THE RELATIONSHIP BETWEEN DENDRITIC CELL MATURATION AND
EXOSOME CONTENT AND FUNCTION

by

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ABSTRACT

Dendritic cells (DCs) are a type of regulatory immune cell that shape the nature and duration of an immune response. DCs exist in an immature state and upon the detection of pathogens via Toll-like receptors (TLR), endocytose the pathogen, mature, and differentiate into sub-classes of cells with immunostimulatory or immunosuppressive roles. DCs are the most potent antigen-presenting cell (APC) to induce T-cell activation through the up-regulation of co-stimulatory molecules, antigen-presenting molecules, and cytokine secretion. Exosomes are nanovesicles secreted by most cells and serve critical roles in cell-cell signaling. DC-derived exosomes initiate the same type and intensity of immune response as the cell from which they originate. Exosomes have the potential to serve as immunotherapeutic cargos for cancer treatments because of their size, stability, and signaling capacity, however, the relationship between DC maturation and exosome content and function remains ambiguous.

In the current work, we present a mouse CD8α+ conventional DC (cDC) line, named MutuDC, as a model for studying the effect of various TLR-ligand (TLR-L) stimulations on DC maturation through the up-regulation of activation markers and cytokine secretion. Cell culture medium contains exosomes from fetal bovine serum (FBS). To ensure only MutuDC-secreted exosomes were characterized, the FBS was 100 kDa filtered. A significant portion of this work was dedicated to comparing the phenotypic expression of MutuDCs cultured in unfiltered FBS verses 100 kDa FBS
filtrate to ensure a similar profile was observed. Variations in surface marker expression between MutuDCs cultured in unfiltered and filtered FBS medium were insignificant. MutuDCs respond strongest to TLR3-L and TLR9-L, indicated by the up-regulation of co-stimulatory molecules CD40, CD54, CD80, and CD86; antigen-presenting molecule MHC class II; and cytokine secretion of IL-12. These data signify the MutuDCs have a T_H1-polarizing helper T-cell response with the ability to activate cytotoxic T-cells (CTLs) for an immunostimulatory reaction. Lastly, exosomes from immature and TLR3-L matured MutuDCs were harvested from cell culture supernatant, purified, and characterized. Preliminary results demonstrated exosomes express tetraspanins CD9, CD63, and CD81, and when activated, up-regulate CD40 and CD86. An increase in CD54, CD80, and MHC class II expression was expected, however results were inconclusive.
DEDICATION

I dedicate this thesis to my family who has supported me through my career and education. For my husband, Charlie, who put up with long days and nights studying, lengthy conversations venting my frustrations, and understanding my desires for personal and professional growth. To my parents Rob and Alyce, for their unconditional love and for providing me with the foundation needed to reach my goals. Most importantly, I dedicate this work to my daughter Lena. Thank you for keeping me in check and to not take life too seriously, to live in the moment, and for the amazing memories we make everyday together. I love you all so much.
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LIST OF ABBREVIATIONS

APC: Antigen-Presenting Cell
BMDC: Bone marrow-derived dendritic cell
CD: Cluster of differentiation
cDC: Conventional dendritic cell
CTL: Cytotoxic T lymphocyte
DAMP: Damage-associated molecular pattern
Dex: Dendritic cell-derived exosomes
ECM: Extracellular matrix
ELISA: Enzyme-linked immunosorbent assay
ER: Endoplasmic reticulum
ESCRT: Endosomal Sorting Complex Required for Transport
EV: Extracellular vesicle
FBS: Fetal bovine serum
FPLC: Fast protein liquid chromatography
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GFP: Green fluorescent protein
HRP: Horseradish peroxidase
Hsp: Heat shock protein
ICAM: Intercellular adhesion molecule
IFN: Interferon
IL: Interleukin
LAMP: Lysosome-associated membrane protein
MHC: Major histocompatibility-complex
MFI: Mean fluorescence intensity
miRNA: micro RNA
mRNA: messenger RNA
MutuDC: Murine tumor dendritic cell
MVB: Multivesicular bodies
NKT: Natural killer T-cell
OVA: Ovalbumin
PAMP: Pathogen-associated molecular pattern
PM: Plasma membrane
PRR: Pattern recognition receptor
RMFI: Relative mean fluorescence intensity
siRNA: small interfering RNA
TAA: Tumor-associated antigens
TC: Cytotoxic T-cell
TCR: T-cell receptor
T_{FH}: Follicular helper T-cell
T_{H}: Helper T-cell
TLR: Toll-like receptor
TLR-L: Toll-like receptor ligand
TNF: Tumor necrosis factor
T_{REG}: Regulatory T-cell
CHAPTER I
INTRODUCTION

The focus of this study is to investigate the relationship between dendritic cell (DC) maturation conditions and exosome content and function in order to advance the use of exosomes as an immunotherapy treatment. DCs exist in an immature state and upon activation by the detection of pathogens via Toll-like receptors (TLRs), up-regulate co-stimulatory molecules, antigen-presenting molecules, and cytokine secretion. Based on the pathogen encountered the DCs can polarize T-cells to induce an immunostimulatory or immunosuppressive response. DCs have been used therapeutically in cancer treatments showing a high efficacy in cancer-specific killing, however the process is invasive, time-consuming, and costly. DCs secrete exosomes, nanovesicles that display similar protein compositions and surface activation markers as the host cell, that can induce a comparable immune response. DC-derived exosomes are an attractive immunotherapeutic treatment alternative because of their size, stability, signaling capacity, and high bioavailability.

Work presented here focuses on examining the effect of various stimulation conditions targeting different classes of TLRs expressed on the DC surface and interior to induce the expression of various genes within the DC. The type of TLR-ligand (TLR-L) encountered dictates the maturation path and cellular fate of the DC, ultimately shaping the immune response. Specifically, the goals of this thesis are to determine the degree of expression of co-stimulatory and antigen-presenting molecules upon maturation with various TLR-Ls. Additionally, the DC cytokine profile will be evaluated to confirm DC maturation and T-cell polarization. MutuDCs require fetal bovine serum (FBS) in the
culture medium; however, exosomes derived from the FBS are present. This necessitates the culture medium be filtered of any FBS-derived exosomes. A significant portion of this study is dedicated to assessing the phenotypic profile of MutuDCs cultured in unfiltered and filtered FBS conditions to ensure a similar expression profile is observed. Finally, exosomes secreted into cell culture supernatant from immature and mature MutuDCs will be collected, purified, and characterized. This will provide evidence exosomes derived from mature DCs are capable of inducing a similar immune response as their cell of origin.

The ensuing chapters within this work provide an explanation and relevance for my research, a description of the experimentation that took place, a summary of results, and a discussion of the results including additional experiments necessary to supplement and confirm our data. Chapter Two is a review of pertinent literature and background information to understand the importance of my research. Chapter Three describes the methods employed including the instrumentation and materials. Chapter Four gives results of each experiment. Chapter Five provides a discussion of results, conclusions, and the future directions of this project.
CHAPTER II

REVIEW OF THE LITERATURE

2.1 Overview of the Mammalian Immune System

The mammalian immune system has two components, the innate immune system and the adaptive immune system. All cells of the immune system develop from hematopoietic stem cells during hematopoiesis. The body’s first lines of defense are physical and chemical barriers to prevent pathogens from entering deep tissues. Once these barriers are breached, however, the next line of defense is the innate immune system. The innate immune system is responsible in providing a rapid, non-specific response to a pathogen, wound, inflammation, or diseased cell using germ-line encoded receptors\(^1\). Innate immune cells consist of natural killer (NK) cells, phagocytes (such as neutrophils, macrophages, and monocytes), other leukocytes (such as eosinophils, basophils, and mast cells), and dendritic cells (DCs)\(^2\). Innate immune cells arise from myeloid progenitor cells\(^2\) except for NK cells, which arise from lymphoid progenitor cells\(^3\). The adaptive immune system consists of humoral (antibodies produced by B-cells) and cellular components (T lymphocytes). The adaptive immune response is highly specific to a particular pathogen and has a diverse repertoire of receptors due to genetic recombination\(^2\). Adaptive immune cells arise from lymphoid progenitor cells and consist of B-cells, T-cells\(^2\), and a subset of DCs\(^4\).

Innate immune cells display germ-line encoded molecules called pattern recognition receptors (PRRs) that recognize generic molecules called pathogen-associated molecular patterns (PAMPs) found on viruses, bacteria, fungi, or parasites; or damage-associated molecular patterns (DAMPs) found on dead, dying, or diseased cells\(^2\).
There are currently four classes of PRR families identified including transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and cytoplasmic proteins such as Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). The sensing of PAMPs or DAMPs by PRRs, except for some NLRs, results in the up-regulation of transcription genes involved in the inflammatory response. Inflammation is a crucial protective response by the body to remove harmful stimuli as well as a healing process to repair damaged tissue.

Adaptive immune cells display randomly generated receptors that bind to specific antigens rather than generic molecules found on the pathogens, thus allowing a highly diverse and specific immune response. Because of the diversity and specificity of adaptive immune cells, the response time is in days compared to the innate immune response, which is measured in minutes or hours. Adaptive immune cells also have persistent memory and a faster response time upon subsequent exposure. The innate and adaptive immune systems work cooperatively through the activation of innate immune response-producing signal molecules, cytokines and chemokines, that stimulate and guide the adaptive immune response.

Antigen-presenting cells (APCs) mediate the adaptive immune response through the phagocytosis, processing, and presenting of antigens for recognition by T-cells. Antigens can be “self” meaning derived from the host, or “non-self”, those derived from outside (foreign) sources. Professional APCs include macrophages, B-cells, and DCs; while all nucleated cells are non-professional APCs. T-cells cannot recognize antigens in the absence of antigen presentation; therefore the T-cell receptor (TCR) is restricted to recognizing antigenic peptides only when bound to the major histocompatibility complex
(MHC) class I or II molecules\textsuperscript{2}. A second type of antigen-presenting molecule is the cluster of differentiation (CD) family 1d (CD1d)\textsuperscript{7}. The CD1d family is a non-classical antigen presenting molecule that displays microbial lipids and glycolipids to regulate T-cells\textsuperscript{7}, and is discussed in more detail further in the literature review. The CD displayed on the T-cell, CD4 or CD8, dictates whether the TCR binds to MHC Class II or I, respectively.

Antigens derived from endogenous (intracellular) sources are displayed on MHC class I molecules\textsuperscript{8}. All nucleated cells express MHC class I molecules on their surface, thereby allowing them to act as non-professional APCs. It is imperative that nucleated cells are permitted to display endogenous antigens via MHC class I molecules in order to alert the immune system to virally infected\textsuperscript{8} or modified cells due to a variety of reasons including but not limited to age, death, or cancer. Endogenous proteins are degraded in the cytosol by the immunoproteasome, transported to the endoplasmic reticulum (ER) to be loaded onto a MHC class I molecule, and exported to the cell surface through the Golgi apparatus\textsuperscript{9}. A cytotoxic T-cell (T\textsubscript{C}) displays the co-receptor CD8 and binds to the region on MHC class I molecules containing endogenous antigenic peptides. Naïve CD8\textsuperscript{+} T-cells cannot directly eliminate transformed or infected cells, but must first be activated by an APC to become effector cytotoxic T lymphocytes (CTLs)\textsuperscript{9}. An example of a pathogen processed through the MHC class I pathway are viruses. Normal, healthy cells display their ‘maintenance’ intracellular peptide fragments via MHC class I molecules, allowing the cell to communicate with CTLs about what is happening internally. If a cell is infected with a virus, the cell will also display viral peptide fragments through their MHC class I molecules\textsuperscript{8}. Certain classes of CTLs monitor cell
surface MHC class I molecules for viral peptide fragments and can then eliminate the infected cell.

Antigens derived from exogenous sources are displayed via the MHC class II molecule. Only APCs are able to display MHC class II molecules to T-cells. Antigenic peptides are generated for MHC class II molecule display by proteolytic degradation in endosomal compartments. Examples of peptides displayed on MHC class II molecules include exogenous material such as apoptotic or necrotic cellular debris, antigens (foreign or self), cytokines, or signaling molecules. These proteins can be internalized through four major pathways: endocytosis, pinocytosis, phagocytosis, and macropinocytosis. The type of pathway an antigen will follow depends on its form, solubility, and whether it is part of an immune complex or associated with a pathogen. MHC class II molecules can also display endogenous components including plasma membrane proteins, components of the endocytic pathway, and cytosolic proteins that access the endosomes by autophagy. The MHC class II molecule-peptide complex binds to the co-receptor CD4 on helper T-cells (T_H). DCs have the unique ability to display exogenous antigens on MHC class I molecules, thereby communicating with and activating CD8^+ T-cells. This mechanism is called cross-presentation and is discussed in more detail further in the review.

DCs are the most potent APCs of the immune system. These cells are a link between the adaptive and innate immune system derived from both lymphoid and myeloid progenitors. DCs are widely distributed throughout the body, particularly in tissues that interface with the environment such as skin and mucosal surfaces, as well as in lymphoid organs. DCs consist of a heterogeneous collection of subsets based on
phenotype, function, and origin, with diverse roles necessary to cover the vast array of pathogens encountered by the immune system. The proceeding sections discuss the diverse and essential roles DCs play in the initiation of antigen-specific immune responses and how this can be exploited for immunotherapeutic treatments.

2.2 Dendritic Cells

DCs develop in the bone marrow from common DC precursors and give rise to plasmacytoid DCs (pDCs) or pre-conventional DCs (pre-cDCs). After exiting the bone marrow, pDCs migrate between the blood and secondary lymphoid organs including the spleen and lymph nodes. Pre-cDCs transiently circulate the bloodstream and relocate into secondary lymphoid organs or non-lymphoid tissues (skin, intestine, lung, skeletal muscle, and liver) where they differentiate into cDCs. cDCs in secondary lymphoid organs are non-migratory tissue resident cells, whereas cDCs in non-lymphoid tissues are migratory cells.

In normal, healthy conditions, DCs reside in a resting, immature state to maintain immune tolerance and constantly monitor for signs of invasion from a foreign body. As previously mentioned, DCs display TLRs that recognize a variety of patterns on pathogenic molecules, such as PAMPs or DAMPs. There are 13 murine (mouse) TLRs and the location of the TLR determines what it encounters and how the cell responds. Different subsets of DCs express distinct classes of TLRs, named TLR1 through TLR13, which contribute to their functional specialization. TLRs are located on the plasma membrane (PM) with the exception of TLR3, TLR7/8, and TLR9, which are localized in the endosomal compartment. PM-expressed TLRs are critical in the recognition of
Gram-positive and Gram-negative bacterial cell wall components, as well as bacterial flagella. Endosome-expressed TLRs are essential in recognizing bacterial and viral nucleic acids. After the recognition of a TLR-ligand (TLR-L), DCs internalize the foreign body and a signal transduction pathway is initiated resulting in transcriptional and protein changes to induce DC maturation in order to prime and activate T-cells. There are essentially “three signals” that indicate DC maturation has occurred including the up-regulation of antigen-presenting molecules, co-stimulatory molecules, and the secretion of cytokines (Figure 1).

**Figure 1**

![Diagram](image.png)

**Figure 1. Dendritic cell and T-cell interaction**. Image shows the three signals required for T-cell activation from a DC: antigen presentation via MHC molecules, co-stimulatory molecules, and cytokine secretion. Image taken from De Koker et al., 2011.

The first signal is the engagement of the TCR with an appropriate peptide-MHC complex, the second signal is co-stimulation (accessory signals) that together with the first signal induce immunity. The up-regulation of antigen-presenting and co-stimulatory molecules occurs nearly simultaneously. Finally, the third signal is delivered from the DC to the T-cell as soluble factors, such as cytokines and chemokines. The
soluble factors control the development of naïve T\(_H\) cells into subsets or the development of CTLs\(^{20}\), and is discussed further later in the review.

2.2.1 **Dendritic Cell-T-cell Interaction: “First Signal”**

DCs are highly efficient at antigen-presentation and adaptive immune cell activation due to their ability to determine if the antigen is endogenous or exogenous\(^1\). As discussed, DCs display endogenous antigenic peptides via MHC class I molecules and exogenous peptides via MHC class II molecules, in order to communicate with CD8\(^+\) or CD4\(^+\) T-cells, respectively. DCs are the only APC with the unique ability to undergo cross-presentation and display exogenous antigens on MHC class I molecules to activate CD8\(^+\) T-cells\(^2\). Naïve CD8\(^+\) T-cells cannot directly eliminate transformed or transfected cells until they are activated by an APC to become a CTL\(^9\). This diversion of exogenous antigens to an endogenous pathway is vital in priming CD8\(^+\) T-cells with information about the cell’s environment, such as a tumor or virus infection, when the DCs are not directly infected or impaired\(^21\).

The intracellular pathway for DC cross-presentation is still under debate, however, two pathways proposed for the cross-presentation of exogenous antigens are the cytosolic and vacuolar pathways\(^9\). In the cytosolic pathway, an exogenous antigen undergoes phagocytosis and is transported to the phagosome followed by exportation to the cytosol where it is degraded to a peptide by the proteasome\(^22\). From there, the antigenic peptide can be re-imported into the phagosome (phagosomal loading) or transported to the ER (ER loading) for MHC class I loading and cell-surface antigen presentation\(^22\). In the vacuolar pathway, exogenous antigens undergo phagocytosis.
followed by transportation to the phagosome for degradation\textsuperscript{23}. The antigenic peptides are loaded on the MHC class I molecule in the phagosome then transported to the cell surface for antigen presentation\textsuperscript{23}.

In addition to MHC molecules, CD1 molecules are another way for cells to present antigens for T-cell activation. The CD1 family are a nonclassical, antigen-presenting molecule of microbial lipids and glycolipids involved in the regulation of T-cells that can also be expressed on DCs\textsuperscript{24}. There are four CD1 proteins (CD1a-d) in humans and one (CD1d) in mice\textsuperscript{24}. CD1d is expressed at higher levels on lymphoid DCs and can be up regulated on myeloid DCs by culturing \textit{in vitro} or stimulating with lipopolysaccharide (LPS). CD1 molecules can present both endogenous and exogenous lipids. While there is much to be learned about these pathways and the molecule’s contributions, studies have shown the two CD1 pathways can contribute to microbial immunity, autoimmunity, and antitumor responses\textsuperscript{24}. The CD1d surface marker is an indicator of the DC phenotype.

In summary, the first signal that occurs during DC maturation and T-cell activation is the presentation of antigenic peptides through MHC molecule presentation. Exogenous antigens are displayed on MHC class II molecules and bind to CD4 T-cells, whereas endogenous antigens are displayed on MHC class I molecules and bind to CD8 T-cells. Cross-presentation is a phenomenon enabling DCs to display exogenous antigens on MHC class I molecules for CD8 T-cell priming. Lastly, a non-classical form of lipid antigen presentation is the CD1 family, however this is not explored in this research project.
2.2.2 Dendritic Cell-T-cell Interaction: “Second Signal”

The signaling of co-stimulatory molecules occurs simultaneously with the presentation of foreign peptides via the appropriate peptide-MHC complex with the TCR and either CD4 or CD8. This is the second signal required for T-cell activation and memory generation. Co-stimulatory molecules ensure an efficient amplification of signaling naïve T-cells\(^{25}\). The main signal occurs through the engagement of CD28 on the T-cell with a member of the B7 family, B7.1 (CD80) and/or B7.2 (CD86) on the DC\(^{25}\). The engagement of CD28 by the B7 family recruits membrane rafts containing kinases and adapters to the synapse, amplifying the signaling process and activation of naïve T-cells up to 100-fold\(^{25}\). Homologous but opposite of CD28 is the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a T-cell receptor that binds to CD80 and CD86, functioning as an immune checkpoint and down-regulates the immune response\(^{26}\). CD80 and CD86 have non-equivalent roles in immune modulation due to differences in interactions with CD28 and CTLA-4\(^{26}\). Based on crystallographic studies and surface plasmon resonance (SPR) measurements, the CD80-CTLA-4 interaction is of higher affinity than CD86-CTLA-4 interaction, while the CD86-CD28 interaction is of higher affinity than CD80-CD28 interaction\(^{26}\). This is due to CD86 existing as a monomer whereas CD80 forms a dimer, and CD28 homodimers are monovalent; demonstrating that CD28 favors binding to CD86 over CD80\(^{7}\).

