing cells to Th2 inducing cells following exhaustion, DCs appear to be capable of retaining the Th1-stimulating capacity, at least under certain conditions. Moreover, while IL-12p70 is useful as a surrogate marker for the Th1-skewing ability of DCs, there appears to be other factors involved that are capable of inducing Th1 immunity despite the lack of IL-12p70 secretion [100]. Relying strictly on the IL-12p70 secreting properties of DCs is ultimately insufficient for the determination of DC polarization or for the evaluation of their Th1-skewing capacities.

The Th1-promoting properties of IL-12p70 are well-established and are likely beneficial for the induction of potent antitumor immunity. Therefore, we sought to design our vaccination strategy in such a way to allow for durable high-level IL-12p70 secretion by DCs during their interaction with antigen-specific T cells. Given the transit times reported for the trafficking of skin injected DCs to the draining lymph nodes in murine and human systems, it is plausible that cutaneously administered DCs will engage CD40L expressing T cells before exhaustion [230, 231]. In addition, direct intranodal injection of DCs would likely enable even more timely interaction with resident T cells, obviating exhaustion concerns. In regard to this issue, we evaluated the kinetics of IL-12p70 secretion in response to the duration of TLR ligand stimulation. Our results suggest that a 4h exposure to TLR ligands results in nearly optimal IL-12p70 secretion. This implies that injection after a 4h *ex vivo* stimulation period will optimize the balance between maximal stimulation of IL-12p70 secretion and the likelihood of DC1–T cell interaction before

exhaustion. Finally, the levels of IL-12p70 secretion we observe are very high and likely super-physiologic. This potentially enables targeted cytokine therapy whereby polarized DC1s serve first as targeted delivery vehicles and then as a lymph node resident source of IL-12p70. High level secreted IL-12p70 may affect surrounding responding T cells in addition to those directly engaging the polarized DC1, enabling amplification of Th1 skewing by resident DCs that may be presenting or cross-presenting tumor antigens without having been directly exposed to polarizing stimuli.

Ultimately, the definition of type 1-polarized dendritic cells lies with their ability to elicit Th1-mediated immune responses. Toll-like receptor synergy has been described to induce DC1 polarization and subsequent Th1 skewing *in vitro* [81]. To our knowledge we are the first to describe the use of *ex vivo*-manipulated DCs to induce antigen-specific Th1 skewing *in vivo* following synergistic TLR ligation (Fig. 4). The ability to specifically direct polarized immune responses represents an important step toward the development of more effective vaccines. Importantly, DC1 polarization models have proceeded in human clinical trials in the absence of an equivalent murine DC1 model. Here we have used ELISPOT and ELISA to detect Th1 skewing following immunization, methods also used to evaluate vaccine efficacy and Th1 skewing in human systems. While DCs between murine and human models are intrinsically different, especially concerning Toll-like receptor expression, we have demonstrated the ability of our murine DC1 immunization strategy to elicit polarized immune responses comparable to those seen in human systems.

The capacity of dendritic cells to efficiently process and present exogenous antigens on MHC class I and class II molecules has allowed for a wide variety of antigen loading strategies. The use of single proteins in tumor vaccines has enjoyed some success, allowing for the stimulation of tumor-specific T cells and reducing the possibility of inducing autoimmune responses [208]. Single protein or single epitope vaccines are limited in that they stimulate a narrow population of T cells and their use increases the chance of immune escape by antigen-loss variants. Moreover, every tumor holds a unique set of mutations that give rise to tumor antigens, rendering single antigen approaches useful in only a limited patient population. While it is possible to identify tumor antigens on a patient-to-patient basis, the process is costly and ultimately inefficient for clinical settings. One way to circumvent issues using single antigens is the use of whole tumor cells as a source of antigen. Vaccines using the entirety of the tumor cell content to load DCs have the potential for eliciting broad populations of T cells against multiple antigens. Importantly, using whole tumor cells as a source of antigen for DC-based vaccines leads to the presentation of MHC/peptide complexes unique to that tumor/MHC combination, directing immune responses toward relevant epitopes.

We compared three antigen loading strategies commonly used in DC-based vaccines; coculture of DCs with live tumor cells, feeding DCs with tumor lysate, and fusion of DCs with tumor cells to form dendritomas. All three methods resulted in the efficient transfer of tumor-derived antigens to DCs as determined by flow cytometry, fluorescence microscopy, and confocal microscopy. Live cell imaging revealed that DCs are capable of acquiring antigens from live tumor cells during coculture. Importantly, antigen transfer was not limited to one tumor type,

but was seen with a variety of murine and human tumors, suggesting these methods of antigen loading may be applicable to a wide variety of tumors.

