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# Effect of Extracellular Survivin and Lymphoma Exosomes on Natural Killer Cells

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LOMA LINDA UNIVERSITY
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in conjunction with the
Faculty of Graduate Studies

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by

Heather R. Ferguson Bennit

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Biochemistry

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

| | |
|---|---|
| AML | Acute Myeloid Leukemia |
| APC | Allophycocyanin |
| APC | Antigen Presenting Cell |
| CLL | Chronic Lymphocytic Leukemia |
| CM | Conditioned Media |
| CTL | Cytotoxic T Lymphocyte |
| DC | Dendritic cell |
| DEX | Dendritic cell Exosome |
| DLCL | Diffuse Large B Cell Lymphoma |
| DLS | Dynamic Light Scattering |
| DNA | Deoxyribonucleic Acid |
| EBV | Epstein Barr Virus |
| EV | Extracellular Vesicle |
| FITC | Fluorescein Isothiocyanate |
| FSCCL | Follicular Small Cleaved Cell Lymphoma |
| HSPG | Heparin Sulfate Proteoglycan |
| IAP | Inhibitor of Apoptosis Protein |
| ICAM-1 | Intercellular Adhesion Molecule 1 |
| IFN-γ | Interferon gamma |
| KIR | Killer-cell Immunoglobulin-like Receptor |
| LFA-1 | Leukocyte Function-Associated antigen-1 |
| LMP | Latent Membrane protein |

| | |
|---|---|
| lncRNA | Long Non-coding RNA |
| MCL | Mantle Cell Lymphoma |
| MDSC | Myeloid-derived Suppressor Cell |
| MHC | Major Histocompatibility Complex |
| MICA/B | MHC class I-related chains A/B |
| miRNA | Micro RNA |
| mRNA | messenger RNA |
| MVB | Multi-vesicular Body |
| NF-κB | Nuclear Factor Kappa Beta |
| NK | Natural Killer |
| NKG2D | Natural Killer Group 2D receptor |
| NKG2DL | Natural Killer Group 2D receptor Ligand |
| TCR | T cell Receptor |
| TLR2 | Toll-like Receptor 2 |
| TNF-α | Tumor Necrosis Factor alpha |
| PBL | Peripheral Blood Leukocyte |
| PBMC | Peripheral Blood Mononuclear Cells |
| PCR | Polymerase Chain Reaction |
| PD-L1 | Programmed Death-Ligand 1 |
| PE | Phycoerythrin |
| qRT-PCR | Quantitative Real-Time PCR |
| RNA | Ribonucleic Acid |
| TEX | Tumor-derived Exosome |

| | |
|---|---|
| TGF-β | Transforming Growth Factor beta |
| TME | Tumor Microenvironment |
| TRAIL | TNF-Related Apoptosis-Inducing Ligand |
| Treg | Regulatory T cell |
| ULBP | UL16 Binding Protein |

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Biochemistry
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Tumors alter their microenvironment to promote survival using methods such as angiogenesis promotion, growth signals, and immune suppression. The immune system becomes unresponsive to transformed neoplastic cells through a variety of methods including T cell suppression, increased myeloid-derived suppressor cells (MDSCs), and reduced natural killer (NK) cell activity. NK cells have inherent killing capabilities and thus are among the first responders in recognizing and destroying abnormal cells. However, many types of cancers inhibit the surveillance and cytotoxic abilities of NK cells by releasing exosomes, vesicles that can modulate the tumor microenvironment (TME) and intercellular communication for the purpose of enhancing tumor malignancy. These 30-150 nm sized lipid bound vesicles are secreted by many cell types, including immune cells and tumor cells, and the specific protein, lipid, mRNA and miRNA contents contribute to the complex intercellular communication occurring between malignant and normal cells. Cancer patients often have increased numbers of exosomes circulating through their body, including patients with hematological malignancies, such as lymphoma. The focus of this research was to determine the interactions between B cell lymphoma exosomes and NK cells, and characterize the resultant effects on NK cell

function. A specific objective of this research was to determine whether Survivin, an Inhibitor of Apoptosis protein recently found to be localized within exosomes, has a role in modulating NK cells similar to previous findings of T cell modulations. We report that lymphoma exosomes have low levels of internalization into NK cells, and no detectable presence of immune modulating proteins MICA/B or TGF-β. Exposure of NK cells to lymphoma exosomes did not result in observable changes in degranulation or cytotoxic ability. However, treatment with recombinant Survivin protein was able to decrease NKG2D receptor levels in NK cells stimulated with target cells, and decrease protein levels of TNF-α, IFN-γ, perforin, and Granzyme B. A better understanding of the underlying processes by which Survivin or exosomes suppress immune cells in the TME may pave the way to more efficacious immunological therapies against cancer.

# CHAPTER ONE

# PERIPHERAL BLOOD CELL INTERACTIONS WITH CANCER-DERIVED EXOSOMES AFFECT IMMUNE FUNCTION

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**Abstract**

Cancer-derived exosomes are constitutively produced and secreted into the blood and biofluids of their host patients providing a liquid biopsy for early detection and diagnosis. Cancer exosomes influence biological mechanisms that are beneficial to the tumor cells where they are produced and the microenvironment in which these tumors exist. Accumulating evidence suggests that exosomes transport proteins, lipids, DNA, mRNA, miRNA and long noncoding RNA (lncRNA) for the purpose of cell-cell and cell-extracellular communication. These exosomes consistently reflect the status as well as identity of their cell of origin and as such may conceivably be affecting the ability of a functional immune system to recognize and eliminate cancer cells. Recognizing and mapping the pathways in which immune suppression is garnered through these tumor-derived exosomes (TEX) may lead to treatment strategies in which specific cell membrane proteins or receptors may be targeted, allowing for immune surveillance to once again help with the treatment of cancer. This chapter focuses on how cancer exosomes interact with immune cells in the blood.

