CHARACTERIZATION OF DENDRITIC CELL HANDLING OF CELL-ASSOCIATED MEMBRANE AND CYTOPLASMIC PROTEINS FROM LIVE AND APOPTOTIC CELLS

by

Sherrianne Maria Gleason

B.S. in Biology, Indiana University of Pennsylvania, 2001

Submitted to the Graduate Faculty of

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

July 2008

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This dissertation was presented

by

Sherrianne M. Gleason

It was defended on

July 31, 2008

and approved by

Dissertation Advisor: Simon M. Barratt-Boyes, BVsc, PhD
Associate Professor
Department of Infectious Diseases and Microbiology, Graduate School of Public Health
Deparment of Immunology, School of Medicine
University of Pittsburgh

Robert L. Hendricks, PhD
Professor
Departments of Immunology and Molecular Genetics and Biochemistry, School of Medicine
University of Pittsburgh

Charles R. Rinaldo Jr., PhD
Chairman and Professor
Department of Infectious Diseases & Microbiology, Graduate School of Public Health
University of Pittsburgh

Russell D. Salter, PhD
Professor
Department of Immunology, School of Medicine
University of Pittsburgh

Simon C. Watkins, PhD
Professor
Departments of Cell Biology and Physiology and Immunology, School of Medicine
University of Pittsburgh

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Dendritic cells (DCs) are a heterogeneous population of immune cells that influence a wide variety of immune responses, including immunity to infectious diseases and malignant tumors, and in the generation of tolerance. In their immature state, DCs are highly specialized at capturing and internalizing exogenous antigens. Cell-associated antigens are of special interest because they play a role in both the induction of immunity and tolerance. This study aimed to add to the field of DC biology by further describing how DCs handle cell-associated proteins from both live and apoptotic cells. We hypothesized that the DCs ability to capture, internalize, and process integral membrane proteins would vary based on the target cell's viability and that the DCs ability to capture cell-associated protein would vary based on the protein's intracellular localization. To quantitatively and qualitatively characterize uptake, we created a biologically relevant system using the Epstein Barr virus latent membrane protein 2 and the melanoma protein gp100, each fused to the enhanced green fluorescent protein (EGFP) and expressed at the outer plasma membrane of a tumor cell line, along with a cell line expressing EGFP in the cytoplasm. We found (1) DCs captured integral membrane proteins but not cytoplasmic protein from live cells; (2) DCs captured membrane and cytoplasmic proteins from apoptotic cells more efficiently and at a faster rate than from live cells; (3) during direct physical interactions DCs transiently surveyed live cells capturing small quantities of membrane, but stayed in prolonged contact with apoptotic cells while continuously internalizing membrane fragments; (4) DC

internalization of membrane protein from live cells was clathrin-dependent while uptake from apoptotic cells was clathrin- and caveolae-dependent; and (5) internalized membrane protein from both live and apoptotic cells was found in early endosomes, late endosomes, and lysosomes. This work has potential broad public health implications as it is important to understand all aspects of DC biology when developing vaccines for both chronic and acute diseases. We hope that by uncovering the intricacies of DC handling of cell-associated proteins we will gain a better understanding of how to possibly manipulate DCs in order to influence the immune response.

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ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Simon M. Barratt-Boyes for his guidance and for giving me the opportunity to develop my project over the past several years. I also want to thank my committee members Dr. Charles Rinaldo, Dr. Russell Salter, Dr. Robert Hendricks, and Dr. Simon Watkins for their valuable suggestions and direction during my graduate career, and with Dr. Watkins I want to thank the staff of the Center for Biological Imaging.

I also want to thank my laboratory members past and present for their daily support and stimulating conversations. I can't imagine go through this process without all of you.

I would like to thank the staff, faculty, and several students, past and present, for their assistance over the years. In particular, I want to thank Monica Tomaszewski-Flick, Kevin Brown, Nada Melhem, Heather Hensler, Jill Montgomery, Shauna Clark, and Milka Rodriguez, all of whom have been with me from the beginning.

To my parents, I can't express in words what your support and faith in my abilities has meant to me. I love you and just saying thank you will never be enough. To my sister, thank you for the moral support, I treasure our long phone conversations more than you know. And to Nick, thank you for sticking with me through this entire experience. Having you there to support me means the world to me. And to my grandma and the rest of my large family, thank you for your thoughts, prayers, and support. It always lifted my heart and spirits to know so many people were on my side.

1.0 INTRODUCTION

1.1 DENDRITIC CELL SUBSETS

DCs are a heterogeneous population of immune cells with the ability to survey and sample peripheral tissue and process and present the captured antigen to naïve T cells. Even though all DCs have a similar function, they can be divided into various subsets. DC subsets are still a highly debated aspect of DC biology. In general, the most common way to categorize DCs is based on the progenitor cell, phenotype, anatomical location, and biological function of the DC (1).

All DCs originate from $CD34^+$ hematopoietic stem cells within the bone marrow (2). From the stem cells two types of DC precursors develop, the myeloid progenitor cells and the lymphoid progenitor cells. The myeloid progenitor cells give rise to myeloid DCs, also called conventional DCs, which exist in at least three compartments: peripheral-tissue-resident DCs, secondary lymphoid-organ resident DCs, and circulating blood DCs. Epidermal Langerhans cells and dermal DCs are found within two distinct layers of the skin, while the interstitial DCs include blood DCs, kidney DCs, liver DCs, and heart DCs. There are also mucosa-associated DCs within the oral cavity, lungs, and intestinal tract, thymic DCs, germinal center DCs, and follicular DCs. The lymphoid progenitor cells give rise to plasmacytoid DCs which are found in the blood (1, 3, 4).

DCs are relatively rare in the body and therefore the isolation procedures are time consuming and cell yields are low. Therefore, it has become and common and well accepted practice to culture human $CD14^+$ cells isolated from peripheral blood mononuclear cells in GM-CSF and IL-4 to differentiate them into human monocyte-derived DCs, which are equivalent to immature myeloid DCs (3). Using this method we can generate enough DCs in culture to study DC biology to gain a better understanding of natural occurring DCs.

1.2 DENDRITIC CELL FUNCTION AND ROLE OF MATURATION

The functional development of DCs is often referred to by their maturation state, either immature or mature. This maturation state is determined in the periphery and by external stimuli from the environment.

1.2.1 Immature dendritic cells

DCs in peripheral tissues, under steady-state conditions, are termed immature DCs. Their main function is to survey the peripheral tissue and capture antigens via macropinocytosis, receptor-mediated endocytosis and phagocytosis. They are specialized to internalize and process antigens, yet they are very poor at stimulating naïve T cells. Phenotypically, they express low extracellular levels of major histocompatibility class II (MHCII), low levels of CD54, CD58, CD80, CD86, CD40, and CD83 (5).

1.2.2 Mature dendritic cells

Mature DCs are often called activated DCs because they are activated by various stimuli. DCs can become activated when they encounter cells in the surrounding tissue that are stressed, virally infected or killed necrotically. The maturation process results in the endocytic and phagocytic capability of DCs substantially decreasing. The DCs then gain the ability to stimulate naïve T cells. Phenotypically, they express high levels of CD40, CD80, CD86, CD83 and MHCII (5).

1.2.3 Semi-mature dendritic cells

A less discussed maturation state of DCs is the semi-mature state. These DCs exhibit characteristics between a fully immature DC and a fully mature DC. Semi-mature DCs have been shown to be involved in both the induction of tolerance and the stimulation of immunity. The determining outcome is based on the maturation stimuli encountered by the DC at the time of antigen uptake. Immature DC can be stimulated with LPS and IFN-γ to produce IL-12 in a semi-mature state, which will decrease as the DC becomes fully mature. During the semi-mature, IL-12 producing state the DC can stimulate immunity (6). In contrast, studies have also shown the involvement of semi-mature DCs in the induction of tolerance, including transplant tolerance (7), and using TNF-α to induce a semi-mature state where the DCs produce no proinflammatory cytokines but result in the induction of IL-10 producing T regulator cells (8).

1.3 ANTIGEN PROCESSING AND PRESENTATION IN DENDRITIC CELLS

DCs are responsible for processing antigens and presenting the resulting peptide in the context of MHC class I and MHC class II molecules to either $CD8^+$ or $CD4^+$ T cells. The proteins from which the peptides originate can either come from within the DC or captured from the external environment. The source of the protein determines the intracellular pathway that the DC uses to process them. Endogenous proteins are synthesized within the cytosol of the DC and can be either self-derived cellular proteins or virus-derived proteins. Exogenous proteins by definition are not synthesized by the DC, but are internalized from the extracellular environment. There are many different kinds of exogenous antigens, including self and foreign, cell-associated, particulate, and soluble (9, 10).

1.3.1 Endogenous proteins and the MHC class I pathway

Proteins synthesized within the cytosol are ubiquitinated (11) then sent to the proteasome for degradation in smaller peptides. DCs constitutively express low levels of the immunoproteasome, which becomes the main type of proteasome in mature DCs (12, 13). The immunoproteasome generates a different peptide repertoire from the standard proteasome (13). The resulting peptides are transferred to the endoplasmic reticulum (ER) via the specialized peptide transporter, TAP. Once in the ER, the resident chaperons tapasin, calnexin, and calreticulin load the peptides onto MHC class I molecules (14, 15). MHC class I molecules have a binding cleft that is closed on both ends and can only accommodate peptides 9 residues in length. Once the peptides bind to the MHC class I molecules they are then transferred through the Golgi apparatus to the plasma membrane to be presented to $CD8^+$ T cells (Figure 1A) (16).

1.3.2 Exogenous proteins and the MHC class II pathway

The classical way that DCs present exogenous proteins is through the MHC class II pathway, resulting in presentation of peptides to $CD4^+$ T cells (15). MHC class II molecules originate in the ER, and shortly after synthesis associate with a trimer of invariant chains (17), which act to stabilize the MHC class II heterodimer. Transport signals in the invariant chain move the MHC class II complex out of the ER, through the Golgi apparatus, then on to the endocytic pathway. Once in the endosomes and lysosomes, the complexes are in an acidic, protease-rich environment, where the invariant chain is degraded by several proteolytic enzymes of the cathepsin family (18, 19), leaving only the invariant chain peptide CLIP that is removed just prior to peptide loading (20). The peptide is derived from soluble antigen that is captured from the external milieu, which is internalized via receptors then sent through the endosomal processing pathway (described in more detail in Section 1.5). The antigens are released from their receptors in the low pH of the early endosomes and the receptors recycle back to the cell surface. Within the endosomal pathway the proteins are degraded, producing peptides of varying length (21). The peptide is loaded onto the MHC class II molecules in the endosomal compartments, then is shuttled to the cell surface for presentation to $CD4^+$ T cells (Figure 1B) (15)

1.3.3 Exogenous proteins and the MHC class I pathway

Cross-presentation is when exogenous proteins are internalized and processed by the DC then presented in the context of MHC class I to $CD8^+$ T cells. This pathway of peptide loading is a combination of the two pathways described above. Both TAP-dependent and TAP-independent

pathways have been described which allow exogenous antigen to presented in the context of MHC class I. In the TAP-independent pathway, exogenous antigens do not need to be processed by the cytosolic machinery, but can be loaded on MHC class I molecules in the endocytic pathway (22-24). This could involve direct endosomal loading of preformed MHC class I molecules in with peptides that are generated within the endosomal compartment (Figure 1C) (23, 25, 26). In the TAP-dependent pathway, the exogenous protein is diverted to the cytosol of the DC in an undefined pathway, where they are digested by the proteasomes and loaded onto MHC class I molecule (22, 27) similar to the pathway to process and present endogenous protein (Figure 1D).

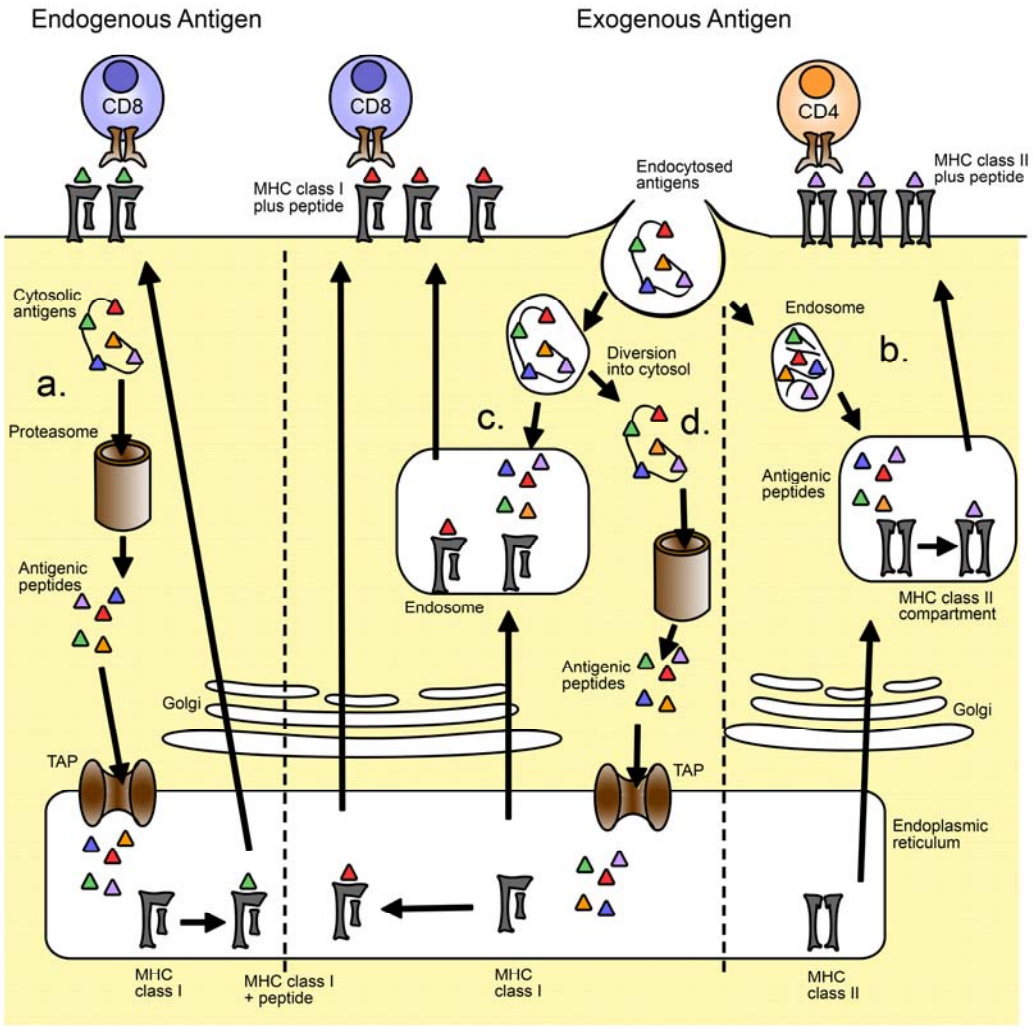

Figure 1. Antigen degradation and presentation pathways in DCs

DCs process and present both endogenous and exogenous proteins. (A) Endogenously synthesized proteins are degraded in the immunoproteasome into antigenic peptides, transported through the TAP into the ER then loaded onto MHC class I molecules. The loaded MHC class I are then transported through the golgi to the plasma membrane for presentation to $CD8^+$ T cells. (B) Exogenous antigens are internalized into endosomal compartments, degraded within the compartments, and then the antigenic peptides are loaded onto MHC class II molecules and transported to the plasma membrane for presentation to $CD4^+$ T cells. (C) Exogenous antigen is internalized into endosomal compartments then degraded within the compartments. MHC class I molecules are transported from the ER, through the golgi, into the endosomal pathway. The processed peptides and MHC class I molecules combine within the endosomal compartments then are transported to the plasma membrane for presentation to $CD8^+$ T cells. (D) Exogenous antigen is internalized into endosomal compartments then is diverted into the cytosol for degradation by the immunoproteasome. The antigenic peptides are then transported via TAP into the ER where they are loaded onto MHC class I molecules. The loaded MHC class I are then transported through the golgi, continuing to the cell surface for presentation to $CD8^+$ T cells. (C) and (D) are both cross-presentation pathways. (Adapted from Heath et al, 2001, Nat Rev Immunol and Villadangos et al, 2007, Nat Rev Immunol)

1.4 ENDOCYTOSIS: PORTALS OF ENTRY INTO DENDRITIC CELLS

Once the DC has captured exogenous protein, no matter what the source, there are various portals of entry into the DC. Endocytosis, by definition, is a plasma-membrane associated process by which a eukaryotic cell internalizes extracellular fluid or particles (28). There are several different endocytic pathways into the DC, they include phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin- and caveolin-independent endocytosis (Figure 2) (29).

1.4.1 Phagocytosis

Large particles, usually such as bacteria and apoptotic bodies, are internalized via phagocytosis. It is a highly regulated process involving specific cell-surface receptors and signaling cascades (29). The scavenger receptors CD36 (30), LOX1, and SR-A, and the integrins $\alpha_v\beta_3$ (31) and $\alpha_v\beta_5$ (30) are all involved in DC phagocytosis of cell-associated protein from apoptotic cells and necrotic cells. Once the appropriate receptors have been engaged, sheet-like plasma-membrane protrusions form a phagocytic cup in an actin-dependent manner, and this cup surrounds and ingests the exogenous antigen forming a phagosome.

1.4.2 Macropinocytosis

Macropinocytosis is morphologically and mechanistically similar to phagocytosis. It also requires actin polymerization, membrane ruffling, and the formation of large vacuoles. It also forms macropinocytic cups, which are similar to the phagocytic cups involved in phagocytosis.

However, unlike phagocytosis, the membrane extensions do not 'zipper up' along a ligand-coated particle, but instead they collapse onto and fuse with the plasma membrane. Its main purpose is to non-discriminately ingest bulk amounts of extracellular fluid, along with any particulate matter in the fluid, as part of the immune surveillance function of the immature DC. In macrophages, macropinocytosis is transiently induced, whereas DCs are constitutively macropinocytic.

1.4.3 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is the major pathway for internalization of receptor-mediated endocytosis. It is involved in the internalization of nutrients, pathogens, antigens, growth factors and receptors. Clathrin is a triskelion, or three-legged structure, formed by three clathrin heavy chains, each with a tightly associated clathrin light chain (32-34). During internalization, soluble clathrin from the cytoplasm is recruited to the plasma membrane. The clathrin triskelia assemble into a polygonal lattice at the plasma membrane to form coated pits that bud off from the membrane in a dynamin-dependent manner, giving rise to clathrin-coated vesicles (32, 35). These vesicles then fuse with the early endosome. Once the cargo enters the endosome, it is sorted and trafficked to its next destination within the cell.

1.4.4 Caveolae-mediated endocytosis

Caveolae have been implicated in endocytosis, transcytosis (36), and numerous signaling events (37) (38-40). At the plasma membrane of cells, caveolae form a functional unit generated by oligomerized caveolin and associated proteins and lipids (38). They are a stable membrane

compartment, which is anchored by the actin cytoskeleton, and not involved in constitutive endocytosis (41). During caveolae-mediated endocytosis, which can be stimulated through a signaling cascade (29), by agents such as SV40 virus (42, 43), sterols and glycolipids (44), flask-shaped invaginations of the plasma membrane form, lined with caveolin (38). These budding caveolae fused with the early endosome or the caveosome, an endosomal compartment with a neutral pH that lack classic endosomal markers but contains markers that are internalized through caveolae (38).

1.4.5 Clathrin- and caveolae-independent endocytosis

There are several different types of cholesterol-rich microdomains on the plasma membrane, also called lipid rafts. Caveolae fall into this category but there are other small structures that are caveolae-independent. The mechanisms that govern caveolae- and clathrin-independent endocytosis are poorly understood. In fact, it is only described as a form, or forms, of uptake that is revealed when the other two endocytosis pathways are blocked (28). These poorly understood pathways are often described using negative terms; they do not contain clathrin, they do not contain caveolae (29). Simian virus 40 (45) and SARS (46) are two agents that have been shown to be internalized via this mechanism.