We next evaluated differences in antigen presentation using chicken egg ovalbumin (OVA) as a model antigen. We compared the ability of coculture, lysate, and fusion in their ability to induce the presentation of cytosolic and membrane-bound forms of OVA in OVA-expressing B16 clones. Previous genetic immunization studies using DNA encoding cytosolic or membrane-bound forms of antigen demonstrated that intramuscular injections of membrane-bound, but not cytosolic antigen, resulted in the induction of CTL activity [232, 233]. However, intradermal genetic immunizations resulted in effective CTL induction for both cytosolic and membrane-bound antigens [232, 233]. The authors concluded that cytosolic antigen expression following intramuscular injections is primarily in muscle cells due to the paucity of DCs in muscle tissues and may be invisible to the immune system, resulting in the absence of detectable CTLs. However, the density of DCs in the skin is high, greatly increasing the possibility that genetic immunizations may result in the direct transfection of DCs, obviating the necessity of cross-priming. Similarly, injection if viable cytosolic OVA-expressing cells resulted in consistently lower levels of CTL induction than seen with injection of viable membrane-bound or secreted forms of OVA [211]. Together, the data suggest that cell-associated cytosolic antigens are less efficient for cross-presentation than membrane-bound antigens *in vivo*. However, when cell lysates or cellular fractions containing the antigens were injected into naïve mice, both cytosolic and membrane-bound forms of the antigen were equally capable of eliciting CTL responses [211]. In our system, cellular location of the antigen had little effect on the resulting presentation of tumor-derived OVA via class I or class II antigen processing pathways *in vitro*. The

differences seen between our model and previous models may be attributed to different factors. First, clonal populations of antigen-expressing cells were cultured *in vitro* with purified dendritic cells, increasing the opportunity for cross-priming. Second, tumor cells in our experiments were treated by psoralen-UVA irradiation that may have induced stress responses, increasing the cross-priming ability of these cells. And third, the dendritic cells in our experiments were treated with DC1 cocktail which has been shown to enhance the cross-presentation ability in these cells.

The method of antigen transfer to DCs greatly affected the ability of DCs to present OVA-derived peptides. We measured OT-I and OT-II activation by antigen-loaded polarized DCs by secretion of IL-2 and IFN-γ, although IFN-γ appeared to be a more sensitive indicator of T-cell activation in our system likely due to the consumption of IL-2 by activated T cells. In our model, tumor lysates appeared far inferior at inducing functional presentation to antigen-specific T cells despite their ability to deliver high levels of tumor-derived antigens to DCs. Interstingly, reports on the efficacy of lysates to induce effective T cell immunity are varied in the literature. For example, Strome et al. compared the ability of DCs pulsed with tumor lysate or cultured with irradiated tumor cells (both using OVA-expressing tumors) to stimulate MHC class I-restricted OT-I T cells. The authors found that DCs cultured with irradiated tumor cells, but not those pulsed with tumor lysate, were capable of stimulating $CD8^+$ T cells and inhibiting tumor growth in protection and therapy models [234]. Moreover, the inability of lysate-fed DCs to stimulate class I-restricted T cell clones has also been demonstrated in human models [186, 234]. Similarly, immunization of mice by direct injection of apoptotic or necrotic tumor cells, DCs loaded with apoptotic or necrotic tumor cells, demonstrated that only apoptotic cells or DCs cultured with apoptotic cells were unable to protect mice from tumor challenge [27, 193]. In

contrast, others have reported that lysate-pulsed DCs are capable of stimulating both memory T cell responses in human systems [184] and protective antitumor responses in mice [183, 189, 191]. There are several factors that may account for the differences in the effectiveness of tumor lysates seen in the literature. First, different tumors may contain varying levels of inhibitory factors, such as IL-10. Second, while levels of heat shock proteins are known to be greater in tumors and cells lines than seen in normal primary cells [185], their expression levels may vary between tumor models, potentially affecting the cross-presentation of antigens. Third, the methods used to generate tumor lysates are varied, including using whole tumor freeze-thaw lysate [27, 189, 193, 234], feeding of DCs with whole freeze-thaw lysate followed by γ-irradiation [183], freeze-thaw lysates that have been filtered or fractionated [184, 192], and hypotonic shock [186]. In addition, both primary necrosis, such as that generated by freeze-thaw lysis, and secondary necrosis, which occurs in the late stages of apoptosis, are both referred to as "necrosis" in the literature, yet represent two distinct pathways of cell death that may be differentially processed by the immune system [235]. Finally, the methods used to evaluate antigen presentation following antigen loading range from *in vitro* to *in vivo*, proliferation to cytokine secretion, and CTL assays to tumor protection. There is clearly a lack of consistency on many levels that can contribute to the differences seen between studies. More extensive studies need to be performed to evaluate the functional relevance of these differences. Our experiments only examined the relative level of antigen presentation on lysate-pulsed DCs compared to DCs loaded by coculture or fusion. The relative capacity of lysate-pulsed DCs to prime immune responses *in vivo* was not tested in our model. While the possibility remains that lysate-pulsed DCs may be capable of eliciting detectable antitumor immune responses, similar data shown by Strome et al. [234] suggest lysates in our system will be inefficient at priming antitumor