Other important co-stimulatory phenotypic markers indicative of DC maturation, but not necessarily categorized as a “second signal”, are CD40 and CD83. CD40, a member of the tumor necrosis family (TNF)-receptor superfamily, binds to CD154 (CD40L) on T-cells and leads to an increased expression of CD80/CD86 and cytokine
release, thereby enhancing DC activation\textsuperscript{27}. The CD40L-CD40 interaction also has the ability to induce high levels of IL-12 to polarize CD4\textsuperscript{+} T-cells toward the T\textsubscript{H1} sub-type, enhance proliferation of CD8\textsuperscript{+} T-cells (CTLs), or activate NK cells\textsuperscript{27}. CD83 is predominately expressed on mature human and mouse DCs of various subsets\textsuperscript{28}. Studies have shown that CD83 is an important activator for CD4\textsuperscript{+} T-cells and is essential for normal immune system development and maintenance\textsuperscript{28}. While research is ongoing in determining the exact role CD83 has in CD4\textsuperscript{+} T-cell development, studies have revealed the requirement of CD83 expression\textsuperscript{28}.

\textbf{2.2.3 Dendritic Cell-T-cell Interaction: “Third Signal”}

The third signal to induce T-cell activation is the production of soluble protein factors called cytokines secreted from DCs. Cytokines regulate the duration and intensity of an immune response. Cytokines secreted by cells are recognized by cytokine receptors and fall into five families: Immunoglobulin (Ig) superfamily receptors, Class I (hematopoietin) family, Class II (interferon, IFN) family, TNF family, and chemokine receptor family\textsuperscript{2}. There are more than 200 different cytokines exhibiting various biological activities including pleiotropy, redundancy, synergy, antagonism, and in some instances cascade induction\textsuperscript{2}. In order to efficiently respond to different types of pathogens resulting in maximum effectiveness and minimal damage, DCs secrete specific cytokines that polarize naïve T\textsubscript{H} cells. The polarizing signal results in the production of T\textsubscript{H} cell subsets, leading to effector immune function control\textsuperscript{20}. Further details of the T\textsubscript{H} subsets are discussed in a proceeding section.
2.2.4 Dendritic Cell: Tissue Migration

Cell targeting and adhesion molecules assist in mediating DC-T-cell clustering and interaction. There are various complexes associated in the interaction of cells with their extracellular environment or with other cells. Cell-cell adhesion molecules allow the cell to “shake hands” with another cell\(^\text{29}\). Cell-matrix adhesion molecules allow a cell to pull itself forward through a matrix\(^\text{29}\) and aids in leukocyte extravasation\(^\text{30}\). Integrins including CD11b and CD11c, are proteins that attach the cell cytoskeleton to the extracellular matrix (ECM) and function as adhesion receptors for extracellular ligands to sense whether adhesion has occurred\(^\text{31}\). Integrins function bi-directionally, meaning they can transmit information from both the outside into the cell and vice versa\(^\text{32}\). CD11b and CD11c expression also help differentiate DC subsets. DCs from the myeloid lineage generally express both CD11b and CD11c, whereas DCs from the lymphoid lineage tend to be low or negative in CD11b expression and high in CD11c expression\(^\text{33}\).

Immunologically important integrin ligands are intercellular adhesion molecules (ICAMs), members of the Ig superfamily\(^\text{34}\). ICAM-1 (CD54), a surface molecule expressed by activated DCs, forms rigid extracytoplasmic rod domains with adhesion molecules on T-cells in order to improve the binding strength and therefore communication between two cells\(^\text{35}\). Additionally, CD54 aids in the extravasation of leukocytes from blood vessels in areas of inflammation\(^\text{2}\). CD54 is a ligand to complement receptor 4, a family of integrins composed of a heterodimer of CD11c or CD11b and CD18, a molecule expressed by all leukocytes\(^\text{36}\). The up-regulation of CD54 expression is indicative of DC maturation.
The migration of DCs through the body is paramount in eliciting a powerful immune response. Chemical signals from small molecules produced endogenously, called chemokines, must be up-regulated in order for DC migration to occur\(^{37}\). The directional movement, or chemotaxis, of DCs is initiated when the chemokine receptors in the plasma membrane are activated\(^{37}\). The chemokine receptor CCR7 is up-regulated in matured DCs, enabling them to access lymph vessels and migrate to draining lymph nodes to activate naïve T-cells\(^{38}\). In addition to chemotaxis, CCR7 also regulates the migratory speed of DCs\(^{38}\) and mediates leukocyte adhesion by activating integrins on the leukocyte cell surface\(^{39}\).

### 2.3 Immunosuppressive verse Immunostimulatory Roles of Dendritic Cells

DCs play a pivotal role in deciding the fate of the immune system’s response to a pathogen. Depending on the type of foreign body (antigen) encountered, the activation state of DCs can lead to immunosuppression (tolerance to an antigen) or immunostimulation (reaction against the antigen). Immunosuppression may lead to anergy, tolerogenicity, or pro-tumorigenicity. There is much debate in the literature as to the nomenclature or terminology used when describing the maturation states of DCs. Previous reports have used the terms “activation” and “maturation” to describe the transition of DCs from an immature state to a mature state, and therefore able to elicit an immune response, interchangeably, however other researchers have deemed these as distinct processes\(^{40}\). Maturation occurs when DCs differentiate from an immature to a mature state and activation is a process requiring additional stimuli, causing mature DCs to go from a tolerogenic state to an immunogenic state\(^{40}\). It is universally accepted,
however, that DCs are matured and activated upon encountering inflammatory cytokines or bacterial or viral products. These may include outer surface membrane components of Gram-negative bacteria known as lipopolysaccharides (LPS); single-stranded synthetic DNA molecules such as oligodeoxynucleotides (ODNs) containing CpG motifs (shorthand for 5’-C-phosphate-G-3’); or synthetic double stranded RNA including polyinosinic:polycytidylic acid (Poly I:C)\textsuperscript{40}.

DCs exist in two main states: steady state immature and fully mature\textsuperscript{1}. The distinction between the states is based on changes occurring at the phenotypic and functional level. Steady state immature DCs exhibit continuous endocytic activity and display “self” antigens to T-cells leading to an immunosuppressive role, or tolerance\textsuperscript{1}. Immunotolerance maintains immune checkpoint pathways that are crucial for self-tolerance and modulation of the duration and amplitude of an immune response\textsuperscript{1}. Self-tolerance is necessary to prevent autoimmunity. Immature DCs exhibit an immunosuppressive role resulting in regulatory T-cells (T\textsubscript{reg}) that further help to spread tolerance toward “self antigens”\textsuperscript{1}. T\textsubscript{reg} cells are a CD4\textsuperscript{+} subset and their role is to suppress, or negatively regulate, the immune response as well as play a role in lymphocyte homeostasis. Tolerogenicity typically occurs when T-cells receive insufficient co-stimulatory molecule signaling or deficiencies in cytokine secretion from DCs\textsuperscript{41}. There are cases where DCs retain their tolerogenic function even when phenotypically matured\textsuperscript{41}, however this is not the focus of this study.

When DCs encounter pathogens or bodies with PAMPs they mature to a state exhibiting characteristic functional and phenotypic markers. Phenotypically mature DCs exhibit the hallmark DC maturation markers CD80, CD83, and CD86 along with the
appropriate MHC class I or II complex. Functionally mature DCs secrete pro-inflammatory, immunostimulatory, or immunosuppressive cytokines based on the nature of their environment\(^1\). These three signals of MHC complex presentation, co-stimulatory molecules, and cytokine secretion prime CTLs or activate naïve helper T-cells and polarizes them to effector cells with a T\(_H1\) or T\(_H2\) subset for antigen-specific elimination. DCs can also polarize a third unique subset of T\(_H\) cells, T\(_H17\) cells, that play a role in host defense against extracellular pathogens by secreting IL-17 and recruiting macrophages and neutrophils to the infected tissues\(^2\).

2.3.1 **CTL Activation**

CTL activation occurs in two ways, both in which the CTL must be able to recognize a processed antigen via MHC class I molecules. In one pathway, CTLs can be activated via CD4\(^+\) T\(_H1\) cells\(^2\). In a second pathway, DC cross-presentation can induce CTLs when the DCs are activated and “licensed” by CD4\(^+\) T\(_H\) cells\(^42\) and present all three signals to the CTL. Once an effector CTL is generated, it recognizes and kills infected cells or tumor cells in two ways. In one way, the CTL secretes cytotoxic enzyme granules such as perforins or granzymes that induce permeability of the target cell leading to apoptosis\(^43\). The second killing mechanism is the interaction of Fas ligand (FasL) on the CTL with Fas on the target cell, leading to the activation of caspases within the target cell and ultimately results in apoptosis\(^43\). Interestingly, even though a cross-presenting DC may express the relevant MHC-peptide complex that a CTL is targeting for elimination, the DC is capable of preventing CTL-induced elimination to prolong the DCs lifespan\(^44\). Proposed explanations include DC maturation signals themselves protect
the DC against CTL lysis, CD4+ T<sub>H</sub>-cells rescue the DC from CTL elimination through T<sub>H</sub>-cell-DC interaction signaling, or DCs up-regulate granzyme and perforin inhibitors upon maturation to prevent CTL-mediated elimination<sup>44</sup>. Lastly, DC cross-presentation can result in CD8<sup>+</sup> T-cell central and peripheral tolerance<sup>9</sup>.

2.3.2 Dendritic Cells Polarize Helper T-Cells

Helper T-cells can be divided into subclasses including T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17, and T<sub>reg</sub>, with each subset producing a different cytokine profile and regulating certain activities in the body (Figure 2). The focus of this study is on the T<sub>H</sub>1 and T<sub>H</sub>2 subsets. The fate of the T<sub>H</sub> cell subset depends on the third signal, cytokine secretion, from the DCs during the interaction between a naïve T-cell and DC. DCs polarize the T<sub>H</sub>1 subset by secreting the cytokines interleukin (IL)-12, -18, -23, interferon (IFN)-γ, and TNF-α<sup>45</sup>. The secretion of IL-4 and IL-10 from DCs promotes T<sub>H</sub>2 differentiation<sup>2</sup>.
DCs are matured and activated upon the contact of PM- or endosome-expressed TLRs to PAMPs and the up-take of antigens. The differentiation of naïve $T_H$ cells to various subsets is regulated by the polarizing cytokines. DCs activate naïve $T_H$ cells through the up-regulation of the antigen presenting molecule MHC class II and co-stimulatory molecules CD80 and CD86 to the corresponding T-cell receptors and CD28 molecule. The secretion of IL-12 polarizes $T_{H1}$ cells whereas the secretion of IL-4 and IL-10 polarizes the $T_{H2}$ cell subset. Image taken from Kindt et al., 201346.

$T_{H1}$ cells secrete IFN-$\gamma$ and TNF-$\beta$ to protect cells against intracellular infections caused by viruses, bacteria, and micro-organisms47. $T_{H1}$ cells are also crucial in eliciting an anti-tumor response and eliminating cancer cells47. $T_{H1}$ cells are characterized by strong IFN-$\gamma$ production leading to class switching to IgG that support phagocytosis and complement fixation, as well as supports differentiation of CD8$^+$ T-cells. $T_{H2}$ cells secrete IL-4, -5, and -13, all of which up-regulate antibody production through the activation of B-cells20 and target parasitic organisms47. IL-4 induces class switching to IgE production, IL-5 activates eosinophils aiding in the release of anti-parasitic
inflammatory mediator molecules, and IL-3 and -4 stimulate mast cell proliferation and degranulation, owing to the development of asthma.

The essential discriminating factor to determine if DCs are going to polarize T\(_H\) cells to either a T\(_H\)1 or T\(_H\)2 subset appears to not be the ability of the DC to secrete IL-12, but the amount. The higher IL-12 production by a DC, the more likely it is to induce a T\(_H\)1 subset. Previous research suggested the molecular maturation marker CD80 was up-regulated in DCs that only produced a T\(_H\)1 response and CD86 was only up-regulated in DCs that lead to a T\(_H\)2 response. Recent research has challenged this hypothesis, however, and has shown either maturation marker can be up-regulated in DCs leading to a T\(_H\)1 or T\(_H\)2 subset. Another proposed T\(_H\)1 polarizing factor is the up-regulation of CD54, but this is still being investigated. Still, the most effective way in determining the T\(_H\) subset outcome is measuring the cytokine profile.

The preceding sections highlight the functional significance of DCs in the activation and differentiation of naïve T\(_H\) cells and CTLs in order to elicit a strong immune response. It is important to note, however, that pre-existing T\(_C\)-cells or memory T\(_H\)-cells are not strongly dependent on fully matured DCs for their effector functions. T\(_C\)-cells or memory T\(_H\)-cells are able to activate faster and with a stronger immune response upon re-exposure to the same antigen or pathogen compared to their naïve counterparts.

2.4 Murine Dendritic Cell Line

The role of DCs in various immunologic functions such as immune tolerance and activation are paramount, and significant efforts have been dedicated in understanding
these cells. As previously discussed, DCs are found in blood, secondary lymphoid tissues, or peripheral tissues as a heterogeneous mixture of different DC subtypes. The complex mixture creates many challenges when isolating DCs for in vivo studies. The following paragraphs discuss common DC isolation procedures and the ease of working with immortalized DC lines.

There are two common methods for preparing murine DCs. The first is to isolate DCs from a mouse spleen resulting in a cell population highly enriched in accessory cells and APC function. A second method uses bone marrow cells cultured in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) to express DC surface markers, called Bone Marrow-derived DCs (BMDCs). Primary DC research is technically challenging because of the natural scarcity of DCs in vivo that affects their functional potency and limits the availability of cell material. The differentiation of DCs in vitro through harvesting and maturation is an expensive process requiring the repetitive sacrifice of mice and yield only moderate quantities of DCs. Additionally, this method is very tedious and time-consuming compared to working with immortalized cell lines.

A number of immortalized DC lines have been generated for research purposes that are oncogene-based (CB1 or D2/SC/1 cell lines), growth factor dependent (D1 and JAWSII cell lines), or oncogene-based plus growth factor–dependent (DC2.4 cell line). Many studies have been conducted using these immortalized cell lines; however there are concerns over their equivalence to natural DC counterparts in vivo and the potential effects of growth factors on functional activities or phenotypic markers. A fairly new immortalized DC line, MutuDC, has been generated from murine splenic tumors.
Although human DC lines have been established (MUTZ-3 cell line), a murine cell line was first utilized in this experiment because the next steps are in vivo research using animal models and mice are the preferred models as they are more accessible.

The MutuDC line is named because they are derived from murine CD8α⁺ DC (cDC subset) tumors harvested from the spleen. The MutuDC line is green fluorescent protein (GFP) positive due to a GFP reporter found in the CD11c:SV40LgT (simian virus forty large tumor antigen) transgene. The MutuDC lines immortalize in vitro spontaneously in complete medium in the absence of growth factors. According to the MutuDC line methods from the cell line generator, “within the first days of culture, a large majority (ca. 90%) of DC tumor cells die, suggesting that DC tumor cells are not as yet immortal. Rather, immortalization is achieved when spontaneous selection of the minority of DC tumor cells that stabilize their survival and growth in culture.” When compared to purified splenic CD8α cDC ex vivo, the immature MutuDC line demonstrated similar characteristic surface markers and a proteomic profile. Some major advantages to using the MutuDC line is the familiarity and ease of maintenance in standard culture medium, high viability, low technical difficulties, and similar immature and mature phenotypic markers as purified splenic cDCs.

MutuDCs express a number of phenotypic markers characteristic of lymphoid cDC subsets including CD11b, CD11c, and CD8α positive. CD8α is a reliable marker of the DC lineage and is expressed as a homodimer on the lymphoid DC but absent from myeloid DC subsets. As with wild-type CD8α DCs, the MutuDC line expresses high levels of TLR3 to detect double-stranded RNA such as viruses (i.e. Poly I:C) and TLR9 to detect viral or bacterial DNA (i.e. CpG DNA). MutuDCs moderately

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express TLR4 to detect bacterial cell wall components (i.e. LPS) \(^{33}\). The cells lack TLR7 expression to detect viral single-stranded RNA (i.e. imidazoquinoline compound R848) \(^{33}\). Lastly, MutuDCs are capable of antigen cross-presentation and are critical inducers of \(T_{H1}\) and CTL responses with corresponding cytokine secretion \(^{33}\).

DCs have demonstrated a key role in the regulation of immune activation and tolerance. Significant efforts are focused on understanding DC maturation and activation for the potential application of DC-based immunotherapy cancer treatments. The natural properties of DCs can be harnessed to identify and target cancer cells for elimination. The proceeding section provides an overview on current research using DC-based treatments to attain the desired immune response in patients.

2.5 Dendritic Cells in Cancer Immunotherapy Treatment

The separation of DC maturation states between self and non-self antigens is straightforward in healthy cells and tissues; however, in a cancer environment, the tumors and tissues are similar to that of healthy cells thereby contributing to a difficult distinction \(^1\). Cancer cells can also interfere with DC behavior and function through the release of immunosuppressive cytokines or complement factors allowing cancer cells and tissues to continue proliferating. Cancer cells can cause DC dysfunction through the inhibition or decrease of antigen recognition, up-regulation of maturation markers and/or co-stimulatory molecules, and cytokine secretion, leading to the DC’s inability to properly mature and signal T-cell activation \(^{52}\).

Current experimental and clinical research have been aimed at killing cancer cells through either non-immunogenic or low-immunogenic cancer cell death, however, this
keeps DCs in an immature state or leads to a DC “semi-mature” state. A schematic representation of DC states interacting with cancer cells, shown in Figure 3, demonstrate how DCs in an immature or semi-mature state can cause T-cell anergy leading to tolerogenicity and a lack of anticancer immunity.

Figure 3. **DC maturation states in response to cancer cells**. Immature DCs are devoid of or have negligible amounts of co-stimulatory and antigen-presenting molecule presentation leading to anergy. DCs interacting with cancer cells can result in functional and phenotypic maturation dysfunction, leading to tolerogenicity or pro-tumorigenicity. Fully mature DCs up-regulate co-stimulatory and antigen-presenting molecules as well as cytokine secretion to polarize T-cells towards a helper behavior with inflammatory or immunogenic properties and anti-tumor roles. Image taken from Dudek et al., 2013.

Semi-mature DCs are able to exhibit two of the three required signals for T-cell activation (discussed previously) but are not able to display all three signals at once.
This causes DCs to be dysfunctional in either phenotypic maturation of antigen-presenting or co-stimulatory molecules, or have disparities in their functional maturation of cytokine secretion\textsuperscript{53}. This leads to T-cell anergy and tolerogenicity\textsuperscript{54}, or a pro-tumor environment\textsuperscript{55}. Even if the semi-mature DCs exhibit at least two of the three signals for T-cell activation, they are typically inconsistent in their up-regulation of phenotypic maturation ligands and/or in cytokine secretion quantity and timepoint\textsuperscript{53}. As discussed, fully matured DCs exhibit all three signals required for T-cell activation and can have an inflammatory or immunogenic role, with both states having the potential to elicit anticancer immunity. The secretion of IL-23 from DCs is a discriminating factor, leading to an inflammatory response and polarizing T-cells toward a helper state and T\textsubscript{H}1 subtype\textsuperscript{56}. If the fully matured DCs also secrete IL-17, it polarizes the T\textsubscript{H}17 subtype, however, the role of T\textsubscript{H}17 on cancer is controversial because there is evidence that T\textsubscript{H}17 can have both a pro- and anti-tumorigenic role\textsuperscript{56}. The absence or decreased secretion of IL-23 or other inflammatory cytokines polarizes a fully mature immunogenic DC\textsuperscript{57}.