**Key Words:** exosome, non-Hodgkin's lymphoma, B cell

Exosomes are small 30-150 nm sized extracellular vesicles (EVs) important in the intercellular communication between cells(Thery *et al.*, 2002, Ratajczak *et al.*, 2006, Iero *et al.*, 2008, Bobrie *et al.*, 2012). Communication can occur both by transfer of nucleic acids and proteins, or by binding cell-surface receptors and inducing cell signaling pathways(Feng *et al.*, 2010, Kalluri, 2016). Both normal and tumor cells release exosomes, although tumor derived exosomes (TEX) have been the subject of a wide range of studies. TEX have been shown to be involved in many aspects of the tumor microenvironment (TME) including immune suppression(Thery *et al.*, 2009, Zhang & Grizzle, 2011), antigen presentation(Wolfers *et al.*, 2001, Mallegol *et al.*, 2005, Hao *et al.*, 2007, Zeelenberg *et al.*, 2008, Zeelenberg *et al.*, 2011), a means of acquiring chemotherapeutic resistance(Shedden *et al.*, 2003, Safaei *et al.*, 2005, Khan *et al.*, 2009, Wang *et al.*, 2014, Yu *et al.*, 2016), as biomarker reservoirs(Skog *et al.*, 2008, Taylor & Gercel-Taylor, 2008, Mitchell *et al.*, 2009, Welton *et al.*, 2010, Keller *et al.*, 2011, Hong *et al.*, 2014a, Turay *et al.*, 2016), inducers of angiogenesis(Deregibus *et al.*, 2007, Kawamoto *et al.*, 2012, Kosaka *et al.*, 2013), and vehicles of niche preparation for metastasis(Graves *et al.*, 2004, Hood *et al.*, 2011, Luga *et al.*, 2012, Peinado *et al.*, 2012, Suetsugu *et al.*, 2013) (**Figure 1**). However, modes and mechanisms of uptake are not completely understood. Cells appear to internalize EVs through several endocytic pathways, including clathrin- and caveolin-dependent endocytosis, phagocytosis, and lipid raft–mediated internalization. It is likely that cells utilize multiple routes to take up EVs, depending on the proteins, glycoproteins, and lipids found on the surface of the vesicles and the target cell itself(Mulcahy *et al.*, 2014). Numerous studies show

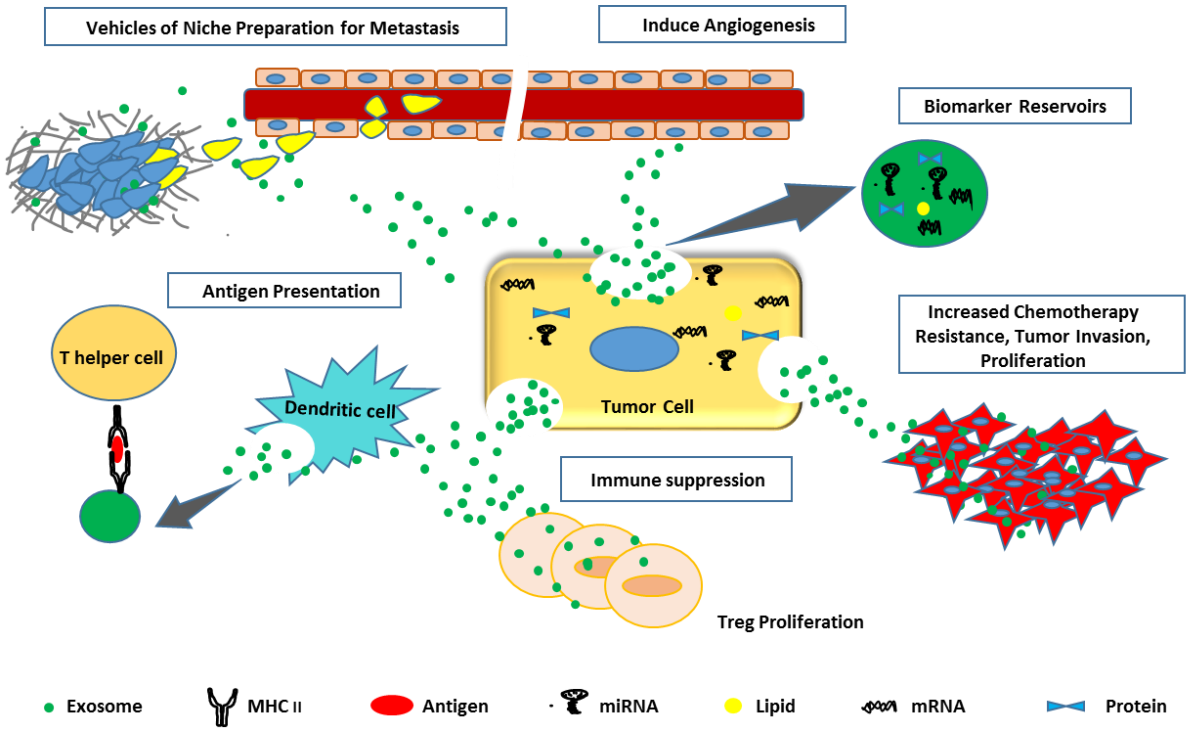


***Figure 1.*** Cancer Exosomes Influence the Tumor Microenvironment. Tumor-derived exosomes (TEX) function in favor of metastasis, support angiogenesis, confer chemoresistance and promote immune-suppression and cellular proliferation. Exosomes from tumor cells were found to release functional biomolecules into the tumor microenvironment (TME) thereby affecting the biology of cancer.

proficient uptake of TEX by endothelial cells(Nazarenko *et al.*, 2010, Rana *et al.*, 2012, Paggetti *et al.*, 2015), epithelial cells(Obregon *et al.*, 2009), fibroblasts(Clayton *et al.*, 2004), myeloid precursors in bone marrow(Peinado *et al.*, 2012, Paggetti *et al.*, 2015), mesenchymal stem cells(Paggetti *et al.*, 2015), and other tumor cells(Franzen *et al.*, 2014) (**Figure 2**).

Cancer cell uptake of exosomes has been well documented and studies even show that TEX can preferentially associate with cancer cells(Parolini *et al.*, 2009, Smyth *et al.*, 2014). Multiple groups have demonstrated that labeled tumor exosomes can be taken up by tumor cells. Endocytic pathways are utilized by ovarian cancer cells to internalize exosomes from the SKOV3 ovarian cancer cell line(Escrevente *et al.*, 2011), by glioblastoma cells(Svensson *et al.*, 2013), and by bladder cancer cells as demonstrated by dose and time dependent uptake of PKH26 labeled bladder exosomes(Franzen *et al.*, 2014). Treatment with heparin can partially block the active and specific mechanism of uptake, implicating receptor-mediated endocytosis involving heparin sulfate proteoglycans (HSPGs)(Franzen *et al.*, 2014). HSPGs were also shown to be critical in the internalization of glioblastoma exosomes by glioblastoma cells(Christianson *et al.*, 2013). Other tumor types have demonstrated exosome uptake, such as colorectal cancer exosomes into lung cancer cells(Chiba *et al.*, 2012) and breast cancer exosomes into breast cancer cell lines(O'Brien *et al.*, Riches *et al.*, 2014), although mechanisms underlying the uptake were not addressed.