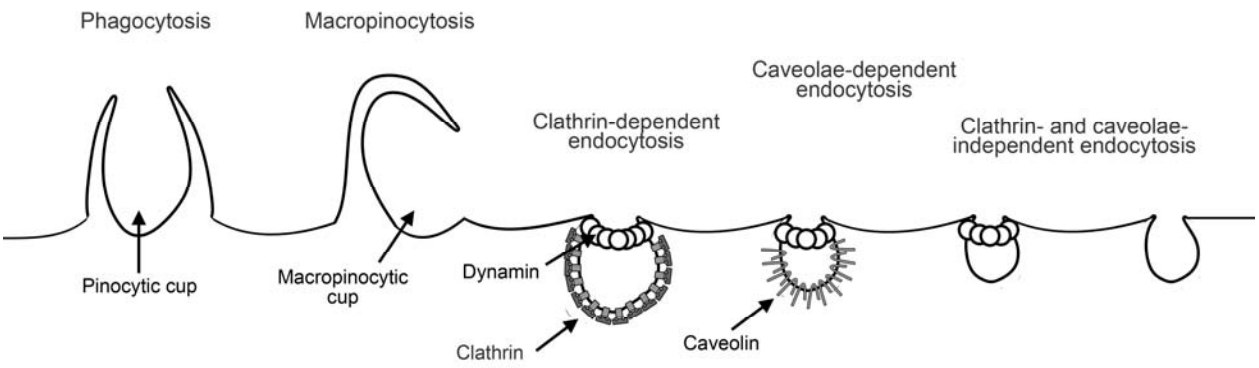

Figure 2. Pathways of entry into DCs

There are several pathways via which extracellular material can enter the DC. Each pathway has certain characteristics which make it distinct from the others. (Adapted from Conner et al, 2003, Nature and Mayor et al, 2007, Nat Rev Mol Cell Bio)

1.5 THE ENDOCYTIC PATHWAY

Following internalization by receptor-mediated endocytosis, most cargo goes directly to the early endosome. Early endosomes contain an acidic environment created by ATP-dependent proton pumps (47) and once the internalized proteins encounter the low pH they dissociate from their receptors (48-50). The receptors are recycled back to the cell surface (48-50) while the released material goes next to either the late endosomes or lysosomes. There are transport intermediates between the early and late endosomes, termed endosomal carrier vesicles approximately 300-400nm in diameter (51, 52), that can fuse with late endosomes in a microtubule-dependent fashion but not with early endosomes or with each other (52). The endosomal carrier vesicles do not contain early endosome-specific proteins or recycling receptors, nor do they contain the main

lipid and protein constituents of late endosomal membranes. Once the endosomal carrier vesicles fuse with the late endosome, the pH continues to decrease. From late endosomes, material can be further delivered to lysosomes for degradation. The processed cargo is then either transported to the golgi, cytoplasm, or recycled to the surface.

1.6 DENDRITIC CELL CAPTURE OF CELL-ASSOCIATED EXOGENOUS PROTEIN

As mentioned previously, the main function of immature DCs is to survey peripheral tissue and internalize antigen. DCs have been shown to capture viral, bacterial, eukaryotic, and cell-associated proteins. Recognition and internalization of pathogen associated proteins is important during an infection. However, during physiological steady-state conditions within the body the capture and processing of cell-associated antigens is the main purpose of DCs. Cell-associated antigens can come from many different cell types including virus-infected cells (53-56), transfected cells, tumor cells (57), and various normal tissue cells (58, 59). In addition, many types of cell-associated protein antigens have been shown to be captured and presented by DCs, including nuclear, cytoplasmic and cell-surface, or membrane-bound protein antigens.

1.6.1 Dendritic cell uptake of protein from apoptotic cells

Apoptosis is a programmed and physiological form of cell death that does not induce inflammation. In general, apoptosis usually affects scattered cells in the tissue and is triggered by both physiologic and pathologic stimuli. Apoptotic cells undergo distinct morphological

changes involving cell shrinkage, retention of organelles and nuclear chromatin condensation, which is often associated with nuclear fragmentation. Recognition of an apoptotic cell is initiated by changes on the cell surface of the apoptotic cell, such as translocation of phosphatidylserine (PtdSer) from the inner to the outer leaflet of the plasma membrane (60).

Immature DCs are capable of large-scale phagocytosis of apoptotic material (30, 31). However, different human DC subsets also exhibit differential capabilities to capture apoptotic material. A direct comparison of three different human DC subsets, found that immature monocyte-derived DCs are three times better at capturing apoptotic material than CD11c+ DCs, and plasmacytoid DCs were hardly able to capture apoptotic cells at all (61).

Several receptors have been implicated in the recognition and clearance of non-self. The scavenger receptors, including the class-B scavenger receptor CD36 (30, 61), the class-A scavenger receptor (SRA) (62), CD68 (63), and the oxidized low-density lipoprotein receptor 1 (LOX1) all recognize oxidized sites on apoptotic cells that mimic oxidized low-density lipoprotein. The PtdSer receptor recognizes phosphatidylserine (64) and the engagement of this receptor leads to macropinocytosis and uptake of the tethered apoptotic cell (65). The integrins $\alpha_v\beta_3$ (31) and $\alpha_v\beta_5$ (30) link the DC to the apoptotic cell via thrombospondin 1 (TSP1) (66, 67).

1.6.2 Dendritic cell uptake of protein from live cells

DCs are capable of acquiring protein antigens from live cells in via several different mechanisms. Exosomes, nanotubules, trogocytosis, and nibbling all result in intact cell-associated protein moving from the originating cell to the DC, however, each has properties that make them distinct from each other.

1.6.2.1 Exosomes

Exosomes are small membrane vesicles 30-100 nm in diameter (68) and are secreted from many different cells types, including epithelial cells (69, 70), mast cells (71), platelets (72), tumor cells (73), B cells (74), and even DCs (75, 76). They are believed to originate from multivesicular bodies by inward budding from the endosomal membrane (68, 77, 78) and the molecular composition varies slightly depending on the originating cell type but all exosomes contain plasma membrane patches and nano-organelles. Cytosolic proteins signal transduction proteins, antigen-presentation molecules, cholesterol and lipids can all be found in exosomes. They have a unique composition of proteins and lipids that distinguish them from other types of cellular vesicles, and the cell type they are generated from often determines their function (77). DCs have been shown to take in cell-associated protein from live cells via exosomes (79-82), but unlike other form of cell-associated protein uptake from live cells this is not a cell-contact dependent mechanism.

1.6.2.2 Nanotubules

Nanotubules are transient membrane bridges or tethers (83) between a wide variety cell types. Neuronal cells, T cells, NK cells, macrophages, DCs, epithelial cells, and fibroblasts have all been observed forming nanotubes (84). They can be formed either by actin-driven protrusions (83) or a cell-contact dependent, time-dependent mechanism (85). Two main functions of nanotubules have been described. First, nanotubes can used to transmit signals between cells (86). Second, are capable of transporting cell surface and intracellular proteins, as well as internal organelles between cells (84). With respect to DCs, there has only been one study. Watkins and Salter demonstrated that nanotubules between myeloid DCs can transmit signals, specifically, the transmission of calcium fluxes. In addition, microinjected dye tracers were also

transferred (86). This suggests the possibility that proteins may be transferred if the nanotubes were large enough, however, no evidence of cell-associated protein transfer via nanotubules to DCs has been found.

1.6.2.3 Trogocytosis

Trogocytosis is the rapid transfer of intact plasma membrane proteins between immune cells (87, 88). B cells (89, 90), T cells (91-94), and natural killer (NK) cells (95, 96), and DCs (97) all have the capability of obtaining cell-associated proteins via trogocytosis, but most research has focused on the APC-T cell interaction (98). It is characterized as an active process that is triggered specifically by antigen receptor signaling and the formation of the immune synapse (87, 88, 98). Transfer of proteins occurs within an hour of co-culture (88). A wide array of proteins can transfer between immune cells because entire membrane patches carrying intra- and transmembrane proteins move from cell-to-cell. The most widely studied interaction is between T cells and APCs. However, it is important to note that when DCs are involved, proteins typically move from the DC to the other immune cell (99, 100) and not vice versa.

1.6.2.4 Nibbling

'Nibbling' is a term used to describe the phenomenon of DCs actively capturing cell-associated proteins from live cells (10, 101). This process is distinct from the other mechanisms of live-cell protein acquisition because it does not involve the formation of an immunological synapse (101, 102) as in trogocytosis (87, 88, 98), it requires cell-to-cell contact (101) unlike uptake of protein from exosomes (103), and it is a receptor-mediated process involving close intimate contact (101, 102) with the target cell unlike the use of a membrane bridge with nanotubules (84). Using dye based methods, DCs have been shown to acquire both membrane and cytoplasmic proteins.

DCs have captured proteins from $CD4^+$ T cells, $CD8^+$ T cells, macrophages, monocytes, B cells, epithelial cells, and other DCs. Antigen capture via this mechanism can result in the stimulation of naïve T cells (101, 102). Whereas uptake from apoptotic cells involves CD36, LOX1, SRA, and certain integrins, nibbling seems to be mediated mainly by scavenger receptors, specifically SRA (102).

1.7 DENDRITIC CELL CROSS-PRESENTATION AND THE INDUCTION OF TOLERANCE AND IMMUNITY

The absence or presence of stimuli from the environment controls the behavior of the DCs surveying the periphery. After capturing, internalizing, and processing cell-associated antigen DC will present the resulting peptide to T cells, resulting in either tolerance (104) or immunity (105) to the presented antigen . DCs are often referred to as professional APCs, as they express 10-100 fold higher levels of MHC molecules than macrophages and B cells (106).

1.7.1 Stimulation of T-cell immunity

Upon infection or tissue damage, immature DCs are rapidly recruited by "danger signals" produced at the site of inflammation. These danger signals include those directly from pathogens, involving the engagement of pathogen recognition receptors which recognize conserved molecules on the surface of the organism (107), as well as endogenous signals from stressed, virally infected or necrotic cells (108). These stimuli can induce maturation of the DCs (described in Section 1.2.2) and the subsequent trafficking to lymphoid tissues and presentation

to T cells. The stimuli produced at the site of inflammation influence the functional properties of the DC. Microbial pathogen-derived signals such as LPS, bacterial lipoproteins, CpG motifs, and double-stranded RNA, as well as T-cell-derived signals such as IFN-γ or CD40 cross-linking, induce IL-12 production by DC, promoting Th1 responses (109). In contrast, pathogens such as fungi, schistosomes, and cholera toxin, as well as anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF-β), provoke DC to induce Th2 responses (110).

Growing evidence supports the idea that DCs are important in generating immunity to tumors. An inherent characteristic of malignant cells is that they are genetically abnormal. This often results in the production of proteins, lipids, and sugars that might then be able to be recognized by the immune system as foreign, or dangerous. DCs can efficiently cross-present present captured tumor antigens from dying cells. However, like the immunity response to infectious diseases, this requires an activation stimulus. *In vivo* mouse studies have shown that this stimuli can come from innate lymphocytes, CD40 ligation, or signaling through Toll receptors (111).

1.7.2 Generation of peripheral tolerance

The generation of immunity is regulated by activated, mature DCs but immature DCs are responsible for tolerance induction (104). In steady state conditions, DCs migrate to the T-cell regions of the lymphoid organs after a defined period of time or following environmental signals. Upon presentation to T cells, antigens that have been capture and processed by DCs in steady state conditions result in tolerance (110). After the DCs interact with the naïve or resting T cells, tolerance can be achieve in a number of ways. First, T cells can be induced to undergo apoptosis upon recognition of peptides bound to MHC molecules on the DC in the absence of or low

levels of costimulation factors, such as IL-1, IL-12, TNF-α, CD40, CD80 and CD86 (104). Second, instead of deletion, the T cell can be induced into anergy, which is a hyporesponsive state in which T cell proliferation and cytokine production are impaired upon subsequent exposures to the presented antigen (112). Third, the DC can stimulate the T cell to become a regulatory T cell. Regulatory T cells display regulatory functions by down-regulating effector T-cell response. Naturally occurring T-regulator cells come from the thymus, while so called 'secondary' suppressor T-cells are induced in the periphery by DCs (110). T-regulatory cells function by suppressing proliferation of effector T cells (113).

1.8 CONCLUDING REMARKS

The biology of DCs has many facets that are important when studying their handling of protein antigens. The subtype and maturation state of the DC determines the general function of the DC, such as the ability of immature myeloid DCs to internalize extracellular material and the ability of mature myeloid DCs to process and present the internalized protein to other cells of the immune system. There are several different pathways the DCs can use to internalize and process these proteins, as well as various presentation pathways to present the antigenic peptides to T cells. With all of these variables, different types of exogenous antigen do not have to be handled in the same manner. Therefore, when characterizing uptake of different kinds of exogenous antigen it is important to look at all of these aspects of DC biology.

2.0 HYPOTHESIS AND SPECIFIC AIMS

Dendritic cells (DC) are antigen presenting cells that internalize self and non-self antigens while surveying peripheral tissues. These internalized antigens are then processed and presented to various kinds of T cells. There are several ways that exogenous antigen can be presented to T cells, including the cross-presentation pathway where exogenous antigen is presented to $CD8^+$ T cells. One type of exogenous antigen is cell-associated antigen which can come from dead and dying cells or from live cells. Until recently, it was thought that DCs were only able to cross-present cell-associated antigens upon acquisition from apoptotic or necrotic cells. However, over the past several years, some focus has turned to characterizing the recently described phenomenon of DC nibbling, where monocyte-derived DC capture proteins from live cells through direct interactions. Previously, we have visualized this event using membrane fluorescent dyes and studied its biological relevance with the tumor antigen gp100 using adenoviral vectors and monkey monocyte-derived DCs. Here we plan to compare both live and apoptotic handling of cell-associated proteins. DCs have been shown to capture protein from apoptotic cells using many different receptors and various processing pathways. The receptors involved in uptake and its subsequent processing may differ when the source of the antigen is a live cell. Little has been studied and learned about live-cell antigen capture and its subsequent internalization and processing by DC. By studying compartmentalized protein uptake using

tumor and an infectious disease protein, for live cells as well as apoptotic cells, we might provide valuable information about the biological significance of this phenomenon.

We hypothesize that the ability of DCs to capture live cell-associated protein will vary based on the cellular localization of the protein. In addition, the profile of protein uptake by DC from the different cellular localities will differ when comparing live cells and apoptotic cells.

2.1 SPECIFIC AIM 1

Develop a system using chimera proteins to examine cell-associated protein uptake, processing, and presentation by DCs from various cellular compartments from both live and apoptotic cells.

Constructs expressing the melanoma tumor protein gp100 fused to EGFP, cytoplasmic EGFP, and the Epstein Barr virus (EBV) latent membrane protein 2 (LMP2) fused to EGFP were constructed using molecular cloning techniques. A HLA-A2$^-$ epithelial cell line was transfected with the constructs using a lipophilic method and analyzed using flow cytometry and confocal microscopy to verify levels of expression and expression patterns of all constructs. Cell sorting methods were used to purify the high expressing, transfected cell populations and the cells lines were kept stable in a drug selection medium.

2.2 SPECIFIC AIM 2

Visualize and determine the efficiency and kinetics of membrane and cytoplasmic protein uptake from live cells versus apoptotic cells.

Human monocyte-derived DC were generated from $CD14^+$ cells isolated from peripheral blood mononuclear cells using a ficoll histopaque gradient and $CD14^+$ bead selection. Immature DCs then were cocultured with the various targets, i.e. the stably transfected cell lines, and protein transfer was quantified and visualized using flow cytometry and live cell microscopy. We compared DC uptake of live cell-associated proteins and apoptotic cell-associated proteins, as well as proteins from both the membrane and cytoplasm of the target cell.

2.3 SPECIFIC AIM 3

Analyze the DC uptake and internal processing pathway of membrane bound protein from live cells versus apoptotic cells.

Human monocyte-derived DCs were incubated with various inhibitors which will be used to distinguish differences between protein uptake from both live and apoptotic cells. Inhibitors of various portals of entry into the DC via endocytosis were used to determine via what endocytic pathway the protein enters the DC. Antibodies to various endosomal compartments in the internal protein processing pathway of DCs were visualized with protein uptake via confocal microscopy in order to track the protein within the DC.

2.4 SPECIFIC AIM 4

Determine the ability of DCs to capture protein from live tumor cells and then stimulate a T cells response.

An HLA-A2 restricted system was used to analyze the ability of DCs to cross-present live cell-associated membrane protein. DCs were incubated with our HLA-$A2^-$ transfected cell line expressing gp100, a HLA-$A2^-$ cell line naturally expressing gp100, or a HLA-$A2^+$ cell line naturally expressing gp100 and then incubated with $CD8^+$ T cell clones specific for a gp100 peptide in the context of HLA-A2. T cell stimulation was measured by detecting IFN-γ production.

3.0 DISTINCT EFFECT OF CELL VIABILITY ON DENDRITIC CELL CAPTURE AND INTERNALIZATION OF CELL-ASSOCIATED PROTEINS

Sherrianne M. Gleason,*[†] Monica Jo Tomaszewski-Flick,* David T. Rowe,* Simon C. Watkins,[§‡] and Simon M. Barratt-Boyes*[†§]

*Department of Infectious Diseases and Microbiology, Graduate School of Public Health, [†]Center for Vaccine Research, Departments of [‡]Cell Biology and Physiology, and [§]Immunology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

This chapter is adapted from a study to be submitted to a peer review journal for publication.

Running title: DC uptake from live and apoptotic cells

Keywords: macropinocytosis, endocytosis, caveolae, clathrin

Correspondence:

Simon M. Barratt-Boyes, 9046 BST3, University of Pittsburgh, 3501 Fifth Avenue, Pittsburgh, Pennsylvania 15261, USA. Phone: (412) 383-9057. Fax: (412) 624-4577. E-mail: smbb@pitt.edu

3.1 PREFACE

This chapter fulfills specific aim 1, specific aim 2, and part of specific aim 3. Sherrianne M. Gleason wrote the manuscript, as well as ran all the experiments. Live-cell microscopy experiments were performed and data collected with the assistance of Simon C. Watkins and Monica Tomaszewski-Flick assisted with the generation of vectors. Simon M. Barratt-Boyes revised the manuscript and provided guidance and financial support. Additional financial support came from the Center for Vaccine Research.

Additional data related to this chapter can be found in Appendix A.

3.2 ABSTRACT

Dendritic cells (DCs) are antigen presenting cells that internalize self and non-self proteins while surveying peripheral tissues. Until recently, it was widely assumed that cell death was required, either via apoptosis or necrosis, before cell-associated protein could be captured and cross-presented by DCs. In the past several years, more studies have focused on the relatively new phenomenon of DCs capturing live cell-associated protein, with several indirect methods used to characterize the process. In this study, we created a biologically relevant system using two different integral membrane proteins fused to EGFP to characterize live-cell associated membrane uptake both quantitatively and qualitatively. We show that uptake of live-cell associated protein, though efficient, is not as efficient as that of apoptotic uptake, and that protein located in the cytosol of live cells is not captured by the DCs. When comparing uptake of live-cell associated membrane protein with apoptotic-cell associated membrane protein using live-cell microscopy we found that DCs capture apoptotic- and live-cell protein with very different dynamics. DCs take in small fragments of membrane from live cells but continuously internalize large quantities of protein from the membrane of apoptotic cells. Finally, we show that captured live-cell associated membrane protein is internalized via a clathrin-dependent endocytic mechanism and goes through the same internal processing pathway as captured membrane from apoptotic cells, traveling through the early endosomal, late endosomal, and lysosomal compartments within the DCs. This study highlights the similarities and differences in the biological process of DCs capturing cell-associated proteins from live cells versus apoptotic cells.