Clinical trials have ensued using DC-based cancer immunotherapy treatments since approximately 1995. The trials have revealed general safety and feasibility of DCs as a cancer vaccine without the toxicity found using other cancer treatments such as chemotherapy or radiation. Anti-cancer therapies like chemotherapy affect not only cancer cells but cells from the tumor microenvironment, systemic hematopoietic cells, and dividing bone marrow cells\textsuperscript{1}. This can lead to immune system dysfunction due to a lack of immune cell production.

The DC-based immunotherapy approach can be accomplished in two ways. Either the DCs can be stimulated directly \textit{in vivo} to mature phenotypically and
functionally or DCs can be stimulated \emph{ex vivo} and then infused back in the host to continue with an anticancer effector function\cite{1}. The \emph{in vivo} approach has yielded failed results because the CD4$^+$ T-cells and T$_{H}2$ type cytokines could not be stimulated or activated\textsuperscript{58,59}. Studies are being conducted following up on the clinical responses by vaccinating patients with different TLR combinations and other stimulating factors to elicit a DC anti-cancer response. The \emph{ex vivo} approach leads to DC-based anti-cancer vaccines by isolating DC precursors from patients (leukapheresis), maturing and stimulating the precursors \emph{ex vivo}, and injecting the fully matured DCs back into the patient\textsuperscript{1}.

DC-based anti-cancer immunotherapy clinical trials are well tolerated, however the clinical responses have been suboptimal\textsuperscript{60}. There are very limited results for clinical DC-based vaccines due to the complexities associated with DCs. Immunologic vaccines have not followed a linear dose-response effect as they do in chemotherapy treatments\textsuperscript{61}. There are many variables to consider when using DC-based cancer vaccines, these include the administration route, minimum and maximum dose needed to achieve effects, scheduling (i.e. weekly versus monthly injections), immunological adjuvant type, and the current state of the patient’s immune system\textsuperscript{61}. The most significant issue when using a DC-based anti-cancer vaccine is targeting the tumor itself. Tumors are heterogeneous tissues with varying physiological sites (primary verse metastatic sites), vascularization, and surrounding tissues\textsuperscript{61}. Another concern is the tumor may have different tumor-associated antigens (TAA) that are unable to be loaded into the DC, thereby the DC-based anti-cancer vaccine not working properly because it cannot target the specific tumor cells\textsuperscript{61}. Many research groups are trying to determine the optimal TAAAs to target
in DC-based vaccines that are expressed only on tumor cells, are immunogenic, and are critical for the tumor cell to survive. Other issues in DC-based anti-cancer vaccine research and development include DC differentiation protocols, maturation cocktail compositions, and the source of the DCs themselves. Headway is being made in this area, however progress needs to be made in ensuring a standardized trial design, investigating the many different variables, and thoroughly monitoring immune assessments.

DCs can be stimulated in various ways ex vivo to elicit an anti-cancer immune response including stimulation with TAAs, tumor lysates, electroporation or transfection of DCs with cancer cell mRNA, or with exosomes. The ability of DCs to acquire antigens and elicit an immune response is paramount in suppressing cancer. DCs can procure antigens via infectious agents, dead or dying cells, or exosomes secreted by cells, leading to the processing and maturation of the DC to respond appropriately. Research has demonstrated that cancer-derived exosomes can induce phenotypic and functional DC maturation, thereby inducing a T1, T2, or CTL response. Other studies have shown cancer-derived exosomes have an immunosuppressive role and cause DC maturation dysfunction. Due to the contradictory results of cancer-derived exosomes and their ability to elicit a DC-based immune response, research has begun focusing on a safer and more consistent approach of using DC-derived exosomes (Dex) that have been loaded with TAAs for cancer immunotherapy treatments.
2.6 Microvesicles: Exosomes

For decades, researchers have been reporting that membrane-enclosed vesicles were present outside cells or other biological fluids\(^68\). In the early 1980s, the secretion pathway for these membrane-bound extracellular vesicles (EVs) was described when researchers observed that late endocytic compartments, called multivesicular bodies (MVBs), in reticulocytes released vesicles into the extracellular space\(^69,70\). Once thought to have no biological significance, EVs have recently demonstrated a role in stimulating the adaptive immune response\(^71\) and have been linked to tumorigenesis\(^72\) and the spread of viruses\(^73\). EVs have also been exploited as potential therapeutic agents for tissue regeneration and immunotherapy treatments\(^74\).

Traditionally, cells were believed to communicate exclusively through cell-cell interaction or the secretion of various biomolecules including growth factors, however, EVs have been shown as a way of long distance cell-cell communication. EVs are membrane vesicles containing cytosol from the secreting cell in a lipid bilayer and are classified based on their size, origin, and/or biological function: exosomes (40-120 nm diameter, 3-5 MDa), microvesicles (100-1000 nm diameter), and apoptotic bodies (500-500 nm diameter). The focus of this study was using exosomes as potential immunotherapeutic agents. Nearly all cells secrete exosomes both \textit{in vitro} and \textit{in vivo}. Exosomes have been found in various biological fluids such as blood, urine, saliva, and breast milk,\(^75\) as well as \textit{in vitro} cell culture medium.
2.6.1 Exosome Composition

Exosomes are composed of various families of proteins, lipids, and nucleic acids and are formed during the endocytic pathway in which dynamic membrane compartments are involved in internalizing extracellular ligands or cellular components\(^{68}\). Exosomes share common features regardless of their cell of origin including their size, structure (bilipidic layer), density, and protein composition\(^{62}\). While there are no known exclusive exosomal protein markers, they do contain general proteins found in MVBs. A schematic representation of the possible composition and components of an exosome is shown in Figure 4.

**Figure 4**

![Schematic representation of the composition, location, and function of proteins in exosomes](image)

Figure 4. Schematic representation of the composition, location, and function of proteins in exosomes\(^{76}\). Exosomes display a variety of targeting and adhesion molecules, antigen-presenting molecules, tetraspanins, and co-stimulatory molecules as the parental cell from which they were secreted. Image taken from Tran et al., 2015\(^{76}\).
The protein content of exosomes has been studied extensively and revealed exosomes contain a specific subset of cellular proteins based on the cell type that secreted them as well as common proteins regardless of their cell of origin. Some commonly used markers for exosomal determination include tetraspanins CD9, CD63, and CD81, MVB formation markers Alix and Tsg101, as well as heat-shock proteins (Hsp) that facilitate peptide loading onto MHC molecules. Other proteins present in exosomes include endosomal sorting complex required for transport (ESCRT), annexins that regulate the membrane cytoskeleton, lysosomal-associated membrane proteins (LAMPs), and metabolic enzymes including peroxidases, pyruvate kinase, lipid kinase, and enolase-1. It is suggested that CD9 is possibly more ubiquitous in EVs compared to the other tetraspanins based on inhibition studies in tumor cells.

Exosomes secreted from APCs, namely DCs, contain cell-specific proteins such as MHC class I and II molecules and co-stimulatory molecules CD80 and CD86. Exosomes can also display adhesion molecules including ICAM-1 and integrins such as CD11b and CD11c in order to facilitate internalization by other cells. Studies show the maturation markers and co-stimulatory molecules up-regulated in mature DCs are also up-regulated in mature exosomes. These findings were imperative in understanding and confirming exosomes as a potential avenue for immunotherapy treatments.

The lipid composition of exosomes include sphingomyelin, phosphatidylserine (PS), cholesterol, and other saturated fatty acids. The sphingomyelin and cholesterol contribute to the detergent-resistance subdomains of the exosome plasma membrane, known as lipid rafts. Unlike cells where PS is restrained to the inner leaflet of the plasma membrane, PS is exposed on secreted exosomes. This is likely due to the
absence of flippase, an enzyme that generates asymmetrical distribution of phospholipids in the plasma membrane of cells. Carayon et al. demonstrated changes in lipid composition during reticulocyte maturation in red blood cells, suggesting exosomes can be modified during biogenesis from their cell of origin.

Exosomes contain nucleic acids including small non-coding, regulatory micro RNA (miRNA) molecules and messenger RNAs (mRNAs). The mRNA contained in exosomes is functional and can be expressed in cells that take up the exosome but contain information that may not necessarily reflect that of the host cell of origin.

Interestingly, three studies have shown that miRNA has exported out of cells through secreted exosomes and can affect gene expression in distant cells in vitro, however this has not been demonstrated in vivo. The shuttling of RNA into vesicles allows for genetically encoded messages to be exchanged between cells at a distance. The mechanisms underlying the sorting and selection of RNAs into exosomes remains unknown. Next-Generation Sequencing (NGS) techniques are aiding in the discovery of how cells transfer non-coding RNA species and modify cellular function through the study of DC-T-cell interactions.

2.6.2 Exosome Biogenesis and Secretion

Exosomes are formed during the endocytic pathway. This pathway consists of highly dynamic membrane compartments that internalize extracellular ligands or cellular components, followed by recycling to the plasma membrane or result in degradation. A schematic representation of this pathway is shown in Figure 5.
Figure 5. The biogenesis of exosomes\textsuperscript{85}. Image depicts the generation of exosomes in the late endosomal compartment. The inward budding of the MVB membrane ensures the exosomes orient the bound proteins in the same manner as the cell. Cellular DNA and RNA can be incorporated into the exosome. The exosome-filled MVBs can either fuse with the lysosome for degradation or fuse with the plasma membrane to release exosomes into the extracellular environment. Image taken from Waldenström and Ronquist, 2014\textsuperscript{85}.

Upon internalization of vesicles through the plasma membrane, sorting endosomes, also known as early endosomes, mature into late endocytic compartments (late endosomes), known as MVBs, and during this process they accumulate vesicles in their lumen\textsuperscript{86}. The vesicles formed during this process sequester proteins, lipids, and cytosol that are then sorted\textsuperscript{68}. The majority of MVBs fuse with lysosomes that contain acidic compartments to degrade the content of the vesicle. Alternatively, EVs displaying markers such as tetraspanins, LAMP1/2, and other molecules present in late endosomes can fuse with the plasma membrane and release their contents into the extracellular environment\textsuperscript{62}. These secreted vesicles are now called exosomes. The mechanism of intraluminal vesicle formation in MVBs to form exosomes rather than MVB-lysosomal
fusion for degradation is not fully understood, however, there are four distinct mechanisms proposed resulting in MVB formation and thus exosomes. Two mechanisms are dependent on the ESCRT complex while the other two are ESCRT complex-independent. The most well described mechanism for MVB formation is ESCRT complex-dependent. The ESCRT machinery is composed of approximately thirty proteins that assemble into five complexes (ESCRT-0, -I, -II, -III, and Vps4) and the machinery assists in the budding and scission of membranes away from the cytosol. ESCRT-0 initiates the MVB pathway, ESCRT-I and –II stabilize the growing vesicles, ESCRT-III aids in budding and vesicle scission, and Vps4 terminates the MVB cargo sorting and vesicle formation. It is unclear if ESCRT components alone can produce exosomes or if lipids and tetraspanins are required in the process. Additionally, various studies have demonstrated that not all ESCRT machinery components are required for MVB formation, leading researchers to conclude there are various processes and pathways that can end in MVB formation.

ESCRT-independent mechanisms may involve an independent process of lipids or tetraspanins generating MVBs and thus exosomes. In this pathway, two metabolism enzymes have been shown to generate lipids that form vesicles including neutral sphingomyelinase (nSMase) and phospholipase D2. The nSMase hydrolyzes the sphingomyelin into ceramide, observed during cellular stress and apoptosis, and is important for the budding of intracellular vesicles into the MVB. The phospholipase D2 hydrolyzes phosphatidylcholine into phosphatidic acid, and is important in membrane trafficking, cytoskeletal reorganization, and cell migration. Lastly, the
tetraspanin CD63 has demonstrated a role in vesicle formation and coordination between the two pathways\textsuperscript{90}.

The release of EVs from the plasma membrane are typically induced by stimuli causing an increase in intracellular calcium and cytoskeleton remodeling\textsuperscript{91}. Studies have revealed that treating cells with calcium ionophores can trigger the release of vesicles as well as extracellular signals\textsuperscript{81}. The proportion of EVs secreted from intracellular compartments verses the cell surface varies by cell type\textsuperscript{68}. Just as intracellular trafficking pathways can be conserved or tissue-specific, exosome biogenesis and secretion mechanisms can differ by cell type. Some cells are able to secrete exosomes spontaneously while other cells, such as murine DCs, secrete exosomes more readily upon their interaction with antigen-specific CD4\textsuperscript{+} T-cells\textsuperscript{92}.

The secretion of exosomes through MVB and plasma membrane fusion is not fully understood, however, research has demonstrated that the Ras-related proteins in brain (RAB) family, essential in intracellular vesicle transport between various compartments, including RAB11, RAB27, and RAB35 play a role in exosome exocytosis\textsuperscript{75}. RABs are known to direct vesicle and organelle trafficking by regulating motor proteins, adhesion factors, and other trafficking mechanisms\textsuperscript{75}. RAB11 and RAB25 are expressed in a variety of cell types and aid in cellular recycling to the plasma membrane\textsuperscript{75}. RAB27 is found in secretory cells and support the secretion of lysosome-related organelles\textsuperscript{75}. There are a variety of other mechanisms that potentially play a role in exosome secretion that may explain differences in exosome content and function that must be explored further.
Both hematopoietic cells (including B-cells, T-cells, and DCs) and non-hematopoietic cells (fibroblasts and tumor cells) have demonstrated the ability to secrete and internalize exosomes in vitro and in vivo. The binding of EVs to the cell surface involves specific receptor-ligand pairs found on both the EV and the plasma membrane of the recipient cell. For example, LFA-1 on DCs or activated T-cells can bind to and capture Dex displaying the ICAM-1 molecule. A variety of tetraspanins, integrins, and other adhesion molecules have all shown to play a potential role in the cellular uptake of exosomes from the extracellular environment. Additionally, internalization may be dependent on the presence of heparin sulfate chains on exosomes and receptors found on the recipient cell surface.

Depending on the action required to induce physiological changes in the recipient cell, secreted exosomes may bind to the cell surface or be internalized. For instance, exosomes would be able to induce an immune response by displaying antigenic peptides via MHC class II molecules to T-cell receptors. In other cases, the contents of exosomes must be transferred into the recipient cell. The recipient cell dictates how exosomes are internalized, either through phagocytic or endocytic pathways. Once internalized, exosomes can gain access to the cytosol in order for gene silencing or nucleic acid induced expression to occur through a fusion step with the plasma membrane or endocytic compartments.

2.6.3 Exosome Functions

Exosomes have various functions and additional responsibilities are still being uncovered. They have demonstrated a biological role by activating cell surface receptors.
and delivering transcription factors, oncogenes, miRNAs and mRNAs, and infectious particles into recipient cells\textsuperscript{93}. These factors contribute to the maintenance of cellular physiology where exosomes have also shown to externalize the transferrin receptor that is required during reticulocyte maturation\textsuperscript{94}. Exosomes support intercellular communication to maintain cell homeostasis or respond to pathogens by allowing the exchange of proteins and lipids between two cells\textsuperscript{68}. This has been especially prevalent and beneficial in the immune system where exosomes have been shown to exchange antigenic peptide information between DCs and T- and B-cells\textsuperscript{76}. Additionally, exosomes serve a pathological role. This has been observed in tumor biology where studies have shown exosomes can promote tumor progression by inducing tumor growth, angiogenesis, metastasis, and prevent T-cell activation thereby promoting tumor immune escape\textsuperscript{93}. Studies have also shown exosomes can contribute to neurodegenerative diseases including Alzheimer’s and Parkinson’s disease because the exosomes are able to influence neuronal networks at a distance\textsuperscript{95}.

Because exosomes are involved in a variety of biological and pathological roles, they have become a potential target for therapeutic interventions. The inhibition of either exosome secretion or uptake could stave off disease progression\textsuperscript{93}. Exosomes could be used for antigen presentation, immune modulation, or tissue repair in regenerative medicine applications\textsuperscript{93}. Lastly, exosomes can be engineered as drug delivery vesicles by loading particular tissue- or cell-specific targeting ligands on their surface\textsuperscript{93}. 
2.6.4 Exosome Isolation Techniques

Exosomes are isolated from cell culture supernatant through various methods based on size, density, or particular exosomal surface markers. A commonly employed technique is ultracentrifugation. There are several variations of this method, but the overarching goal is to separate the smallest molecules, including exosomes, from tissue culture conditioned medium. Prior to ultracentrifugation, the larger vesicles are eliminated through successive centrifugations with increasing speeds in order to prevent creating small vesicles from the larger ones\(^96\). Other means of ridding the culture medium of larger vesicles is through filtration or size-exclusion chromatography\(^96\). Once the larger vesicles are depleted, the sample undergoes higher-speed ultracentrifugation (as high as 140,000x \(g\))\(^97\). Ultracentrifugation is not a proper purification technique, however, because vesicles other than exosomes that are similar in size (including impurities, protein aggregates, or cellular debris) can cosediment\(^68\). This technique could also possibly cause damage to the exosomes.

Another method of exosome purification is using magnetic beads coated with monoclonal antibodies specific to exosome antigens, such as tetraspanins. ThermoFisher Scientific and other manufacturing companies have designed protocols and products designed to decrease the time and possible contaminates that are normally seen when isolating exosomes via ultracentrifugation. Using antibodies should restrict the range of vesicles obtained during isolation. Further research, however, is needed to compare the processes and ensure the vesicles obtained are pure.

A variety of liquid chromatography techniques can also be employed to isolate exosomes from culture medium including ion exchange, size exclusion, hydrophobic, or
affinity chromatography. Affinity chromatography exploits the interactions between molecules and is widely used for protein purification. When a mixture is passed over a functionalized resin, the protein of interest is immobilized on the resin and contaminating proteins are washed away. When a buffer with a high salt concentration, pH shift, or competing ligand are added to the column, the protein of interest is released from the resin and collected for further analysis. A heparin gel can be used as an affinity support to purify a wide range of proteins, including exosomes, due to its stability as a result of the heparin coupled by amide bonds to cross-linked agarose beads. Fast-protein liquid chromatography (FPLC) separates proteins by controlling the buffer flow rate and varying the buffer composition\textsuperscript{98}. During this process, the proteins dissociate from the column and appear in the effluent. The effluent passes through detectors that measure the salt concentration and protein concentration, and display peaks to measure the concentrations\textsuperscript{98}. The effluent can then be collected for further analysis. As with any separation technique, there are advantages and disadvantages. Some advantages to using this process include high selectivity and resolution, concentration of the sample, and it utilizes the protein’s structure or function for purification\textsuperscript{99}. Disadvantages include buffer/elution limitations, column packing sensitivity, and the possibility of harsh elution conditions that could denature the proteins\textsuperscript{99}.

2.7 DC-Derived Exosomes and Immunotherapy Treatments

Exosomes are an endogenous and biostable form of long-distance cell-cell communication\textsuperscript{75}. Because exosomes display key antigenic peptide information via MHC class II molecules and are readily internalized by nearly all cells, they have become
an attractive vector for cancer immunotherapy treatments. As discussed, Dex have demonstrated the ability to induce an antigen-specific T-cell immune response when loaded with a particular antigen\textsuperscript{100,101}. Table 1 (adapted from Pitt et al., 2016) summarizes functional differences between DC-based vaccines verse Dex-based cancer immunotherapy treatments.

<table>
<thead>
<tr>
<th>Potential functional differences</th>
<th>DC-based treatments</th>
<th>DC-derived exosome treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular composition</td>
<td>Fluctuates, difficulty in defining quality control parameters</td>
<td>Strictly defined and enforced through bioengineering</td>
</tr>
<tr>
<td>Amount of antigenic peptide-MHC II on surface</td>
<td>Lower yield/fewer complexes</td>
<td>Highly enriched in peptide-MHC II complexes (10-100x that of DCs)</td>
</tr>
<tr>
<td>Stability of vaccine preparation</td>
<td>Limited storage and stability over extended periods</td>
<td>High stability (&gt;6 months) at -80°C</td>
</tr>
<tr>
<td>Localization</td>
<td>Relies on chemokines and other signals</td>
<td>Relies on surface membrane receptor topography</td>
</tr>
<tr>
<td>Resistance to immunosuppression</td>
<td>Susceptible to immunosuppressive molecules and tumor microenvironment</td>
<td>Not responsive to immunosuppressive molecules</td>
</tr>
</tbody>
</table>

Table 1. Summary of DC-based verse exosome-based cancer immunotherapy treatment. Table adapted from Pitt et al., 2016.