Diffuse large B cell lymphoma (DLCL), an aggressive form of lymphoma representing over 40% of adult lymphoma patients, has not until recently been investigated. In an attempt to close the gap in knowledge concerning lymphoma TME

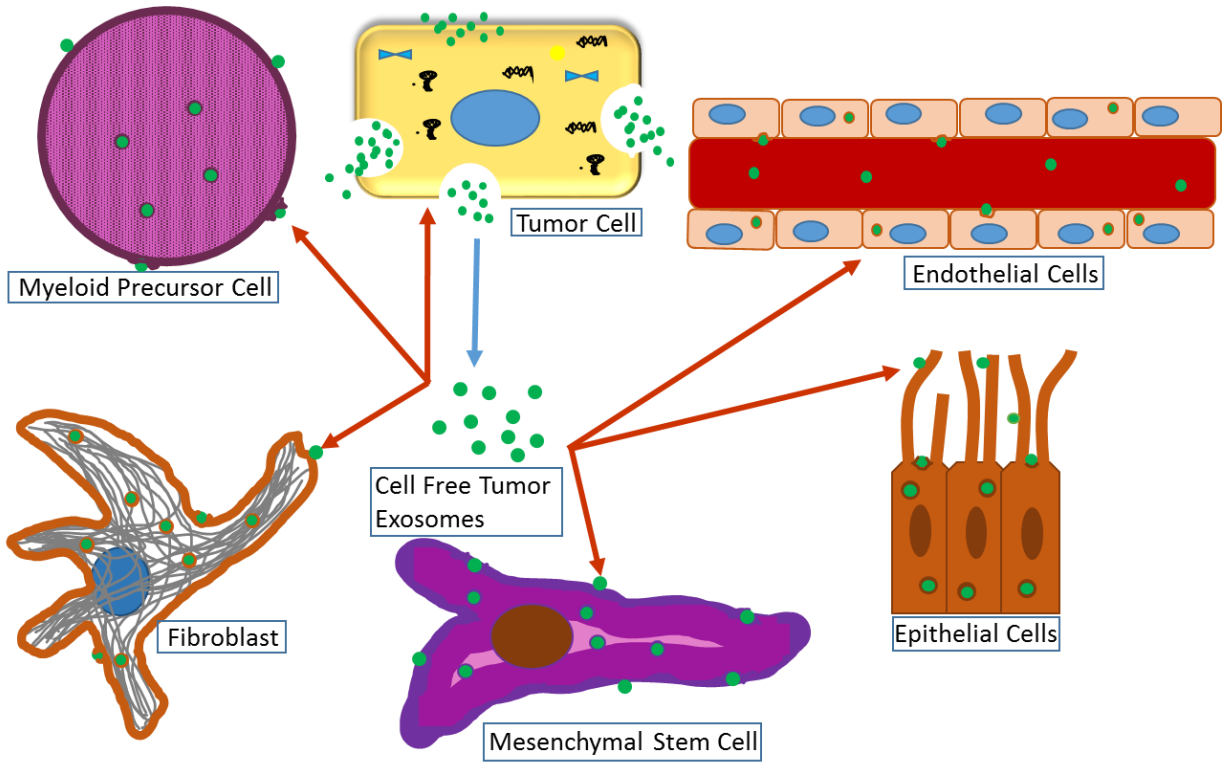


***Figure 2.*** Uptake of TEX by Cells in the Tumor Microenvironment. Cancer cells release exosomes which are taken up by other cancer cells, endothelial cells, epithelial cells, fibroblasts, bone marrow myeloid precursor cells, and mesenchymal stem cells. Exosomes from tumor cells were found to release functional biomolecules (protein, RNA, miRNA) into many cell types.

immunosuppression, normal human peripheral blood leukocytes were treated with PKH67-labeled lymphoma exosomes and monitored for uptake by measuring fluorescence at different time points using flow cytometry and fluorescent microscopy. Results show that of the four populations examined, B cells and monocytes demonstrated uptake of PKH67 labeled lymphoma exosomes, while T cells and NK cells displayed significantly less uptake(Ferguson Bennit *et al.*, 2017).

As TEX have exhibited multiple forms of influence within the immune system, immune cells have also been investigated regarding their ability to interact with exosomes. Macrophages exhibit specialized capacity for internalizing exosomes, although they typically reside within tissue, not in circulation. Their blood counterpart, monocytes, also appear to have a relatively high level of exosome internalization. Consequently, numerous investigations have focused on exosomal interactions with these myeloid-derived cell types. Macrophages have been shown to internalize exosomes from both normal(Lässer *et al.*, 2011) and malignant sources(Chow *et al.*, 2014), with differing effects. Macrophages exposed to breast cancer exosomes, but not normal exosomes, activated NF-κB pathways and released pro-inflammatory cytokines like IL-6 and TNF-α, possibly due to TLR2 interacting with palmitoylated protein ligands on the exosomes(Chow *et al.*, 2014). There are reports of multiple ways macrophages use to interact with exosomes, such as CD169 (SIAGLEC) to bind exosomes carrying sialic acids, as seen with B cell-derived exosomes expressing α2, 3-linked sialic acids(Saunderson *et al.*, 2014). Macrophages also utilize a dynamin-dependent phagocytic pathway to internalize vesicles(Barrès *et al.*, 2010, Feng *et al.*, 2010). Leukemia exosomes were found to be efficiently internalized via phagocytosis by

macrophages, while non-phagocytic cells such as T cells show few intracellular exosomes, with most interaction being with surface-attached vesicles(Feng *et al.*, 2010).

Monocytes also utilize phagocytic mechanisms to internalize exosomes, perhaps relying on tetraspanin targeting, as was shown by Rana et al(Rana *et al.*, 2012). Vesicle internalization by monocytes can induce changes such as production of cytokines like TNF-α, which has important downstream ramifications on T cells(Danesh *et al.*, 2014). Like macrophages and monocytes, other cells of the myeloid lineage such as neutrophils and dendritic cells (DCs) have the ability for exosome uptake. As one of the major infiltrators of the TME, neutrophil interactions with exosomes have been of interest. Investigations in leukemia demonstrate communication between tumor cells and neutrophils, transferring genomic DNAs (gDNAs) of the BCR/ABL hybrid gene from K562 cells to normal neutrophils(Cai *et al.*, 2013) and even promoting leukemia tumorigenesis in rats(Cai *et al.*, 2014).

Early investigations in the exosome field recognized follicular DCs as interactors with exosomes(Morelli *et al.*, 2004), and even though no specific receptors had been demonstrated yet, it seemed that alpha 4-integrin on B cell-derived exosomes was important(Wubbolts *et al.*, 2003). Integrin complexes with CD9 and CD81 tetraspanins, externalized phosphatidylserine (PS), and CD11a (LFA-1)/ICAM-1 interactions all participate in the binding and uptake processes of DCs(Morelli *et al.*, 2004, Segura *et al.*, 2007). Uptake can be through endocytic mechanisms(Xie *et al.*, 2010), including phagocytosis(Bastos-Amador *et al.*, 2012) and DCs may be even more efficient than macrophages at picking up exosome-sized particles(Sheng *et al.*, 2011, Czernek *et al.*, 2015). DCs are affected by their interactions with vesicles. Uptake of mast cell exosomes

can induce immature DCs to acquire antigen presenting capacity to T cells(Skokos *et al.*, 2003), and CD11b+ and CD11c+ cells in mice began releasing IL-6 and TNF-α and upregulated CD86 and MHC class II after exposure to exosomes(Sheng *et al.*, 2011). Uptake of TEX by immature DCs can block maturation and promote induction of myeloid-derived suppressor cells(Yu *et al.*, 2007, Xiang *et al.*, 2009).