3.3 INTRODUCTION

Dendritic cells (DCs) are cell of the immune system that specialize in internalizing exogenous proteins and then processing the internalized proteins, load the resulting antigenic peptides onto MHC class I and II molecules, and present them to naïve $CD4^+$ and $CD8^+$ T cells to illicit an immune response (114). Several receptors have been implicated in capturing cell-associated self proteins from apoptotic cells, such as the scavenger receptors CD36, SR-A, and LOX1and the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (115, 116). Cell death, either via apoptosis or necrosis, was thought to be a requirement before cell-associated protein could be captured, internalized and processed by DCs (117), and this process has been well described. However, a growing body of literature has shown that DCs are capable of capturing cell-associated plasma membrane from live, intact cells, in a process termed "nibbling" (10). Monkey DCs captured both membrane and cytoplasmic protein from other DCs, T cells, B cells, macrophages, and epithelial cells through direct cell-to-cell contact (101), viable donor cells were required for murine DCs cross-present *Listeria*-derived $CD8^+$ T cell epitopes (118), human tonsil DCs took up fluorescent membrane dye from intact, necrotic, and apoptotic cells (119), human monocyte-derived DCs cross-presented HIV Ags from both live and apoptotic $CD4^+$ T cells (120), and both macrophages and DCs were observed to cross-present Ag from live, vaccinia-infected cells (121). Thus far, only the scavenger receptor SR-A has been directly implicated in uptake of protein from live-cell associated protein (102).

All of the studies documenting DC uptake of protein from live cells, observed uptake using indirect methods. Live-cell protein uptake was documented by the exclusion of cell death and subsequent cross-presentation assays, however uptake was never directly visualized (118, 121), but the most common method used was visualization using dye transfer for microscopy and

flow cytometry (101, 102, 119, 120), sometimes with the inclusion of some cross-presentation assays (102, 120). Even though scavenger receptors have been implicated in protein uptake from live cells (102, 120), thus far, no one has looked at what route the captured proteins are internalized. There are multiple endocytic routes that extracellular material can enter the DC, which include phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and a less defined clathrin-caveolae independent endocytosis (28, 29). Once the protein enters the DC, the internalized protein can go through the endocytic processing pathway which consists of the early endosome, late endosome, and lysosome, however it does not necessarily have to travel through all compartments. Although some have determined that protein captured from live cells is found in the early endosome and late endosome, they visualized this transfer via antibody staining of the captured protein or again, uptake of membrane labeled dye, and did not look at the lysosomal compartment (102, 120).

We hypothesize that the dynamics and characteristics of DC uptake of proteins from both live and apoptotic cells will be different when studying the transfer of integral membrane proteins instead of diffuse membrane and cytoplasmic dyes. In this study, we created a biologically relevant system, using various integral membrane proteins fused to EGFP, to study the uptake and processing of cell-associated proteins. With this system, we were able to directly characterize quantitatively and qualitatively DC uptake of integral membrane proteins without the use of dyes, and found that though efficient, uptake of cell-associated protein from the membrane of live cells was not as efficient as uptake of membrane from apoptotic cells. In addition, no uptake of cytoplasmic protein was observed when the target cells were living. With the fluorescently tagged proteins, we were able to directly visualize the dynamic interactions of the DCs and target cells, observing brief DC-live tumor cell interactions but prolonged DC-

apoptotic tumor cell interactions, as well as continuous, substantial membrane uptake when the target cell was apoptotic. We found that uptake of apoptotic membrane material was clathrin- and caveolae- dependent; however uptake of live-cell membrane material was only clathrin-dependant. With our system of fluorescently tagged integral membrane proteins, our study revealed that DC uptake of apoptotic membrane proteins occur with different efficiency, kinetics, and entered the DC through different mechanisms than DC uptake of cell-associated membrane, proteins, but all captured protein traveled through an identical internal processing pathway.

3.4 MATERIALS AND METHODS

3.4.1 Cell culture

The stable cell lines MSgp100egfp, MSN1egfp, and MSlmp2egfp were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 10mM HEPES, 10% FBS, and 600 ug/ml geneticin (Invitrogen, Carlsbad, CA). PBMCs were isolated from leukocyte-rich buffy coats from healthy donors as previously described (122), then $CD14^+$ cells were positively selected using Ab-coated microbeads and magnetic separation (Miltenyi Biotec, Gladbach, Germany). Monocyte-derived DCs were generated by culturing human $CD14^+$ monocytes in RPMI-1640 media containing 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 10 mM HEPES, 10% FBS, 10mM non-essential amino acids, and 10mM sodium pyruvate (Invitrogen) supplemented with recombinant human (rh) GM-CSF (1000 U/ml) and rhIL-4 (1000 U/ml) (Schering-Plough Kenilworth, NJ), for 5-7 days.

3.4.2 Generation of cell lines

The target cells for DC protein uptake consisted of the stable cell lines MSgp100egfp, MSN_1egfp, and MSlmp2egfp, all generated from the breast adenocarcinoma cell line, MS, previously described (123). For the cell line MSgp100egfp, a replication-defective recombinant adenovirus encoding the melanoma gp100 (Ad-gp100), which has been previously described (101), was used as a template for cloning gp100. The PCR product, encoding the full length gp100, was amplified then inserted into the pN_1-EGFP vector (Clonetech, Mountain View, CA). The resulting construct was transfected into MS cells using GeneJuice™ Transfection Reagent

(Novagen, Madison, WI), resulting in cells expressing gp100 with a transmembrane region across the outer plasma cell membrane with EGFP fused to the N_1 terminal end of the protein on the inner side of the plasma membrane. Other cell lines were created similarly with MSN_1egfp containing the empty pN_1-EGFP vector, resulting in a cell line expressing EGFP in the cytoplasm, and MSlmp2egfp containing the pN_1-EGFP vector expressing a truncated version of the EBV latent membrane protein 2 (lmp2) containing the first 3 loops of the full version protein with EGFP expressed on the inner side of the plasma membrane, as well as a flag tag in loop 1, previously described (124). MSgp100egfp and MSN_1egfp were sorted on a MoFlo High Speed Sorter (Dako Cytomation, Fort Collins, CO) gating on high EGFP expression to obtain a highly pure, bright population. All transfected cell lines were maintained as stable cell lines in the selection media, as described above, containing geneticin (600ug/ml; Invitrogen).

3.4.3 Uptake assay and flow cytometry analysis

For live-cell assays, live, adherent target cells were washed 3 times with PBS to remove any dead cells, then harvested with 5 mM EDTA and washed 2 more times with PBS to remove any residual EDTA. Trypan-blue staining and cell morphology was used to verify cell viability. For apoptotic assays, cells were generated by seeding target cells at a concentration of 1 X 10^6 cells/ml in RPMI-1640 with no serum and no phenol red, in 60 x 15 mm tissue culture plates (Falcon). The lid was removed and cells were treated with UVB at 0.6 mJ/cm^2/sec for 15 min then placed at 37°C. Apoptotic cells were harvested with the next day. Apoptosis was confirmed with Annexin V staining (BD Biosciences, San Jose, CA) and cell morphology. 5 x 10^5 DC were cocultured with equal numbers of live or apoptotic target cells in 24-well plates at 37°C or 4°C then harvested at various time points, fixed with 2% paraformaldehyde (PFA), and

placed at 4°C until stained for flow cytometry analysis, as previously described (102). Briefly, cells were labeled with unconjugated Ab specific for human HLA-DR (BD Pharmingen, San Diego, CA) then Cy5-conjugated Fab fragment goat anti-mouse (Jackson ImmunoResearch, West Grove, PA) and kept at 4° C. Nucleic acid stains can be used to distinguish doublets by plotting pulse-area versus pulse-height (125), therefore, immediately before analysis, cells were incubated with hexidium iodide (10 μg/ml; Invitrogen), a nucleic acid stain, for 15-20 min at room temperature (RT) in the dark then washed once with FACS buffer. Data was collected on a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed using CellQuest Software (Becton Dickinson) using a $\log_{10}$ fluorescence. After doublet exclusion, percent uptake was calculated with the following equation: DC with green protein / total number of DC x 100.

3.4.4 Confocal microscopy

DC were co-cultured with MSgp100egfp target cells at 37°C for 1hr, then the inhibitors chloroquine (100uM), bafilomycin A1 (400nM), or NH_4Cl (50mM) (Sigma-Aldrich, St. Louis, MI) , were added and cells incubated another 2 hr. Cells were harvested, resuspended in PBS and, following a 30-60 min adherence period at 37°C, were fixed in 2% PFA for 20 min at RT. Cells were permeabilized with 0.1% Triton-X (Sigma-Aldrich) and stained with Ab specific for early endosomal Ag 1 (EEA1) (BD Biosciences), mannose-6-phospate (Man-6-Ph) (Abcam, Cambridge, MA), lysosome associated membrane protein 1 (LAMP1) (Abcam), and CD40-PE (Ancell, Bayport, MN), then stained with Cy5 goat anti-mouse or Cy-3 goat-anti mouse (JacksonImmuno Research). Alexa-488 anti-EGFP (Molecular Probes, Eugene, OR) was used to visualize any EGFP that had been quenched. Alternatively, MSgp100egfp were stained alone

with Ab specific for gp100 (BioGenex, San Ramon, CA), while kept on ice, and then fixed with 4% PFA before using Cy3 goat ant-mouse (Jackson ImmunoResearch). MSlmp2egfp were fixed with 2% PFA, permeabilized with 0.1% Triton-X, and then stained with anti-flag (Sigma-Aldrich) then Cy3 goat anti-mouse (JacksonImmuno Research). Slides were then cover slipped and analyzed on an Olympus FV 500 Confocal Scanning Microscope (Olympus, Tokyo, Japan). Images were obtained using 60x objectives and final composites were constructed using Metamorph software (Molecular Devices, Sunnyvale, CA) and Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA).

3.4.5 Live-cell microscopy

For live-cell membrane uptake studies, target cells were seeded onto glass cover slips in 60 X 15 mm tissue culture dishes (Falcon), placed at 37°C, and allowed to adhere overnight. The next day, the cell-coated coverslip was placed in the chamber of an Olympus 1X70 and kept at 37° C. DCs, resuspended at a concentration of $1x10^6$/ml, were then injected into the closed system. Images from several random fields were collected every 4-6 min for each of the illuminated conditions with Metamorph software. For apoptotic-cell membrane uptake studies, DC were injected into the microscope chamber and allowed to adhere for 30-60 min at 37°C. Harvested, resuspended, apoptotic cells were then injected into the closed system and images taken as described above.

3.4.6 Inhibition assays

DCs were preincubated for 30 min at 37°C with sucrose (0.5M), methyl-β-cyclodextrin (10mM), filipin (2ug/ml), or chlorpromazine (10ug/ml); all obtained from Sigma-Aldrich, in a 24-well plate. An equal number of live or apoptotic target cells were then added to the wells and incubation continued for 3 hrs. Cells were then harvested with PBS, fixed with 2% PFA, and stained as described above for transfer assays. Percent change in uptake was calculated as follows: 100 – (percent uptake of inhibitor / percent uptake with no inhibitor * 100).

3.5 RESULTS

3.5.1 DCs capture integral membrane proteins from apoptotic cells with greater efficiency and at a faster rate than from live cells

For our studies, we focused on the characterization of uptake of immature DCs, as they are the cells that have been shown to be involved with live-cell associated membrane uptake (101), as well as uptake of apoptotic material (30). Our previous experiments utilized various dyes to observe live-cell associated uptake (101), so the next logical step was to create a system to visualize the uptake of integral membrane proteins from live and apoptotic cells. Various stable cells lines were created from the breast epithelial cell line, MS (123), since its adherent characteristic made it easier to exclude dead and dying cells from the live-cell assays. The first target cell line, MSlmp2egfp, expressed the first six loops of the EBV latent membrane protein 2 with EGFP fused to the N-terminal end, resulting in EGFP expressed on the inner side of the

plasma membrane, with a flag-tag inserted in one of the extracellular domains (124). The second cell line, MSgp100egfp, expressed the full length melanoma protein gp100 at the cell surface, again with EGFP fused to the N-terminal end of the protein on the inner side of the cell membrane. Co-expression of each protein with EGFP at the cell membrane was verified via confocal microscopy by staining for the flag-tag in MSlmp2egfp and for gp100 in MSgp100egfp (Figure 3A). This enabled us to use EGFP as a marker for both the EBV LMP2 and gp100 proteins. Uptake assays were conducted by coculturing DCs with live or apoptotic target cells for 4 hr, then analysis using flow cytometry to quantify the uptake of membrane bound protein from the target cells. DCs were labeled with HLA-DR to distinguish them from the target cells and a DNA stain was used to exclude doublets from the analysis, therefore all double positive cells were DCs that had captured membrane protein and not a DC and target cell sticking together. When DCs were incubated with apoptotic MSlmp2egfp, after 1 hr 23.4% of DCs had taken in membrane-associated protein. This percentage continued to increase over time with 47.2% at 2 hrs then up to 59.4% at 4 hrs (Figure 3B). In contrast, only 10.6% of DCs incubated with the live target cell had captured membrane-associated protein after 1 hr, increasing to only 9.2% after 2 hrs, then rising to 16.3% after 4 hrs (Figure 3B). The phenomenon of capturing integral membrane proteins from both live and apoptotic cells was an active process. The DCs were not acquiring membrane protein passively because at 4°C, a temperature that halts all active movement of the cell, no membrane uptake was seen after 4 hrs (Figure 3B). Similar results in the differences between uptake of integral membrane proteins from live and apoptotic cells were seen when the target cell was MSgp100egfp, with percent uptake at 22.6% with apoptotic target cells and 16.4% with live target cells after 4 hrs (Figure 3C). In both instances, membrane uptake from apoptotic cells occurred at a greater level than from live cells, regardless of the

target protein (Figure 3B and 3C). When comparing the rate of DC uptake from both cell lines over a 4 hr time period, we saw that they followed a similar pattern, with apoptotic uptake increasing rapidly then slightly leveling off at 4 hrs, and uptake from live cells increased much slower, never reaching the same level as with apoptotic uptake at 4 hrs (Figure 3D). All live-cell, membrane uptake we measured was due to direct, cell-to-cell interaction because when DC were incubated with supernatant from donor cells there was no uptake visible at 4 hrs (Figure 3E), suggesting that exosomes were not contributing to our calculations. These data show that uptake of integral membrane proteins from live cells was not as efficient as previously thought, and that uptake from dead cells was more efficient than uptake of proteins from live cells.

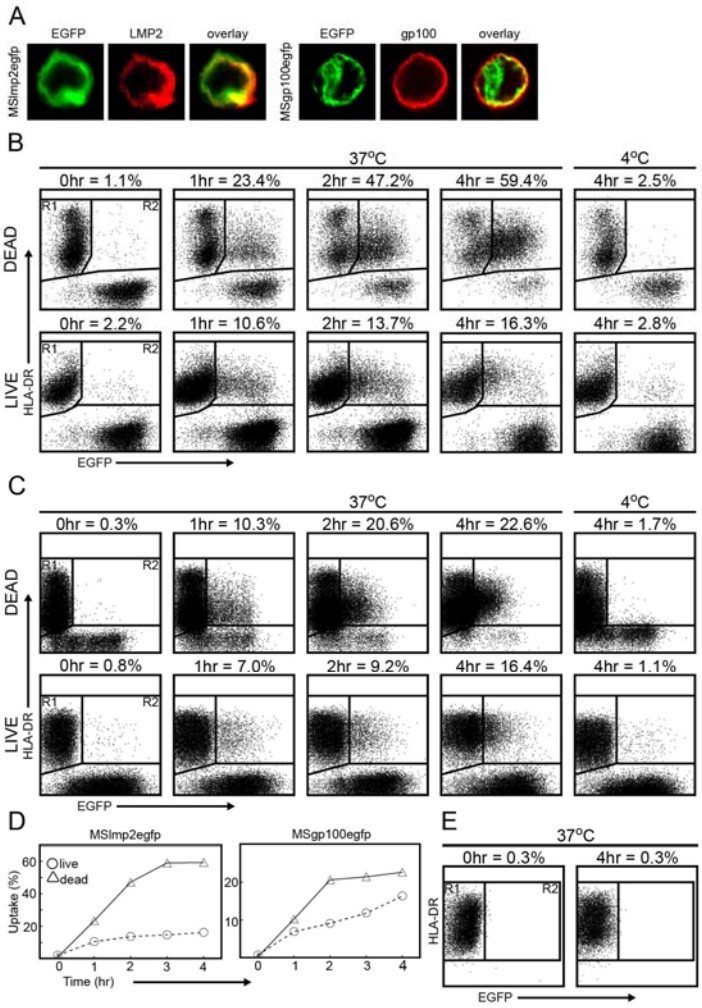

Figure 3. DCs capture membrane-associated protein from apoptotic cells at a faster rate and greater efficiency than from live cells.

(A) Confocal microscopy verifying membrane-localized protein expression in the stable cell lines MSlmp2egfp and MSgp100egfp. The truncated LMP2 protein expressed MSlmp2egfp was stained with anti-flag Ab (red) and the gp100 protein expressed in MSgp100egfp was stained with anti-gp100 Ab (red). Colocalization (yellow) of both proteins with EGFP (green) occurs at the plasma membrane. (B-C) DC capture membrane protein from dead cells more efficiently than from live cells, from both MSlmp2egfp (B) and MSgp100egfp (C). Immature human DCs were incubated with equal numbers of target cells at 37°C and 4°C. Cells were analyzed immediately after mixing (0 hr) and every hour for 4 hrs. DCs were identified using HLA-DR and target cells were identified using EGFP. Percent uptake was calculated by dividing the number of DC with captured membrane (R2) by the total number of DC (R1+R2) then multiplying by 100. Cells included in the analysis were first gated on forward-side scatter, then hexidium iodide was use to exclude doublets. (D) Kinetics of DC uptake of live (open circle) and apoptotic (open triangle) membrane bound protein from MSlmp2egfp (left panel) and MSgp100egfp (right panel). Percent uptake was plotted against time over 4 hrs. (E) Observed uptake is not due to exosomes. Supernatant from live MSgp100egfp was incubated with DC. DC were analyzed immediately (0 hr) and after 4 hrs after incubation with supernatant from MSgp100egfp cells, which had been spun to remove cellular debris but not exosomes and aggregate proteins. DC were stained with HLA-DR and analyzed as in B.

3.5.2 Dendritic cells capture cytoplasmic protein from apoptotic target cells but not live target cells

We next wanted to look at uptake of a cytoplasmic protein, in order to determine if the cellular localization of the fluorescent protein had an effect on the efficiency of DC uptake. We again used the MS cell line, this time with EGFP targeted to the cytoplasm, resulting in the stable cell line MSN1egfp. Localization of EGFP was verified using confocal microscopy (Figure 4A). DC were coincubated with live or dead MSN1egfp and stained similar to the cells in Fig. 3B. At 4 hrs there was no DC uptake of cytoplasmic protein from live cells (Figure 4B). However, we did see uptake when MSN1egfp were induced to undergo cell death, with 18.4% of DCs capturing cytoplasmic protein (Figure 4B), illustrating that uptake was possible with this cell line. In fact, when observing the kinetics of the uptake of cytoplasmic protein we saw a similar pattern as with the membrane-bound proteins. Apoptotic uptake increased rapidly, with uptake climbing to 23.8% at 2 hrs, then decreasing slightly before leveling off at 4 hrs at 18.4%. In contrast, with a live target cell, no cytoplasmic protein uptake was seen at 1 hr and still nothing at 4 hrs, with an uptake of 2.6% (Figure 4C). This data illustrated that transfer of cytoplasmic protein from live cells does not occur. This also supported that all uptake seen with MSlmp2egfp and MSgp100egfp from live cells was only due to membrane-bound protein and not due to any protein in the cytoplasm.