Exosomes are highly advantageous for numerous reasons. They are inexpensive and easily reproduced in high yields\textsuperscript{75}. Since exosomes contain endogenous proteins and RNA materials, they are able to have exogenous biological materials loaded into them through bioengineering processes\textsuperscript{75}. Since the goal of cancer immunotherapy treatments is to cause a systemic immune response of the host to eradicate tumor cells, it is important exosomes remain in circulation as long as possible. Tumor cells escape
detection through the secretion of cytokines and other proteins leading to immune cell dysfunction. Exosomes have the advantage over DC-based cancer immunotherapy treatment because they can evade the suppressive cytokines IL-10 and TGF-β secreted by tumor cells that normally cause DC dysfunction\textsuperscript{102}. Exosomes are also capable of evading clearance by phagocytic cells due to their size, allowing a longer time in circulation to induce an immune response\textsuperscript{76}. Lastly, Dex are resistant to complement factors because they express CD55 and CD59\textsuperscript{103}. CD55 and CD59 (along with CD46) are glycosylphosphatidylinositol (GPI)-anchored membrane regulators of the complement system\textsuperscript{103}. The complement system is a group of serum proteins that help link the innate and adaptive immune response by circulating in an inactive form and upon activation, aids in cell membrane lysis, chemotaxis, and opsonization to enhance phagocytosis\textsuperscript{2}. Expression of CD55 and CD59 decreases C3 deposition during the classical complement pathway, thereby inhibiting complement-mediated lysis\textsuperscript{103}.

Both immature and mature DCs are able to secrete exosomes that display a similar morphology as well as surface and internal proteins common to all exosome types. As previously mentioned, mature Dex up-regulate similar maturation markers, co-stimulatory molecules, and adhesion molecules (CD54) as seen in the parent DC\textsuperscript{62}. These adhesion molecules along with integrin α and β chains allow for exosomes to target and dock to a recipient cell\textsuperscript{102}. The internal vesicles of MVBs in immature DCs function as a storage site for MHC class II molecules, but during DC maturation the internal vesicles that are carrying the MHC class II molecules are transferred to the MVB membrane and results in morphological changes to the MVB\textsuperscript{104}. Cell maturation facilitates MHC class II antigen loading and movement of the MVB to the surface for
exosomal secretion. This results in a decrease in endocytic activity and therefore decreases exosome production, by two to three times less than immature DCs\textsuperscript{105}. It has also been shown that exosomes are mainly internalized by immature DCs and not mature DCs\textsuperscript{106}. This is likely due to immature DCs having a high endocytic ability to sequester their exosomes and prevent the matured, less phagocytic DCs from doing so\textsuperscript{106}.

Numerous studies are being conducted investigating the potential role of Dex inducing an immune response. In one study, Dex were able to activate naïve T-cells \textit{in vitro} but required two distinct DC populations. One DC population produced and secreted exosomes displaying the peptide-MHC class II complex or intact antigen\textsuperscript{107}. The second DC population (one that had not seen the antigen) internalized and reprocessed the antigen contained in the exosome or acquired the peptide-MHC class II complex in order to stimulate T-cells\textsuperscript{107}. This concluded that exosomes could mediate the transfer of MHC class II molecules between two DC populations, one of which had not seen the antigen previously. This mechanism of indirect T-cell activation from Dex may increase the number of DCs containing the antigenic peptide to amplify the immune response resulting in an immunostimulatory effect.

Two studies resulted in the inhibition of tumor growth when Dex loaded with tumor specific peptides activated CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells or induced CTLs\textsuperscript{108}. Numerous \textit{in vitro} studies have shown Dex are more efficient at inducing T-cell lines, activated T-cells, and memory T-cells, whereas Dex are inefficient at naïve T-cell activation and typically require the presence of APCs\textsuperscript{102,109}. A third study showed exosomes can suppress the immune system through the activation of T\textsubscript{reg} cells thereby inhibiting the activation of immune cells\textsuperscript{110}. Alternatively, exosomes derived from cancer cells have
been shown to prepare a suitable pre-metastatic microenvironment, stimulate angiogenesis, enhance growth and survival of cancer cells, promote cancer invasion, and be drug resistant\(^{111}\). Clinical trials utilizing Dex are discussed in a proceeding section.

2.7.1 DC-Derived Exosome Immune Stimulation Pathways

There are two ways Dex containing antigenic peptide-MHC molecule complexes can elicit an immune response. This can be achieved through direct MHC presentation or indirect MHC presentation\(^{102}\), summarized in Figure 6.

**Figure 6**

![Diagram of DC-Derived Exosome Immune Stimulation Pathways](Image)

*Figure 6. Schematic representation of the interaction of Dex and activation of the immune response\(^{102}\).* Dex activate T-cells through direct or indirect antigen presentation. Direct antigen presentation occurs through MHC class I or II molecules and the TCR. Indirect presentation occurs through Dex cross-dressing or through the endocytosis of Dex by the DC and reprocessing/transfer of peptides to MHC molecules. Image taken from Pitt et al., 2016\(^{102}\).
Dex display both MHC class I and II and co-stimulatory molecules on their surface, allowing for direct activation of CTLs and CD4⁺ T-cells, respectively. Direct activation has been demonstrated only in vitro. Results showed Dex were able to stimulate weak T-cell clones or T-cell lines, but not naïve T-cells. Direct Dex-to-T-cell stimulation is highly unlikely to occur in vivo possibly due to the exosomes small size, membrane composition, or low ability to cross-link TCRs and deliver co-stimulation. Dex have also demonstrated the ability to directly induce NK cell activation and proliferation; however, this is not the focus of the study.

Evidence suggests that Dex stimulate T-cells more efficiently via indirect MHC-antigen complex presentation and require the presence of DCs. The transfer of antigenic peptides/MHC complexes to other APCs can happen in two ways. The first route occurs when a Dex merges with a DC and the exosome transfers their peptide-MHC complex to the DC membrane surface, known as “cross-dressing.” This allows DC-T-cell communication without the need for further antigen processing. Thery et al. demonstrated cross-dressing when MHC class II-deficient DCs were cultured in the presence of exosomes secreted by DCs displaying MHC class II molecules loaded with antigenic peptides. The MHC class II-deficient DCs subsequently displayed MHC class II molecules from the Dex and were able to stimulate T-cells and induce T-cell proliferation, whereas MHC class II-deficient DCs alone were unable to do so. The results suggest MHC class II-deficient DCs that are unable to present antigenic peptides to T-cells can still stimulate T-cells with antigenic peptide complexes derived from exosomes. The second possible indirect route happens following internalization by phagocytosis of Dex by bystander DCs. The Dex peptide/MHC complex is then
reprocessed via endosomal pathways in the DCs (or other APCs) and results in the transfer of antigenic peptides from Dex MHCs to the DC MHCs\textsuperscript{109}. The MHC/peptide complex is subsequently transported back to the DC surface for presentation to T-cells\textsuperscript{102}. A possible third indirect route results from exosomes being internalized by tumor cells, converting the tumor cells into immunogenic targets\textsuperscript{102}, but this theory remains ambiguous.

2.7.2 DC-Derived Exosome Immunotherapy Clinical Trials

Several \textit{in vitro} and pre-clinical mouse studies have been conducted using DC-derived exosomes showing promising results. To date, there have been three phase I and one phase II human clinical trials that have been completed investigating the therapeutic effects of exosomes in cancer patients. The phase I trials consisted of patients with non-small cell lung cancer (NSCLC)\textsuperscript{112}, metastatic melanoma\textsuperscript{113}, or advanced colorectal cancer\textsuperscript{114}. Peripheral immune cells were harvested from patients with NSCLC or metastatic melanoma, cultured \textit{ex vivo} to expand their DCs, and the culture supernatant containing exosomes secreted from the DCs was harvested. The MHC class I and II molecules on the exosomes were loaded with melanoma-associated antigen (MAGE) peptides either during culture or proceeding exosome purification. The patients received the exosome vaccine weekly for four weeks. Antigen-specific immune responses were modestly detected and no T-cell effects were observed in the peripheral blood\textsuperscript{112,113}. Interestingly, both studies revealed an increase in NK cell activation, possibly attributed to NK-specific surface ligands detected on exosomes being able to engage and activate NK cells\textsuperscript{102}. The NK cell activation also attributes to the lack of T-cell responses.
observed in the patients. The third phase I clinical trial used ascites-derived exosomes in lieu of DC-derived exosomes to examine the therapeutic effects\textsuperscript{114}. Exosomes were prepared from malignant ascites of the patients and were found to display high amounts of MHC class I and II molecules and were non-toxic and stable, however results were similar in that only a minor response was observed\textsuperscript{114}.

The phase II clinical trial treated advanced NSCLC patients with IFN-\( \gamma \) DC-derived exosomes weekly for four weeks\textsuperscript{115}. IFN-\( \gamma \) was used to stimulate the DCs \textit{ex vivo} in hopes to achieve an improved T-cell stimulation \textit{in vivo}. Overall the DC-derived exosome vaccinations resulted in poor T-cell stimulating activity and little patient response, however there was disease stability in 32\% of the patients\textsuperscript{115}.

Although human clinical trials involving DC-derived exosomes have shown limited efficacy, they have demonstrated promising results in animal studies and have proven their stability and safety in humans. There are many factors contributing to the suboptimal results including but not limited to: small trial size, inability for the exosomes to elicit T-cell responses possibly due to antigenic peptide-MHC complex employed, and it is questionable if the exosomes even reached the intended tumor site\textsuperscript{102}. With many questions still unanswered, scientists are continuing to develop strategies in order to improve the efficacy of exosome vaccines. The future of exosome-based cancer vaccines may involve the bioengineering of exosomes in order to enhance the immunostimulatory effect. While the methodologies and results of bioengineering exosomes is not broached in this work, it is the ultimate goal of this research lab and is discussed in the proceeding section.
2.8 Bioengineering Exosomes

By controlling the contents and functions of exosomes, one is able to develop a more targeted and potent immune response. Many research groups have advanced in therapeutic immunotherapy treatments, regenerative medicine, and small molecule delivery (i.e. drugs) through the manipulation of exosome content. Exosome bioengineering could result in a specifically cultured cell type with a specific immunoregulatory function by loading antigens or cell-targeting proteins to produce an immune response \textit{in vivo}\textsuperscript{75}. Synthetic liposomes (phospholipid vesicles) have been investigated as a drug delivery system but proteins must be loaded manually onto and into the liposome for adequate bioavailability\textsuperscript{116}, so exosomes have emerged as a promising delivery system tool. Proteins can be loaded into and onto exosomes in two ways: direct or indirect exosome-loading.

2.8.1 Direct Exosome Loading

Direct exosome-loading can be accomplished by fusing the protein of interest to known exosomal proteins or exosome-binding proteins, exosome-binding lipid anchors, or random exosome loading of transmembrane G-coupled protein receptors (GPCR)\textsuperscript{75}. In one study, Alvarez-Erviti et al. engineered DCs to express LAMP-2b by fusing a protein made with N-terminal truncated LAMP-2b to C-terminus of a 29-mer peptide from a rabies glycoprotein (RVG) that binds acetylcholine receptors on neurons\textsuperscript{117}. The purified exosomes were loaded with exogenous siRNA by electroporation to target mRNA and proteins involved in Alzheimer’s disease\textsuperscript{117}. The results from this study demonstrated the therapeutic potential of exosome-mediated siRNA delivery, however continued research
revealed that engineered chimeras would require modification to engineer glycosylation sites to protect from lysosomal degradation\textsuperscript{118}.

In another study, researchers directly loaded exosomes with non-native soluble, membrane-bound, trans-membrane, or multimeric antigens that could be expressed on the exosome surface in a native confirmation using a technology called Exosome Display\textsuperscript{119}. In the soluble antigen method, the lipid-binding C1C2 domain of Lactadherin was fused with a protein of interest to generate a chimeric protein\textsuperscript{119}. The C1C2 domain binds to lipids on the outer leaflet of the exosome membrane and anchors to secreted exosomes, thereby displaying the antigen of interest on the exosome surface\textsuperscript{119}. Another example using Exosome Display technology was loading transmembrane proteins into exosomes during exosome biogenesis in the MVB. Mouse DCs were transfected with plasmid encoding the trans-membrane protein HLA-A2 (MHC molecule specific to humans) and resulted in the expression of HLA-A2 on Dex\textsuperscript{119}. Results showed, however, the protein expression was found in multiple locations within the cells and not just the exosomes, most notably the plasma membrane\textsuperscript{119}. These results indicate this type of exosome loading may not afford the control of protein content and expression as one would prefer for therapeutic applications, but is still a potential application in exosome drug development.

Many studies have focused on loading exosomes with MHC class I or II restricted peptides to stimulate the immune system, but Gabrielsson et al. has demonstrated that only whole antigen-loaded exosomes (not peptide-loaded) are able to induce a CD4\textsuperscript{+} and CD8\textsuperscript{+} response \textit{in vivo}\textsuperscript{120}. The stimulating effect of Dex could be enhanced by adding the natural killer T (NKT) cell ligand $\alpha$-galactosylceramide ($\alpha$GC) onto exosomes, resulting
in significantly reduced tumor growth\textsuperscript{121}. Continued research showed αGC in conjunction with the model antigen Ovalbumin (OVA), produced a more potent immune response than OVA alone in OVA-expressing cancer in mice\textsuperscript{122}. The researchers concluded that exosomes could stimulate T-cells in the recipient by three mechanisms (previously discussed and shown in Figure 5): direct MHC/peptide complex binding to T-cells, recycling of exosomal MHC onto the surface of APCs, or exosome degradation and full reprocessing by recipient APC\textsuperscript{102}. If the third mechanism were correct, then a match between the exosome donor and patient MHC molecules would not be required. This is especially important because it is not optimal to perform leukapheresis on a patient with an already immunocompromised system\textsuperscript{120}. The possibility of using MHC-mismatched exosomes when designing exosome-based therapy treatments would therefore be a more feasible option\textsuperscript{120}. This was confirmed by demonstrating exosomes lacking MHC class I expression induce OVA-specific CD8\textsuperscript{+} T-cells and IFN-γ expression to the same degree as wild type exosomes\textsuperscript{120}. Direct T-cell stimulation by exosomes \textit{in vivo} is therefore insignificant when whole antigen is present because DCs are internalizing and reprocessing the exosomes for antigen T-cell and B-cell responses\textsuperscript{120}.

2.8.2 \textit{Indirect Exosome Loading}

Indirect exosome-loading is accomplished by altering the cell type or maturation conditions to modify exosome content. Different immune cells can activate various pathways and signal other immune cells to elicit an immune response. The initially discovered B-cell derived exosomes were shown to display MHC class II molecules in order to activate CD4\textsuperscript{+} T-cells\textsuperscript{71}. Continued research discovered that Dex display both
MHC class I and II molecules, permitting the activation of CD4$^{+}$ T-cells and CTLs. Human Dex also contains the NKG2D ligand that allows for the activation of NK cells$^{123}$, contributing to a more comprehensive immune response.

The alteration of maturation conditions \textit{in vitro} can lead to indirect exosome-loading. After a certain incubation time of cells with specific tumor antigens, the exosomes can be isolated and purified from the cell culture supernatant, followed by co-culturing with T-cells and measuring their activation potency. One study demonstrated their success by culturing DCs in the presence of a lymphocytic leukemia cell antigen and Poly I:C$^{124}$. The exosomes secreted by the DCs were isolated, purified, and their anti-tumor activity was assessed. Results showed an increase in spleen cell proliferation and enhanced cytotoxic effect of spleen cells on lymphocytic leukemia cells both \textit{in vitro} and \textit{in vivo}$^{124}$. In another study, DCs were matured with LPS and IFN-$\gamma$, their secreted exosomes harvested, and results revealed a significant increase in CTL activation and response \textit{in vivo}$^{63}$. Even with successful results, indirect exosome-loading by altering culture conditions is potentially problematic because the medium is a heterogeneous mixture of proteins and the culture conditions would affect more than just the specific antigen loading$^{75}$. Other concerns lie in the fact that this type of loading relies on the exosome sorting mechanisms within the cell and this is not fully understood$^{75}$.

2.8.3 Future of Bioengineered Exosomes in Immunotherapy Treatments

The overarching goal of bioengineered exosomes is to obtain the desirable components and have minimal negative effects \textit{in vivo}. The advent of bioengineered exosomes allows for the control of exosome content and function, and the manipulation
of an immunostimulatory or immunosuppressive role in treatments. Continued research will need to address concerns including how to ensure specific antigens or cell-targeting factors are loaded into or onto the exosome, as well as reliably reproducing exosome-loading. The mechanism of exosome content loading and sorting, and the molecules responsible for generating exosome-specific immune responses are not fully comprehended. The understanding of the methods to control the function of cell-specific exosomes in immune responses is making headway and has the potential to result in an entirely new class of immunotherapeutic. Although bioengineering exosomes is not the focus of my work, others are investigating it in the lab as well as our collaborators.

2.9 Thesis Statement

In order to develop novel immunotherapeutic strategies using bioengineered exosomes, the relationship between DCs and the exosomes they secrete must be fully understood. This thesis presents preliminary data on the effects of maturation conditions on the expression of immune activation markers and cytokine secretion in the MutuDC line. This will be accomplished by analyzing immature and various TLR-L-matured MutuDCs via flow cytometry to determine cell phenotype. Additionally, the cytokine profile will be assessed via direct ELISA to establish if the cells are polarizing a T\textsubscript{H}1- or T\textsubscript{H}2-type T-cell subset. Based on the up-regulation of co-stimulatory molecules, antigen presenting molecules, and IL-12 secretion, the more potent TLR-L will be utilized to induce MutuDC activation for exosome harvesting and characterization. The exosomes produced by MutuDCs and secreted into the culture medium will be isolated, purified, and their protein contents evaluated, with a particular focus on the characteristic exosome
proteins, antigen presenting molecules, and immune co-stimulatory molecules. Ideally, the MutuDC-derived exosomes will display a similar phenotypic profile and bear the immune surface molecules specific to their cell of origin in order to initiate the same type and intensity of immune response as the mature MutuDCs themselves.
CHAPTER III

METHODOLOGY

3.1 Cell Culture Conditions

The immortalized cell line MutuDC (Murine Tumor DC) was derived from spleen tumors in CD11c:SV40LgT-transgenic C57BL/6 mice and generously provided by Hans Acha-Orbea, Department of Biochemistry, Center of Immunity and Infection Lausanne, University of Lausanne, Epalinges, Switzerland beginning at passage 22. The cells were kept in culture at 37°C in a humidified incubator with 5% CO₂. The complete medium composition was Iscove’s Modified Dulbecco’s Medium (IMDM) with 25 mM HEPES (ThermoFisher, 12200) adjusted with NaHCO₃ and supplemented with 8-10% heat inactivated fetal bovine serum (FBS) (Rocky Mountain Biologicals, FBS-BBT), 50 µM beta-mercaptoethanol (BME) (Sigma Aldrich, M7154), 50 U/mL penicillin (Gibco, 15140), and 50 µg/mL streptomycin (Gibco, 15140). No additional growth factors were required in the cell culture medium. The MutuDCs were harvested by incubation in phosphate buffered saline (PBS) (Sigma, P4417) with 5 mM ethylenediaminetetraacetic acid (EDTA) (Sigma, E5134) for 10 minutes and centrifuged at 200x g for 5 minutes.