immunity. However, *in vivo* experiments need to be performed to verify or refute this hypothesis.

While the frequency of observable DC/tumor cell fusions was relatively low, the ability of these cells to stimulate T cell responses was very high in our model, suggesting these cells have an enhanced capacity to present tumor-derived peptides. Coculture demonstrated a relatively high capacity to stimulate OT-I and OT-II T cells although not to the extent seen with fusion. While increased levels of presentation are believed to be correlated with an increased ability to stimulate immunity, to our knowledge no experimental data has thus far supported that conclusion. It is reasonable to conclude that an increased number of specific MHC/peptide complexes on the surface of a DC may lead to a higher avidity for antigen-specific T cells, increasing the likelihood of a stable and productive interaction. However, previous work in our lab has demonstrated the ability of fusion and coculture to elicit equally effective antitumor immunity [188]. Importantly, noticeable levels of cell death was observed in our fusion preparations and may have contributed to the efficacy of the antigen loading strategy, possibly through the induction of high levels of heat shock proteins in stressed tumor and dendritic cells or through the induction of apoptosis, which has been demonstrated to be effective at delivering tumor antigens to dendritic cells for class I and class II presentation [27, 186, 189, 191, 192, 234, 236-238].

The primary purpose of this study is to examine a therapeutic approach combining an antigen loading method with a DC polarization signal. Many current DC-based vaccines are administered following irradiation of the total vaccine, possibly hindering the ability of the DC

to function properly. In order to ensure we utilized DCs with the full capacity to secrete IL-12p70, we compared the effect of antigen loading on the ability of DCs to secrete IL-12p70 following stimulation with DC1 cocktail. While fusion resulted in very high antigen presentation, the capacity for IL-12p70 secretion by DCs following the fusion process was greatly inhibited. Conversely, coculture of DCs with tumor cells resulted in intermediate/high levels of antigen presentation and maintained the full IL-12p70-secreting capability of DCs in response to DC1 cocktail. The mechanisms underlying the impaired IL-12p70 secretion by DCs following fusion are unknown. While fusion resulted in some cell death, equal numbers of live cells were used for DC1 cocktail stimulation, eliminating the possibility that the decreased secretion was due to a lower number of viable cells. Moreover, DC/tumor fusion is an inefficient process. Many of the remaining DCs are unfused, suggesting the suppression was not due to tumor-induced mechanisms following fusion. However, there is a distinct possibility that the decreased IL-12p70 secretion may be due to diminished DC health or possibly a direct effect of polyethylene glycol (PEG) on the DCs. Based on our results, we decided the optimal method of antigen loading for immunotherapy in our system was coculture of live tumor cells with DCs. Experiments examining the effect of PEG on DCs alone would give insight into the effect of this chemical on the abilities of DCs to respond to TLR ligation and could have implications on the development of PEG fusion-based vaccine strategies.

To evaluate tumor immunotherapy, we chose the B16 melanoma, a tumor with multiple well-established mechanisms of tolerance induction and immune escape [150, 155, 223]. We have previously shown that DCs loaded with tumor antigens by coculture with live tumor cells, even without polarization, can induce effective therapeutic tumor immunity against multiple model