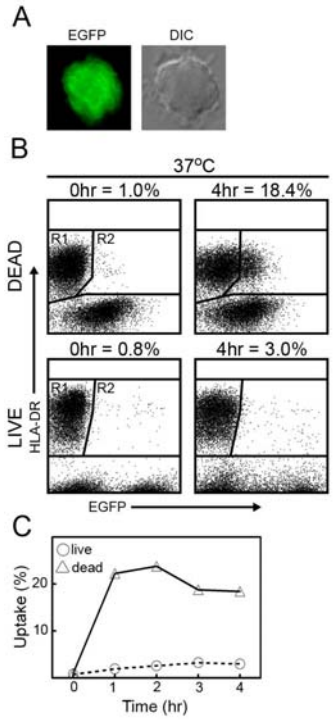

Figure 4. DCs do not capture cytoplasmic protein from live, intact tumor cells.

(A) Visual confirmation of cytoplasmic EGFP. Expression of EGFP (green) in MSN1egfp was visualized using a confocal microscope. DIC was used to see the morphology of the cell. (B) DCs do not capture cytoplasmic protein from live cells. Immature human DC were incubated with an equal number of live or apoptotic MSN1egfp for 0 – 4 hrs, then stained and analyzed as in Fig. 3B. (C) Uptake of cytoplasmic protein from dead cells increases over time. Kinetics of DC uptake of live (open circle) and dead (open triangle) cytoplasmic protein from MSN1egfp. Percent uptake was plotted against time over 4 hrs.

3.5.3 Extended interaction of dendritic cells with apoptotic cells and brief interaction with live cells

Since DCs were not observed to capture proteins from the cytoplasm of live cells, we decided to focus further studies on uptake of membrane-associated protein. In order to directly visualize DC uptake of membrane-bound proteins from various populations of target cells we

used live-cell microscopy techniques, which allowed real-time imaging of cellular interactions. Cover slips containing live MSgp100egfp were inserted into a microscope chamber, and then unlabeled DCs were injected into the closed system. Differential interference contrast (DIC) was used so the dynamic movement of the DCs could be observed while they interacted with the green target cells. The target cells were deliberately overexposed to detect any captured protein with maximum sensitivity. Immediately, the DC approached the donor cell (Figure 5A, 6 min), then the DC started to extend its membrane processes to interact with the target cell (Figure 5A, 26 min). The membrane taken up by the DC from the live MSgp100egfp can be followed by the arrow at the various time points (Figure 5A). After taking in membrane protein, we saw the DC retract its processes and move on (Supplementary Video 1). The DCs were also visualized capturing membrane from MSlmp2egfp. In this instance, the DC started to take in membrane before it settled down on the cover slip within the focus range of the microscope, with the DC on top of the target cells (Figure 5B, 19 min). As the DC came into focus we clearly saw the membrane taken in by the DC as a green punctuate structure (Figure 5B, 1 hr 36 min). The DC interacted with other tumor cells, though did not capture any more protein (Supplementary Video 2), and can be tracked over time by following the arrow pointing to the captured membrane (Figure 5B). With live-cell microscopy, we saw that uptake of integral membrane protein from live cells by DCs was a dynamic process, with the DC actively taking in membrane, while continuing to move from cell-to-cell. An additional example of a DC capturing membrane from live cells can be found in the Appendix (Figure 12), along with the Supplementary Video 5.

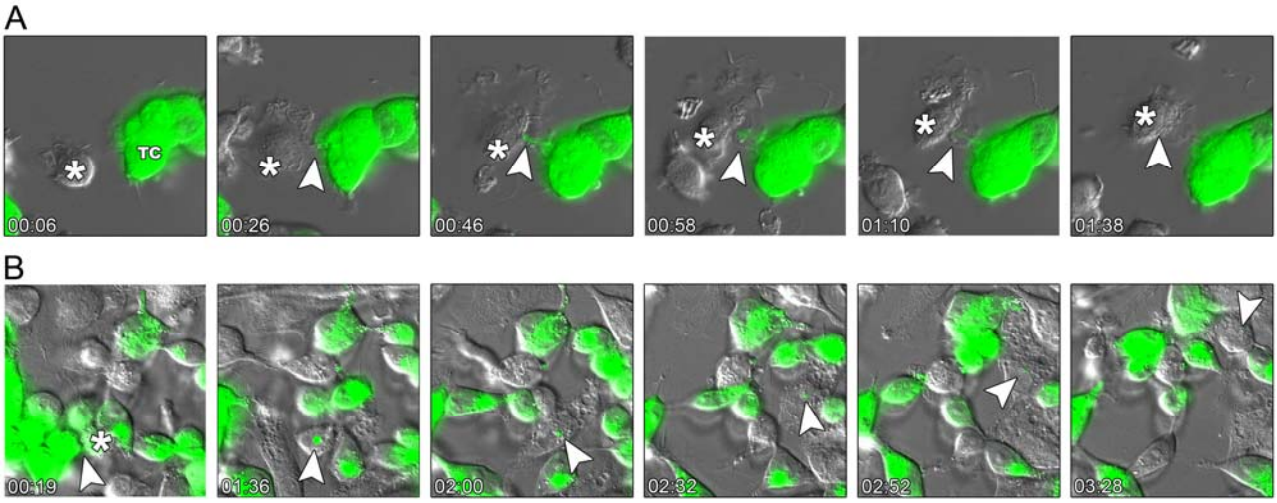

Figure 5. Direct visualization of DC uptake of membrane-bound protein from live cells.

Immature human DCs were cocultured with live MSgp100egfp (A) and MSlmp2egfp (B), in a live cell microscopy chamber. Images of EGFP and DIC were collected every 4-6 min, from which a montage of selected frames was generated. EGFP (green) was overlaid onto the DIC (grey) to visualize the dynamic movement of the DC capturing membrane. The DC capturing membrane (asterisk) is identified in the first frame. In (A) the donor cell (TC) is indicated in the first frame and starting in the second frame (26 min) captured membrane can be followed (arrow). In (B) the captured membrane and DC can be followed throughout the montage, starting in the first frame (arrow, 19 min), with a green punctuate structure becoming distinct as the DC came into focus in the second frame (1 hr 36 min).

Next, to visualize DC uptake of membrane-bound proteins from apoptotic cells, we first injected the DC into the closed microscope chamber, which permitted them to adhere to the cover slip, then injected the apoptotic cells. We saw the target cells floating above the DCs in the microscope chamber and they remained that way for the entire assay, the dead cells never settled so they remained out of focus above the DCs. Initially, with apoptotic MSgp100egfp, we saw a DC next to and under the target cells (Figure 6A, 7 min), then the DC started to take in large amounts of membrane, as visualized by numerous green punctuate structures (Figure 6A, 28 min). Over time, we saw numerous fragments of captured membrane streaming into the DC, while the DC stayed in contact with the target cells, even as it stretched away from them (Figure

6A). The internalized fragments of membrane can even be seen flowing around the nucleus of the DC (Figure 6A, 1 hr 43 min and Supplementary Video 3). We saw even more activity with apoptotic MSlmp2egfp target cells. Two DCs both interacted with a large mass of dead cells, with one DC staying in constant contact and the other internalizing membrane protein and moving away (Supplementary Video 4). Each DC can be followed using color coded arrows, as well as the associated captured protein (Figure 6B). An additional example of a DC capturing membrane from apoptotic cells can be found in the Appendix (Figure 13), along with the Supplementary Video 6.

Interestingly, the DC was seen to stay in contact with the apoptotic target cells for a much longer length of time than with the live target cells. The main body of the DC can be observed to gradually move away from the apoptotic target cells, however, a long process was still in association with the apoptotic cells for the entire 3 hrs (Figure 6A and Supplementary Video 3) and 6 hr 30 min (Figure 6B and Supplementary Video 4) of the assays. In fact, in one experiment, the DCs were observed moving out of the stage position, taking the tumor cells with them (Supplementary Video 4). This was not seen when the DC interacted with live target cells. DC simply captured small fragments of membrane from the live cells while interacting for only around 60 min, then moved away to interact with other cells (Figure 5 and Supplement Videos 1 & 2). This live-cell imaging supported our previous findings with flow cytometry, that DC captured more membrane protein from apoptotic cells than from live cells. This was possibly in part due to the extended contact of the DC with the apoptotic cells.

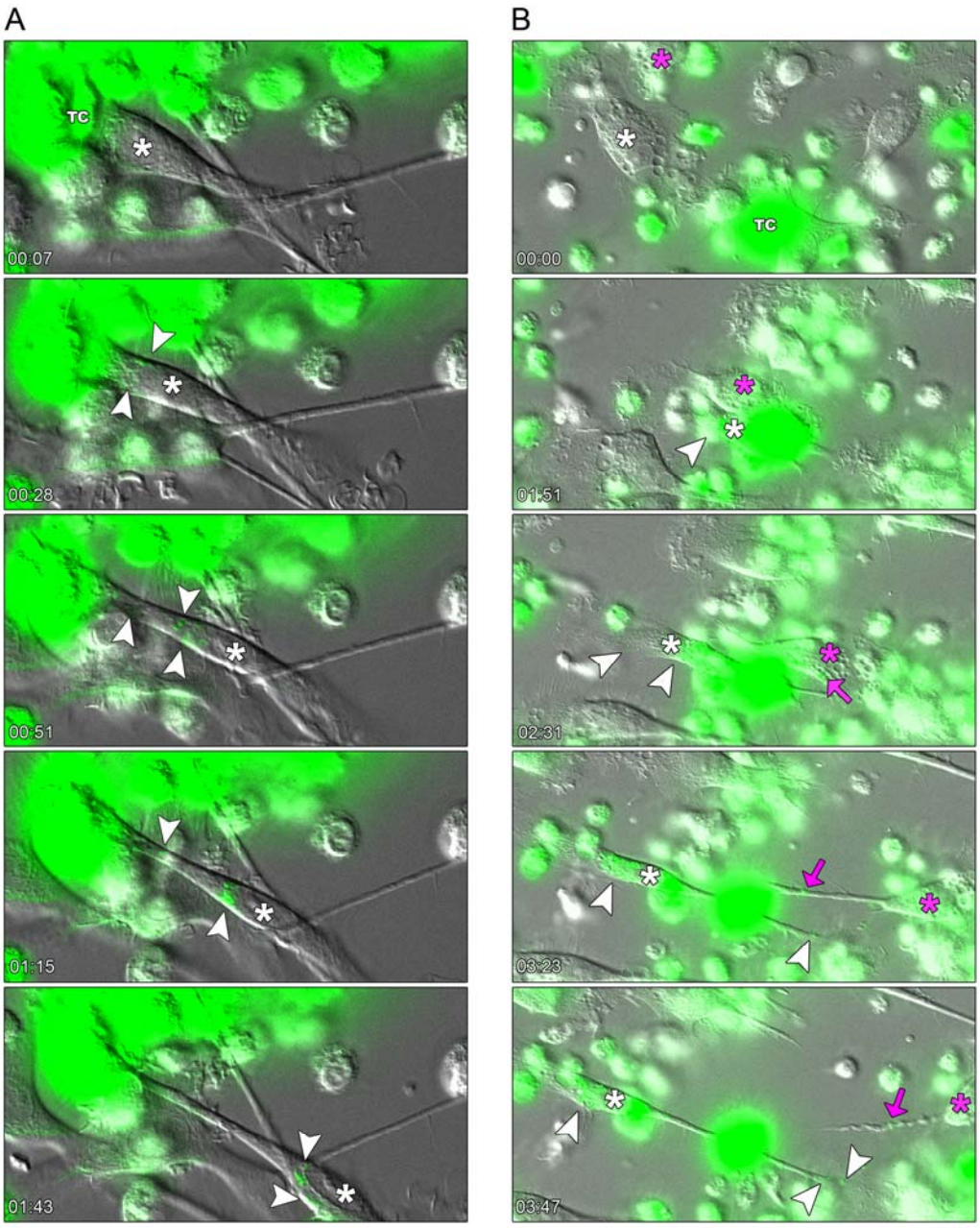

Figure 6. Direct visualization of DC uptake of membrane-bound protein from apoptotic cells.

Immature human DCs were cocultured with apoptotic MSgp100egfp (A) and MSlmp2egfp (B) in a live cell microscopy chamber. Images were collected and constructed as in Fig. 3. Donor cells (TC) were indicated in the first frames. In (A) the DC capturing apoptotic protein (asterisk) is identified in the first frame and throughout the montage. In the second frame (28 min) the DC starts to take in large amounts of apoptotic protein from the donor cell (arrows), and large amounts of protein can be seen streaming into the DC throughout the experiment. Captured protein can be seen moving around the nucleus of the DC (1 hr 43 min). In (B), two DC are identified in the first frame (0 min) with asterisks, one white and one pink, that will be followed throughout the experiment, as well as the donor cells (TC). Capture membrane will be indicated using arrows of corresponding colors. Over time, both DCs can be observed physically taking in large amounts of apoptotic protein.

3.5.4 DCs capture protein from live cells via clathrin-coated pits but not caveolae

In previous studies, we have shown that scavenger receptor A is involved in acquisition of cell-associated membrane from live target cells (102). We wanted to examine the endocytic pathway in more detail by determining the portal of entry for captured membrane proteins from both live and dead target cells using inhibitors that block various types of endocytosis. Sucrose, a strong inhibitor of clathrin-mediated endocytosis (126), shut down almost all uptake of both dead-cell (Figure 7A, second row) and live-cell membrane proteins (Figure 7B, second row), resulting in over 90% decrease in uptake compared to no inhibitor (Figure 7C, first row). We next used filipin, a known blocker of caveolae-dependent uptake (127, 128). Here, we saw a dramatic difference in live-cell membrane uptake and apoptotic-cell membrane uptake. There was a significant decrease in membrane uptake from apoptotic cells from 58.5% to 19.3% (Figure 7A, third row) with an average percent change of over 60% (Figure 7C, second row), whereas with live-cell membrane uptake, filipin had no effect, indicating that caveolae were not involved in live, cell-associated membrane uptake (Figure 7B, third row) (Figure 7C, second row). Methyl-β-cyclodextrin (MβCD), which works by extracting cholesterol from the plasma membrane, affecting both clathrin and caveolae-mediate endocytosis (129, 130), decreased apoptotic uptake from 58.5% at 3 hr to 29.0% (Figure 7A, forth row), with an average percent change of over 50% (Figure 7C, third row). Surprisingly, the opposite was seen with live-cell membrane uptake, with an average percent increase of almost 60% (Figure 7C, third row). This may be due to either an increase in membrane fluidity or the result of receptors being more evenly distributed across the membrane of the DC, as was suggested when another study on uptake of high density lipoprotein by scavenger receptor SR-BII was increased with the use of MβCD (131). Taken together, the results illustrated a main difference between DC uptake of membrane-associated protein from

apoptotic cells and uptake of cell-associated membrane protein from live cells. Apoptotic uptake occurs through multiple endocytic pathways, in both caveolae-dependent and clathrin-dependent mechanisms, whereas uptake from live cells appeared to be a clathrin-dependent, caveolae-independent pathway.

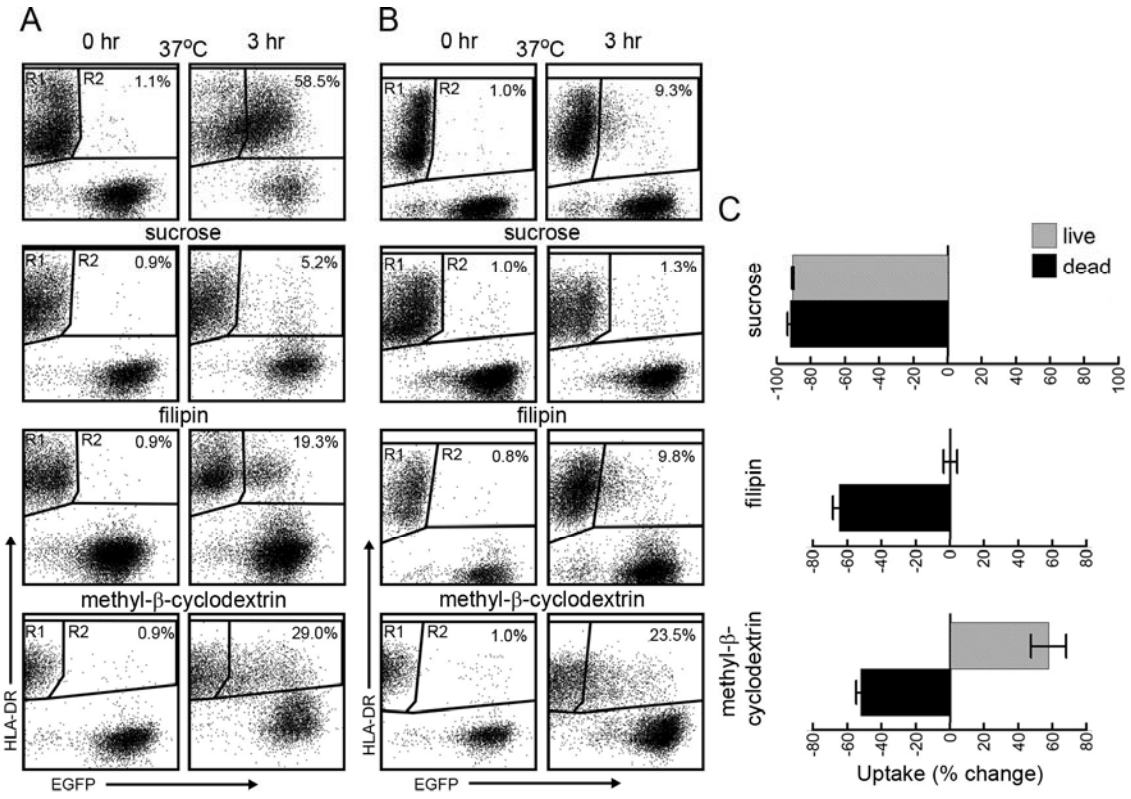

Figure 7. DCs capture cell-associated protein from live cells in a clathrin-dependent manner.

Dot plots quantifying DC uptake of apoptotic (A) and live (B) MSlmp2egfp with various inhibitors of endocytosis. DCs were incubated at 37°C with nothing, sucrose, filipin, or methyl-β-cyclodextrin then analyzed as in Fig 3B. Percent uptake R2/(R2+R1)*100 is indicated in the upper right hand corner of each dot plot. Gates were based on 0 hr for each individual inhibitor. Representative plots of triplicates are shown. (C) Change in percent uptake by DC of apoptotic (black bar) and live (hashed bar) MSlmp2egfp caused by each inhibitor. Percent change in uptake of each inhibitor used in A & B, is calculated based on the 37°C positive control. Data in bar graph illustrate standard error of triplicates. Percent change = 100 – (% uptake of inhibitor / % uptake of control x 100).

3.5.5 Captured proteins from live and apoptotic cells are found in similar compartments within the internal endocytic processing pathway of DCs

Given that internalized protein can go through different processing pathways within the DCs, we next wanted to determine where the internalized protein localizes within different compartments of the endocytic pathway of DCs. In order to visualize EGFP with the various vesicles, DCs were incubated with different inhibitors to prevent the acidification of the early endosome, late endosome, and lysosome. After incubating with the target cells for 1 hr, the various inhibitors were added and permitted to co-incubate for 3 hrs more. Cells were then harvested and stained for each of the organelles. We observed DCs that were incubated with dead target cells had internalized green protein which localized to the early endosome, late endosome, and lysosome (Figure 8A), as indicated by the arrows. We saw similar results when the target cell was a live cell. In all cellular compartments visualized, internalized membrane from live cells can be observed as small green punctuate structures (Figure 8B). Therefore, even though live-cell associated membrane protein was captured and taken in via clathrin-coated pits, and apoptotic protein was taken in via both clathrin-coated pits and caveolae, we saw internalized protein from both types of target cells in the same intracellular compartments.