MutuDCs were seeded at approximately 2.5-5.0 x 10⁵ cells per cm² and not split lower than 10⁴ cells per cm². MutuDC stocks were continually prepared at 1.0-2.0 x 10⁶ cells per mL in 50% FBS and 10% DMSO in complete medium and kept in liquid nitrogen. MutuDC cultures were not kept past passage 43 due to changes in morphology and potential loss of function.
3.1.1 Exosome-Depleted FBS

In order to isolate and characterize exosomes secreted only from the MutuDCs, the FBS was filtered to rid the cell culture medium of FBS exosomes. Tangential flow using a Vivaflow 50R (Sartorius Stedim Lab, Ltd.) 100,000 HYS (100 kDa) filter was used to deplete the exosomes from the FBS with IMDM. A 100 kDa filter was utilized because exosomes are approximately 3-5 MDa, therefore this size of filter ensures exosome depletion from the FBS and the filtrate (flow through) containing exosome-free FBS and IMDM was harvested for further processing. Tests were also conducted using a 10 kDa filter to deplete the FBS of exosomes, however in the remainder of this paper, FBS-filtered media utilized the 100 kDa filter unless otherwise stated. Following tangential flow, the exosome-depleted FBS filtrate and IMDM was 0.22 µm filtered (Sartorius, 180C2) and the remaining BME, penicillin, and streptomycin were added at the stated concentrations (section 3.1) to the culture medium.

3.2 Cell Stimulation and Maturation

MutuDC stimulations were performed using the following TLR-ligands (TLR-Ls): LPS, TLR4-L (5 µg/mL, Invivogen, ttrl-pb5s); Poly I:C, TLR3-L (5 µg/mL, Invivogen, ttrl-pic); Class B CpG ODN 1826, TLR9-L (1 µM, Invivogen, ttrl-1826); Class C CpG ODN 2395, TLR9-L (1 µM, Invivogen, ttrl-2395); Flagellin from Bacillus subtilis, TLR5-L (0.5 µg/mL, Invivogen, ttrl-bsfla); Imidazoquinoline compound R848, TLR7/8-L (1 µg/mL, Invivogen, ttrl-r848); 19 mer S. aureus 23S rRNA (ORN Sa19), TLR13-L (1 µg/mL, Invivogen, ttrl-orn19); Peptidoglycan from Staphylococcus aureus, TLR2-L (5 µg/mL, Invivogen, ttrl-pgns2); or combinations thereof using the indicated
concentrations. Cells were seeded in new flasks 24 hours prior to stimulation. Cells were incubated in various TLR-Ls or TLR-L combinations for 20-24 hours in complete cell culture medium and regular culture conditions. Immature control cells were prepared and cultured in the same conditions as stimulated (matured) cells without TLR-L addition.

3.3 Cell Surface Immunophenotyping

For phenotypic analysis, MutuDCs were detached from culture flasks using 5 mM EDTA in PBS for 10 minutes at room temperature. Cell count and viability analysis was performed using trypan blue and a hemocytometer. The threshold deemed acceptable to continue phenotyping the cells were viability of $\geq 90\%$ and confluency of $\geq 80\%$, equating to approximately $10^5$ cells per cm$^2$. MutuDCs were centrifuged at 200x g for 5 minutes and re-suspended in PBS at 250,000 cells per vial to use for immature and mature DC antibody staining. Phycoerythrin (PE)-conjugated monoclonal antibodies and isotype controls were used to assess MutuDC phenotypic markers. All antibodies and isotype controls were purchased from BioLegend and diluted in PBS immediately before use. The antibodies used were specific to CD83 (0.25 µg/µL, 121507), CD8α (0.25 µg/µL, 100707), CD9 (0.25 µg/µL, 124805), CD63 (0.5 µg/µL, 143903), CD86 (0.25 µg/µL, 105007), CCR7 (CD197) (0.5 µg/µL, 120105), CD40 (0.25 µg/µL, 124609), CD1d (0.25 µg/µL, 123509), CD11b (0.25 µg/µL, 101207), CD54 (0.2 µg/µL, 116107), MHC-II (I-A/I-E) (0.25 µg/µL, 107607), MHC-I (H-2D$^d$) (0.25 µg/µL, 110607), CD36 (0.25 µg/µL, 102605), CD80 (0.5 µg/µL, 104707), CD81 (0.25 µg/µL, 104905), and CD11c (0.25 µg/µL, 117307). The isotype-matched monoclonal antibodies were used as controls in
every experiment and included Rat IgG1, κ Isotype Control (0.25 µg/µL, 400407), Rat IgG2a, κ Isotype Control (0.25 µg/µL, 400507), Rat IgG2b, κ Isotype Control (0.25 µg/µL, 400607), Mouse IgG2a, κ Isotype Control (0.25 µg/µL, 400211), and Armenian Hamster IgG Isotype Control (0.25 µg/µL, 400907).

For cell surface staining, 50 µL of primary diluted antibody and 50 µL of cell suspension (from the 250,000 cells/100 µL vial) was added to microcentrifuge tubes for a final volume of 100 µL. Microcentrifuge tubes were gently pulse vortexed to mix. Cell and antibody solution incubated for 30 minutes at 4°C in dark. Cells were washed twice in 500 µL PBS and pelleted by centrifugation at 300×g for 5 minutes with supernatant discarded in between washes. Cells were re-suspended in 100 µL of 1 mM EDTA in PBS for flow cytometry analysis.

3.4 Flow Cytometry Analysis

Flow cytometric analysis was performed using a Millipore Guava easyCyte Instrument (EMD Millipore). Parameters were Blue laser (488 nm) with Yellow-B (583/26) filter for PE-conjugated antibodies. Live cells were gated on followed by GFP positive cells using Yellow-B Fluorescence (YEL-B-HLog) on y-axis and Green-B Fluorescence (GRN-B-HLog) on x-axis. The GFP positive cells were gated on in order to determine the fluorescent intensity. Gain controls were set to FSC: 14.7, SSC: 38.1, GRN-B: 8.00, Yel-B: 8.00, Red-B: 8.00, with 5 Decade Acquisition of blue, red, and violet. The flow rate was medium and each flow test had a count of 30k events. The data was exported using FCS 2.0 and programmed using GuavaSoft version 3.1.1. All data was processed using Cytobank Community software program, version 5.3.1 (Cytobank,
3.5 Statistical Analysis

Statistical analyses were performed using GraphPad Software, Inc. Where indicated, p-values were obtained using two-tailed unpaired $t$-tests with 95% confidence intervals (ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001). Grubbs’ test was performed on data points to determine outliers using GraphPad Software, Inc. Error bars, where shown, indicate standard deviations.

3.6 Quantitation of Cytokine Secretion

3.6.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Cell supernatants were assayed for IL-12/23 p40 (BioLegend, 505201) and TNF-α (BioLegend, 510801) production by ELISA using an indirect protocol adapted from Abcam, Inc. The wells of a PVC microtiter plate were coated with 100 µL of undiluted cell supernatant from either immature or matured cells. The plate was sealed with parafilm and incubated overnight at 4°C followed by three washes with PBST (PBS + 0.05% Tween20). The remaining protein-binding sites were blocked with 200 µL of a 1x blocking solution: 5% non-fat dry milk in PBST overnight at 4°C followed by two washes with PBST. The primary antibody (IL-12/23 p40 or TNF-α) was diluted according to manufacturers directions in 1x blocking solution to reduce non-specific binding and 100 µL was added to the wells. The plate was sealed with parafilm and incubated overnight at 4°C followed by four washes with PBST. Horseradish-peroxidase
(HRP)-conjugated secondary antibody (HRP Goat anti-rat IgG, BioLegend, 405405) was diluted in 1x blocking solution according to manufacturers directions immediately before use and 100 µL was added to corresponding wells. The plate incubated at room temperature for two hours with gentle rocking followed by four washes with PBST.

Detection was accomplished in two ways to determine peroxidase enzymatic activity. For colorimetric reagent detection using a TMB solution: TMB substrate kit (Thermo Scientific, 34021) was prepared according to manufacturers directions. The absorbance of each well was measured at 450 nm using a BioTek Synergy HT Multi-Mode Microplate Reader (Gen 5, version 2.09). For fluorogenic substrate detection using an Amplex Red assay: Amplex UltraRed Reagent (Invitrogen, A36006) was diluted according to manufacturers recommendations and provided protocol, using PBS as the buffer for the analyte system. The absorbance was measured at 570 nm on a Nanodrop 2000c Spectrophotometer (ThermoScientific) and the fluorescence was measured at 530/590 nm using the BioTek Microplate Reader.

3.6.2 Intracellular Cytokine Staining

Cells were stimulated with various TLR-Ls for 20 hours. The intracellular protein transport inhibitor brefeldin A (10 µg/mL, Biolegend, 420601) was added to cell culture 4 or 16 hours after the addition of the TLR-L. Cells were fixed and permeabilized with Fixation Buffer (BioLegend, 420801) and Intracellular Staining Perm Wash Buffer (BioLegend, 421002) according to manufacturers directions. Cells were indirectly stained for intracellular IL-12/23 p40 (BioLegend, 505201) and TNF-α (BioLegend, 510801) for 30 minutes in the dark at 4°C followed by secondary goat anti-rat IgG PE-
conjugated antibody (Abcam, 7010) for 30 minutes in the dark at 4°C. Antibodies were diluted according to manufacturers directions in PBS immediately before use. Cells were re-suspended in 100 µL of 1 mM EDTA in PBS and analyzed on the flow cytometer.

3.7 Exosome Isolation and Purification

3.7.1 Tangential Flow

A Vivaflow 50R (Sartorius Stedim Lab, Ltd.) 100,000 HYS (100 kDa) filter was used to concentrate cell supernatant for exosome purification, isolation, and characterization, where exosomes are retained in the 100 kDa concentrate. Immature and TLR3-L matured cell culture supernatant was collected after MutuDC stimulation. The supernatant was concentrated using PBS by pumping the liquid through the system at a recirculation rate of approximately 200 mL/min. When the desired volume of approximately 8 mL was reached, the recirculation rate was reduced to 20 mL/min and the concentrated sample was re-circulated for 2 minutes. To recover the concentrated exosome sample, the feed line was removed from the sample reservoir and the residual system liquid was pumped back into the reservoir container for collection and analysis. A Nanodrop 2000c Spectrophotometer (ThermoScientific) at 280 nm was used to determine the protein (exosome) concentration for further analysis.

3.7.2 Fast Protein Liquid Chromatography (FPLC)

Following tangential flow concentration, cell culture supernatant containing MutuDC exosomes were purified and further concentrated using fast protein liquid chromatography (FPLC) technique. Prior to FPLC, exosomes from immature and
matured MutuDC culture supernatant was concentrated using tangential flow (as described in 3.7.1). The exosome mixture was purified and concentrated further via FPLC (ÄKTAexplorer, GE Healthcare Life Sciences, 18111241) technique utilizing a HiTrap Heparin HP column (5 mL, GE Life Sciences, 17-0407-01) equilibrated with 10 mM Na$_2$HPO$_4$, eluted with 2 M NaCl, and the eluted exosomes were collected for further analysis. Contents from the Heparin column elution were further concentrated using a 30 kDa filter (VivaSpin 20, GE Healthcare, 28-9323-60) and centrifuged at 3500x g for 15 minutes at 4°C until the final volume reached approximately 0.5 mL. The concentrated exosome mixture was re-suspended in 1.5 mL PBS with 10% glycerol and 1 mM EDTA and transferred to an eppendorf tube. The protein (exosome) concentrations were determined using the Nanodrop spectrophotometer for exosome surface characterization. An Amplex Red assay was utilized to determine the peroxidase enzymatic activity. Amplex UltraRed Reagent (Invitrogen, A36006) was diluted according to manufacturers recommendations and provided protocol, using PBS as the buffer for the analyte system. The absorbance was measured at 570 nm on a Nanodrop 2000c Spectrophotometer (ThermoScientific) and the fluorescence was measured at 530/590 nm using the BioTek Microplate Reader.

3.8 Exosome Surface Characterization

Exosomes secreted from MutuDCs were purified and concentrated via tangential flow or tangential flow in combination with FPLC, as described in 3.7.1 and 3.7.2. Exosomes were re-suspended in PBS with 10% glycerol and 1 mM EDTA. Because exosomes are too small to be detected by the flow cytometer, they were conjugated to
aldehyde/sulfate latex beads, 4% w/v, 4 µm (ThermoFisher, A37304). Exosomes were either directly conjugated to the beads or antibodies were coated to the latex beads followed by incubation with exosomes. The latex bead/exosome mixture was stained with various antibodies (as described in 3.3) and characterized via flow cytometry analysis.

3.8.1 Direct Latex Bead Conjugation

A 10 µL solution of latex beads were washed twice with PBS and centrifuged at 10,000x g for five minutes at room temperature. The supernatant was aspirated and the beads were re-suspended in 100 µL PBS. To eppendorf tubes, 1.0 µL of bead solution was added followed by the addition of varying exosome solution concentrations. The volume was brought to 500 µL with PBS and rotated overnight at room temperature. The solution was centrifuged at 10,000x g for five minutes and re-suspended in 1 mL of 1 mM glycine to block remaining latex bead sites. The solution incubated 30 minutes at room temperature while rotating. After incubation, the mixture was washed in 1 mL PBS and spun down at 10,000x g for 10 minutes, followed by re-suspension in 50 µL PBS. Antibodies were diluted according to manufacturers directions and 50 µL of antibody mixture was added to the beads/exosomes. The bead/exosome and antibody mixture incubated at 4°C in darkness for 30 minutes. Upon incubation completion, 100 µL PBS was added to beads to wash followed by centrifugation at 2,000x g for five minutes. The pellet was re-suspended in 150 µL PBS and ran on the flow cytometer.
3.8.2 Sandwich Latex Bead Conjugation

A 10 µL solution of latex beads was washed twice with PBS and centrifuged at 10,000x g for five minutes. The supernatant was aspirated and the beads were re-suspended in 10 µL PBS. To the beads, 10 µg of CD9 antibody (Santa Cruz Biotechnology, SC-13118) was added and the volume was adjusted to 50 µL with PBS. The mixture incubated at room temperature for 15 minutes. PBS was added to reach a total volume of 500 µL and rotated overnight at room temperature. The bead/antibody mixture was spun down at 10,000x g for five minutes followed by the addition of 1 mL of 1 mM glycine to block the remaining sites on the beads. The solution incubated 30 minutes at room temperature while rotating. The beads were washed in PBS containing 1% bovine serum albumin (BSA) (Sigma Aldrich, A9647), centrifuged at 10,000x g for five minutes, and re-suspended in 100 µL (10x more volume than initial bead volume) PBS containing 1% BSA. From the 10x bead solution, 1.0 µL was transferred to eppendorf tubes and incubated with varying concentrations of exosome solution generated from the procedures described in 3.7.1 or 3.7.2. The bead-exosome mixture incubated for 30 minutes at room temperature. The mixture was scaled up to 500 µL with PBS and incubated overnight at room temperature while rotating. The beads were spun down at 10,000x g for 10 minutes and re-suspended in 50 µL PBS. Antibodies were diluted according to manufacturers directions and 50 µL of antibody mixture was added to the beads/exosomes. The bead/exosome and antibody mixture incubated at 4°C in darkness for 30 minutes. Upon incubation completion, the solution was washed with 100 µL PBS followed by centrifugation at 2,000x g for five minutes. The pellet was re-suspended in 150 µL PBS and ran on the flow cytometer.
3.8.3 Flow Cytometry Analysis of Exosomes

Flow cytometric analysis was performed using a Millipore Guava easyCyte Instrument (EMD Millipore). Parameters were Blue laser (488 nm) with Yellow-B (583/26) filter for PE-conjugated antibodies. The latex beads were gated on to determine fluorescent intensity. Gain controls were set to FSC: 14.7, SSC: 38.1, GRN-B: 8.00, Yel-B: 8.00, Red-B: 8.00; with 5 Decade Acquisition: blue, red, and violet. The flow rate was medium with a count of at least 10k events. The data was exported using FCS 2.0 and programmed using GuavaSoft version 3.1.1. All data was processed using Cytobank Community software program, version 5.3.1 (Cytobank, Inc.). Where indicated, the mean fluorescent intensity (MFI) was used to determine the fluorescent difference between samples.
CHAPTER IV

EXPERIMENTAL RESULTS

4.1 Maintenance of the MutuDC Line

The MutuDC line was derived from mouse CD8$\alpha^+$ DC splenic tumors and undergoes immortalization in vitro through the spontaneous selection of the DC tumor cells that are able to stabilize their survival in culture$^{33}$. Unlike some immortalized cell lines, MutuDCs cannot be cultured indefinitely, however, published results showed MutuDCs retained their ability to up-regulate activation markers even at passage number 98$^{33}$. It was recommended not to conduct experiments on the cells after passage 50 however, due to the diminished capacity of the cells to secrete IL-12 p70 and up-regulate the co-stimulatory molecules to the same degree as lower passaged cells$^{33}$. We received the MutuDCs at passage number 22 and used the cells for experimentation until passage number 42 to avoid any potential loss of co-stimulatory up-regulation or cytokine secretion. A loss of co-stimulatory up-regulation was not observed at passage 42 cells, but we used this as a limit to mitigate variation in results due to aging cell cultures.

4.2 Gating Strategy for Flow Cytometry Analysis

MutuDCs are GFP positive due to the GFP reporter in the CD11c:SV40LgT transgene. Cells were gated based on live eGFP expression cells and identified on the FSC and SSC plot as indicated (Figure 7A). The mean fluorescence intensity (MFI) value was determined by the mean log channel fluorescence value of the antibody-reacted cell samples (Figure 7B).
Figure 7. **Gating strategy for flow cytometry analysis of MutuDC line.** (A) MutuDCs were identified on the FSC and SSC plot that represented single cells, circled. The circle denotes the region selected for gating in all experiments. (B) Histogram profile shows a representative sample for determining the mean fluorescence intensity (MFI) of surface CD antigens on MutuDCs.

The relative mean fluorescence intensity (RMFI) was determined by dividing the MFI value by that of the isotype control. This eliminated non-specific binding that may have been a result of the immunoglobulin binding to Fc receptors on the cell surface. Dividing the matured MutuDC RMFI by that of the immature MutuDC RMFI, yielding a fold to immature cell value, normalized the data.

4.3 *MutuDC Phenotypic Data Validation*

Prior to collecting phenotypic data from different TLR-L stimulations, it was important to ensure our immature and mature MutuDCs exhibited an expression profile that was similar to those observed in the published results. Marraco et al. generated the novel MutuDC line and published their results in 2012 with very few publications since
then, making it even more vital that we validate our phenotypic results with theirs. The activation profile of MutuDCs in response to TLR3-L (Poly I:C), TLR4-L (LPS), TLR7/8-L (R848), and TLR9-L (CpG 1826) stimulations display the characteristic maturation markers as wild type splenic CD8α+ cDCs (Figure 8A)33. MutuDCs strongly up-regulated the co-stimulatory molecules CD40, CD80, CD86, and MHC class II and this was especially prevalent in response to the TLR9-L. The phenotypic results of MutuDCs stimulated in this laboratory (Figure 8B) coincide with the fold to medium, or fold to immature cells, as observed in the published data using the same TLR-L concentrations and treatment time.
Figure 8

Figure 8. The activation profile of MutuDCs in response to TLR-L stimulations for data validation. (A) Data published from Marraco, et al. showing MutuDCs, labeled as DC lines (n=4), and purified splenic cDC subsets (n=2 per subset) stimulated with various TLR-Ls and analyzed for activation markers CD40, CD80, CD86, and MHC class II. Data are shown with the corresponding Spearman’s rank correlation coefficient comparing three types of DCs: MutuDCs and wild type CD8α+ and CD8α− cells. The data are presented as mean ± SD. (B) Data obtained from our experiments stimulating MutuDCs with TLR3-L (n=7), TLR4-L (n=8), TLR9-L (n=3), and TLR7/8-L (n=1) to compare and validate the maturation markers to the published results. Data are presented as mean ± SD and their corresponding p-values (ns = not significant, * = p < 0.05, ** = p < 0.01, *** p < 0.0001).
Stimulation of cells with TLR9-L CpG displayed up-regulation of co-stimulatory molecules to a higher degree than the other TLR-Ls. Because MutuDCs are CD8α+ they do not express TLR7/8, as observed in a lack of co-stimulatory and MHC class II up-regulation. Overall, the data indicated MutuDCs stimulated in this laboratory strongly responded to TLR3-L, TLR9-L, and to a lesser extent TLR4-L, coinciding with the published results of the MutuDC line and characteristic CD8α+ cDC subset.