In addition to antigen presenting cells (APCs) like macrophages and DCs, B cells are also capable of internalizing exosomes. B lymphocytes interact with exosomes containing MHC class II and ICAM-1 from mature DCs and acquire the ability to prime naïve T cells and trigger antigen-specific effector responses(Segura *et al.*, 2005a, Segura *et al.*, 2005b). B cells may need specific surface proteoglycans (HSPGs) such as syndecans and glypicans to aid exosome uptake. It was demonstrated that chronic lymphocytic leukemia (CLL) exosomes can be internalized via active uptake by various benign cell populations found in the TME such as endothelial cells, myeloid cells, bone marrow mesenchymal stem cells, and even some leukocytes. However, CLL B cells themselves did not show uptake of labelled exosomes – possibly due to a lack of surface HSPGs(Paggetti *et al.*, 2015). In a separate study however, malignant B cell exosomes showed a natural specificity for B lymphocytes. Mantle cell lymphoma (MCL) exosomes were efficiently internalized by both healthy and diseased B lymphocytes utilizing a cholesterol dependent pathway independent of clathrin and caveolin(Hazan-Halevy *et al.*, 2015). Very little uptake was recorded in bone marrow stroma cell lines, T cell leukemia cells, or NK cells.

While effector cells such as T cells and NK cells are less equipped to internalize vesicles, there is still evidence for a variety of interactions with exosomes. T lymphocytes

are affected by exosomes from APCs harboring antigen in MHC class I and II molecules, and constitute an important aspect of immune system communication(Raposo *et al.*, 1996, Arnold & Mannie, 1999). The mechanisms of T cell internalization or binding of exosomes from APCs have been posited to involve the T cell receptor (TCR), CD28, and LFA-1 (CD11a)(Hwang & Ki, 2011). Activated T cells use LFA-1 (leukocyte function-associated antigen-1) for binding of DC exosomes containing MHC class II(Nolte-'t Hoen *et al.*, 2009). This LFA-1/ICAM interaction is critical for priming of naïve T cells by exosomes from mature DCs(Segura *et al.*, 2005a). CD4+ T cells can internalize DC exosomes and stimulate antigen-specific CD8+ CTL while overcoming Treg suppression, with a resultant shift in immune responses(Hao *et al.*, 2007). In contrast to T cell priming effects of exosomes, tumor exosomes (TEX) can also suppress T cells. Surface ligands such as TGF-β1, TRAIL, PD-L1, FasL result in exosome-mediated cell death(Chen *et al.*, 2010, Stenqvist *et al.*, 2013, Wang *et al.*, 2016).

Evidently, despite low exosome internalization, T cells are still subject to exosome-mediated effects. Likewise, even with little uptake, NK cells are influenced through exosomal interactions. NK cell cytotoxicity is frequently seen to be inhibited after exposure to exosomes derived from solid tumors and even EBV-immortalized B cells. It has been surmised that MICB and TGF-β1 expressed on exosomes are responsible(Clayton & Tabi, 2005, Clayton *et al.*, 2008, Ashiru *et al.*, 2010). One mechanism is through the downregulation of NK activating receptor NKG2D, as exemplified by plasma exosomes from AML patients(Hong *et al.*, 2014a). In contrast to TEX, DEX can promote NK activation and proliferation through copresentation of NKG2DL with IL-15Ra(Viaud *et al.*, 2009). Some studies have found evidence of NK

cell uptake of exosomes in a time dependent fashion, perhaps utilizing PS located on vesicle membranes as demonstrated in ovarian cancer model(Keller *et al.*, 2009).

There have been few studies investigating uptake of exosomes by peripheral blood cell populations. In one study, rat pancreatic adenocarcinoma exosomes could be taken up by all leukocyte subpopulations examined, with CD11b+ cells demonstrating higher internalization than T or B cells(Zech *et al.*, 2012). At this time there is only one other publication addressing peripheral blood uptake of lymphoma exosomes - a study by Hazan-Halevy et al., looking at MCL exosomes and their preferential uptake by B lymphocytes(Hazan-Halevy *et al.*, 2015). In this study, it was shown that exosomes isolated from a MCL cell line, when administered to B lymphocytes, NK cells, and various T lymphocytes, preferentially internalized into B lymphocytes.

TEX have shown immunosuppressing properties and are involved in regulating peripheral tolerance in cancer patients. AML and solid tumor patients downregulated CD3 zeta and JAK3 expression in activated T cells(Taylor *et al.*, 2003, Kim *et al.*, 2006), mediated FasL apoptosis of CD8+ T cells(Huber *et al.*, 2005, Kim *et al.*, 2006), and promoted Treg conversion and proliferation(Wieckowski *et al.*, 2009). After exposure to TEX, Tregs expressed IL-10, TGF-β1, CTLA-4, Granzyme B/perforin and had enhanced suppressor activity(Szajnik *et al.*, 2010, Hong *et al.*, 2014a). AML TEX were positive for TGF-β1 and could decrease NK cell cytotoxic activity, inducing Smad phosphorylation and NKG2D downregulation(Hong *et al.*, 2014a, Hong *et al.*, 2014b). Another study investigating TEX from mesothelioma cells effect on lymphocyte responses to IL-2 revealed inhibition of proliferation in all unfractionated lymphocytes, with CD4+ T cells

being most sensitive. Treg function was enhanced, while NK cell cytotoxic ability was directly inhibited, possibly through TGF-β1 dependent mechanisms(Clayton *et al.*, 2007).

Exosomes have been relatively well-studied in EBV-positive transformed human B cell lines, as these cells constitutively produce large numbers of MVBs and MHC class II molecules(Raposo *et al.*, 1996, Escola *et al.*, 1998, Dolcetti, 2015). The WSU-DLCL2 B cell lymphoma cell line used in our own study(Ferguson Bennit *et al.*, 2017) as a source of exosomes, is EBV-free. This may be of interest because the virus has been known to hijack and alter exosomes in infected cells. The internalization and subsequent effects of these exosomes may involve viral factors, such as latent membrane protein (LMP). One group examined epithelial uptake of exosomes from EBV-infected B lymphocytes and found uptake was through a dynamin and caveolae-dependent process, and type III latency-derived exosomes were able to induce proliferation and upregulation of ICAM-1 in recipient cells(Nanbo *et al.*, 2013). LMP-1 was also harbored on exosomes from a Burkitt's lymphoma cell line, and could mimic CD40 signaling to induce stimulatory changes in the B cells that efficiently bound them(Gutzeit *et al.*, 2014). LMP-1 can also produce an immunosuppressive effect by inhibiting T cell proliferation and NK cytotoxicity(Dukers *et al.*, 2000). Furthermore, EBV-infected cells can produce exosomes with FasL capable of inducing apoptosis in surrounding cells(Ahmed *et al.*, 2015).