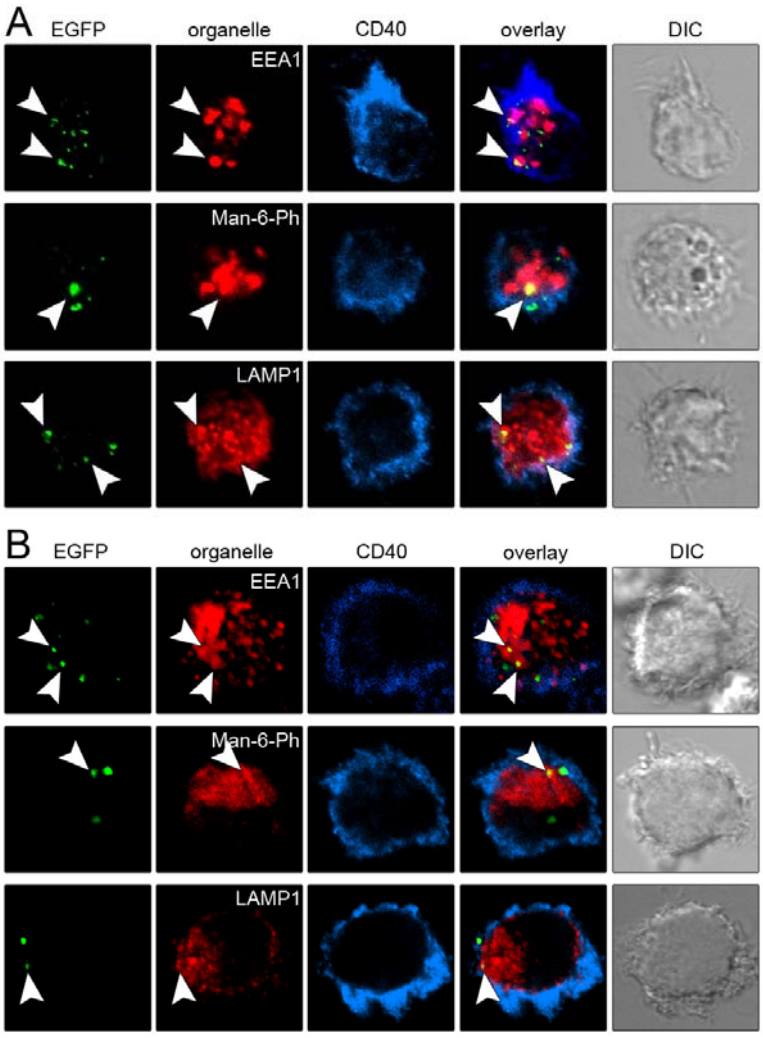

Figure 8. Membrane internalized from live and dead cells travels through the same processing pathway in DCs.

Immature human DCs were incubated with dead (A) or live (B) MSgp100egfp with bafilomycin, chloroquine, or NH_4Cl to inhibit acidification of vesicles and break down of protein. DC were identified with CD40 (blue), and the early endosomes, late endosomes, and the lysosomes (all red) were identified with the early endosomal Ag 1 (EEA1), mannose-6-phosphate (Man-6-Ph), and lysosome associated protein 1 (LAMP-1), respectively. The internalized protein, seen as punctuate green structures, colocalized with each endocytic compartment (yellow) and is highlighted with an arrowhead.

3.6 DISCUSSION

This was the first study to create and employ a biologically relevant system using two applicable integral membrane proteins fused to EGFP to directly compare, quantitatively and qualitatively, DC uptake of cell-associated proteins from both live and dead cells. Using this system, we have demonstrated that DCs capture various integral plasma membrane proteins with greater efficiency and faster kinetics from apoptotic cells than from live cells. Additionally, in contrast to membrane-associated proteins, DCs were only able to capture cytoplasmic proteins from apoptotic cells and not live cells. When examining the method of protein internalization and intracellular processing, live-cell associated membrane protein entered the DC via a clathrin-dependent, caveolae-independent mechanism, whereas apoptotic material entered the DC via clathrin- and caveolae- dependent mechanisms. However, once inside the DC, both live cell and apoptotic cell-associated membrane proteins traveled through the same internal endocytic compartments.

DC uptake of membrane protein from live cells has been observed in a variety of experimental situations. Harshyne, et al. first illustrated that monkey DC capture of cell-associated membrane protein from live cells was an efficient process, with over 90% of DC capturing fluorescently labeled plasma membrane proteins from various populations of live target cells (101). Freshly isolated human tonsil DCs have also been shown to capture dye labeled membrane from a variety of live-cell lymphocyte targets with an efficiency of almost 40%, depending on the type of target cell (119). Another study used the membrane dye PKH67 to label a HIV-infected human T lymphoblastoid cell line, and incubated them with DC, with or without apoptosis induction. They found that almost 100% of DC captured cell-associated membrane after an overnight culture, regardless if the target cell was dead or alive (120). Unlike

the previous studies which used membrane dyes to visualize DC uptake of membrane-associated protein, our system used two different integral membrane proteins fused to EGFP. With this more biologically relevant system, we found that DC uptake of cell-associated membrane protein from live cells only reached an efficiency of almost 17% at 4 hrs, regardless of the target protein. This difference in efficiency from the studies using dye-based methods may be because with the current system the DC was required to capture an entire integral membrane protein and was not simply capturing fragments of dye-stained plasma membrane. The membrane dyes diffuse through the entire membrane, whereas EGFP was expressed on the internal side of the plasma membrane, fused only to one particular protein. Therefore, this system was more stringent than the dye based systems, requiring the DC to capture entire trans-membrane proteins for uptake to be observed. We then directly compared the efficiency of DC uptake of live-cell associated membrane protein to DC uptake of apoptotic-cell associated membrane protein. Not only did we determine that DC uptake of apoptotic membrane was over twice as efficient for the truncated EBV latent membrane protein 2 cell line (MSlmp2egfp), when we analyzed the other cell line expressing the melanoma gp100 protein (MSgp100egfp) we found that even though uptake of membrane-associated apoptotic protein was more efficient, the efficiency depended on the purity of the target cell population. This difference was probably due to the difference in protein expression in both cell lines. MSlmp2egfp was over 95% high expressers, while MSgp100egfp was between 85 – 90% (Appendix A, Figure 14). The observation that DC uptake of membrane-associated protein was more efficient when the target cell was apoptotic and had a high level of protein expression coincides with studies demonstrating that cell death and high Ag expression was needed for MHC class I-restricted cross-presentation and activation of naïve $CD8^+$ T cells (132). In addition, by observing uptake efficiency over time, we have demonstrated that DCs

started capturing live-cell associated protein immediately, though not in as large a quantity as when the target cell had undergone cell death. This finding is supported by studies of the kinetics of cross-presentation of live-cell associated proteins which found that DCs, as well as macrophages, cross-present Ag from live, vaccinia-infected cells within a few hours of the target cells becoming infected. They determined this by excluding cell death, not by actually visualizing uptake (120). Interestingly, while quantifying uptake via flow cytometry we observed two possible populations of DC when staining with HLA-DR, some cells had higher levels of HLA-DR than others (Figure 1B). This may indicate some spontaneous maturing of a portion of our DCs. It is of note to point out that the cells with high levels of HLA-DR did not capture membrane protein as efficiently as the DCs expressing lower levels HLA-DR. This observation corresponds with previous studies indicating that only immature DCs are capable of capturing cell-associated membrane and not DCs which have undergone maturation (101).

In contrast to membrane-associated proteins, cytoplasmic protein was not captured by DCs when the target cell was living, only when it had been induced to undergo apoptosis. This illustrates why pathogens may utilize an immune evasion mechanism wherein they reside within the infected cell without causing cell death, essentially hiding their antigenic proteins (133). If the pathogen remains intracellular the DC is less likely to detect the infection. Studies have shown when CTL interact with cells in the environment they ignore them or kill them, based on the target cells' extracellular protein expression. For example, in vivo, studies using fibroblasts expressing chicken OVA constructs at either the plasma membrane or cytosol, found that CTL had very low level of lytic activity when the OVA was cytosolic versus the other forms of OVA (134).

Though there are several mechanisms which have been proven to transfer cell-associated proteins from live cells to DCs, none seem to be involved in our assays. Exosomes are membrane vesicles that contain lipids, and other membrane proteins from the cell from they originate (78). Recently, migrating DC, generated either in vitro or from allografts, were demonstrated to transfer gfp-expressing exosomes to spleen-resident DCs (79). And even though tumor cells have been shown to produce exosomes (73, 135) they do not appear to be involved in any DC uptake in our assays because when incubated with our target EGFP expressing cells' supernatants, there were no DCs visualized that captured EGFP-containing exosomes. Another possible way DC may take up protein from other cell types is via tunneling nanotubules (TNT), which are long membrane tethers between cells, and have been shown not only to be a physical method for cells to communicate (86) but also a way for cell surface proteins to transfer from one viable cell to another (136). Although we did not do high resolution imaging to rule out TNTs between the tumor cells and DCs, if transfer of membrane proteins did occur via this mechanism it was likely to have a minor contribution. A few studies have looked into the transfer of proteins fused to EGFP via TNTs, and have found a small amount of membrane protein transfer via TNTs was observed after 24 to 48 hours (83, 136). We saw large amounts of capture proteins in the first few hours of DC-to-target cell contact.

We observed uptake of live, cell-associated membrane protein that was a dynamic process with DC interacting directly with the target cells. In addition to the requirement for direct cell-to-cell contact, the absence of cytoplasmic protein transfer from live cells would suggest that the mechanism of uptake of membrane from live cells involves the physical process of stripping proteins from the plasma membrane rather than budding off of the membrane. The physical process of budding is likely to carry within it cytoplasmic proteins within the bud.

Our data using inhibitors of different portals of entry into the DC via endocytosis, indicated DC capture of membrane protein from live and apoptotic cells involved different mechanisms. Endocytosis can be divided into phagocytosis, which is the uptake of particles, and pinocytosis, which is the uptake of fluid. There are four basic mechanisms for pinocytosis, which are clathrin-mediated endocytosis, caveolae-mediate endocytosis and a less know clathrin- and caveolae-independent endocytosis (28, 29). We discovered that DC uptake of live-cell associated protein occurs via clathrin-coated pits. This is not entirely unexpected, because ligand binding to SR-A has been shown to occur via clathrin coated pits (137, 138). However, other studies have shown that SR-A does not always colocalize with clathrin, as when SR-A is involved in receptor-mediated adhesion (139), so there was uncertainty as to which endocytic pathway the DC utilized to internalized live-cell associated membrane proteins. In addition, other receptors than SR-A may be involved in live cell-associated membrane uptake. We have previously shown that SR-A is involved, though SR-A antibodies do not block all uptake (102). Using fucoidan, a general blocker of all scavenger receptors, we were able to block over 90% of live cell-associate membrane uptake indicating that scavenger receptors other than SR-A are involved in uptake via the clathrin-coated pits (Appendix A, Figure 15).

Interestingly, the inhibitor MβCD actually increased the live-cell uptake capability of DC. This was possibly due to the mechanism by which that particular inhibitor works. MβCD actually extracts the cholesterol from the plasma membrane of cells, which in some cases, can result in the redistribution of receptors across the plasma membrane that are normally isolated within specific microdomains. This more even distribution of receptors across the membrane could ultimately result in an increase in protein uptake because of the greater surface area for protein to bind to its receptor (140). The profile of DC internalization of membrane-associated

protein from apoptotic cells was more complex. All inhibitors blocked the internalization of the apoptotic protein to a certain extent, with the most inhibition occurring with sucrose, the inhibitor of clathrin-mediated endocytosis. Apoptotic uptake has been studied to a greater extent and several receptors are involved, not just SR-A, including CD36, $\alpha_v\beta_5$, and $\alpha_v\beta_3$, to name a few (30, 31, 115, 116). Interestingly, apoptotic uptake has not been studied with direct relation to clathrin or caveolae. CD36, a class B scavenge receptor involved in internalization of apoptotic protein, has been implicated in pathways that are clathrin- and caveolae independent (141), as well as concentrated in caveolae microdomains (142). The integrins $\alpha_v\beta_5$ and $\alpha_v\beta_3$ are usually associated with caveolae, however they have been shown to bind to several pathogens that enter the cell through clathrin-dependent routes (143). Phagocytosis is another mechanism by which apoptotic protein can enter a DC, however, we are dealing with intact, membrane proteins and small pieces are entering the DC, as was visualized with the live-cell microscopy. In general, phagocytosis involves the engulfment of larger particulate matter (29).

After DCs capture and internalize protein, it can travel through the endocytic pathway, first entering the early endosome, then the late endosome, and lastly the lysosome. However, it does not have to go to every compartment. Sometimes the internalized exogenous protein travel through the pathway, yet exit the endosomal compartment before entering the lysosome (28). When we looked at the processing pathway of captured membrane protein we found that even though apoptotic-cell associated membrane protein entered via difference portals than live-cell, membrane-associated protein they travel though the same compartments within the DC. We found captured protein from both live and dead target cells in the early and late endosomes, as well as the lysosomes.

This was the first study to look quantitatively and qualitatively at the uptake of live-cell associated integral membrane proteins and directly compare it to the uptake of apoptotic-cell membrane associated protein. Live-cell, membrane uptake may not be as efficient as previously thought, but it still occurs at a high enough level to induce cross-presentation, as observed by other supporting studies. By highlighting the detailed similarities and differences in DC uptake of both live and apoptotic cell membrane-bound proteins we gain a greater understanding into how different characteristics of target cells in the body can influence an immune response, based not only on their viability, but also on the cellular localization of their various target proteins.

4.0 PRELIMINARY STUDIES ON THE ABILITY OF DENDRITIC CELLS TO STIMULATE INTERFERON-GAMMA PRODUCTION FROM $CD8^+$ T CELLS AFTER CAPTURING CELL-ASSOCIATED MEMBRANE PROTEIN FROM LIVE CELLS

Sherrianne M. Gleason*† and Simon M. Barratt-Boyes*†§

*Department of Infectious Diseases and Microbiology, Graduate School of Public Health, †Center for Vaccine Research, Departments of §Immunology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

4.1 PREFACE

The following work completes the requirements for specific aim 4. Sherrianne M. Gleason ran all experiments and wrote this chapter. The cell lines Mel397 and Mel526 were generously provided by Walter Storkus. The T cell clone R6C12 was generously provided by Steven Rosenberg.

4.2 ABSTRACT

Dendritic cells (DCs) internalize many types of antigens in the periphery including exogenous, cell-associate proteins. The ultimate purpose is thought to be cross-presentation to T cells, resulting in either immunity or tolerance. Previously we have shown that DCs are very efficient at capturing integral membrane proteins from live cells. In this study we wanted to examine the immunological significance of this observed transfer by examining the ability of immature human DCs to cross-present an epitope processed from an integral membrane protein captured from live cells. In order to distinguish cross-presentation from direct presentation, we used an HLA-A2 restricted system, with two T cell clones, TIL620 and R6C12 that recognize a specific epitope of the melanoma protein gp100 in the context of HLA-A2. Using the HLA-A2 negative melanoma cell line Mel397 which naturally expresses gp100 at the cell surface as a live donor cell for DCs, we found that DCs were unable to stimulate R6C12s to produce IFN-γ. The same result was seen with another HLA-A2 negative donor, the breast epithelial cell line MSgp100egfp stably transfected to express gp100 fused to EGFP at the cell surface, which failed to stimulate TIL620s. In contrast, the T cell clones produced massive amounts of IFN-γ via direct presentation with the HLA-A2 positive, gp100-expressing cell line Mel526 or with the gp100 peptide alone. Our preliminary results suggest that in the absence of any external stimuli, DCs are unable to cross-present antigens from protein captured from live cells.

4.3 INTRODUCTION

Dendritic cells (DCs) are the primary antigen presenting cell (APC) capable of cross-presentation, which is defined as the presentation of exogenous antigen in the context of MHC class I or MHC class II to naïve T cells (5, 10). The existence of a cross-presenting APC was first proposed by Bevan in 1987 (144), based on his discovery that minor histocompatibility antigens that were synthesized in one cell could be captured as and presented in the context of MHC class I to prime T cells (58). Since then, cross-presentation has been found to be important for the initiation of immune responses to viruses and tumors, often called cross-priming, as well as in the maintenance of peripheral tolerance to self, often called cross-tolerance (10, 145). Cell-associated antigens, in particular, have been shown to play a role in both cross-priming and cross-tolerance. Virus-infected cells, transfected tumors, normal tissue cells, and protein-coated cells have all been sources of cell-associated antigen for cross-presentation (146).

A lot of research into DCs and cross-presentation has been done in the field of tumor immunology, particularly with melanoma. Immature DCs have been shown to induce tolerance in metastatic melanoma (147). To generate an cellular immune response, a frequently used strategy is loading DCs with exogenous peptides from tumor-associated antigens (148). However, more interest has been shown in using whole tumor cells as a source for cell-associated antigen because it permits a wide range of antigens to be processed and presented by the DCs (149, 150),thereby increasing the likelihood of a positive immune response. Apoptotic and necrotic cells have both been used as effective donors of cell-associated antigen for DCs. For example with melanoma, DCs loaded with killed melanoma cells can cross-prime $CD8^+$ T cells against the melanoma antigens (151, 152). Clinical trials have also shown that DCs loaded with killed allogeneic melanoma cells can induce CD8+ T cell specific responses (153).

However, killed cells are not the only source of cell-associated antigen. Several studies have demonstrated the ability of DCs to capture protein from live, intact cells. Previous studies from our lab using DCs infected with an adenovirus expressing the melanoma protein gp100 demonstrated cross-presentation of the cellular antigen to a $CD8^+$ T cell clone, stimulating the production of IFN-γ (101).

Previously, we have shown that DCs are capable of efficiently capturing and internalizing cell-associated integral membrane proteins from live cells (Chapter 3). In this study, we looked at the cross-presentation capability of these DCs which have captured the cell-associated membrane protein from live cells, and compared it to direct presentation, using an HLA-A2 restricted system. We found that DCs were able to present directly to a T cell clone specific for an HLA-A2 positive restricted epitope of gp100, when pulsed with the gp100 peptide. In addition, the T cell clones were efficiently stimulated via direct presentation from a HLA-A2 positive tumor cell expressing gp100, in the absence of DCs. However, the HLA-A2 positive DCs were unable to induce IFN-γ production when the DC captured the gp100 protein from live, HLA-A2 negative cell lines expressing gp100. Attempts were made to influence the DCs ability to stimulate the T cells, via maturation stimuli, but confounding factors such as high background, made it impossible to determine if maturation would have increased cross-presentation of cell-associated antigen from live cells. Our results support the notion that immature DCs are very poor at stimulating a T cell response, even in a population of pure T cell clones specific for one epitope. This finding suggests that the phenomenon of live cell-associated protein uptake may play a role in the tolerance of T cells to cells in the periphery, and that live cells alone would be a poor source of antigen for *ex vivo* loading of autologous DCs for vaccination purposes, in the absence of any external adjuvant or stimuli to induce DC maturation.

4.4 MATERIALS AND METHODS

4.4.1 Cells

Immature DC were generated by culturing normal human $CD14^+$ monocytes in GM-CSF (1000 U/ml) and rhIL-4 (1000 U/ml) for 5-7 days, as previously described (122). The melanoma cell lines Mel526 and Mel397 were generously provided by Walter Storkus (University of Pittsburgh, Pittsburgh, PA) and maintained in complete RPMI-1640, with pen-strep and L-glutamine. The breast adenocarcinoma cell line MSgp100egfp, expressing gp100 fused to EGFP at the outer plasma membrane (described in Chapter 3, Materials and Methods) was maintained in selection media containing 600 mg/ml geneticin. The oligoclonal T cell line TIL620, which recognizes two epitopes of the melanoma antigen gp100 (154) was derived in the laboratory of Dr. Steven Rosenberg (National Institutes of Health, Bethesda, MD). The $CD8^+$ T cell clone R6C12, recognizing the melanoma peptide epitope gp100:209-217, and was isolated from a melanoma patient vaccinated with the gp100 peptide G9-209M (155) was generously provided by Steven Rosenberg.