4.4 Exosome-Depleted FBS

Like most mammalian cells, MutuDCs require the presence of fetal bovine serum (FBS) in culture medium to provide a variety of macromolecular proteins, nutrients, and various insoluble components necessary for cell survival. FBS contains bovine-generated exosomes, however, making it necessary to deplete the culture medium of any FBS exosomes present in order to ensure the purification and characterization of exosomes contains only those secreted from MutuDCs. The viability and efficacy of MutuDCs in various culture conditions was determined. First MutuDCs cultured in FBS-free media containing only IMDM, β-mercaptoethanol (BME), and penicillin/streptomycin were tested. The MutuDCs displayed a different cellular morphology after 24 hours, appearing to have longer outgrowths from the main cell body. The MutuDCs in FBS-free media were also strongly adhered to the cell culture flask and would not detach even after 20 minutes of incubation with trypsin and 5 mM EDTA. Within 48 hours, 100% of the MutuDCs were dead. Next, MutuDCs were cultured in the serum-free media OptiMEM. This culture medium contained only OptiMEM, BME, and penicillin/streptomycin. Culturing MutuDCs in OptiMEM resulted in approximately 50% cell death after 24
hours. The remaining 50% of the cells were also strongly adhered to the cell culture flask and could not be detached even in the presence of trypsin and 5 mM EDTA. Within 48 hours, MutuDCs incubated in OptiMEM resulted in 100% cell death.

Based on the results obtained from culturing MutuDCs in FBS-free and OptiMEM medium, another approach to depleting the FBS of exosomes and evaluating MutuDC viability was required. We decided to filter the FBS via tangential flow using a 10 kDa and 100 kDa filters to rid the serum of exosomes. Tangential flow was the preferred method of filtration because it prevented filter cake formation, thereby increasing the lifespan of the filter as well as allowing a large amount of fluid to be filtered continuously. Because exosomes are approximately 3-5 MDa, both the 10 kDa and 100 kDa FBS filtrate would be depleted of exosomes, thus ensuring the cell culture supernatant collected for exosome purification and characterization contains only MutuDC-secreted exosomes. Cells were cultured in IMDM containing 10 kDa or 100 kDa FBS filtrate, BME, and penicillin/streptomycin. MutuDCs cultured in 10 kDa FBS filtrate were over 90% viable after 24 hours although they exhibited a different cellular morphology with longer outgrowths from the main cell body as observed in MutuDCs cultured in serum-free media. After 48 hours, however, MutuDCs cultured in 10 kDa FBS filtrate resulted in over 75% cell death. On the other hand, MutuDCs cultured in 100 kDa FBS filtrate remained over 90% viable and were able to be kept in culture in excess of four weeks. A decrease in viability was not observed even at the four week mark, but cells were discarded by that time point because they had reached too high of a passage to be kept for further experiments. MutuDCs in 100 kDa FBS filtrate also displayed a similar cellular morphology and were able to be detached from cell culture
flasks easily after 10 minutes of incubation with PBS and 5 mM EDTA, as observed with MutuDCs cultured in regular conditions using unfiltered FBS. To ensure the MutuDCs cultured in 100 kDa FBS filtrate display similar DC characteristic surface markers and activation markers upon stimulation with various TLR-Ls as those in unfiltered FBS medium, experiments were conducted using both culture conditions for all TLR-L stimulations.

4.5 TLR-L Stimulations

A significant portion of this research project was dedicated to investigating the effects of various maturation conditions on MutuDCs by analyzing the expression of surface immune markers. As discussed previously, DCs display different classes of TLRs that recognize TLR-Ls, known as pathogen-associated molecular patterns (PAMPs) found on bacteria, viruses, or parasites that determines the maturation path and function of the DC. Stimulations were conducted on DCs targeting TLRs expressed on the plasma membrane and intracellularly on the endosome. PM-expressed TLRs include TLR2, TLR4, and TLR5, and endosome-expressed TLRs include TLR3, TLR7/8, TLR9, and TLR13. All of the TLR-L stimulations were completed in both unfiltered FBS medium and 100 kDa FBS filtrate medium, annotated as unfiltered FBS and filtered FBS, respectively.

MutuDCs were assessed for the expression of a panel of lineage surface markers characteristic of the splenic CD8α+ cDC subset including CD8α, CD11b, and CD11c. The activation profile of MutuDCs to various TLR-L stimulations was analyzed and included the adhesion molecule CD36, chemokine receptor for DC homing CCR7, and
tetraspanins CD9, CD63, and CD81 for their role in DC maturation and motility. Most importantly, the antigen presentation molecules CD1d and MHC classes I/II, as well as the co-stimulatory molecules historically up-regulated during DC maturation including CD40, CD54, CD80, CD83, and CD86 were evaluated.

4.5.1 Plasma Membrane-Expressed TLR Stimulations

The PM-targeting TLR2-L (PGN-SA), TLR4-L (LPS), and TLR5-L (Flagellin) were used to stimulate MutuDCs for 20 hours and their surface antigen expression was assessed via flow cytometry as described in sections 3.2, 3.3, and 3.4. The data are represented as the RMFI (Figure 9) and normalized to show the fold to immature cells (Figure 10). Similar to the CD8α+ cDC subset, MutuDCs responded to TLR-L stimulations through the up-regulation of co-stimulatory molecules and increased antigen presentation via MHC class II molecules.
**Figure 9**

Relative Mean Fluorescence Intensity (RMFI) of PM-Expressed TLR Stimulations

**Cells Cultured in Unfiltered FBS**

**Cells Cultured in Filtered FBS**

\[ \text{RMFI} \]

\[ \text{MHC I} \quad \text{MHC II} \quad \text{CD1d} \]

\[ \text{RMFI} \]

\[ \text{CD40} \quad \text{CD54} \quad \text{CD80} \quad \text{CD83} \quad \text{CD86} \]

\[ \text{RMFI} \]

\[ \text{CD40} \quad \text{CD54} \quad \text{CD80} \quad \text{CD83} \quad \text{CD86} \]

\[ \text{ns} = \text{not significant, } * = p < 0.05, ** = p < 0.01, *** p < 0.0001 \]
Figure 9. The RMFI of characteristic surface and activation markers of MutuDCs in response to PM-expressed TLR stimulations. MutuDCs were stimulated for 20 hours with different TLR-Ls as indicated and analyzed for characteristic DC surface and activation markers. Data are presented as mean ± SD with their corresponding p-values (ns = not significant, * = p < 0.05, ** = p < 0.01, *** p < 0.0001).
Figure 10

Fold to Immature Cells of PM-Expressed TLR Stimulations

Cells Cultured in Unfiltered FBS

Cells Cultured in Filtered FBS

ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001
Figure 10. The fold to immature cells of characteristic surface and activation markers of MutuDCs in response to PM-expressed TLR stimulations. MutuDCs were stimulated for 20 hours with different TLR-Ls as indicated and analyzed for characteristic DC surface and activation markers. Data are presented as mean ± SD with their corresponding p-values (ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001).

MutuDCs cultured in unfiltered and filtered FBS displayed an overall uniformity in their lineage surface markers, with a CD11c\textsuperscript{high} and CD11b\textsuperscript{low} expression regardless of
the TLR-L stimulation (Figure 9). Consistent with published results\textsuperscript{125}, a slight decrease in CD11c expression was observed upon DC activation. An approximate 2.25-fold increase of CD8α expression was detected in TLR2-L stimulated cells cultured in unfiltered FBS (Figure 10). It is not uncommon to see CD8α fluctuations \textit{in vitro} and this can be possibly attributed to the culture conditions or passage number at the time of stimulation. For all immature and TLR-L stimulated MutuDCs, little expression of CD36 and CCR7 was observed. Lastly, a similar tetraspanin profile was noted between the unfiltered and filtered FBS cultured MutuDCs, exhibiting CD63\textsuperscript{low} and CD81\textsuperscript{high}.

Consistent with DC maturation profiles, the antigen presenting molecule MHC class II and co-stimulatory molecules CD40, CD54, CD80, and CD86 were strongly up regulated upon stimulation. As expected, the antigen-presenting molecule CD1d did not have an increased expression because none of the TLR-Ls were glycolipids. The data confirms a trend in surface molecule expression between MutuDCs cultured in unfiltered or filtered FBS upon stimulation with various PM-targeting TLR-Ls, with a stronger DC activation response to TLR4-L.

\subsection*{4.5.2 Endosome-Expressed TLR Stimulations}

The endosome-expressed TLRs were targeted using TLR3-L (Poly I:C), TLR9-L (CpG 1826 and 2395), TLR13-L (23S rRNA), and TLR7/8-L (R848) for 20 hours and their surface antigen expression was assessed via flow cytometry as described in sections 3.2, 3.3, and 3.4. The data are represented as the RMFI (Figure 11) and normalized to show the fold to immature cells (Figure 12). Similar to the PM-targeting TLR-Ls, MutuDCs responded to endosome-targeting TLR-L stimulations through the up-
regulation of co-stimulatory molecules and increased antigen presentation via MHC class II molecules, albeit to a higher degree. Because MutuDCs lack TLR7/8 expression, the co-stimulatory molecules CD40, CD80, CD83, and CD86 and antigen presentation molecules MHC class I/II for cells cultured in unfiltered FBS were the only surface markers tested in order to validate a lack of expression and up-regulation.
Figure 11

Relative Mean Fluorescence Intensity (RMFI) of Endosome-Expressed TLR Stimulations

- Immature Cells
- TLR3: Poly I:C
- TLR9: CpG 1826
- TLR9: CpG 2395
- TLR13: 23S rRNA
- TLR7/8: R848

Cells Cultured in Unfiltered FBS

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Cells Cultured in Filtered FBS

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ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001
Figure 11 (cont’d)

Relative Mean Fluorescence Intensity (RMFI) of Endosome-Expressed TLR Stimulations

Cells Cultured in Unfiltered FBS

Cells Cultured in Filtered FBS

Figure 11. The RMFI of characteristic surface and activation markers of MutuDCs in response to endosome-expressed TLR stimulations. MutuDCs were stimulated for 20 hours with different TLR-Ls as indicated and analyzed for characteristic DC surface and activation markers. Data are presented as mean ± SD with their corresponding p-values (ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001).
Figure 12

Fold to Immature Cells of Endosome-Expressed TLR Stimulations

|----------------|---------------|----------------|----------------|-----------------|-------------|

- **Cells Cultured in Unfiltered FBS**
- **Cells Cultured in Filtered FBS**

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ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001
Figure 12 (cont’d)

Fold to Immature Cells of Endosome-Expressed TLR Stimulations

Figure 12. The fold to immature cells of characteristic surface and activation markers of MutuDCs in response to endosome-expressed TLR stimulations. MutuDCs were stimulated for 20 hours with different TLR-Ls as indicated and analyzed for characteristic DC surface and activation markers. Data are presented as mean ± SD with their corresponding p-values (ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001).
Based on the data in Figure 11, the RMFI of the lineage surface markers remained consistent between unfiltered and filtered FBS cultured MutuDCs as well as between immature and mature cells. The RMFI displayed CD11b^{low} and CD11c^{high} expression across the various culture and stimulation conditions as expected. As observed with TLR2-L stimulated MutuDCs (Figure 10), TLR3-L also exhibited an approximate 2- and 3-fold increase in CD8α expression in unfiltered and filtered FBS cultured cells, respectively. Again, fluctuations in CD8α expression are not uncommon in DCs.

MutuDCs cultured in both FBS conditions exhibited a similar tetraspanin profile with CD63^{low} and CD81^{high} and overall low expression of CD36 and CCR7.

Consistent with the DC maturation profile, TLR-Ls increased the expression of co-stimulatory molecules and MHC class II. Interestingly, TLR3-L stimulation up-regulated the CD1d molecule to a higher degree than the other TLR-Ls. As expected, TLR7/8-L did not activate MutuDCs due to the lack of TLR7/8 expression in the cells. Overall, the data suggests TLR3-L and both TLR9-Ls induce stronger DC activation through the up-regulation of CD40, CD80, CD86, and MHC class II when compared to the other TLR-Ls.

4.5.3 Combination TLR Stimulations

In order to induce a superior immune response, we wondered if the simultaneous activation of different TLRs would produce a stronger up-regulation of co-stimulatory and antigen presenting molecules. A synergistic activation of MutuDCs by combining TLR3-L (Poly I:C) and TLR9-L (CpG 1826) was tested using the same TLR-L concentrations and stimulation times when added alone. These TLR-Ls were chosen
based on the superior up-regulation of co-stimulatory and antigen-presenting molecules compared to the endosome-expressed TLR-L stimulations. The data are represented as the RMFI (Figure 13) and normalized to show the fold to immature cells (Figure 14). The data from TLR3-L and TLR9-L (CpG 1826) from Figures 11 and 12 was included in order to discern any similarities or differences in the RMFI and fold increase of surface molecule expression.
Figure 13

Relative Mean Fluorescence Intensity (RMFI) of Combination TLR Stimulations

Cells Cultured in Unfiltered FBS

Cells Cultured in Filtered FBS

ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001
Figure 13. The RMFI of characteristic surface and activation markers of MutuDCs in response to simultaneous TLR stimulations. MutuDCs were stimulated for 20 hours with different TLR-Ls as indicated and analyzed for characteristic DC surface and activation markers. Data are presented as mean ± SD with their corresponding p-values (ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001).
Figure 14

Fold to Immature Cells of Combination TLR Stimulations

Cells Cultured in Unfiltered FBS

Cells Cultured in Filtered FBS

ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001
Figure 14 (cont’d)

**Fold to Immature Cells of Combination TLR Stimulations**

- Immature Cells
- Poly I:C + CpG 1826
- TLR3: Poly I:C
- TLR9: CpG 1826

Cells Cultured in Unfiltered FBS

![Graph](image1)

Cells Cultured in Filtered FBS

![Graph](image2)

Figure 14. The fold to immature cells of characteristic surface and activation markers of MutuDCs in response to simultaneous TLR stimulations. MutuDCs were stimulated for 20 hours with different TLR-Ls as indicated and analyzed for characteristic DC surface and activation markers. Data are presented as mean ± SD with their corresponding p-values (ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001).

The combination stimulation of MutuDCs reveals consistent lineage surface molecules CD8α and CD11b/c, adhesion molecule CD36, and tetraspanin expression as...
observed in the other TLR-L stimulations and between the unfiltered and filtered FBS cultured cells. Interestingly, the combination TLR3/9-L stimulation in the filtered FBS cultured MutuDCs up-regulated the chemokine receptor CCR7 expression compared to the TLR-Ls alone, with an approximate two-fold increase.

The simultaneous activation of TLR3 and TLR9 caused a significant fold increase in co-stimulatory molecules CD80 and CD86, and a moderate fold-increase in MHC class II expression (Figure 14). This was observed in both unfiltered and filtered FBS culture conditions. An increased expression of co-stimulatory and antigen-presenting molecules was observed in the synergetic activation; however, it was not statistically significant when compared to the expression of the individual TLR-Ls. The remaining co-stimulatory and antigen presenting molecules exhibited a similar trend in up-regulation upon stimulation between the unfiltered and filtered FBS cultured cells, and the single versus synergistic TLR stimulations.

4.6 Varying the TLR-L Concentrations and Stimulation Times

The concentrations of the TLR-Ls used in these experiments to stimulate the MutuDCs were chosen based on published literature and/or the manufacturer’s recommendations. We wanted to know if MutuDCs would increase the up-regulation of co-stimulatory and antigen presenting molecules if they were cultured for longer than 20 hours with a TLR-L. The longer incubation period would also allow more time for the MutuDCs to secrete exosomes that could be harvested for further experimentation and characterization. Additionally, we wanted to assess MutuDC surface antigen expression after doubling the amount of TLR3-L to see if co-stimulatory and/or antigen presenting
molecule expression would increase, allowing for a more potent immune response.

TLR3-L (Poly I:C) was chosen because a vast amount of data was available to compare the results. Furthermore, there was a large stock in the laboratory and the synthetic ligand is relatively inexpensive. MutuDCs were cultured in 100 kDa filtered FBS medium for 48 hours in the presence of 5.0 µg/mL Poly I:C and for 20 hours with 10 µg/mL Poly I:C (Figure 15). The standard concentration of Poly I:C for all the experiments was 5.0 µg/mL.
Figure 15. The activation profile of MutuDCs in response to TLR3-L stimulations in various conditions. Graphs show MutuDCs cultured in 100 kDa FBS filtered medium stimulated with the average 5 µg/mL of Poly I:C for 20 hours (pink bars), double the Poly I:C concentration at 10 µg/mL for 20 hours (green bars), and the standard Poly I:C concentration of 5 µg/mL for 48 hours (purple bars). Bar graphs show the RMFI (A) and fold to immature cells (B) of MutuDCs in response to Poly I:C in various conditions.

The activation profile of MutuDCs in response to Poly I:C stimulation at double the TLR-L concentration and at 48 hours verses 20 hours both reveal a decrease in co-stimulatory and antigen presenting molecules. The viability of the MutuDCs also decreased upon changing the two variables. MutuDCs were 87% viable in 10 µg/mL Poly I:C and 76% viable after 48 hours of stimulation in 5 µg/mL Poly I:C, compared to
the typical 90% viability achieved after 20 hours of stimulation with 5 µg/mL Poly I:C.

Cells cultured for 48 hours in the presence of Poly I:C had significant amounts of cellular debris and were difficult to detach from the cell culture flasks. The results from this experiment revealed that a 20 hour stimulation in Poly I:C at 5 µg/mL was sufficient for MutuDC activation while maintaining cellular viability and function.

4.7 MutuDC Cytokine Profile

To assess whether the TLR-L stimulations were activating MutuDCs to become T\(_{H1}\) verses T\(_{H2}\) polarizing, it was vital to determine the cytokine profile. IL-12 production is one of the characteristic features of CD8\(^+\alpha\) cDCs, resulting in a T\(_{H1}\)-polarizing and CTL-inducing function. Another signature T\(_{H1}\)-polarizing DC cytokine is TNF-\(\alpha\). Additionally, the cytokine IL-4 was evaluated, as the detection of this cytokine would indicate the MutuDCs were T\(_{H2}\)-polarizing. Two cytokine detection methods were employed: intracellular cytokine staining and direct ELISA. The intracellular cytokine detection method was unsuccessful and none of the cytokines were detected (results not shown). The addition of brefeldin A the last two hours of stimulation significantly reduced the confluency of MutuDCs from 90% to 50% confluent. The residual live cells however, remained above 90% viable. The addition of Brefeldin A for 16 hours during stimulation resulted in nearly 100% cell death.

MutuDCs cultured in 100 kDa FBS filtrate were activated with several TLR-Ls and combination TLR-Ls for 20 hours. The cell culture supernatant was collected and assayed via direct ELISA for IL-12/23p40, IL-4, and TNF-\(\alpha\) secretion (Figures 17 and 18). Recombinant IL-12/23p40 was used to generate a standard curve (Figure 16) and...
determine the concentration of IL-12/23p40 secreted from MutuDCs (Figure 17). No recombinant IL-4 or TNF-α was available to quantify the MutuDC cytokine secretion, so the graph represents fold to immature cells based on the fluorescence intensity value (Figure 18). The fluorogenic Amplex UltraRed reagent was used for detection in the ELISA because it offered a more sensitive and robust reaction product compared to the colorimetric reagent TMB (data not shown).