Exosomes are important mediators and regulators of cellular communication. Although they are involved in active immunosuppression and can facilitate tumor progression, they are also a good source of tumor antigens. Therefore, until a more full understanding of the interplay between the tumor microenvironment and the exosome

occurs, effective strategies to mobilize the immune system as an effective anticancer modality will be limited. Recognizing the luminal and surface contents of the exosome is not enough to design exosome-associated therapy; understanding the communication patterns is also essential. Moreover, identifying which exosome populations are communicating and which are providing additional ligands or receptors in order to facilitate communication will prove necessary to potentiate the immune response.

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# CHAPTER TWO

# UPTAKE OF LYMPHOMA-DERIVED EXOSOMES BY PERIPHERAL BLOOD LEUKOCYTES

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**Abstract**

Exosomes are nano-sized lipid vesicles secreted into blood and other body fluids, and serve as vehicles for intercellular communication. Despite being an important component of the tumor microenvironment, exosomal targeting and uptake into recipient cells are still not fully understood. Few studies have looked at lymphoma exosomes and their interactions with circulating blood cells. In this work we examine the exosomal uptake distribution among peripheral blood leukocytes (PBL) using vesicles derived from a diffuse large B cell lymphoma cell line, WSU-DLCL2. Lymphoma cells survive, proliferate and are protected from the cytotoxic effects of chemotherapy agents by soluble factors or by direct contact with inflammatory and stromal cells within the tumor microenvironment (TME). In an attempt to close the gap in knowledge concerning lymphoma TME immunosuppression, we have treated normal human peripheral blood leukocytes with PKH67-labeled lymphoma exosomes and monitored the uptake by measuring fluorescence at different time points using flow cytometry and fluorescent microscopy. Our results show that of the four populations examined, B cells and monocytes demonstrated uptake of PKH67 labeled exosomes, while T cells and NK cells displayed significantly less.

**Key Words:** exosome, non-Hodgkin's lymphoma, B cell

## Introduction

Diffuse large B cell lymphoma (DLCL) is an intermediate grade and the most common form of non-Hodgkin lymphoma (NHL), affecting 40-50% of adult lymphoma patients in the United States(Solimando *et al.*, 2016). Chemokines, cytokines, and growth factors are critical for the growth and survival of these malignant B cells(Grivennikov *et al.*, 2010). In addition, specific oncogenes such as c-Myc have been shown to have proliferation regulatory ability in malignant B cells both ex vivo and in vivo(Miller *et al.*, 2012). The cross talk between the tumor microenvironment and the DLCL cells is mainly mediated by direct cell-to-cell interactions but has recently been shown to be facilitated through extracellular vesicle trafficked soluble factors(Khan *et al.*, 2015, Valenzuela *et al.*, 2015).

Exosomes are small 30-150 nm sized extracellular vesicles important in the intercellular communication between cells (Thery *et al.*, 2002, Ratajczak *et al.*, 2006, Iero *et al.*, 2008, Bobrie *et al.*, 2012). Communication can occur both by transfer of nucleic acids and proteins, or by binding cell-surface receptors and inducing cell signaling pathways. Both normal and tumor cells release exosomes, although tumor-derived exosomes (TEX) have been the subject of a wide range of studies. TEX have been shown to be involved in many aspects of the TME including immune suppression (Thery *et al.*, 2009, Zhang & Grizzle, 2011), antigen presentation (Wolfers *et al.*, 2001, Mallegol *et al.*, 2005, Hao *et al.*, 2007, Zeelenberg *et al.*, 2008, Zeelenberg *et al.*, 2011), a means of acquiring chemotherapeutic resistance (Shedden *et al.*, 2003, Safaei *et al.*, 2005, Khan *et al.*, 2009, Wang *et al.*, 2014, Yu *et al.*, 2016), as biomarker reservoirs (Skog *et al.*, 2008, Taylor & Gercel-Taylor, 2008, Mitchell *et al.*, 2009, Welton *et al.*, 2010, Keller *et al.*,

2011, Hong *et al.*, 2014a, Turay *et al.*, 2016), inducers of angiogenesis (Deregibus *et al.*, 2007, Kawamoto *et al.*, 2012, Kosaka *et al.*, 2013), and vehicles of niche preparation for metastasis (Graves *et al.*, 2004, Hood *et al.*, 2011, Luga *et al.*, 2012, Peinado *et al.*, 2012, Suetsugu *et al.*, 2013).

However, modes and mechanisms of uptake are not completely understood. Cells appear to internalize exosomes through several endocytic pathways, including clathrin- and caveolin-dependent endocytosis, phagocytosis, and lipid raft–mediated internalization. It is likely that cells utilize multiple routes to take up exosomes, depending on the proteins, glycoproteins, and lipids found on the surface of the vesicles and target cell itself (Mulcahy *et al.*, 2014). Numerous studies show proficient uptake of TEX by endothelial cells (Nazarenko *et al.*, 2010, Rana *et al.*, 2012, Paggetti *et al.*, 2015), epithelial cells (Obregon *et al.*, 2009), fibroblasts (Clayton *et al.*, 2004), myeloid precursors in bone marrow(Peinado *et al.*, 2012, Paggetti *et al.*, 2015), mesenchymal stem cells(Paggetti *et al.*, 2015), and other tumor cells (Franzen *et al.*, 2014).
There have been few studies investigating uptake of exosomes by peripheral blood cell populations. Zech et al. found that rat pancreatic adenocarcinoma exosomes could be taken up by all leukocyte subpopulations examined, with CD11b+ cells demonstrating higher internalization than T or B cells(Zech *et al.*, 2012). At this time there is only one other publication addressing peripheral blood uptake of lymphoma exosomes - a study by Hazan-Halevy et al. looking at MCL exosomes and their preferential uptake by B-lymphocytes(Hazan-Halevy *et al.*, 2015). Diffuse large B cell lymphoma (DLCL), an aggressive form of lymphoma representing over 40% of adult lymphoma patients, has not been investigated. It is therefore important to investigate these interactions between the

lymphoma cells and the TME in order to find and exploit new prognostic factors and to design new therapeutic approaches.