tumors [188]. Here we sought to evaluate the capacity of this general strategy, combined with DC polarization, to induce effective immunity against a tumor shown to possess many of the immune evading mechanisms described for human cancers [10-13, 150, 155, 223]. In initial experiments, we established that exposure of DCs to B16 tumors in culture does not inhibit DC maturation or IL-12p70 secretion. Furthermore, these DC1s were capable of inducing DTH responses when injected *in vivo*. In our model, tumor growth was slowed significantly by the DC1/melanoma vaccine, and the anti-tumor immune response was characterized by tumor-specific IFN-γ producing T cells and brisk tumor infiltrates containing $CD4^+$ T cells and macrophages. We found no evidence of $CD8^+$ T-cell immunity, neither by traditional ^{51}Cr release assays nor by localization of $CD8^+$ T cells in tumor infiltrates. This is consistent with recent results demonstrating partial tumor inhibition in a B16 protection model, without evidence of $CD8^+$ T cells in ^{51}Cr release assays [10]. Previous data from our lab [188] as well as OT-I T-cell proliferation experiments evaluating cross-presentation of tumor-derived antigens in this model (Fig. 14) suggest the absence of a CTL response is not due to a lack of class I presentation. These and other studies suggest that induction of more efficacious B16 melanoma rejection and potent $CD8^+$ T-cell responses may require elimination of naturally occurring $CD4^+CD25^+$ regulatory T cells [10, 14]. The need to deplete regulatory T cells, or to systemically administer adjuvants and/or CD40 ligands to overcome regulatory T-cell mediated suppression has been demonstrated in several tumor systems [9-14, 138]. There are two primary types of Tregs. The first are naturally occurring Treg cells. These cells have been demonstrated to be necessary for the inhibition of autoimmune disorders. Naturally occurring Tregs originate in the thymus and express high levels of the IL-2R α-chain, CD25 [210, 239-241]. They are distinguished by their expression of Foxp3 and their survival and proliferation is dependent on

CD28 stimulation [210, 239, 240]. Importantly, naturally occurring Tregs are believed to be specific for antigens they were exposed to in their thymic development [241]. The exact mechanism by which these Tregs inhibit immune responses in the periphery isn't entirely known. In one model, naturally occurring Tregs compete directly with other T cells for self antigen, limiting the self-reactive responses. In other models, Tregs are believed to have a higher affinity for self antigens than effector T cell populations and are able to respond more quickly to stimulation, subsequently inhibiting other "bystander" T cells that recognize self antigens in the same tissue through the secretion of suppressive cytokines such as IL-10 and TGF-β [241]. The second type of Tregs is IL-10 secreting Tregs or "adaptive" Tregs. These cells are antigen-inducible and believed to be generated from fully mature T cells in the periphery as a result of insufficient antigenic stimulation. These Tregs do not express Foxp3, express intermediate levels of CD25, and appear to survive independent of CD28 stimulation [210, 239]. The exact mechanisms by which Tregs are able to exert immune suppression are not entirely clear. However, both subsets appear to have contact-dependent mechanisms of suppression (possibly through membrane-bound TGF-β as well as through expression of CTLA-4 and GITR) and contact-independent mechanisms (such as the secretion of IL-10 and TGF- β) [210, 239-241]. *In vitro* studies have demonstrated that adaptive Tregs can be generated through antigen stimulation in the presence of IL-10 [210]. Importantly, IL-10 is known to be secreted by both murine and human tumors [140-145], suggesting that tumors may be inducing the generation of tumor-specific Tregs. In addition, IL-10 can induce the generation of tolerogenic DCs, potentially aiding in Treg stimulation [147, 148]. The mechanisms described above suggest that the lack of CD8-mediated antitumor activity may be due to the activity of either or both types of regulatory T cells, possibly through the immunosuppressive activity of IL-10 and the generation of

tolerogenic DCs. These DCs would likely to be presenting tumor antigens from the tumor site, subsequently stimulating the production of tumor-specific adaptive Tregs. Notably, we observed suppression in the $CD8^+$ T cell compartment, but not in $CD4^+$ T cells. This difference in suppression may be due to differences between T cell subsets in their susceptibility to Treg-mediated suppression or possibly their responsiveness to activated DCs. However other possibilities also exist that may explain the presence of $CD4^+$-mediated responses and the lack of $CD8^+$ T-cell immunity. First, tumor cells are more likely to induce the anergy or depletion of $CD8^+$ T cells through presentation of tumor-derived peptides on MHC class I molecules in the absence of proper costimulation. $CD4^+$ T cells are immune to this direct depletion due to the lack of MHC class II molecules on the tumor cells. Interestingly, recent studies indicate B7-H1 expression in tumor cells is capable of inducing apoptosis in $CD8^+$ T cell in human and murine models [242]. Expression of B7-H1 on the surface of tumor cells has been demonstrated to increase following exposure to IFN-γ [242]. Notably, $CD4^+$ T cells may be more resistant to this type of inhibition due to a higher production of IL-2 by these cells [243]. Due to the lack of MHC class II expression on tumor cells, $CD4^+$ T cells cannot directly interact with tumors. Instead they recognize peptide/MHC complexes found on APCs. Tumors in our model demonstrated moderate DC infiltration (data not shown). It is possible that antigen-bearing DCs are stimulating IFN-γ secretion by tumor-specific $CD4^+$ T cells, resulting in the upregulation of B7-H1 expression in tumor cells. Other tumor-associated factors, such as FasL, may also have a role in selectively depleting $CD8^+$ T cells but not $CD4^+$ T cells, again due to the lack of MHC class II on tumor cells. Further investigation is required to support or refute these possibilities.