Figure 16

**Standard Curve of Recombinant IL-12/23 p40**

\[
y = 449.02x + 690.19 \\
R^2 = 0.99184
\]

**Figure 16. Recombinant IL-12/23 p40 standard curve.** The recombinant IL-12/23 p40 underwent serial dilutions and a standard curve was generated. The trendline was calculated and the equation was used to determine the concentration of IL-12/23 p40 secreted by MutuDCs under various TLR-L stimulation conditions.
**Figure 17.** IL-12/23 p40 expression level of MutuDCs stimulated with various TLR-Ls. Cell culture supernatant from 100 kDa FBS filtrate cultured MutuDCs stimulated with various TLR-Ls underwent direct ELISA to determine the concentration of IL-12/23 p40 secretion. **(A)** The concentration of IL-12/23 p40 (ng/mL) was determined based on the standard curve in Figure 15. The cytokine results of stimulated MutuDC were all in filtered FBS. The immature MutuDCs cultured in unfiltered FBS is shown to compare the cytokine concentration. **(B)** The fold to immature cells of IL-12/23 p40 secreted from MutuDCs. Data are presented as mean ± SD with their corresponding p-values (ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001).
Figure 18. IL-4 and TNF-α expression levels of MutuDCs stimulated with various TLR-Ls. Cell culture supernatant from 100 kDa FBS filtrate cultured MutuDCs stimulated with various TLR-Ls underwent direct ELISA to determine the fold-increase of IL-4 (A) and TNF-α (B) secretion. Data are presented as mean ± SD with their corresponding p-values (ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.0001).

Based on the standard curve of recombinant IL-12/23 p40 (Figure 16), MutuDCs secreted significantly higher concentrations of IL-12/23 when stimulated via their
endosome-expressed TLRs, as observed using TLR3-L Poly I:C and TLR9-L CpG. Cells secreted relatively low amounts of IL-12/23 p40 when their PM-expressed TLRs were stimulated with TLR4-L flagellin and TLR5-L LPS. TLR9-L CpG 2395 had the highest fold increase of IL-12/23 p40 secretion, approximately 126.4 (Figure 17B). MutuDCs had little to no secretion of IL-4, indicating the cells were not Th2 polarizing (Figure 18A). Unexpectedly, the Th1 polarizing cytokine TNF-α was not detected in the MutuDC cell culture supernatant (Figure 18B).

4.8 Exosome Purification and Characterization

4.8.1 Exosome Quantitation

To investigate the immunotherapeutic vehicle potential of exosomes secreted from MutuDCs, we isolated, purified, and concentrated exosomes from TLR3-L (Poly I:C)-stimulated MutuDC culture supernatant as described in section 3.7. Cells were cultured in medium containing 100 kDa FBS filtrate to ensure no FBS-derived exosomes were present. Approximately 80 mL of immature and TLR3-L matured cell culture supernatant was concentrated via tangential flow using a 100 kDa filter (as described in section 3.7.1) and further purified by FPLC (as described in section 3.7.2). An enzymatic assay using Amplex UltraRed reagent determined the moles of peroxidase in the purified exosomes. A standard curve (Figure 19A) was generated by serial dilution of HRP-conjugated antibody. The approximate moles of peroxidase for the exosomes were calculated based on the line of best fit (Figure 19B).
Figure 19. **Amount of exosomes secreted based on enzymatic activity.** MutuDCs in 100 kDa FBS filtrate culture medium were stimulated with TLR3-L. Exosomes were harvested by concentrating the cell culture supernatant via tangential flow and purified using FPLC. The moles of exosomes from immature and mature cells was determined and validated based on their enzymatic activity. A standard curve was generated by serial dilutions of HRP-conjugated antibody (A) and the equation was utilized to determine the moles of peroxidase of the exosomes (B).
Immature MutuDCs secreted approximately seven times the amount of exosomes compared to TLR3-L matured MutuDCs. Additionally, the same sample of purified exosomes was quantified using a Nanodrop (Figure 20).

**Figure 20.** Quantitation of exosomes based on Nanodrop. MutuDCs in 100 kDa FBS filtrate culture medium were stimulated with TLR3-L. Exosomes were harvested by concentrating the cell culture supernatant via tangential flow and purified using FPLC. The exosomes from immature and matured cells were quantified using a Nanodrop.

Based on the results of the Nanodrop, immature MutuDCs secreted approximately 5.6-times the concentration of exosomes compared to TLR3-L matured MutuDCs. Data from both the enzymatic assay and the Nanodrop exhibit a similar trend displaying a decrease in MutuDC exosome production when stimulated with TLR3-L. The exosome concentration obtained via the Nanodrop was used to determine the amount of product required to characterize exosomes using flow cytometry.
4.8.2 Exosome Characterization

Exosomes exhibit a similar phenotypic profile of the host cell and should display the classic DC maturation markers CD40, CD54, CD80, and CD86, the antigen presenting molecule MHC class II, and several tetraspanin proteins including CD9, CD63, and CD81. Exosomes were indirectly or directly conjugated to aldehyde/sulfate latex beads followed by flow cytometry analysis as described in section 3.8. During the bead conjugation process it was imperative to aspirate during the wash steps versus flick the vials, as this could lead to accidentally discarding the bead pellet. Numerous attempts were made at indirectly conjugating the exosomes to beads using anti-CD9 antibody, however it consistently resulted in either little to no signal or an abundant amount of debris and bead aggregates (data not shown). The tetraspanin CD9 was chosen because it was consistently expressed in MutuDCs compared to CD63. The anti-CD81 antibody was not available for bead conjugation.

The direct conjugation of exosomes to aldehyde/sulfate latex beads achieved more promising results. Again, numerous attempts at assessing surface markers on exosomes via flow cytometry were made and it proved to be a difficult task, providing very limited data. The direct conjugation method yielded a signal and displayed the classical latex bead grouping on the dot plot used for gating (Figure 21). The RMFI and fold-increase of exosomes secreted from immature and TLR3-L matured MutuDCs was assessed (Figure 22).
Figure 21. Gating strategy of aldehyde/sulfate latex beads directly conjugated to exosomes. The beads were identified on the FSC and SSC plot that represents single beads (circled), bead aggregates, and debris. The circled region of single beads denotes the region selected for gating in all experiments.
Figure 22. Profile of surface CD antigens on purified exosomes directly conjugated to aldehyde/sulfate latex beads. The RMFI (A) for exosomes was assessed and the fold to immature exosomes (B) was determined for various co-stimulatory molecules, the antigen presenting molecule MHC class II, and tetraspanins.
Based on the results shown in Figure 22, TLR3-L matured MutuDC-secreted exosomes conjugated to aldehyde/sulfate latex beads exhibited a phenotype with higher expression of co-stimulatory molecules CD40 and CD86, as well as the tetraspanin CD63. Immature exosomes displayed a significantly higher expression of the tetraspanin CD9 compared to mature exosomes. The remaining co-stimulatory and antigen presenting molecules were similarly expressed, with a slightly lower expression in mature exosomes. The exosome phenotype was significantly lower in surface marker expression compared to cell phenotype. All surface markers yielded RMFI values lower in exosomes than RMFI values for cells, except immature CD9. The tetraspanin CD9 expressed at approximately twice the level on immature DC-derived exosomes compared to the cells based on the RMFI value.
CHAPTER V
DISCUSSION

5.1 The MutuDC Line Displays Similar Characteristics of the CD8α+ cDC Subset

Dendritic cells have enormous immunotherapy potential to fight infectious diseases or cancer. There are various subtypes and classes of DCs that capture pathogens and present antigenic peptides to T-cells that induce an immunosuppressive or immunostimulatory response. Primary DC lines such as BMDCs would be ideal in studying DC differentiation in vitro, however this approach is not practical due to the lack of animal facilities in this laboratory as well as the cost, low yield of DCs, the number of mice required to be sacrificed for experiments, high variability, and a slow and tedious process. A number of immortalized murine DC lines have been established but many are growth factor-dependent and there are concerns the DC maturation response differs from what is observed in vivo or using BMDCs.

Previous research utilizing the murine cell line DC2.4 has shown results of different TLR-L stimulations and exosome content and function with satisfactory results. This cell line, however, is growth factor dependent and the up-regulation of maturation markers are highly variable. The MutuDC line is an attractive alternative cell line because it does not require additional growth factors, can be grown in regular culture conditions, and are easily maintained. MutuDCs display the characteristic surface markers and proteomic profile of splenic CD8α+ cDCs. The cell line responded to TLR3-L, TLR4-L, and TLR9-L through the up-regulation of co-stimulatory molecules and showed no response to TLR7-L, a trait observed in wild type CD8α+ cDCs. Marraco et al. also found MutuDCs were capable of MHC class II restricted antigen presentation.
and antigen cross-presentation similar to CD8α⁺ cDCs. Lastly, MutuDCs efficiently secreted the cytokine IL-12 when cultured with various TLR-Ls, a characteristic feature of splenic CD8α⁺ cDCs, resulting in Th1-polarizing and CTL-inducing functions. These observations and results led to the selection of MutuDCs as the cell line for our research.

After selecting the MutuDC line, we had to ensure our data coincided with published results, as there is very little data on this cell line compared to other immortalized lines with only three published papers to date. Our results exhibited a similar trend to that of Marraco et al. in that MutuDCs strongly up-regulated maturation markers and the antigen presenting molecule MHC class II upon stimulation with TLR3-L and TLR9-L, moderately responded to TLR4-L, and did not respond to TLR7/8-L (Figure 8). The fold-increase of surface marker expression varied slightly under different stimulation conditions between our results and Marraco et al. This could be attributed to passage number, culture conditions, gating strategy, or calculations used to obtain the fold to immature cells. Regardless of the value obtained for the fold-increase, a similar trend was observed for both data sets.

Mouse CD8α⁺ DCs are non-migrating tissue resident DCs that continuously seed lymphoid organs from bone marrow and during infection become major presenters of pathogen antigens to promote CD8⁺ T-cell responses. Not to be confused with CD8 expression on CD8⁺ T-cells, DCs express mRNA for CD8α in the form of a CD8αα homodimer whereas T-cells express CD8αβ heterodimer. Similar to wild type CD8α⁺ DCs, the MutuDC line exhibited fluctuations in CD8α expression, most notably when stimulated with TLR2-L (Figure 10), TLR3-L (Figure 12), and combination TLR3/9-L
The up-regulation of surface marker CD8α upon stimulation may be attributed to the fact that CD8α expression appears later in DC development\textsuperscript{128}, so at the time of stimulation with these TLR-Ls some of the cells may not be expressing the marker even if they are functional. Another likely explanation for the increased expression of CD8α on TLR3-L stimulated MutuDCs is because the cells strongly respond to viral ligands and undergo cross-presentation to activate CD8\textsuperscript{+} T-cells, thereby increasing their CD8α expression.

The other classic cDC surface lineage markers exhibited a CD11b\textsuperscript{low} and CD11c\textsuperscript{high} expression, a trend that coincides with wild type CD8α\textsuperscript{+} cDCs. CD11c has been shown to associate with CD18 on T-cells\textsuperscript{129} and works in conjunction with CD11b for cellular adhesion to T-cells, playing an important role in immune response regulation. During DC activation \textit{in vitro}, CD11b expression can change from low to high spontaneously, just as we observed upon stimulation with TLR4-L, TLR5-L, and TLR13-L (Figures 9 and 11). Because the expression of CD11b can drastically change from almost negative to moderately positive by overnight culture\textsuperscript{129}, it was not used as a definitive lineage marker but valued as simply one marker of the CD8α MutuDC line. CD11c expression remained very stable between immature and matured cells with an overall higher expression indicating the CD8α DC subset.

\section*{5.2 MutuDCs Exhibit a Similar Phenotypic Profile Regardless of Culture Conditions and Respond Stronger to Endosome-Expressed TLR Stimulations}

Once the MutuDC line was established and a similar trend of DC maturation marker up-regulation between our results and those published were verified, the
phenotypic results of cells stimulated with various TLR-Ls cultured in unfiltered and 100 kDa filtered FBS was compared. This was a crucial step to address whether the culture conditions affected the activation markers of the MutuDC line. There have been numerous studies conducted investigating the effects of culture medium on DC maturation marker expression and cytokine secretion levels with varying degrees of success. Many studies use serum-free media, but these conditions were deadly to the MutuDC line. Our primary concern was depleting the FBS of bovine-derived exosomes. Tangential flow using a 100 kDa filter proved to be a rapid, cost-effective method with high throughput while maintaining consistent cell viability and maturation marker expression. All of the experiments were completed in at least triplicates in both unfiltered and 100 kDa filtered FBS culture conditions for the various TLR-Ls to ensure a representative sample of surface marker expression was assessed.

Additionally, our aim was to examine the effect of individual and combination TLR-Ls on DC maturation to induce the most potent response. As a refresher, PM-expressed TLR2 detects cell wall components of Gram-positive bacteria (PGN-SA), TLR4 recognizes cell wall components of Gram-negative bacteria (LPS), and TLR5 identifies flagella from both Gram-positive and Gram-negative bacteria (flagellin). The endosome-expressed TLR3 responds to viral double stranded RNA (Poly I:C), TLR9 detects oligodeoxynucleotides with un-methylated CpG dinucleotides in viral and bacterial DNA (CpG 1826 and 2395), and TLR13 detects viral double stranded RNA (bacterial 23S rRNA). Two classes of CpG DNA were utilized including B-class (1826) and C-class (2395). Both have shown to be potent DC stimulators producing high
amounts of cytokines and are structurally similar possessing a full phosphorothioated backbone, and the classes only differ in that CpG 2395 contains a palindromic motif\textsuperscript{130}.

The PM-targeting TLR2-L, TLR4-L, and TLR5-L exhibited an approximate 2- to 3-fold increase in MHC class II expression with moderately expressed co-stimulatory up-regulation (Figure 10). TLR4-L stimulated MutuDCs had higher co-stimulatory molecules CD40, CD80, and CD86 expression compared to TLR2-L and TLR5-L. Between the PM-targeting TLR-L stimulations, the expression of CD80 and CD86 were comparable. The targeting of endosome-expressed TLR3 and TLR9 resulted in an increased expression of MHC class II and co-stimulatory molecules CD40, CD80, and CD86 (Figure 12). The combination TLR3/9-L stimulation yielded moderately higher CD80, CD86, and MHC class II expression compared to TLR3-L or TLR9-L alone, however it was not a statistically significant difference (Figure 14).

We concluded endosome-targeting TLR stimulations most notably TLR3-L, TLR9-L, and a combination thereof, amplified up-regulation of antigen presenting and co-stimulatory molecules compared to PM-expressed TLR stimulations. These observations are likely attributed to the expression of distinct repertoires of TLRs that contribute to the functional specialization of the CD8$\alpha^+$ cDC subset. The CD8$\alpha^+$ cDC subset are preferentially designed to respond to viral and bacterial nucleic acids through TLR3 and TLR9 in order to undergo cross-presentation and enhance CTL activation\textsuperscript{14}. In particular, CD8$\alpha^+$ cDCs uniquely express high levels of endosome-expressed TLR3 to recognize viral double stranded RNA and lower levels of PM-expressed TLRs involved in the recognition of components of bacterial cell walls and flagella including TLR2, -4, and -5\textsuperscript{14}. Additionally, studies have revealed that DCs cultured from C57BL/6 mice,
where the MutuDC mouse cell line was generated from, express much higher levels of TLR3 and TLR9, and lower levels of TLR2, -4, and -5. While TLR13 is expressed in these cells, it is to a lesser extent than TLR3 and TLR9, indicated by an overall lower RMFI of co-stimulatory and antigen presenting molecules (Figure 11). In summary, with regard to their TLR expression pattern, MutuDCs appear to be more equipped for intracellular pathogen detection and this is confirmed by a higher expression of co-stimulatory and antigen presenting molecules upon stimulation. Moreover, the synergistic stimulation of TLR3 and TLR9 increased surface maturation marker expression even further. This observation has been confirmed in studies showing that combined TLR3/9 stimulation enhances the activation and signaling pathway in terms of pro-inflammatory cytokines, thereby activating more DCs.

As expected, none of the PM-targeting TLR-Ls up-regulated MHC class I expression on MutuDCs because cross-presentation primarily occurs during viral infections in order to prime CTLs. Endosome-targeting TLR-Ls can induce DC cross-presentation and therefore increased MHC class I expression in vitro, however this was not observed in our experiments because additional molecular signals are required. Research has shown that phagocytosis of cellular material from virally infected cells, as well as their signals generated, are required for DCs to favor cross-presentation. In one study, Vero cells loaded with Poly I:C via electroporation were co-cultured with CD8α+ DCs. The DCs were only able to undergo cross-presentation if phagocytosis of the virally infected Vero cells occurred in conjunction with cytokine secretion, including type I interferons. In agreement with published results, MutuDCs do not undergo cross-presentation when cultured with free viral particles. This requirement is important to
consider when harvesting exosomes from cross-presenting MutuDCs to use as an immunotherapeutic treatment through the activation of CTLs to elicit an anti-tumor response.

When experimenting with various TLR-Ls we unfortunately did not test with lipid antigens to investigate the expression of CD1d on MutuDCs. The CD1d molecule has a similar structure to MHC class I molecules, presents both self and foreign lipid and glycolipid antigens to T-cells, and has the responsibility of selecting NKT-cells\textsuperscript{134}. Unlike MHC class II molecules that are rapidly delivered to the DC surface upon maturation for increased expression, CD1d molecules do not necessarily show a change in cell-surface expression upon DC maturation\textsuperscript{134}. While not the focus of this study, it would be interesting to activate DCs via a CD1d-L as studies have reported increased NKT cell activation and cytokine production, suggesting a potential anti-tumor efficacy using this type of ligand\textsuperscript{135}. A marginal up-regulation of CD1d was observed when stimulated with endosome-targeting TLR-Ls, most notably TLR3-L and TLR9-L (Figures 11 and 12). Research has shown that TLR-mediated activation of DCs can alter the cell’s lipid biosynthetic pathway, resulting in an increased expression of CD1d molecules\textsuperscript{136}.

The co-stimulatory molecules CD80 and CD86 assist in strengthening intercellular interactions and amplifying T-cell activation during an immune response\textsuperscript{137}. Upon DC maturation, a higher expression of one co-stimulatory molecule over the other and their polarizing $T_H$-cell response remains ambiguous with conflicting results. We observed a consistent up-regulation between both co-stimulatory molecules across all TLR-L stimulations with a slightly higher expression of CD86. Research suggests that CD86 strengthens the relationship between DCs and T-cells more markedly than CD80,
however, the DC-T-cell interaction still occurs likely due to functional redundancies of these co-stimulatory molecules\textsuperscript{137}. Additionally, CD86 expression is up-regulated sooner than CD80\textsuperscript{137} and this possibly attributes to the increased expression we detected. Regardless of the co-stimulatory molecule expression level observed during MutuDC activation, the molecules were up-regulated indicating the cells were matured.

The third co-stimulatory molecule, CD83, was only marginally up-regulated during MutuDC activation. There is still a limited understanding of the function of CD83 in DC-T-cell interactions but studies show CD83\textsuperscript{high} DCs are able to prime significantly larger populations of CD8\textsuperscript{+} T-cells and increase T-cell proliferation\textsuperscript{138}. The research group concluded CD83\textsuperscript{low/neg} DC subpopulations, along with CD80 and CD86 expression, predominantly influenced the phenotype of the T-cells, but had limited capacity to promote T-cell expansion\textsuperscript{138}. This is important for us to keep in mind as MutuDCs displayed CD83\textsuperscript{low} expression upon stimulation, so we achieved DC activation but may not be able to induce T-cell proliferation to the degree desired for a heightened immune response.

The remaining co-stimulatory molecules CD40 and CD54 were up-regulated under all stimulation conditions to various degrees. The increased CD40 expression indicates mature MutuDCs that can play a central role in T-cell activation, as well as aiding in the regulation of lymphocytes\textsuperscript{139}. As expected, the adhesion molecule CD54 was moderately expressed in immature cells and up-regulated after TLR-L stimulation (Figures 9, 11, and 13). This is important for mediating adhesive contacts for DC migration and DC-T-cell interactions. It was also desirable to assess CD54 expression
because studies have shown the importance of this molecule in exosome communication with APCs and T-cells during the immune response\textsuperscript{63}.

Agreeing with other research groups, mature MutuDCs did not up-regulate CD36, the B class scavenger receptor that recognizes oxidized phospholipids and lipoproteins\textsuperscript{140}. Immature DCs preferentially phagocytize apoptotic cells and capture antigens to undergo cross-presentation and CD8\textsuperscript{+} T-cell activation compared with mature DCs\textsuperscript{11}. DCs selectively express CD36 and upon maturation, the receptors as well as the phagocytic capacity of DCs are down-modulated. While some studies have suggested CD36 is required for DC cross-presentation, other research groups have shown the ability of DCs to capture and prime CTLs in the absence of CD36 expression, indicating the molecule is not required for the process\textsuperscript{141}. We found that CD36 expression was relatively low on immature and mature MutuDCs, although a slight decrease in CD36 expression was observed on mature cells.