## Materials and Methods

### *Cell Culture*

Human lymphoma cell lines WSU-DLCL2 and WSU-FSCCL were developed at Wayne State University and are EBV-negative(Al-Katib *et al.*, 2009). Cell lines were grown in RPMI 1640 media supplemented with 10% USDA-sourced heat-inactivated fetal bovine serum (FBS) (Mediatech, Manassas, VA), 4 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 units/ml penicillin and incubated at 37°C and 5% $CO_2$. Trypan blue staining was used to measure cell density (confluent at $1x10^6$/ml) and viability (above 90%).

Peripheral blood from healthy apheresis blood donors were obtained from the Life Stream Blood Bank (San Bernardino, CA) according to our approved Loma Linda University IRB protocols. The red blood cells were lysed using an ACK lysis buffer containing 8.3g/L $NH_4Cl$, 1g/L $KHCO_3$, and 1.8 ml 5% EDTA and centrifuged for 5 minutes at 1,500 rpm at 4°C in a Beckman Coulter Allegra X-15R centrifuge, equipped with a SX4750 rotor to obtain a pellet of peripheral blood leukocytes (PBL). These were allowed to rest overnight before exosome treatment at $5x10^6$/ml complete RPMI, with or without 100 IU/ml IL-2. This study, in its entirety, was approved by Loma Linda University's institutional review board.

### *Exosome isolation*

Lymphoma cells were cultured for 24 hours in media depleted of exosomes from fetal bovine serum (FBS, Hyclone, Logan, UT) by overnight ultracentrifugation at 100,000 x g. The cell conditioned medium (CM) was subjected to serial centrifugation, removing cells (300 × *g*, 5 min) and removing non-cellular debris (2000 × *g* for 10 min). The supernatant was then centrifuged at 10,000 × *g* for 30 min. Exosomes were isolated with the commercially available ExoQuick-TC™ (System Biosciences, Mountain View, CA) at a 1:5 ratio of reagent to CM and incubated overnight at 4°C. A low speed spin at 1500 x g for 30 minutes was sufficient to pellet the precipitated vesicles. Exosome pellets were resuspended in 40-70 µl PBS and protein quantified by BCA protein assay (Pierce/Thermo Scientific, Rockford, IL). Size of the vesicles was examined using dynamic light scattering (DLS) with a Nicomp N3000 nanoparticle sizing instrument (Particle Sizing Systems, Port Richey, FL).

### *Uptake of Exosomes*

The exosome pellet, obtained from ExoQuick isolation, was labeled with PKH67 Green Fluorescent Cell Linker Kit (Sigma Aldrich, St Louis, MO) as per manufacturer's protocol, with modifications. Briefly, exosomes in PBS corresponding to 200 µg were added to 250 µl of diluent C. As a control, the same volume of PBS was also added to 250 µl of diluent C (no exosome control) and processed in parallel. The exosome suspension was added to an equal volume of 2X PKH67 dye mixture, and mixed well for 4 minutes. The dye reaction was stopped by addition of 9 ml of media depleted of bovine exosomes by ultracentrifugation and then spun for 90 minutes at 110,000 x g using an

SW41 rotor. The pellet was washed in PBS with a second ultracentrifugation. The PKH67-labelled vesicles were incubated either with PBL for 1 hour, 4 hours, or 24 hours for dose –curve experiments or with NK cells for 30, 60, or 240 minutes. Cells were washed with PBS, stained with surface marker antibodies, and fixed in 2% paraformaldehyde before proceeding with further analysis by flow cytometry or microscopy.

### *Flow Cytometry*

Antibodies directed against the following markers and directly labeled with indicated fluorophore were used to stain PBLs for flow cytometry analysis: CD3-PE, CD1a-PE (Becton, Dickinson (BD) Biosciences, San Diego); CD14-APC (M5E2),) (BioLegend, San Diego, CA); CD56-APC (MY31, Tonbo Bioscience, San Diego, CA); and CD19-APC (H1B19). Live cell exosome binding was distinguished from dead cells using fixable viability dye eFluor 780 (eBioscience, Grand Island, NY). Cells were run on MACSQuant Analyzer (Miltenyi Biotec, Auburn, CA) and data analyzed using FlowJo software (Tree Star, Ashland, OR). The percentage of cells positive for PKH67+ exosomes was determined for each cell population. The gating strategies used to assess uptake of PKH67-labeled exosomes by leukocyte populations are shown in **Supplementary Figures 2 and 3**.

### *Microscopy*

Samples were spun onto poly-L-lysine slides using StatSpin CytoFuge 2 (Beckman Coulter) set at 800 rpm for 4 minutes. One drop of mounting medium

containing 4′,6-diamidino-2-phenylindole (DAPI) stain to visualize nuclear structures (Vectashield, Vector Laboratories, Burlingame CA) was placed onto the glass slide before adding glass coverslip and sealing with nail polish. Slides were imaged using a fluorescence light microscope (Keyence BIOREVO BZ7000) and a Zeiss LSM 710 NLO confocal microscope.

### *Statistical Analysis*

All the quantitative data of this study were expressed as the mean ± SD and statistical analysis was conducted using GraphPad Prism software v.5.01 for Windows (San Diego, CA). To test for statistical significance, nonparametric two-tailed Mann–Whitney analysis was performed. Comparisons between groups were performed using student's t-test with probability $p < 0.05$ considered to indicate a statistically significant difference. Each experiment was repeated at least twice to assess the level of reproducibility.

## Results

### *Cellular Uptake Characterization of DLCL2 Exosomes*

Exosome uptake by peripheral blood cells was measured using flow cytometry and fluorescent microscopy, with demonstrated uptake differing between cell lineages and in a dose and time dependent manner. Specifically, to study the uptake of lymphoma-derived exosomes by peripheral blood leukocytes, vesicles were isolated from the conditioned media from the WSU-DLCL2 cell line. Vesicle size was evaluated by

dynamic light scattering (DLS) and confirmed to be consistently in the reported range of exosomes (30-150 nm) **(Supplementary Figure 1)**.

The exosomes were labeled with a lipophilic dye, PKH67 after which 25, 50, 100, 200 or 400 μg of these exosomes were incubated with peripheral blood leukocytes (PBLs) for 1, 4 and 24 hours. Internalization by confocal microscopy was performed and analyzed. Exosomal internalization was observed as early as 1 hour post incubation with longer incubation times and higher concentrations resulting in higher accumulation of exosomes inside the cells (**Figure 1**).