Recent studies demonstrate that activated $CD4^+$ T cells and macrophages can inhibit tumor growth in the absence of $CD8^+$ T-cell responses, and that this immunity depends in large part on IFN-γ production [179, 220, 221]. In our studies, inhibition of tumor growth is associated with IFN-γ production by antigen-specific $CD4^+$ T cells and tumor infiltrates dominated by $CD4^+$ T cells and activated macrophages. Although the DC1/melanoma vaccine we describe did not appear capable of overcoming suppressive/evasive effects of B16 tumors, as evidenced by only partial tumor growth inhibition and the lack of tumor-specific $CD8^+$ effector T cells, the vaccine did stimulate Th1-skewed tumor-specific $CD4^+$ T-cell immunity and corresponding IFN-γ secretion that likely contributed to macrophage activation. The importance of these DC1 inducible tumor control mechanisms may be underappreciated. These studies suggest that non-$CD8^+$ T cell-dependent Th1-skewed immunity can significantly contribute to tumor therapy, and that measurement of these responses should be included in clinical immune monitoring. A rational approach to the further development of DC1-based tumor vaccines should include strategies to maintain the benefits of Th1-skewed tumor immunity observed here and add enhancements designed to improve stimulation of tumor-specific $CD8^+$ T cells in the setting of immunosuppression or be combined with strategies to the eliminate regulatory T-cell activity. The murine polarized DC1 model we describe may provide a useful tool to achieve these goals.

6. CHAPTER SIX

6.1. Materials and methods

6.1.1. Mice and cell lines

Female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the Central Animal Facility at the University of Pittsburgh and used according to institutional guidelines. B16 is a C57BL/6-derived murine melanoma obtained from American Type Culture Collection (ATCC, Rockville, MD). The human melanoma line, Colo38, and the murine 3-methylcholanthrene-induced sarcoma of BALB/c origin, MethA, were kindly provided by Dr. Walter Storkus, University of Pittsburgh. The CD40L-transfected cell line J558 was a generous gift from Dr. Pawel Kalinski, University of Pittsburgh. 293T cells were purchased from ATCC. OT-I cells were obtained from OT-I transgenic mice (Jackson Laboratories) and recognize the class I-restricted chicken egg ovalbumin-derived peptide SIINFEKL when presented by H-2K^b molecules. OT-II T cells were obtained from OT-II Rag-/- transgenic mice (Taconic, Germantown, NY) and recognize an I-A^b-presented C-terminal peptide derived from chicken egg ovalbumin, $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) [206].

6.1.2. Reagents

Chicken egg ovalbumin (OVA) (Sigma Chemical Co., St. Louis, MO) was dissolved in AIM-V medium (GIBCO, Carlsbad, CA) at 30 mg/ml and sterile filtered. Cytosolic and membrane-bound OVA constructs are previously described [177, 211]. CpG is phosphorothioate CpG-ODN 1668 with sequence 5'-tccatgacgttcctgatgct-3' (Sigma Genosys, The Woodlands, TX),

which was previously described to activate murine immune cells [72]. Poly[I:C] (Sigma) is a double-stranded RNA mimic known to stimulate maturation in both murine and human DCs. IFN-γ (PeproTech, Rock Hill, NJ) was used at a concentration of 20 μg/ml. LPS (Sigma) is used at a concentration of 1 μg/ml to induce maturation of DCs.

6.1.3. Preparation of DCs

DCs were generated from bone marrow as previously described [188]. Briefly, bone marrow cells were depleted of RBC and lymphocytes and cultured for 5 days in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS, L-glutamine, 2-ME, HEPES, and antibiotics and containing 10^3 U/ml of GM-CSF and IL-4 (DC medium). 75% of cell cytokine supplemented culture medium was replaced every other day and loosely adherent cells were collected on day 5. $CD11c^+$ cells expressed MHC class I, MHC class II, CD40, CD80, and CD86 but did not express B220 as determined by flow cytometry (data not shown).