4.4.2 Flow cytometry analysis

Immature DCs, Mel397, Mel526, MSgp100egfp, and MS were stained for flow cytometry analysis as previously described (122). Briefly, the cell lines were labeled with either an Ab specific for gp100 (BioGenex), HLA-A2 (BD Biosciences, Mountain View, CA) , or an isotype control Ab (BD Biosciences), and the DCs were labeled with the HLA-A2 specific Ab. Cy5 goat anti-mouse (JacksonImmuno Research Laboratories, Inc.) was used to visualize surface staining.

Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Data files were analyzed utilizing CellQuest software (BD Biosciences) using $\log_{10}$ fluorescence.

4.4.3 IFN-γ enzyme-linked immunospot (ELISPOT) assay

TIL620 cells or R6C12 cells (3×10^4) were co-cultured with the same number Mel526 cells or immature human DCs cocultured at a 1:1 ratio with MSgp100efp, MS, or Mel397 cells on Multiscreen-HA plates (Millipore) coated with anti-human IFN-γ Ab (1-DIK; MABTECH, Stockholm, Sweden). After 18-24 hr incubation, IFN-γ production was detected by labeling with a second anti-human IFN-γ Ab (7-B6-1; MABTECH) as described previously (156). Spots were enumerated with an Immunospot S4 Pro Analyzer (Cellular Technology Ltd., Shaker Heights, OH).

4.5 RESULTS

4.5.1 The two cell lines MSgp100egfp and Mel397 express similar amounts of the melanoma protein gp100 on the cell surface.

Previously in our lab, we generated and characterized the stable cell line, MSgp100egfp, which expresses gp100 fused to EGFP at high levels on the cell surface (see Chapter 3, Figure 1). For this study, we wanted to determine the ability of immature DCs to cross-present antigen from this cell line and compare it to a melanoma cell line, Mel397, which naturally expressed gp100 (157). Our T cell clones, TIL620 and R6C12 both recognize the gp100 epitope 209-217 in the

context of HLA-A2 (154, 155). To demonstrate cross-presentation our cell lines expressing gp100 had to be HLA-A2 negative. Staining with an anti-HLA-A2 antibody confirmed that both MSgp100egfp and Mel397 were HLA-A2 negative (Figure 9A). In addition, a control cell line, Mel526 expressing gp100 (158) was used for direct presentation and was confirmed to be HLA-A2 positive (Figure 9A). In order to make a valid direct comparison of MSgp100egfp with Mel397, we stained both cell lines with an anti-gp100 antibody to determine their level of gp100 expression, since the quantity of protein can influence the T cell response (132). When stained with an anti-gp100 antibody, both Mel397 and MSgp100egfp were shown to express similar levels of gp100 on the cell surface (Figure 9B). In addition, Mel526 expressed very high levels of gp100. We verified the validity of making a direct comparison between our system using a transfected cell line and the naturally expressing cell line with regards to T cell stimulation.

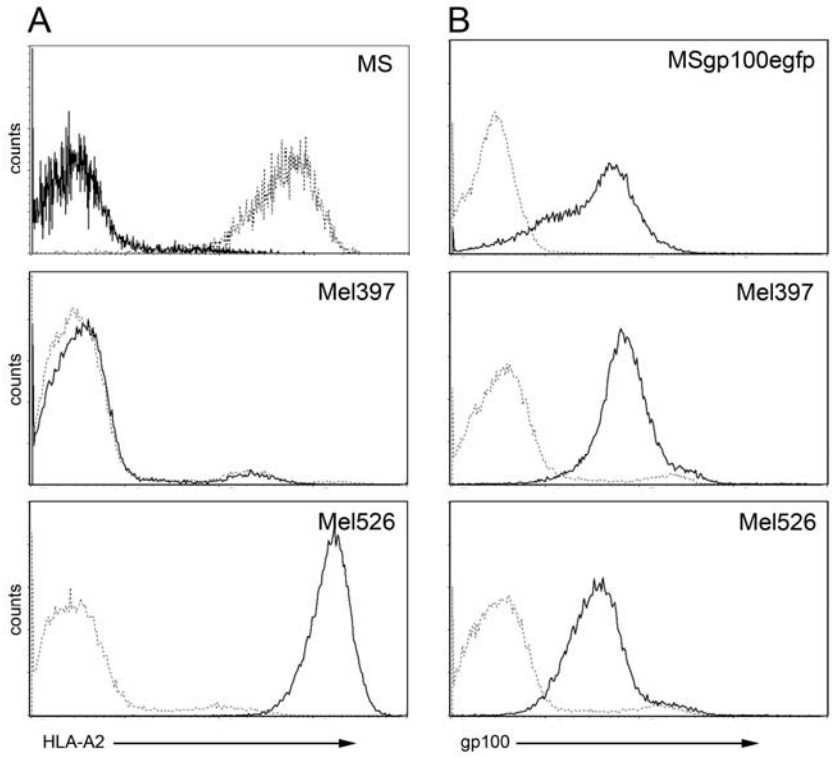

Figure 9. gp100 expression and HLA-A2 phenotype of target tumor cells.

(A) Targets cells were stained for HLA-A2 to determine HLA phenotype. Solid line = target cell, light dotted line = negative or positive control (cell line stained with IgG or Croft cell stained with HLA-A2). The Croft B cell line was used as a positive control for HLA-A2 staining (data not shown in all plots). (B) Target cells were stained with anti-gp100 antibody, and then counterstained with Cy-5. Solid line = target cell, light dotted line = negative control (cell line stained with IgG or MS cell).

4.5.2 Cell-associated membrane protein captured from live cells by DCs does not stimulate $CD8^+$ T cells to produce IFN-γ.

Now that we had both $HLA\text{-}A2^+$ and $HLA\text{-}A2^-$ cell lines that express gp100 we could determine the ability of DCs to stimulate T cell clones after capturing cell-associated Ag from the live tumor cells. A commonly used functional assay to measure the responsiveness of T cells to

stimulation by DCs is the ELISPOT assay. This involves filter-bottom plates pre-coated with anti-cytokine capture antibodies (159). For our experiment, we were measuring the ability of the TIL620s, a HLA-A2-restricted CTL line specific for the melanoma Ag gp100 (154) to produce IFN-γ upon interaction with HLA-$A2^+$ human monocyte-derived DCs. DCs and live MSgp100egfp cells were cocultured in the wells of an ELISPOT plate, along with the TIL620s for 18 to 24 hrs. A widely used method of stimulating T cells with DCs is to pulse the DC with a synthetic peptide (160, 161), therefore we used a gp100 peptide pulsed HLA-$A2^+$ DC, as well as peptide alone as positive controls. As expected, the peptide-pulsed DCs induced the TIL620s to produce IFN-γ, as well as the peptide alone (Figure 10). Cytokine production was not attributed to the DCs, as incubation of the HLA-$A2^+$ DCs with the TIL620s alone did not yield a positive response (Figure 8). In contrast to direct presentation, when TIL620s were cocultured with HLA-$A2^+$ DCs which had presumably captured protein from live, gp100-expressing MSgp100egfp cells, no significant cytokine production was observed (Figure 10). We know that this is not due to a defect in the DCs or the TIL620s, as the DCs were able to stimulate the TIL620s when directly pulsed with the gp100 peptide (Figure 10). To determine if the inability of the DCs to stimulate cytokine production via cross-presentation, was perhaps due to some structural change in the gp100 expressed by a vector or by a confounding factor created by the EGFP, we used the HLA-$A2^-$ cell line Mel397, which naturally expressed gp100 at the cell surface (157), as a membrane protein donor for the HLA-$A2^+$ DCs. For this assay, we used the T cell clone R6C12, which also recognized an HLA-$A2^+$ restricted epitope of gp100. Similar results were seen with the Mel397s as donor cells. R6C12s coincubated with HLA-$A2^+$ DCs and the Mel397 cells were unable to produce a significant amount of IFN-γ (Figure 11). Again, this was unlikely due to a deficiency in the DCs, as these DCs were generated with those incubated

with the MSgp100egfp cells (Figure 9), or a defect in the R6C12s, as they produced substantial amounts of cytokine when coincubated with the HLA-A2^{+} cell line Mel526 which naturally expressed gp100 (158) (Figure 11A). The R6C12s were so sensitive to direct presentation by the Mel526 cells that the number of spots indicating IFN-γ production, counted at approximately 700 spots per 30,000 T cells was an undercount (Figure 11A). The well was almost confluent with spots indicating IFN-γ production that the spot reader was unable to count them accurately (Figure 11B). The glaring difference between the ability of the T cells to respond to the gp100 epitope via direct and cross-presentation can be seen by visually comparing the ELISPOT wells (Figure 11B). An attempt was made to mature the DCs to promote their capability to cross-present the captured protein. However, high background and confounding factors such as incubation time, high background, and timing the addition of the maturation stimulus, inhibited our ability to determine if maturation of the DCs would have promoted cytokine production (data not shown). Therefore, our only finding is that these two experiments using the Mel397 cells and the MSgp100egfp cells illustrated an inability of the HLA-A2^{+} DCs to stimulate cytokine production via cross-presentation of protein captured from the membrane of live cells.

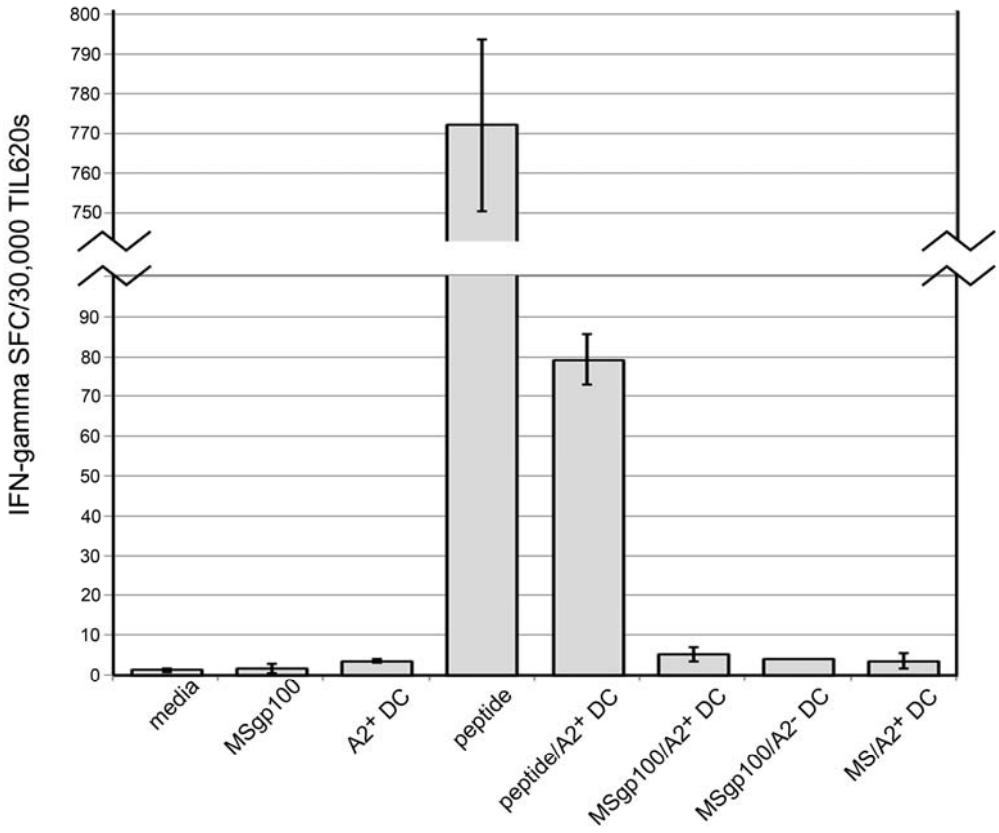

Figure 10. DCs were unable to stimulate cytokine production from a MHC class I-restricted CTL via cross-presentation of live-cell associated membrane Ag from a transfected cell line.

HLA-A-restricted CTL TIL620s were coincubated with HLA-A2+ DCs and gp100 expressing, HLA-$A2^-$ MSgp100egfp tumor cells. The ability of the DCs to cross-present and activate CTLs was determined by measuring the frequency of CTLs stimulated by IFN-γ ELISPOT. gp100-peptide pulsed HLA-$A2^+$ DCs and peptide alone with the CTLs were used as positive controls. HLA-A2- DCs with MSgp100egfp, MS with HLA-2^+ DCs, A2+ DCs, MSgp100egfp, and media were all used as negative controls.

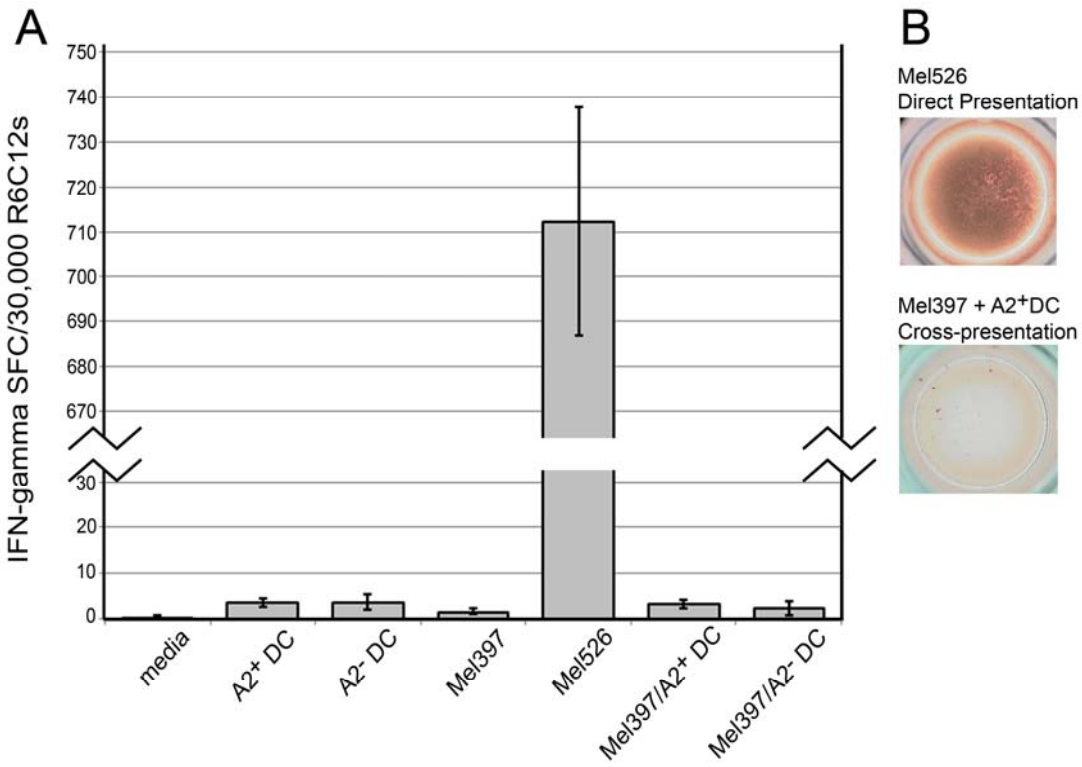

Figure 11. HLA-A-restricted CTLs responded vigorously to direct presentation but not to cross-presentation of cell-associated membrane gp100 Ag from live cells.

HLA-A-restricted CTL R6C12s were coincubated with HLA-$A2^+$ DCs and gp100 expressing, HLA-$A2^-$ Mel397 tumor cells. (A) The ability of the DCs to cross-present and activate CTLs was determined by measuring the frequency of CTLs stimulated by IFN-γ ELISPOT. gp100-expressing, HLA-$A2^+$ Mel526 cells were used to determine stimulation via direct presentation, as well as a positive control for the CTLs. HLA-A2- DCs with Mel397, HLA-$A2^+$ DCs, HLA-$A2^-$ DCs, Mel397s, and media were all used individually as negative controls. (B) Representative wells illustrating the frequency of CTLs producing IFN-γ via direct presentation (*top panel*) and cross-presentation (*lower panel).*

4.6 DISCUSSION

DCs are specialized at internalizing Ag in the periphery. In previous studies, we have shown that DCs are capable of efficiently internalizing membrane protein from live cells (101, 102), including integral membrane proteins (Chapter 3). In this study, we investigated the ability of immature DCs to stimulate IFN-γ production from HLA-A2 restricted T cell lines after capturing cell-associate membrane protein from live target cells. We determined that immature human monocyte-derived DCs were unable to proficiently cross-prime the HLA-A-restricted CTL lines after acquisition of the melanoma integral membrane protein gp100 from live tumor cells. This was not due to a defect in the CTLs because they were able to efficiently respond to the gp100 Ag via direct presentation from the gp100 peptide or from a HLA-$A2^{+}$, gp100-expressing cell line.

This data is in contrast to a previous study in our lab demonstrating that immature DCs were in fact able to stimulate the CTLs to produce IFN-γ (101). Recent studies involving the semi-maturation state of DCs provide a possible explanation that can account for the contrasting observations. The maturation state of DCs is determined by the microenvironment in which the DC captures exogenous antigens. DCs mature in response to danger signals such as products released from necrotic cells, inflammation cytokines, heat shock proteins, and pathogen-associated molecular patterns, such as LPS, DNA rich in CpG motifs, and double-stranded RNA (133). DCs have always been categorized as either immature or mature, with presentation in the mature state resulting in immune stimulation and presentation in the immature state resulting in tolerance. However, there is a semi-mature state, in which the DC can either induce tolerance or immunity, depending on the maturation stimuli. In particular, stimuli that induce the production of IL-12 results in the stimulation of an immune response, and when IL-12 production declines

as the DC fully matures the DC loses its ability to efficiently stimulate T cells (162). Our previous study used an adenoviral vector expressing gp100 to illustrate the ability of DCs to cross-present cell-associate antigen from live cells (101). Vujanovic et al, recently demonstrated that DCs transduced with recombinant adenoviral vectors developed a phenotype and cytokine profile of semi-mature DCs (163), and this semi-mature state may explain some of the IFN-γ production we saw in our previous study. Here, we eliminated the use of adenoviral vectors and then some of the risk of inducing semi-maturation in the DCs. The lack of semi-maturation may explain our lack of T-cells stimulation, though this remains to be proven.

In addition, immature DCs have low levels of costimulatory molecules (5), and if DCs present captured and processed antigen to T cells in the absence of costimulation, tolerance or anergy can result, so no IFN-γ will be produced (164). We provided no maturation stimulus, attempting to replicate the in vivo scenario of a DC interacting with a tumor cell in absence of inflammatory conditions. If we consider the maturation state of the DC, as well as its microenvironment in the assay, we would expect to see no IFN-γ production from the responding T cells, but perhaps induce tolerance or anergy in the CTLs. The induction of tolerance by DCs is important in avoiding autoimmune diseases caused by self-reactive T cells. Cross-tolerance is necessary because DCs do not produce tissue-associated antigens themselves (146). Several studies have provided evidence that cross-presentation is involved in tolerance induction using transgenic mice expressing model tissue-associated antigens (55, 59, 165, 166). Luckashenak was the first to demonstrate that constitutive cross-presentation of cell-associated tissue antigens was responsible for inducing CD8+ T cell tolerance in vivo (167). In contrast to tolerance induction, cross-priming stimulates an immune response against cellular antigens. This would be important when a cell other than a DC is infected by a pathogen, such as a virus, bacterium, or

parasite (145), or when an immune response is needed against antigens specific to different kinds of tumor cells (57), for example the melanoma antigen gp100.

Our preliminary findings suggest that immature DCs are unable to stimulate IFN-γ production from $CD8^+$ T cells specific for an HLA-A2 restricted epitope of gp100 when the antigen is captured from the membrane of live, intact target tumor cells in the absence of any external stimuli. This was demonstrated using more than one target cell line and was not due to an inability of the T cells to produce the cytokines because IFN-γ was produced efficiently when the gp100 peptide was presented via direct presentation. It is important to note that our study did not prove that DCs are unable to cross-present Ag obtain from the membrane of live cells under inflammatory conditions or with other maturation stimuli. It still may be possible for DCs to present cell-associated antigen from live cells to CTLs, resulting in IFN-γ production, if the appropriate maturation stimuli are present. In fact, studies using opsonized intact tumor cells have shown DCs are capable of efficiently cross-priming tumor specific T cells (168), so future studies may prove that live, intact tumor cells may provide a good source of tumor antigens to DCs to stimulate an anti-tumor immune response.