To study the kinetics of exosome accumulation, we performed quantitative flow cytometry. PKH67 labeled DLCL2-exosomes were incubated with PBLs and the fluorescence intensity was detected. Uptake was most prominent by B cells and myeloid-derived cells and less so in T cells and NK cells (**Figure 2**). The uptake of exosomes from healthy B cells was rapid in the higher exosome concentrations, with 28% positive after 1 hour at 400 μg/ml (**Figure 2A and 2B)** and increasing to 69% at 24 hours at 400 μg/ml (**Figure 2B**). Cell specificity for uptake of DLCL2-derived exosomes was further studied in a single experiment using B cells (CD19+), T cells (CD3+), NK cells (CD56+) and monocytes (CD14+) (**Figure 2C**). Compared to B cells where 28% to 70% of cells showed exosome uptake in the 400 μg/ml exosomes over the 24 hour study, NK cells, monocytes and T cells only maximized 6, 8 and 3 % uptake respectively (**Figure 2C**). After a second independent experiment was concluded, CD19+ B cells and CD14+

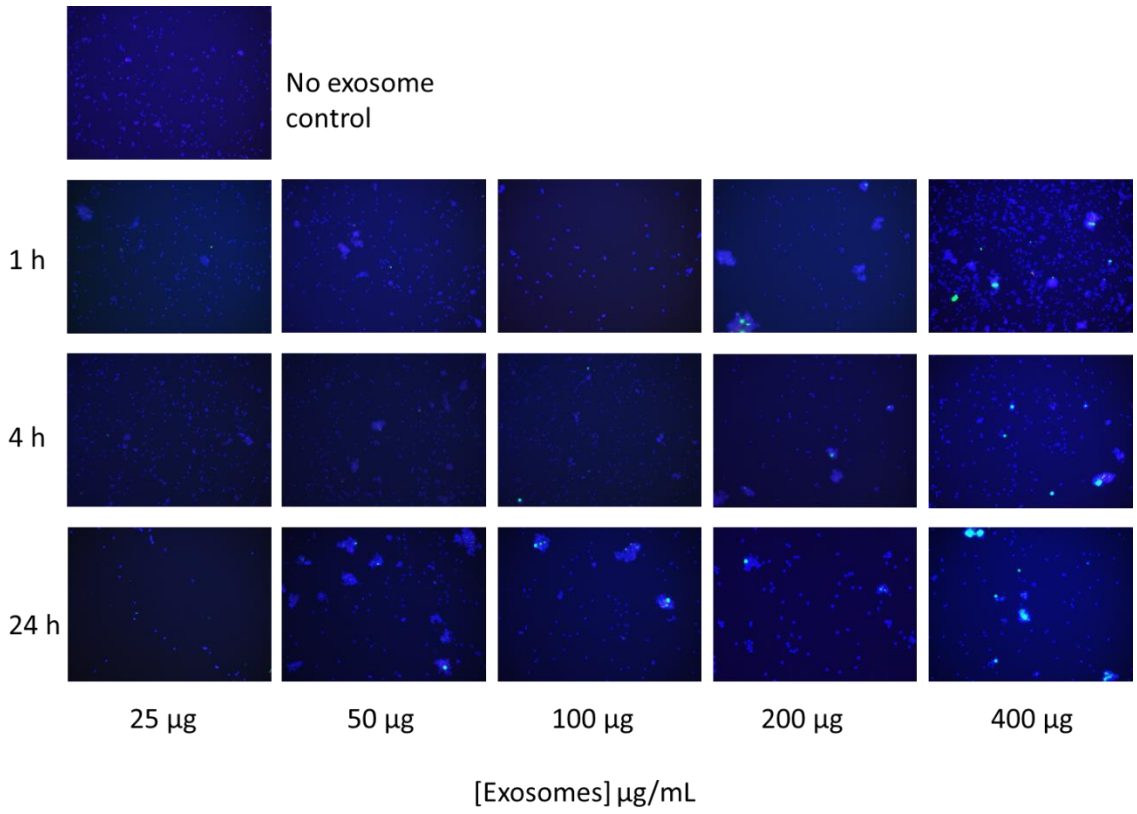


***Figure 1***. Peripheral blood leukocytes take up DLCL2 exosomes. Peripheral blood leukocytes were treated with DLCL2 exosomes (25-400 µg/ml) and harvested at 1, 4, and 24 hours. Cells were cytospun onto poly-L-lysine coated slides. Microscopy images acquired with BIOREVO BZ7000 fluorescent microscope (Keyence), 20X magnification. Nucleic acids are stained with DAPI (blue), and exosome bind and internalization is visualized with PKH67 (green). Cells without the addition of exosomes were used as a negative control. Several fields were analyzed for each labeling condition, and representative results are presented. The data are representative of two independent experiments.