6.1.4. Phenotypic analysis and cytokine production by DCs

Day 5 DCs were purified by immunomagnetic bead cell sorting using anti-CD11c-conjugated magnetic beads (purity >80% as demonstrated by flow cytometry analysis) (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified DCs were suspended in DC medium at a concentration of $5x10^5$ cells/ml and cultured 18h with or without LPS or a combination of CpGs (1μM), poly[I:C] (20 μg/ml), and/or IFN-γ (20 ng/ml). Staining was performed using PE-conjugated anti-CD11c antibody and FITC-conjugated anti-$I\text{-}A^b$, -CD40, or -CD86 antibodies (all from Pharmingen, San Diego, CA) and cells were analyzed by flow cytometry. Marker expression was assessed on gated $CD11c^+$ cells. Cytokine measurement was determined by ELISAs

(Pharmingen) according to manufacturer protocols. Plates were developed with TMB substrate (Sigma) and absorbance read using a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA). In some experiments B16 tumor cells were co-cultured with DCs at a DC: B16 cell ratio of 3:1. In some experiments J558 cells were added at 5h or at 24h at a DC:J558 ratio of 1:2.5 and supernatants collected as described.

To determine the effect of the length of stimulation with DC1 cocktail (1μM CpGs, 20 μg/ml poly[I:C], and 20 ng/ml IFN-γ) DCs were suspended in DC medium with DC1 cocktail (DC1 medium) at a concentration of 10^6 cells/ml. Cells were maintained in this cocktail or washed after 2h or 3h of stimulation and resuspended in DC medium. At 4h, all samples were washed and resuspended in DC medium (2h, 3h, and 4h stimulation groups) or DC1 medium (8h stimulation group) and incubated for 4 additional hours. Supernatants, containing cytokines secreted between 4h and 8h, were then collected and frozen for ELISAs. For controls, cells were maintained in DC medium or DC1 medium for 18h before collecting supernatants for ELISAs.

6.1.5. Antigen transfer

Day 5 DCs were loaded with tumor antigen using coculture, lysate, and fusion. DC:tumor ratios for all loading methods was 3:1. For flow cytometry and microscopy, tumor cells were labeled with CFSE or CMFDS (Molecular Probes, Eugene, OR). For cocultures, DCs were cultured overnight with tumor cells, stained with PE-conjugated anti-CD11c antibodies, and fixed with paraformaldehyde. Labeled tumor cells were brought to 10^6 cells/50 μl in RPMI and lysates were generated by 3-5 rounds of freeze/thaw using liquid nitrogen. DCs were suspended at $3x10^6$ cells/100 μl and incubated with lysate at a DC:tumor cell equivalent ratio of 3:1 for 2

hours. RPMI was added to the cultures to create a final DC concentration of $3x10^6$ DCs/mL for overnight culture. Lysate-fed DCs were washed and stained with PE-conjugated anti-CD11c antibodies and fixed with paraformaldehyde. Fusions were performed as previously described [188]. Briefly, DCs and tumor cells were pelleted and 1 mL of 50% PEG (Sigma) added drop-wise over 45 seconds. Cells were allowed to incubate for an additional 45 seconds before gradual dilution with PBS. Fused cells were gently pelleted and washed with PBS. Fusions were stained with PE-conjugated anti-CD11c antibodies and fixed using paraformaldehyde. Samples were analyzed by flow cytometry and fluorescence microscopy to determine antigen transfer. In some experiments, antigen loading was performed as before using CFSE-labeled tumor cells. Following antigen transfer, cells were labeled with LysoTracker Red (Molecular Probes). Coculture and lysate groups were then stained with anti-CD11c (Pharmingen) and a Cy5-conjugated 2º antibody (Molecular Probes) and then fixed with paraformaldfehyde. Fusions were stained with LysoTracker Red and then fixed with paraformaldehyde prior to staining for CD11c. Samples were then analyzed using confocal microscopy.

6.1.6. Live cell imaging

CFSE-labeled B16 tumor cells were allowed to adhere to a coverslip and placed in a chamber for live cell microscopy. Bead-purified day 5 DCs were labeled with CMTMR (Molecular Probes) and pumped into the chamber and allowed to coculture with the tumor cells. Images of random fields were taken every two minutes to generate live cell movies which were then analyzed by the Center for Biological Imaging facility at the University of Pittsburgh. Fluorescence and DIC images were used to generate a composite demonstrating antigen delivery to DCs from live tumor cells.