5.0 OVERALL DISCUSSION

"I shall endeavor still further to prosecute this inquiry, an inquiry I trust not merely speculative, but of sufficient moment to inspire the pleasing hope of its becoming essentially beneficial to mankind." -- Edward Jenner

Immunology, the study of all aspects of the immune system, can be traced back to Dr. Edward Jenner's discovery of a vaccine for smallpox in the late 18th century. Dr. Jenner's initial inquiry into the observation that milkmaids exposed to cowpox did not get sick from smallpox has developed into an area of study that plays a central role in the field of medicine (169). Several years later in 1972, Michael Sela defined immunogenicity as "the capacity of an antigen to provoke an immune response" (170). It was Dr. Ralph Steinman who, while trying to understand immunogenicity, discovered cells in the mouse that had the capability to form and retract processes, and because of these processes, or dendrites, they named these new cells dendritic cells (DCs) (171). Over the past several decades, DCs have come to the forefront as initiators and modulators of the immune response to antigens, playing a role in several branches of medicine, including infection, transplantation, cancer, autoimmunity and chronic inflammation, allergy, and vaccines (172). Studies into the basic biology of DC capture and processing of proteins abound and can lead to a better understanding of how DCs interact with the immune system and influence the immune response. Of particular importance, is how DCs

capture, internalize, and process exogenous antigens. After the recognition of antigen, these processes are the fundamental beginnings of our adaptive immune response.

DCs have the ability to regulate primary immune responses by directing T-cell mediated immunity (15). T cells are either stimulated resulting in expansion, deleted or suppressed, or tolerized (172). DCs do this by presenting endogenous antigen to $CD8^+$ T cells in major histocompatibility (MHC) class I, exogenous antigens to $CD4^+$ T cells in MHC class II and exogenous protein antigens to $CD8^+$ T cells in MHC class I (5, 15). Cross-presentation, which is the presentation of exogenous antigen to $CD8^+$ T cells, is essential in initiating a T cell response, be it immunity or tolerance (10, 145). It enables T cells to respond to pathogens which have a tropism for cells other than DCs but do not always induce death in the cells (54) or pathogens that functionally compromise DCs, impeding their ability to present antigens to T cells (10). Cross-presentation is also important in the generation of peripheral tolerance, or tolerance to self. DCs are capable of cross-presenting a wide spectrum of antigens, including soluble proteins (173, 174), immune complexes (175, 176), intracellular bacteria (177), parasites (178), and cellular antigens (146). The ability of DCs to cross-present antigens, as well as their influence on the T cell response, has made them the focus of studies to develop vaccines for the treatment and prevention of many kinds of diseases.

Cell-associated antigens are a major source of exogenous proteins for the cross-presentation pathway and as a result, have become a major focus of study (146). Cell-associated antigens were the first type of antigen reported to undergo cross-presentation. Bevan found that minor histocompatibility antigens that were synthesized in one cell could be captured and presented in the context of MHC class I to prime T cells (58). Since this discovery, numerous cell types have been shown to be cross-presented, including virus-infected cells (54, 56, 120,

121, 179), normal tissue cells (9, 55, 58, 59), and tumor cells (53, 57). The source of the cell-associated proteins can be from both apoptotic (30, 132, 179-183) and live cells (101, 102, 118-121).

This project aimed to add to the field of DC biology by further characterizing immature DC handling of cell-associated antigens from live cells and apoptotic cells. To do this, we created a biologically relevant, all inclusive system, using fusion proteins instead of artificially dye stained target cells, to directly characterize DC uptake of cell-associated proteins from live cells quantitatively and qualitatively. A method that has been well established to visualize protein dynamics in live cells involves the use of fluorescent tags (184). The most commonly used fluorescent protein is the enhanced green fluorescent protein (EGFP), which is extremely bright and has excellent photostability (185). Careful consideration was taken in choosing which proteins to use in our model system. Ultimately, we chose to fuse both the Epstein Barr Virus (EBV) latent membrane protein 2 (LMP2) which relates DCs and infectious diseases, and the melanoma protein gp100 which relates DCs and cancer, to EGFP. The results of our research will be presented in the context of the role of DCs in infectious diseases, as well as cancer.

5.1 DENDRITIC CELL CAPTURE OF CELL-ASSOCIATED VIRAL PROTEINS

Dendritic cells play an important role in the immune response generated against infectious diseases. With the wide variety of pathogens and their diverse mechanisms of pathogenesis, DCs must present both endogenous and exogenous pathogen proteins to T cells. Direct presentation occurs when pathogen infects the DCs without impairing the DCs ability to process and present peptides (186-188). However, many pathogens have developed mechanisms that interfere with

the antigen-presentation pathways of the cells they infect (189). They can do this by blocking DC maturation, directly killing the DC with toxins or inducing cell death, or altering expression of cell surface molecules (172). This interference, as well as the situation where pathogens have a tropism for cells other than DCs but don't induce cell death (190), requires that DCs use the cross-presentation pathway to stimulate $CD8^+$ T cells against several pathogens (10, 191). The capture and processing of exogenous cell-associated antigen is a major way for DCs to cross-present infectious disease peptides to T cells. Numerous studies have demonstrated that using cell-associated antigen from both live and apoptotic cells is an efficient way to load DCs. However, it isn't the only way DCs can capture cell-associated viral protein antigen from whole cells.

Over the past several years, the phenomenon of DC nibbling (101), the mechanism of which will be described in the Section 5.3, which is the active acquisition of proteins from live cells, has been shown to be another source of cell-associated antigen. It is a relatively new area of study in the field of DC biology, and there is limited knowledge about the actual process of live cell-associated protein uptake. To study this, we chose the EBV LMP2 as our model infectious disease protein. EBV is a viral pathogen that preferentially infects B cells and epithelial cells. It has the ability to persist in cells in a latent state without causing cell death. Several proteins are involved in the latent phase, including LMP2 (190). Our transfected cell line, MSlmp2egfp, expressed a truncated version of LMP2 on the cell surface fused to EGFP. With this cell line, we were able to quantitatively and qualitatively, directly compare DC uptake of a biologically relevant, viral, integral membrane protein from both live and apoptotic cells, something to our knowledge that has never been done.

We found striking differences in the ability of DCs to capture cell-associated protein from live and apoptotic cells; a decrease in cell viability led to an increase in DC uptake of cell-associated membrane protein. DCs were able to capture cell-associated protein from the apoptotic MSlmp2egfp cells with much greater efficiency than from live cells. Almost four times as many DC were able to capture cell-associated membrane protein from apoptotic cells than from live cells. Previous studies analyzing cell-associated protein uptake from live cells have shown almost 100% of DCs were capable of capturing membrane from live cells (120). Harshyne, et al. established that over 90% of immature monkey DCs captured labeled plasma membrane from various populations of live target cells, including T cells, B cells, macrophages, and other DCs (101). Freshly isolated human tonsil DCs captured dye labeled membrane from a variety of live-cell lymphocyte targets with an efficiency of almost 40%, depending on the type of target cell (119). When we used an integral membrane protein to visualize uptake, the result was a much lower percentage of DCs capturing live cell-associated plasma membrane protein, with approximately 16% capturing membrane. These percentages were much lower than the uptake percentages from the previous studies using dyes. A possible explanation may lie in the inherent properties of dye labeling. Membrane dyes diffuse through the entire membrane so the DC would only have to capture small fragments of the plasma membrane to visualize uptake. However, our EGFP marker was on the inner side of the plasma membrane, fused to only one kind of membrane protein. This would force the DC to take entire proteins, not just plasma membrane fragments, before membrane capture could be visualized.

The process of DC capture of cell-associated protein from live cells is an active process, as no plasma membrane transfer is seen when the cells were kept at 4°C. DCs were in fact very dynamic in their interactions with MSlmp2egfp cells, with differences in how they handled both

live and apoptotic target cells. Immediately upon encountering the apoptotic cells, the DCs started to investigate by extending long processes and touching the target cells. Very rapidly, they started to internalize the integral membrane protein, with fragments of captured membrane continuously streaming through the DC. Most DCs stayed in direct contact with individual groups of apoptotic cells once they started to take in protein, even attaching to the apoptotic cells and dragging them with them as they continued to move along. The lively movement of the DCs was also seen when they were surveying live MSlmp2egfp cells, but the dynamics appeared to be different. The DCs were constantly moving from cell-to-cell, feeling them with their processes. They would capture small membrane fragments from only some of the target cells, and then continue on, surveying other live cells. This difference in how DCs interacted physically with live and apoptotic cells may be an influencing factor in why we see so many more DCs capturing apoptotic protein, over 50% after only 4 hours of contact, than the number of DCs internalizing cell-associated membrane protein from live cells.

In contrast to pathogens that directly impede the immune response by affecting DC function; others simply evade the immune system by remaining intracellular and not killing the host cells. In fact, when we visualized DC uptake of protein from the cytoplasm, using another cell line which expressed EGFP intracellularly, we found that DCs were unable to internalize protein from the cytosol when the cells were living. Only upon the induction of apoptosis were DCs able to capture the cytoplasmic protein. This also supports studies that have compared the effect of protein localization on T cell stimulation. *In vivo* studies using fibroblasts expressing chicken OVA constructs at either the plasma membrane or cytosol, found that the CTL had very low level of lytic activity when the OVA was cytosolic versus the other forms of OVA (134).

Using our cell line expressing the EBV protein LMP2, we demonstrate that live cells are yet another way to efficiently supply protein antigens to DCs. The resulting effect of the mode of protein capture remains to be seen, however several studies suggest that this phenomenon is capable of stimulating T cell responses, sometimes even more efficiently than DCs loaded with apoptotic cells, at least *in vitro (120)*. This may be another way to load DCs for vaccination purposes to illicit a broad range of immune responses, including both $CD4^+$ and $CD8^+$ T cells.

5.2 DENDRITIC CELL CAPTURE OF CELL-ASSOCIATED MELANOMA PROTEIN

DCs have become a primary focus in vaccination protocols for cancer (5, 192-194). They can be used for the prevention and treatment of cancer (195), because tumors express several potential antigen targets, or tumor-associated antigens (TAAs), that can become immunogenic when presented by DCs. Researchers are continually trying to find the best, most effective way, to deliver exogenous antigen to DCs to stimulate a potent T cell response to the TAAs. Several vaccines utilizing DCs either by targeting them *in vivo* or *ex vivo* are being studied for many different types of cancers. There are three main ways that DCs can be targeted for cancer vaccines. DCs can be randomly targeted *in vivo* using various methods such as viral vectors, gene guns, and transduced tumor cells, they can be targeted *in vivo* specifically using anti-DC antibodies, or DCs can be used as vectors themselves by loading generating autologous DCs *ex vivo*, loading them with antigen, then returning them to the host (194).

At lot of research has been done with melanoma, a potent kind of skin cancer, because several TAAs have been identified as effective targets for DCs. Proteins of interest include

MART-1, tyrosinase, MAGE-3, and gp100; all tissue differentiation antigens. The melanoma protein gp100 is a good example of how antigens can be delivered in many different ways to DCs. DNA vaccination has shown mixed results in activating DCs to stimulate either $CD4^+$ or $CD8^+$ T cells (196-203). Viral vectors have also been used, but to a limited extent (204-208). Loading DCs with melanoma specific peptides is the most commonly used strategy (195, 209). $CD8^+$ T cell immunity has been generated by pulsing DC with different gp100-derived peptides (210-214). Recently, there have even been several clinical trials using gp100 peptides to induce an antitumor response, both by targeting DCs *in vivo* and loading them *ex vivo* (212, 215-220). The disadvantage of these methods however is that the generation of an immune response is based solely on the one or a few defined antigens. Therefore, several studies have looked into the use of cell-associated protein antigens from whole cells to illicit a T cell response.

When exposing DCs to the wide spectrum on antigens from a whole cell, there is chance to stimulate a broader immune response to more than one protein antigen. Loading DCs with total antigen preparations may allow proteins to be processed as they would be naturally in the body, allowing the DC to present natural epitopes as opposed to synthetic peptides. Tumor cell lysates are one way to expose DCs to all the proteins on a tumor cell. DCs loaded with allogeneic tumor lysate (221, 222) stimulated T cells in melanoma patients. Recently in a clinical trial, DCs loaded with killed, whole tumor cells stimulated T-cell immunity in some of the patients (153). Though the clinical study found T cells reacting to MART-1, an different independent study found that DCs loaded whole apoptotic and necrotic cells presented both MART-1 and gp100 melanoma antigens to $CD8^+$ T cells *in vitro* (152).

Another possible way for DCs to stimulate gp100 specific T cells is by capturing antigen from live cells. Previously in our lab, we demonstrated that DCs were capable of stimulating

gp100 specific T cell clones by capturing the gp100 protein from other DCs infected with an adenovirus expressing the gp100 protein (101). In this study, we wanted to determine the ability of DCs to cross-present a gp100 epitope to a cell clone using live cells as donors, but without the confounding factor of an adenovirus. For this, we used the melanoma cell Mel397 that naturally expressed gp100 on the cell surface, but in an HLA-$A2^-$ context. We also used Mel526 which expressed gp100 in an HLA-$A2^+$ manner as a direct presentation comparison. Surprisingly, we found that DCs were unable to stimulate the T cell clones via cross-presentation, even though they were able to very effectively stimulate the T cell clone with the Mel526. We knew that cross-presentation of live cell-associated antigens was less effective than direct presentation of the same antigens (101); however, we weren't expecting to see no amount of T-cell stimulation.

One possibility was that the DC wasn't actually capturing Ag from the live Mel397. We did not do any concurrent flow cytometry experiments to confirm that the DCs were capturing protein from the Mel397 cells, even though we have shown that DCs are able to capture protein from a wide variety of cell types. Even though we had already shown that DCs were capable of efficiently capturing cell-associated membrane protein from live cells using a cell line expressing EBV LMP2, we decided to also create a cell line, MSgp100egfp, expressing gp100 at the cell surface fused to EGFP. This would also help illustrate that DCs were capable of capturing more than one type of integral membrane protein. After confirming that Mel397 and MSgp100egfp expressed equivalent levels of gp100 at the cell surface, we again performed a T-cell stimulation assay and obtained the same results with the second cell line, with no cross-presentation of the gp100 protein. The lack of response was not due a lack of protein uptake by the DC. By visualizing gp100 transfer by following the fused EGFP, we found that over 16% of DCs were capable of capturing protein from the live MSgp100egfp cells at just 4 hours, and that the DC

were extremely active, continually extending their processes and surveying the surrounding tumor cells. The DCs were able to internalize cell-associated membrane gp100 with the same efficiency as they internalized the membrane LMP2. DCs also interacted physically with the live cells, just as they did with the MSlmp2egfp cell line. Since the DC are presumably capturing intact proteins, in order to stimulate T cells, the proteins need to be internalized and sent through the endocytic pathway to be processed. Using EGFP as a marker for gp100, we determine that the captured membrane protein was internalized, as was suggested by the live cell microscopy, and traveled through the early endosome, late endosomes, and lysosomal compartments. This demonstrated that the internalized protein was entering the appropriate internal compartments to be processed and cross-presented to the T cell clone.

Given the DC efficiently captured gp100 from the live cells and that the protein was internalized and moved through the endocytic processing pathway, why did we not see stimulation of IFN-γ production from the T cells. One possibility is that the DC remained immature after interacting with the Mel397s. In the absence of any danger signals, such as inflammatory cytokines, the DCs may not mature, and mature DCs are the kind that present to T cells (114). In our past study, it is possible that the adenovirus had a small effect on the maturation state of the DC, just enough to allow it to present effectively to the T cell clones.

This finding suggests that even though DCs are capable of capturing, internalizing and processing protein from live cells, without external stimuli, they may not be a good source of antigen when trying to stimulate an immune response against cell-associated antigens.

5.3 DENDRITIC CELL NIBBLING

There are several ways for DCs to capture protein from live cells. Exosomes, trogocytosis, tunneling nanotubules, and nibbling have all been shown to transfer intact proteins from a donor cell to a DC. Each has its own unique properties distinguishing them from each other. The main focus of our study was the phenomenon of DC nibbling of proteins from live cells. We believe our findings support the belief that nibbling is likely to be distinct mechanism from the other methods of protein capture.

Exosomes are released from many different cells types into the surrounding environment, which can then be internalized by DCs. This transfer of intact membrane protein via exosomes does not require cell-to-cell contact (78). In contrast, studies using transwells demonstrated that DC nibbling of intact proteins seemed to require direct interactions between the DC and the target cell. Harhsyne et al, demonstrated that DCs were unable to capture dye labeled membrane proteins from other live DCs when separated with a transwell (101). In a separate study, Maranon et al determined that although DCs were able to cross-present HIV antigens that had been captured from live infected $CD4^+$ T cells to CTLs, when the DCs were separated from the HIV-infected cells with a transwell, no cross-presentation was observed (120). In our current study, exosomes are not likely involved with our observed membrane capture because we did not see DC uptake from supernatant taken from the target tumor cells (Chapter 3), even though tumor cells have been shown to produce exosomes (223). If exosomes were present, they would have been in the supernatant.

Intact proteins from live cells are also capable of moving to DCs via tunneling nanotubules (TNTs). TNTs are tubular membrane bridges, connecting live cells, capable of transmitting intercellular signals (86), as well as transferring organelles (83, 224, 225), plasma

membrane components (83, 136), and cytoplasmic molecules (86, 136). Although we did not do high resolution imaging to rule out TNTs between the tumor cells and DCs, if transfer of membrane proteins did occur via this mechanism it was likely to have a minor contribution. EGFP-fusion proteins have been used to study the movement of membrane proteins across TNTs. By coculturing unlabeled PC12 cells with PC12 cells expressing an EGFP-fusion protein at the plasma membrane, Rustom et al, observed that the EGFP-fused plasma membrane proteins could travel across TNTs to be found in the membrane of unlabeled cells (83). Önfelt et al, also demonstrated transfer of a membrane EGFP-fusion protein from an EBV-transformed human B cell line to natural killer (NK) cells (136). However, the small amount of membrane protein transfer via TNTs was observed after 24 to 48 hours (83, 136). We observed DC capturing membrane proteins from live cells almost immediately upon coculture, with large amounts already captured at 4 hrs (Chapter 3).

DC nibbling is probably most similar to trogocytosis, both of which involve the direct physical contact of immune cells and the transfer of intact fragments of plasma membrane. Trogocytosis involves the formation and engagement of the immune synapse on the immune cells, including NK cells, B cells, T cells, and DCs (98) DC nibbling does not require an immunological synapse to occur. The DC has been shown to capture protein from live cells other than immune cells, including vaccinia antigen from a mouse fibroblast cell line (121) and protein from a transduced human adenocarcinoma cell line (102). Here, we directly demonstrated the ability of DC to capture two different integral membrane proteins from a transfected, intact, viable epithelial adenocarcinoma breast cell line (Chapter 3).

Not only does DC nibbling differ from other forms of live cell-associated protein antigen uptake, it has different characteristics from the uptake of cell-associated proteins from apoptotic

cells. Not only were proteins internalized more efficiently and with faster kinetics from apoptotic cells than from live cells, as discussed in Sections 5.1 and 5.2, there was a difference in the portal of entry into the DC. We found that DC captured cell-associated membrane proteins in a clathrin-dependent manner, whereas DC captured cell-associated membrane protein from apoptotic cells in both clathrin and caveolae-dependent mechanisms. These results coincide with what we know about the receptors involved in the capture of cell-associated proteins. DCs have a wide spectrum of receptors that internalize apoptotic associated-material, including integrins and scavenger receptors (116), and these receptors can be found in both caveolae and clathrin microdomains on the cell surface. In contrast, DCs use mainly scavenger receptors when capturing proteins from live cells, which are located within clathrin-coated vesicles. Our studies using various blockers of uptake, including fucoidan for scavenger receptors, with our system of characterizing uptake using integral membrane proteins and not dyes, supported previous research into the role of scavenger receptors in the acquisition of antigen from live cells.