The chemokine receptor CCR7 controls the DC migration and homing to draining lymph nodes. Agreeing with published results, immature MutuDCs were largely devoid of CCR7 expression and the receptor was marginally up-regulated upon stimulation. Similar to the DC activation markers, cells stimulated with endosome-targeting TLR-Ls (Figure 12) had a higher expression compared to cells stimulated with PM-targeting TLR-Ls (Figure 10). We hypothesize the low fold-increase of CCR7 expression is attributed to the cell line being non-migratory, tissue resident CD8\textsuperscript{+} DCs. Our findings coincide with other research groups who have established lymph-node resident DCs exhibit CD8α\textsuperscript{+}, CCR7\textsuperscript{low} results\textsuperscript{142}. 

Tetraspanins CD9, CD63, and CD81 were assessed because they not only have diverse functions such as cell activation, proliferation, adhesion, and motility\textsuperscript{143}, but are also used to identify exosomes. A higher expression of CD9 and CD81 was observed in all culture and stimulation conditions while a low CD63 expression. CD63 is primarily localized intracellularly but has been shown to be expressed on the plasma membrane, whereas CD9 and CD81 are predominantly located at the cell surface\textsuperscript{143}, elucidating our findings. Also in agreement with published results\textsuperscript{143}, the tetraspanin expression on mature MutuDCs did not significantly differ from their expression on immature cells. Upon maturation, tetraspanin CD63 follows the endocytic pathway through early endosomes reaching MHC class II compartments to aid in peptide loading\textsuperscript{144}. DCs moderately internalize CD81 and do not internalize CD9 upon maturation\textsuperscript{144}. This was an interesting point to consider as we continued our experiments phenotyping exosomes for tetraspanin markers because the vesicles traverse through the endocytic compartments during biogenesis. Because DCs do not internalize CD9 upon maturation, it should affect the surface expression level on exosomes and we hypothesized CD9 expression would be down regulated in mature exosomes.

A plethora of cytokines is secreted during DC maturation that would reveal if the cells were immunogenic verses tolerogenic. Some examples include but are not limited to the CTL and T\textsubscript{H}1-polarizing IL-12, type I-IFNs, IFN-\(\gamma\), and TNF-\(\alpha\); T\textsubscript{H}2-polarizing IL-4; or T\textsubscript{H}17 and T\textsubscript{Reg} polarizing IL-6 and TGF-\(\beta\)\textsuperscript{145}. The cytokines IL-12/23 p40, TNF-\(\alpha\), and IL-4 were assessed to determine if the DCs were T\textsubscript{H}1- verses T\textsubscript{H}2-polarizing. It was important to evaluate the cytokine profile in conjunction with surface maturation marker expression to verify MutuDC activation and T-cell polarization, as the surface markers
alone do not fully confirm DC maturation. We also wanted to validate TLR3-L, TLR9-L, and TLR3/9-L was inducing the strongest MutuDC response as observed in their high maturation marker expression compared to the other TLR-L stimulations.

The cytokine levels secreted by MutuDCs were assessed via two methods including intracellular cytokine staining and direct ELISA. Unfortunately, the intracellular staining method was unsuccessful. We hypothesize one reason was due to the addition of brefeldin A added at the wrong time point. Brefeldin A is a protein transport inhibitor that prevents formation of COPI-mediated transport vesicles thereby preventing cytokines being transported from the ER to the Golgi apparatus for secretion into the extracellular matrix. There is conflicting literature when to add brefeldin A to the cell culture and it is also cytokine dependent. When brefeldin A was added with 16 hours remaining of DC stimulation, it resulted in cell death. When brefeldin A was added the last two hours it may have been too late into the stimulation, after the cells produced and secreted vast amounts of the cytokines, so none were detected. Another possible explanation for a lack of cytokine recognition is this detection method is a snapshot in time for stimulated cells whereas ELISA detects cytokines released over a longer period of time, in this case 20 hours. It’s possible only a small percentage of MutuDCs with cytokines located intracellularly were available at the time of staining. Fortunately, the detection of cytokines secreted into the culture supernatant via ELISA was successful and DC maturation and T-cell polarization was confirmed, which was the main goal in our research.

The high presence of IL-12 secretion in response to endosome-targeting TLR-Ls confirmed the MutuDCs are polarizing a T\textsubscript{H}1-type helper T-cell response (Figure 17).
Cells stimulated with TLR9-L, TLR3/9-L, and to a lesser extent TLR3-L produced substantial amounts of IL-12/23 p40 compared to the other ligands and corresponds to the trend we observed that overall, these ligands induced a stronger DC response. Although we did not test the ability of MutuDCs to undergo cross-presentation, Marraco et al. demonstrated it does occur in this cell line. This information, along with the high secretion of IL-12 suggests MutuDCs are capable of also inducing a CTL response. The IL-12 cytokine is composed of 40 kDa (p40) and 35 kDa (p35) homodimer subunits linked covalently via disulfide bonds giving a 70 kDa heterodimeric molecule. IL-23 is a heterodimeric molecule consisting of a 19 kDa (p19) and 40 kDa (p40) subunit.

The p40 subunit from IL-12 and IL-23 is shared so when we assay for the IL-12 cytokine, indicating a T<sub>H</sub>1-polarizing DC, we are really detecting both cytokines. Something else to keep in mind is only the entire cytokine consisting of the p70 subunit (IL-12/23 p70) has biological activity, but the p40 subunit (IL-12/23 p40) alone, while biologically inactive, is secreted in excess and is easily detected in the ELISA. This is not a concern in our work because the detection of IL-12, whether as a p40 or p70 subunit, is our objective and is the best parameter in determining DC maturation and T-cell polarization.

Next the pro-inflammatory cytokine TNF-α was assessed, albeit with little success. We were unable to quantify the amount of cytokine secreted so the fold-increase between immature and mature MutuDC culture supernatant was compared (Figure 18B). TNF-α was very difficult to detect and if we wish to measure TNF-α secretion in future experiments, utilizing a manufactured kit may provide better results. A possible reason higher levels of TNF-α in mature MutuDC culture supernatant was not detected is because the profile release of this cytokine is typically shorter, peaking at approximately
4-6 hours post stimulation\textsuperscript{148}, and we collected cell culture supernatant at approximately 20 hours. By this time the TNF-\(\alpha\) secreted from the MutuDCs may have been taken up by TNF-receptors on other cells as the binding affinity is high\textsuperscript{149}. The high binding affinity is essential in further promoting DC maturation but it also decreases the ability to detect the cytokine.

The third cytokine assayed was IL-4, an inducer of T\(_h\)2 cells. Similar to TNF-\(\alpha\), we were not able to quantify the amount of IL-4 secreted from MutuDCs following stimulation and only assessed the fold-increase from immature to mature cells. As expected, IL-4 was not observed following MutuDC stimulation (Figure 18A). DCs can switch from producing IL-12 to IL-4 after exposure to various immunosuppressive viruses or fungi\textsuperscript{150}, permitting a T\(_h\)2-polarizing immune response. The MutuDC line however preferentially polarizes a T\(_h\)1 response and our goal was to confirm the cells were not secreting IL-4. It would be interesting to further explore the ability to polarize a T\(_h\)2 response, as this would activate B-cell antibody production, thereby enhancing the overall immune response.

Based on the maturation surface marker expressions up-regulated and cytokine profile, we concluded TLR3-L, TLR9-L, and combination TLR3/9-L induced the strongest MutuDC response with a T\(_h\)1-polarizing effect. These data coincide with the CD8\(\alpha^+\) cDC subset that exhibit a strong TLR3 and TLR9 expression, high secretion of IL-12, and strong inducers of T\(_h\)1 cells. MutuDCs cultured in the presence of unfiltered and 100 kDa filtered FBS yielded similar phenotypic profiles between various TLR-L stimulations. These data gave us the confidence that cells cultured in filtered FBS would have a similar immunostimulatory response and generate exosomes with comparable
characteristics as those cells, and ultimately their exosomes, that are cultured in unfiltered FBS. Our next step was to isolate and purify exosomes secreted from immature and mature MutuDCs in order to compare their phenotypic profile.

5.3 MutuDC-Derived Exosome Production and Characterization

Once it was confirmed the MutuDC line was matured by TLR3-L stimulation through the up-regulation of antigen presenting molecules, co-stimulatory molecules, and IL-12 secretion we began collecting culture supernatant for exosome purification and characterization. Although TLR9-L and combination TLR3/9-L stimulation induced a stronger MutuDC response, the TLR3-L Poly I:C was used to continue in the exosome studies because an abundant stock was available and the chemical is relatively inexpensive compared to TLR9-Ls. Approximately 80 mL of immature and TLR3-L mature MutuDC culture supernatant was collected. The cell culture supernatant underwent centrifugation, concentration via tangential flow, and purified using FPLC with a Heparin column, as described in section 3.7. As discussed in the literature review section, the most common method of extracting exosomes from cell culture supernatant is differential ultracentrifugation. It is becoming increasingly evident, however, that this method can negatively affect the yield, purity, and/or cause exosome structure damage\[^{151}\]. FPLC is an attractive alternative as research has shown it increases the purity, yield, retains exosome integrity, is scalable, and the column is reusable for a cost saving benefit\[^{151}\]. The downside to this technique is that it has not been fully expounded for exosome purification and little is known about how it compares to the ultracentrifugation
technique. Part of our research goals was to optimize the FPLC technique to isolate and purify exosomes.

MutuDC exosomes were purified in a non-selective manner using a Heparin column, which uses a combination of affinity chromatography and cation exchange chromatography. Heparin, a highly sulfated glycosaminoglycan with a high negative charge density, is coupled to a Sepharose base matrix where the exosomes interact with the Heparin and allow other molecules to flow through. The exosomes are eluted with a high salt concentration solution for further characterization. Balaj et al. isolated exosomes from cell culture supernatant by Heparin affinity purification and demonstrated the exosomes maintain a diverse RNA profile, had lower levels of protein contamination, and were functional at the binding to and uptake into cells\textsuperscript{152}. Using a Nanodrop, the concentration of proteins before and after Heparin purification was determined and as expected, a decreased protein concentration was observed. A Nanodrop is not selective against exosomes so before Heparin purification, there are likely an abundance of impurities and other proteins besides exosomes. Theoretically, once the cell culture supernatant is Heparin purified, exosomes should be the only molecules remaining for quantification by the Nanodrop.

When comparing the amount of immature and mature exosomes secreted using the Nanodrop, an approximate 5.6-fold decrease was observed after TLR3-L stimulation (Figure 20). The decrease in exosome production in mature MutuDCs was confirmed by conducting an enzymatic assay using Amplex UltraRed reagent (Figure 19). Amplex UltraRed reacts with hydrogen peroxide in a 1:1 stoichiometric ratio and is amplified by peroxidases to produce a brightly fluorescent product. Serially diluting HRP-conjugated
antibody to generate a standard curve (Figure 19A), the moles of peroxidase in immature and mature exosomes was determined (Figure 19B). Our results yielded a 7-fold decrease in exosome production upon maturation with TLR3-L. A difference in the amount of exosomes secreted between the two methods was expected as one is detecting proteins and the other enzymatic activity. Caution should be taken when using the enzymatic activity of proteins to indirectly determine the amount of exosomes in samples, as it is not a specific marker of exosomes. In the future, Western blotting must validate the presence of exosomes. Regardless of the specific amount of exosomes determined in the two methods, a decrease in exosome secretion in mature MutuDCs was noticed.

One would believe exosome production would increase after DC activation in order to escalate exosome presence in the body to heighten an immune response, but the opposite was observed. This coincided with results from other research groups that all detected a decrease in exosome production and secretion upon DC maturation, likely due to a reduction in the endocytic activity. In immature DCs, the majority of MHC class I and II molecules are located in late endocytic compartments and upon maturation, the compartments disappear and the molecules are redistributed to the plasma membrane. Théry et al. found the number of MHC molecules in endocytic compartments decrease while the MHC molecules on the plasma membrane increase when activated. The decreased endocytic activity correlates with decreased exosome production, therefore, exosome biogenesis is developmentally regulated and their formation is an active process. These data are important to consider for future experiments where larger quantities of cell culture supernatant will be required for
exosome production. Additionally, exosome production may improve if aliquots of cell
culture supernatant containing the exosomes were harvested at various time points
throughout MutuDC maturation. Research has shown certain proteins, such as p53,
encode genes that regulate the production of exosomes. If cells sense a decrease in
exosome production or changes to certain stress proteins, the cells may increase their
exosome production.

We proceeded to characterize exosomes based on their surface marker expression
using flow cytometry. The exosomes were either directly or indirectly conjugated to
aldehyde/sulfate latex beads because naked exosomes are too small to be detected by the
flow cytometer. For indirect conjugation, anti-CD9 antibody was chosen because of its
availability and higher presence in MutuDCs compared to the other tetraspanins. The
process of exosome conjugation and characterization via flow cytometry proved to be a
difficult task. Often the sample would be high in bead aggregates and debris, would not
display the typical single bead region for gating purposes, or we were unable to detect
any exosomes/beads in the sample. In the experiments where we succeeded in obtaining
a single bead region for gating, a change in the fluorescence intensity between the isotype
control and the target CD antigen was not observed. In fact, we frequently observed a
higher fluorescence intensity of the isotype control. Finally in one experiment using the
direct conjugation method, we achieved low aggregates and debris, a satisfactorily
clustered single bead region (Figure 21), and observed a change in the fluorescence
intensity between immature and mature secreted exosomes (Figure 22).

Commonly expressed exosomal surface markers phenotyped include tetraspanins
CD9, CD63, and CD81; co-stimulatory molecules CD40, CD54, CD80, and CD86; and
the antigen-presenting molecule MHC class II (Figures 21 and 22). The RMFI of CD9 in immature exosomes was extremely high but down regulated after maturation (Figure 21). The CD9 expression in the mature exosomes was comparable to the RMFI of the remaining surface molecules assessed. The high RMFI of CD9 expression on immature exosomes is not completely surprising, as DC-derived exosomes have been shown to be particularly rich in this plasma membrane-associated tetraspanin. The significant down-regulation of CD9 observed in mature exosomes is possibly due to the high rate of immature DCs actively internalizing and recycling membrane proteins during maturation\textsuperscript{62}. Mature DCs have reduced internalization abilities, thus produce less exosomes that are bearing the CD9 marker compared to immature DCs\textsuperscript{62}. The noteworthy difference in CD9 expression between immature and mature exosomes cannot be fully explained, and further experiments are necessary to confirm or disprove these findings.

CD63 and CD81 expression remained unchanged between immature and mature exosomes. This trend was also observed during cell phenotyping. The results were expected based on previous data showing insignificant differences in CD63 and CD81 expression between immature and mature exosomes. As anticipated, the expression of CD40 and CD86 were up regulated in mature exosomes. Contrary to our expectations, CD54 expression was marginally down regulated, and CD80 and MHC class II expression remained unchanged between immature and mature exosomes. These results were not anticipated as numerous studies have proven DC-derived exosomes display a similar phenotypic profile as their parent cell and should yield an increased CD54, CD80, and MHC class II expression when matured. With that being said, however, the protein
composition on exosomes remains ambiguous and it is unclear what immune maturation proteins are passed to exosomes from the parental cells during exosome biogenesis. Recently, CD54 (ICAM-1) has demonstrated a vital role in the binding of exosomes to APCs, including DCs and B-cells, as well as assisting in T-cell binding even if there are little to no co-stimulatory molecules, such as CD80 or CD86, present\textsuperscript{63}.

There were many questions concerning the techniques used to quantify and characterize exosomes secreted from MutuDCs. As previously mentioned, the methods used to estimate the exosome quantity are relying on the measurement of total protein present in the sample preparations. This approach would permit the measurement of other secreted proteins and vesicles even after Heparin column purification, thereby skewing the results. To correct for the characterization of non-exosome vesicles and proteins analyzed via flow cytometry, the conjugation of anti-CD9, or other molecular markers found on exosomes, to the bead followed by incubation with exosomes would help deter this issue. This method was ineffective however, and we were only successful in direct exosome-bead conjugation. This results in a less selective method of detection as other vesicles or proteins may have coupled to the beads. A significant portion of our continued research is optimizing the characterization protocol of exosome surface markers.

5.4 Conclusions

There are several studies showing the immunotherapeutic uses of exosomes derived from DCs, however, the bio-engineering of exosomes and their potentially increased immune stimulation response has yet to be elucidated and published. Prior to
manipulating the content and function of exosomes, we must first understand the response of DCs to various TLR-Ls. This step is essential because the maturation conditions induce changes in DC phenotype and exosomes produced by these cells, which impacts the immune stimulatory capacity of the exosomes. At the beginning of this study, we chose the immortalized MutuDC line and validated our results with those published. This cell line is a suitable compromise between a primary CD8$^+$ DC line and using an immortalized line, as it is necessary to perform many experiments. This allows us to expand the range of parameters and DC maturation conditions while reducing the time, materials, and mice needed for \textit{in vivo} experiments. The MutuDC line was subjected to various TLR-L stimulations to induce activation and study the subsequent T$_{H}$-polarizing responses. The endosome-targeting TLR3-L, TLR9-L, and a combination thereof up-regulated CD40, CD80, CD86, MHC class II, and secreted substantial amounts of IL-12 to a higher degree than the other TLR-Ls assessed. MutuDCs were activated with TLR3-L and their exosomes were collected, purified, and characterized. The exosomes were phenotyped for the classical maturation surface markers, although there is a lack of definitive results and further studies must be conducted to supplement our data. Preliminary results, however, suggest MutuDC-secreted exosomes display a similar phenotypic profile when matured with TLR3-L compared to their cell of origin.

5.5 \textit{Future Studies}

In these studies, we have just touched the surface investigating the MutuDC line and understanding their exosome content and function, and there are many gaps in the research that remain to be explored. In addition to optimizing the purification protocol in
order to ensure only exosomes are obtained, a Western blot must be conducted to confirm
the content and function of the exosomes remain unchanged. Lastly, the flow cytometry
procedure to assess exosome surface marker expression must be further developed. Until
these data are obtained, it will be difficult to move forward and address the overarching
goal of this laboratory: to bio-engineer exosomes with specific proteins in order to
increase immune stimulation and therapeutic potency.

The MutuDC line will be advantageous to investigate the potential therapeutic
uses of exosomes. As a non-migrating CD8\(^+\) DC subset, they have been shown to
preferentially capture and internalize exosomes \textit{in vivo} compared to other DC subsets.
The CD8\(^+\) DC subset has displayed the simultaneous presentation of antigens on MHC
class II molecules and the transfer of antigen via cross-presentation on MHC class I
molecules for CD4\(^+\) and CD8\(^+\) T-cell priming, respectively\(^{154}\). One would expect to see
this mechanism translate to their exosomes secreted, thereby greatly enhancing the
immune response and improve exosome-based therapeutic strategies. It will be
interesting to stimulate MutuDCs co-cultured with a T-cell line to investigate the cross-
presentation and potentially greater T-cell activation capacity of this cell line.

Additionally, there are numerous unanswered questions on the ability to apply
protein-engineering techniques to directly load exogenous proteins onto the surface of
exosomes and subsequently determine if the exosomes can be loaded inside cells. This
would allow for antigen-specific immune cell targeting with the goal of increasing the
overall immune response. The research team in this lab is currently making headway in
the covalent attachment of proteins and antigens via chimeras followed by the
transfection into a murine cell line to determine the efficiency of protein loading.
Subsequent experiments will investigate the loading of exogenous proteins onto the surface of exosomes and testing cell uptake, viability, and expression. These data will lead to the ultimate application of exosomes as a means of cancer immunotherapy treatments. Undoubtedly, of the greatest importance is ensuring the safety and efficacy of bio-engineered exosomes *in vivo* for therapeutic uses.
REFERENCES