6.1.7. Generation of OVA-expressing clones

Verification of cytosolic or membrane expression using OVA-489 and OVA-TR489 constructs was performed using 293T cells. Briefly, 293T cells were transfected with either OVA-489 or OVA-TR489 using Lipofectamine 2000 (Gibco, Carlsbad, CA) and incubated for 24 hours. Cells were then fixed and stained with or without permeabilization using the 1º antibody mouse anti-OVA and the 2º antibody FITC-conjugated goat anti-mouse antibody (both from Sigma). Samples were then washed, suspended in paraformaldehyde, and analyzed by flow cytometry. Lentiviral vectors encoding cytosolic (OVA-138) and membrane-bound (OVA-TR489) forms of OVA were generated using the pLenti6/V5 Directional TOPO Cloning Kit (Invitrogen, Carlsbad, CA). Briefly, OVA DNA was amplified from expression vectors by PCR. For OVA-138 we used the primers 5'-CACCATGGTAGAAAGTCAGACAAATG-3' and 5'-AAGCCATAG AGCCCACCGCA-3'. For OVA-TR489 we used the primers 5'-CACCATGATGGATCAAGT CAGAT-3' and 5'- AAGCCATAGAGCCCACCGCA -3'. Bands were cut from gels and purified using a QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA) and ligated into the TOPO cloning kit expression plasmid. Bacteria were transformed using the plasmids and colonies selected for genetic confirmation using PCR and the primers above. Selected colonies were grown and plasmid DNA purified using a Miniprep Kit (Qiagen). Purified DNA plasmids were used to generate OVA-encoding replication-deficient lentiviruses per kit protocols. To test the accuracy of lentiviral vectors, 293T cells were infected with the viruses and placed in selection with media containing 10 μg/mL of blasticidin (Invitrogen) for 7 days. Cells were then solubilized and a western blot performed to detect OVA expression in these cells. OVA bands were detected using rabbit anti-OVA (1º, 1:2000 dilution) and HRP-conjugated goat anti-rabbit

(2º, 1:10,000 dilution) antibodies (both from ICN Laboratories, Irvine, CA) and developed using Sigma Fast BCIP/NBT tablets (Sigma). The viral constructs were then used to infect B16 tumor cells followed by selection and cloning in medium containing 10 μg/mL of blasticidin. mRNA was isolated from clones using RNAwiz (Ambion, Austin, TX) and tested for expression using an Access RT-PCR Kit (Promega, Madison, WI).

6.1.8. OVA-based assays

Coculture, lysate, and fusion were performed as before using OVA-expressing and non-expressing B16 clones. DCs were then purified using CD11c magnetic beads and stimulated with DC1 cocktail for 4 hours. Cells were washed thrice in PBS and used to stimulate OT-I or OT-II T cells *in vitro* at a ratio of 1:10 for 3 days. Supernatants were collected and frozen for IL-2 and IFN-γ ELISAs to determine the levels of class I or class II OVA presentation by DCs.

For other experiments, purified DCs were loaded with OVA (1 mg/ml) for 6h in DC medium with DC1 cocktail added after 2h to some groups. The proliferative response of OT-II T cells to DCs was determined by titrating the DCs in duplicate in a 96-well round-bottom plate and OT-II T cells in RPMI were added at a concentration of $5x10^4$ cells per well, with the final volume being 200 μl/well. Thymidine (1 μCi/well) was added on day 2 of incubation and plates harvested on day 3. Proliferation is reported as mean c.p.m. of duplicate microcultures. For cytokine production OT-II T cells and DCs were cultured at a ratio of 10:1 in a 96-well round-bottom plate in 200 μl RPMI. Following 3 days of incubation supernatants were collected and frozen for ELISAs.

For *in vivo* immunizations, DCs were suspended in PBS at a concentration of 10^7 cells/ml. Naïve C57BL/6 mice were immunized by footpad/haunch injections on days 1, 7, and 14 with a total of $3x10^6$ DCs delivered per immunization. Spleens were harvested on day 21 and $CD4^+$ T cells purified with magnetic beads. Splenic APCs from naïve C57BL/6 were loaded with OVA (1 mg/ml) for 2h, washed 3x in PBS, and used as target cells for 24h IFN-γ or 48h IL-5 ELISPOT assays (both plates and antibody sets from Pharmingen) and for 48h ELISA assays (Pharmingen). For both assays, $4x10^4$ APCs/well and $2x10^5$ $CD4^+$ T cells/well were suspended in either 150µl (ELISPOTs) or 200 µl (ELISAs) of AIM-V medium. ELISPOT plates were incubated at 37°C for the indicated time and developed as described in the manufacturer's protocols. For ELISAs, supernatants were collected after 48h of incubation, pooled for each group, and frozen for later analysis.