5.4 PUBLIC HEALTH SIGNIFICANCE

Public health, as defined by the American Public Health Association, is "the science of protecting and improving the health of communities through education, promotion of healthy lifestyles, and research for disease and injury prevention" (APHA 2008). In order to protect and improve health we must first understand what influences our health, including our immune system. The immune system has two main functions: to recognize diverse substances (called antigens) and to generate some kind of response to the substance (172). These functions are involved in many areas of the public's health, including infection, cancer, autoimmunity, allergy,

and vaccines. A central cell of the immune system which can play a role in the immune response in all these areas is the DC. Because DCs are responsible for eliciting or suppressing responses from CTLs, they have become a main focus in vaccination research for both cancer and infectious diseases. The only way to develop safe and effective vaccines is to understand how DCs interact with other cells in the body and handle the various antigens they encounter. Our study looked in detail at how DCs handle cell-associated antigen from live cells, as well as apoptotic cells. Both sources of protein antigens have been shown to be important in immunity to infectious agents and tolerance to self antigens. By highlighting and understanding the differences in how DCs handle cell-associated antigen from both live and apoptotic cells, we added to the basic knowledge of DC biology, which is important to understand when developing vaccines for the prevention of diseases, both chronic and acute.

6.0 FUTURE DIRECTIONS

This dissertation has expanded on the understanding of DC handling of cell-associated protein antigens from both live and apoptotic cells. Our *in vitro* studies have highlighted differences in the uptake from live and apoptotic cells, with a strong focus on uptake of cell-associated antigen from live cells. Our observations lead to other questions that can be addressed in future studies. The following section lists some of the key areas that could be studied further, which would address some of the questions that have remained unanswered.

6.1 DETERMINATION OF RECEPTORS INVOLVED IN DC UPTAKE OF CELL-ASSOCIATED ANTIGEN PROTEIN FROM LIVE CELLS

Very few studies have been done to elucidate the DC receptor repertoire that is responsible for the uptake of cell-associated antigen from live cells. Thus far, SR-A has been shown to play a major role, though other receptors may be involved (102). In contrast, mannose receptors and α integrins do not play a role (120). This study supported data that scavenger receptors are the main receptors involved in uptake of material from live cells.

A technique that is increasingly used is called RNA interference which uses dsRNA to silence gene expression (226). dsRNA is a key intermediate structure in the replication cycle of many viruses, but is not found in eukaryotic cells; therefore it is regarded as foreign and thus

degraded in higher cells. Short strands of interfering RNA (siRNA), 20-25 nucleotides in length, are introduced into cells which signal the destruction of homologous mRNA molecules of similar sequences (227). A panel of siRNA could be used to silence various scavenger receptor expressions in our human DCs. Expression of the scavenger receptors could be monitored by antibody labeling and the ability of these the altered DC populations to capture antigen from live cells could be monitored in fluorescence assays.

6.2 DETERMINATION OF LIGAND ON DONOR CELLS TO WHICH THE DC BINDS TO INTERNALIZE PROTEIN FROM LIVE CELLS

Once the receptors have been determined, the next step is to determine the ligand on the live target cell to which the receptors are binding. DCs have been shown to capture cell-associated protein from many kinds of mammalian cells, so the ligand may perform a universal function in all cell types and may be conserved across species, as protein has been captured from both monkey and human cells, or the scavenger receptors are recognizing a molecular pattern on the target cells. Scavenger receptors are known to bind to poly anionic ligands. They are defined by their ability to bind chemically modified, by acetylation, oxidation, maleyated, and sulfated molecules (228). The most effective way to determine the ligand is to clone each scavenger receptor and the Biacore system could be used in the experiment. The scavenger receptor would be bound to glass plates, and then cell lysates would then be cycled over the plate. Following several washes of increasing salt concentrations, ligands with low binding affinity would be eluted first. Molecules with increasing binding affinities would follow the subsequent washing

steps. The proteins would then be separated on a gel and sequence analysis performed to determine the ligand or ligands.

6.3 EXAMINATION OF T CELL RESPONSES TO PROTEIN CAPTURED BY DCS FROM LIVE CELLS

Our greatest interest lies in the effect of DC capture of cell-associated protein from live cells affects the immune response to the protein. Our experiments with immature human DCs illustrated that the DCs were unable to stimulate IFN-γ productions from $CD8^+$ T cell clones. This coincides with our knowledge of how immature DCs are specialized at phagocytosis and not presentation. This situation resembles the steady state condition in the body where there are no danger signals affecting DC maturation. The environment in which the DC captures cell-associated protein from live cells may affect the response of the T cells. We attempted to determine the ability of DCs to stimulate IFN-γ production by maturing the DCs with CD40L. However, due to high background, and the limited number of T cells to optimize the assay, we were unable to interpret the results. We could try using cytokines, for example IL-1β, IL-6, and TNFα, in the coculture of DCs with live cells, simulating an inflammatory condition, to stimulate a response from the T cells.

APPENDIX A

SUPPORTING DATA FOR CHAPTER 3

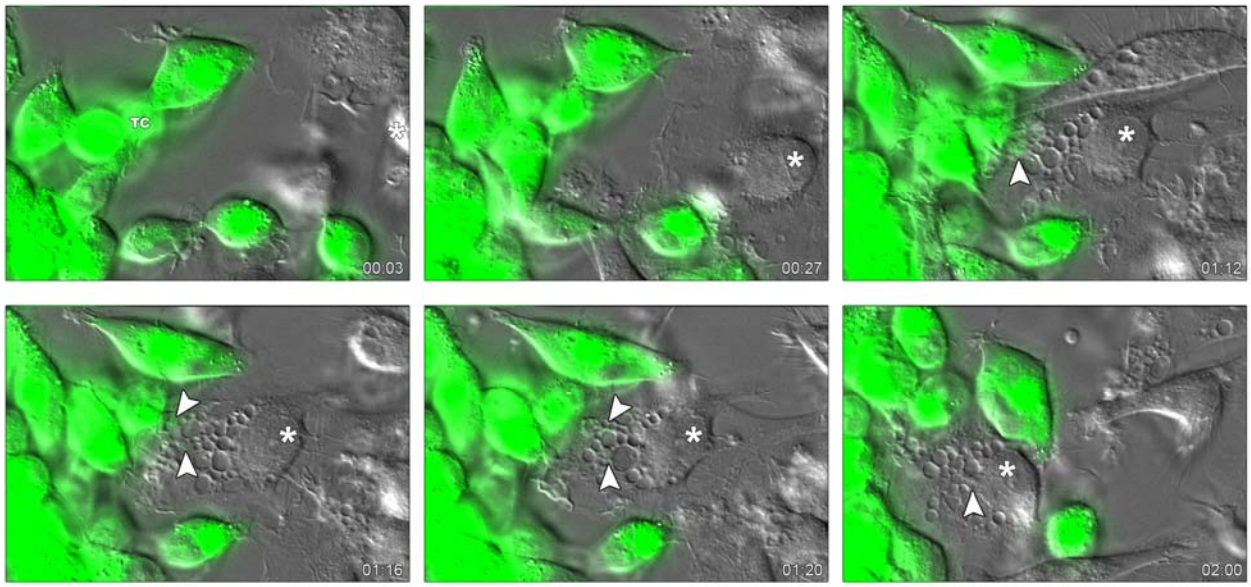

Figure 12. Live-cell imaging of DCs capturing cell-associated antigen from live MSlmp2egfp cells.

Live DCs were injected into a closed microscope chamber where MSlmp2egfp cells were adherent on a coverslip. Images were taken every 4-6 minutes then the desired field and frames were selected and stitched together in MetaMorph to create the movies. EGFP has been overexposed to detect the small punctuate structures within the DCs and overlayed onto the DIC. TC = donor tumor cell; * = DC capturing membrane; arrows = captured membrane.

Note: The stills in Figure 12 are taken from Supplementary Video 5

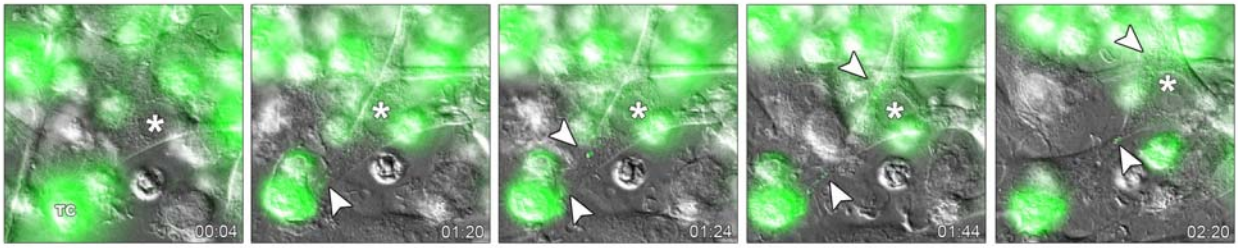

Figure 13. Live-cell imaging of DCs capturing cell-associated membrane protein from apoptotic MSgp100egfp cells.

DCs were permitted to adhere to a coverslip in a closed microscope system then apoptotic MSgp100egfp cells were injected into the system. Images were taken every 4-6 minutes then the desired field and frames were selected and stitched together in MetaMorph to create the movies. EGFP has been overexposed to detect the small punctuate structures within the DCs and overlayed onto the DIC. TC = donor tumor cell; * = DC capturing membrane; arrows = captured membrane.

Note: The stills in Figure 13 are taken from Supplementary Video 6

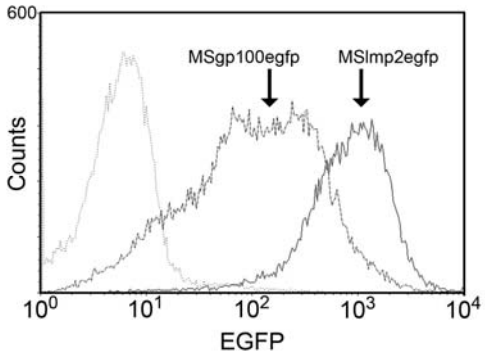

Figure 14. The EBV LMP2 cell line expresses higher levels of EGFP than the melanoma cell line MSgp100egfp.

Cells were harvested and EGFP expression was examined via flow cytometry. No antibodies were used. Solid line = target cells, light dotted line = negative cell line MS. MSlmp2egfp express higher levels of EGFP than the MSgp100egfp cells.

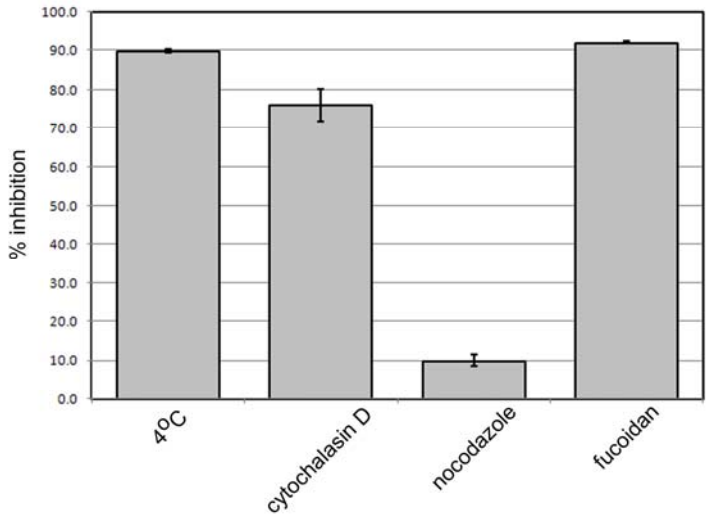

Figure 15. DC uptake of live-cell associates membrane protein is mediated by scavenger receptors and occurs in an actin dependent manner.

DCs were incubated with cytochalasin D, fucoidan, nocodazole, or at 4° for 30 min then live target cells were added for a coincubation time of 3 hours. Cells were harvested, stained, and analyzed as in Figure 1A. Percent inhibition was calculated by 100 – (uptake with inhibitor / uptake at 37°C x 100).

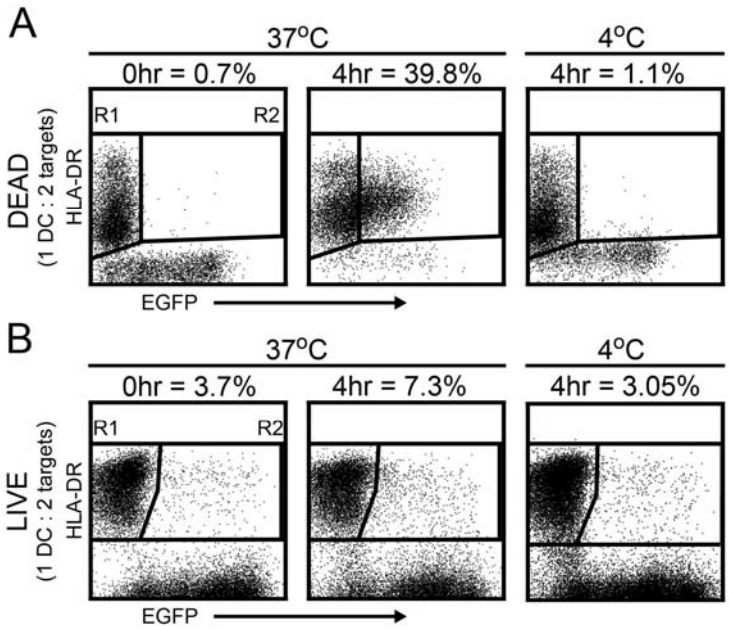

Figure 16. An increase in uptake efficiency of cell-associated membrane protein from apoptotic cells with an increase in the DC to target cell ratio.

DCs were incubated at a ratio of 1 DC to 2 tumor cells for 4 hours. Cells were then harvested and stained with pure HLA-DR they Cy-5 to detect HLA-DR expression. (A) DCs were incubated with apoptotic MSgp100egfp cells. (B) DCs were incubated with live MSgp100egfp cells. Uptake increased with more apoptotic MSgp100egfp cells. However, we were unable to determine the uptake efficiency with more live cells because the addition of more live cells resulted in the clumping of tumor cells and DC. A large number of events were excluded during the doublet analysis.

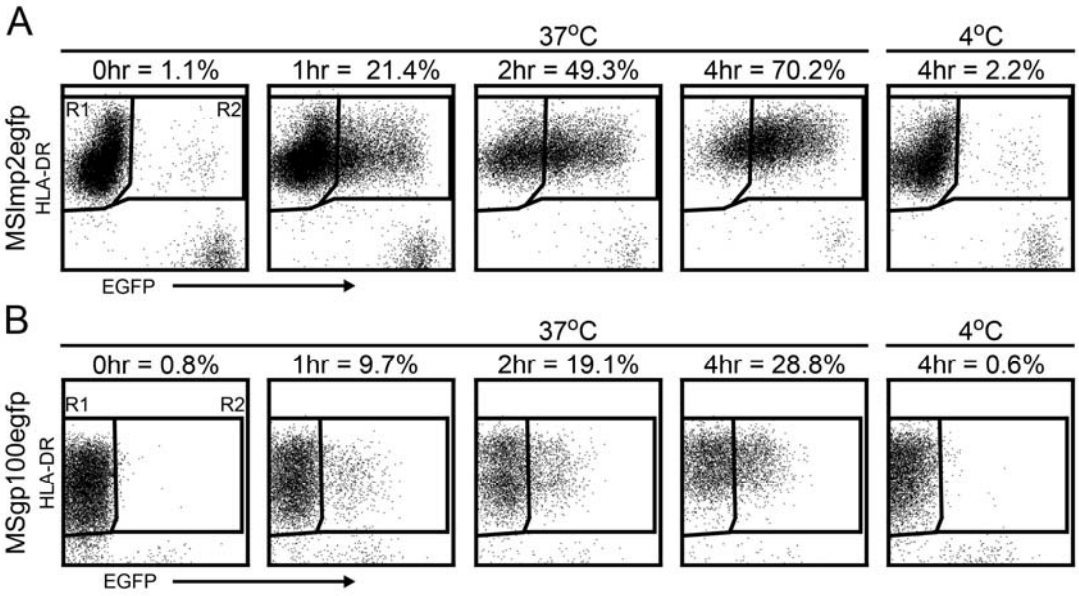

Figure 17. Maximum DC uptake capability of membrane protein determined using target cell lysates.

DCs were incubated at a 1:1 ratio with target cells that had been lysed by freeze-thawing in liquid nitrogen to determine the maximum amount of uptake possible with each target cell line. The DCs were incubated for 4 hours then cells were harvested every hour and stained with HLA-DR to visualize the DCs (A) DC were incubated with lyses MSlmp2egfp cells. (B) DCs were incubated with lyses MSgp100egfp cells.

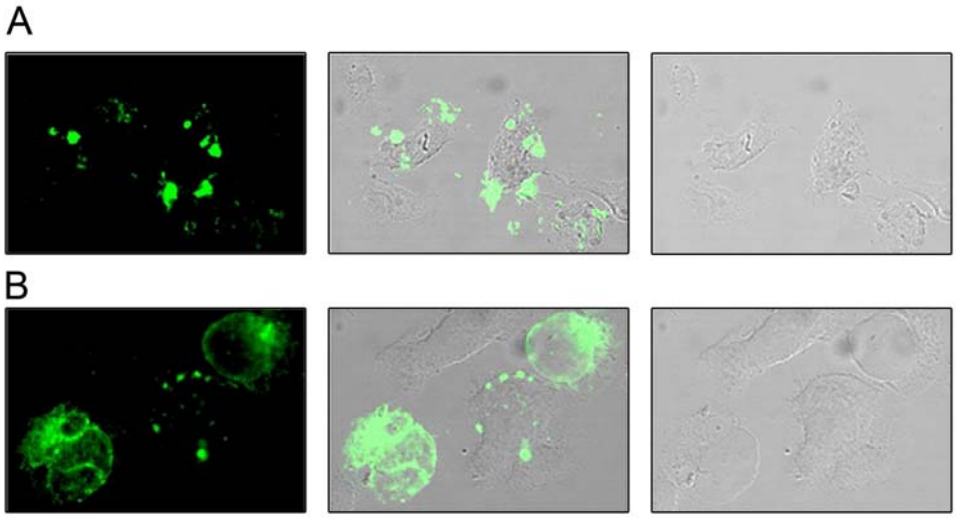

Figure 18. Physical characteristics of captured membrane-associated proteins from live and apoptotic cells.

DCs were incubated at a 1:1 ratio with MSlmp2egfp cells that were (A) apoptotic or (B) live. Cells were harvested after 3 hours then cytospun onto glass slides and fixed. EGFP expression was visualized using a confocal microscope and overlaid onto the DIC image. (A) Apoptotic-cell associated protein was captured in both large and small fragments, while (B) live cell-associated protein was captured in small fragments.

APPENDIX B

ABBREVIATIONS

Ab.....antibody

APC.....antigen presenting cell

DC.....dendritic cell

DNA.....deoxyribonucleic acid

EBV.....Epstein Barr Virus

EDTA.....ethylene diamine tetra-acetic acid

EGFP.....enhanced green fluorescent protein

ELISPOT.....enzyme linked immunospot

ER .....endoplasmic reticulum

GM-CSF.....granulocyte macrophage colony-stimulating factor

HI .....hexidium iodide

HLA .....human leukocyte antigen

IFN-γ.....interferon-gamma

IL.....interleukin

LMP2 .....latent membrane protein 2

MβCD .....methyl-β-cyclodextrin

MHC .....major histocompatibility complex

PBS .....phosphate buffer saline

PFA .....paraformaldehyde

SR-A .....scavenger receptor A

TAA .....tumor associated antigen

TIL .....tumor infiltrating lymphocyte

TNF.....tumor necrosis factor

TNT.....tunneling nanotubule

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