6.1.9. Measurement of anti-tumor responses *in vivo*

For generation of tumor-loaded DCs, B16 tumor cells were suspended in PBS at a concentration of 10^6 cells/ml and placed in a T-75 culture flask. Cells were treated with 17 µl/ml UVADEX (Therakos, Exton, PA) and irradiated with 4.5 Joules UVA using an Ultralite phototherapy process controller (Ultralite Enterprises, Inc., Lawrenceville, GA) and then washed 3x with PBS and suspended in DC medium. Purified DCs were suspended in DC medium and loaded with tumor antigen by co-culture at a DC:tumor ratio of 3:1.

For ELISPOT assays, $3x10^5$ B16 cells in 50 µl PBS were injected intradermally (i.d.) into the shaved abdomen of naïve female C57BL/6 mice on day 0. Tumor-bearing mice were immunized with $3x10^6$ tumor-loaded DCs by footpad/haunch injections on days 7 and 14.

Spleens were harvested on day 21 and $CD4^+$ T cells purified using magnetic beads and used for ELISPOT assays. B16 were centrifuged and pellets subjected to 3 rounds of freezing and thawing using liquid nitrogen to generate tumor lysate. Naïve splenic APCs were pulsed with B16 lysate at an APC:tumor equivalent ratio of 3:1 for 2h, and washed 3x in PBS for use as target cells.

For DTH responses, naïve female C57BL/6 mice were primed by injecting $5x10^6$ DCs, in 150 µl PBS into the left footpad and haunch. 5 days later DTH responses were elicited by injecting $5x10^6$ DC, tumor-loaded DCs, or tumor-loaded DC1s in 50 µl PBS into the contra lateral footpad, using the same cells to elicit the response that were used to prime the mice. After 48h, footpad thickness was measured using a dial thickness gauge and compared to footpad thickness just prior to elicitation.

6.1.10. Immunotherapy and tumor microscopy

Naïve female C57BL/6 mice were challenged on day 0 with $5x10^4$ B16 cells in 50µl PBS by i.d. injection into the shaved abdomen. Mice were then immunized on day 1 and every 7 days thereafter. Tumor size was measured 3 times weekly using digital display calipers and the tumor area calculated by multiplying the widest diameter of the tumor by the diameter 90° to that measurement. Mice were sacrificed when tumor size reached 20 mm in diameter or mice became moribund.

For microscopy, some mice were sacrificed at day 8 and tumors were excised and fixed in 4% paraformaldehyde in PBS. Samples were processed and frozen sections stained with H&E or

stained with rat antibodies to detect cells expressing F4/80-like receptor (macrophages) (Pharmingen), iNOS/NOS2 (Pharmingen), CD4 (BIODESIGN International, Saco, ME), or CD8 (BIODESIGN International) and visualized using Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Nuclei were visualized by staining with Hoechst stain (Sigma). Staining and imaging was performed by the Center for Biological Imaging facility at the University of Pittsburgh.

6.1.11. Evaluation of class I presentation using OT-I T cells

Day 5 DCs were purified and cocultured for 6 hours or 18 hours with OVA-expressing clones, receiving DC1 cocktail stimulation over the final 4 hours of coculture. Naïve C57BL/6 mice were immunized as before via footpad/haunch immunizations on day 0 or day 1. On day 2, purified OT-I T cells were collected and stained with CFSE and injected into immunized mice. On day 5, mice were sacrificed and draining lymph nodes collected and T cells stained using PE-conjugated Vα2 and biotin-conjugated Vβ5.1 antibodies (both from Pharmingen) to identify T cells with the TCR that recognized the OVA-derived peptide, SIINFEKL, when presented in H-2K^b molecules. Samples were then stained with streptavidin-CyChrome, washed, and fixed for flow cytometry.

6.1.12. Statistical analysis

Data are expressed as means ± SEM. Statistical significance of differences was assessed by a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test to evaluate differences between specific groups. An unpaired t-test was used to evaluate differences when only two groups were being compared. For tumor growth analysis, a one-way

ANOVA was used to compare tumor size on day 21. A value of $p<0.05$ was considered to be significant. Statistical analyses were performed using Prism 4.02 software (GraphPad Software, San Diego, CA).

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