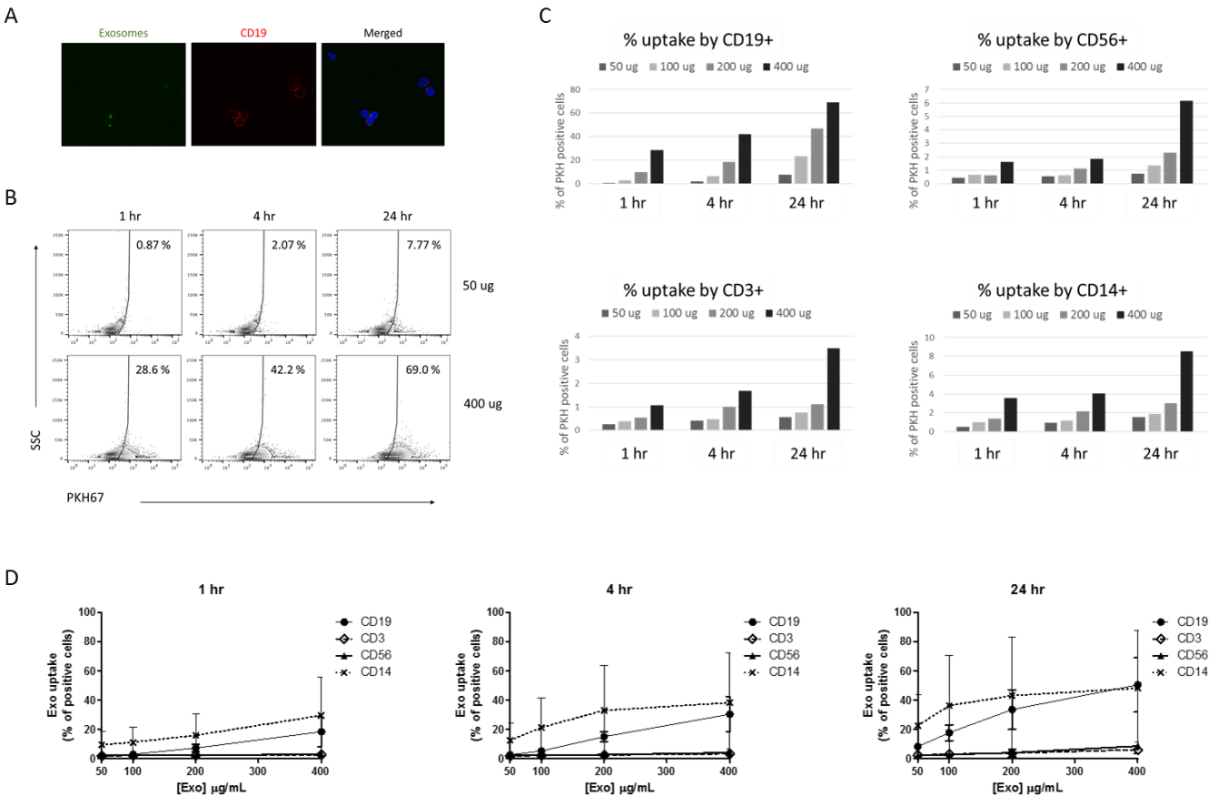


***Figure 2.*** Uptake of PKH67 labeled exosomes in a time- and dose-dependent manner. DLCL2 exosomes were labeled with PKH67 and then added to peripheral blood cells for various lengths of time (1, 4, and 24 hours) and treatment amounts (25, 50, 100, 200 and 400 ug/ml). As measured by flow cytometry, the uptake of labeled exosomes proceeded in a time- and dose-dependent fashion. **A.)** Uptake of PKH67 labeled exosomes after 4 hours by CD19-APC cells (red). Microscopy images acquired with Zeiss LSM 710 NLO confocal microscope. 60X magnification. **B.)** Representative flow cytometry data of CD19+ cells. **C.)** Graphical representation of the percentage of PKH+ cells in each of the four lineages derived from one donor: B cells (CD19+), monocytes (CD14+), NK cells (CD56+), and T cells (CD3+). **D.)** Combined data from two separate experiments depicting the disparity in uptake between each cell population. Results are expressed as mean ± SD.

monocytes maximized near 40% in the 400 μg/ml incubation while CD3+ T cells and CD 56+ NK cells only proved to be able to uptake near 10% (**Figure 2D**).

The specificity of DLCL2 exosomes was further tested using exosomes derived from FSCCL and HeLa cells. Co-culture of B cells and NK cells with 200 μg/ml PKH67 labeled DLCL2, FSCCL and HeLa-derived exosomes for 1 hour and 4 hour time points showed very similar results as were previously recorded using DLCL2 exosome (**Figure 3**). In this study B cells were able to rapidly and preferentially take up PKH67 stained DLCL2, FSCCL and HeLa cell derived exosomes in a time dependent manner while the NK cells were significantly less capable of this internalization. Four hours post incubation, 22% ±8, 20% ± 10, and 12% ± 9 of B cells had taken up DLCL2, FSCCL and HeLa exosomes respectively (**Figure 3**).

## Discussion

Although the spleen, liver and lymph nodes take up the majority of exosomes produced by organs and hematopoietic cells, plasma and other body fluids still contain large quantities of exosomes(Srinivasan *et al.*, 2016). Cancer cells in particular secrete large quantities of TEX, which can be found in peripherally circulating blood(Sun & Liu, 2014). Consequently, blood cells are exposed to many exosomes from both normal and malignant cells(De Toro *et al.*, 2015), which play key roles in modulating the immune system(Iero *et al.*, 2008). While previous studies have investigated exosomal interactions with leukocytes in lymphoid organs, such as antigen presenting cells (APCs) in the spleen(Morelli *et al.*, 2004) and follicular dendritic cells (DCs) in the lymph

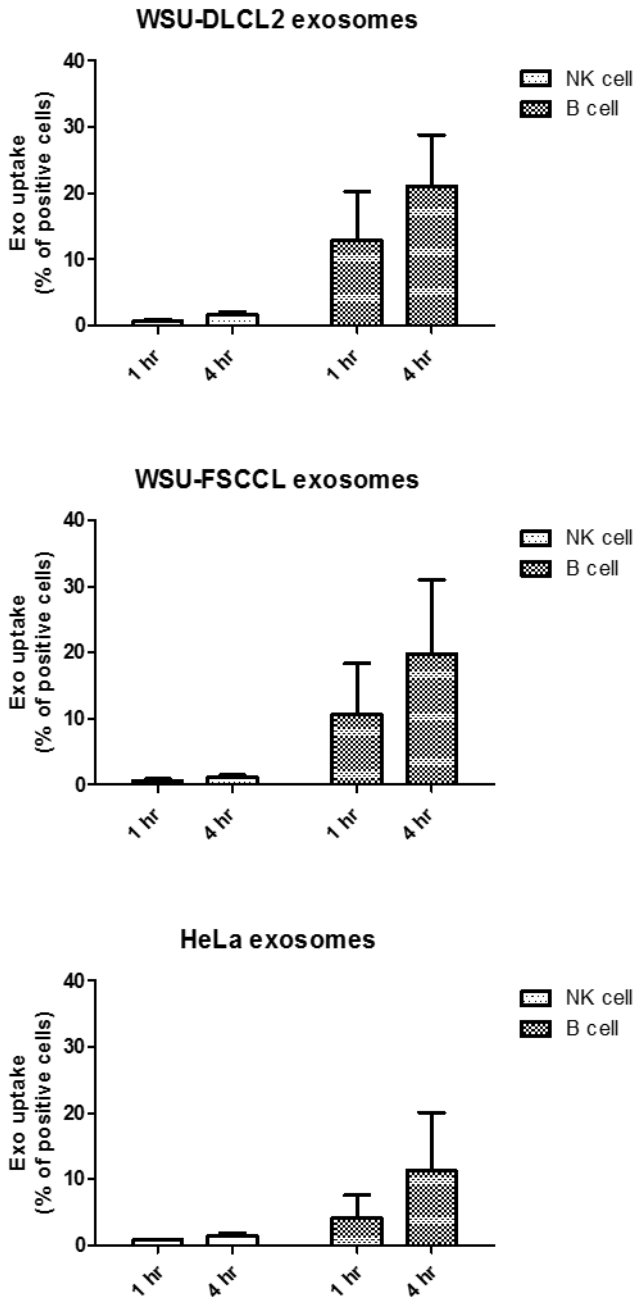


***Figure 3.*** Differences in uptake by NK cells and B cells is not unique to DLCL2 exosomes. DLCL2, FSCCL and HeLa cell-derived exosomes were labeled with PKH67 and then added to peripheral blood for 1 and 4 hours at 200 µg/ml. As measured by flow cytometry, the uptake of labeled exosomes proceeded in a time- and dose-dependent fashion as before in lymphocyte lineage B cells but not the NK cells.

nodes(Denzer *et al.*, 2000, Wubbolts *et al.*, 2003), there is less work done regarding uptake of exosomes by peripheral blood cell populations.

In this study, we tested the hypothesis that some populations of white blood cells will be more receptive to interacting with B cell lymphoma exosomes, and therefore will be more vulnerable to tumor microenvironment modulating effects of these vesicles. In an attempt to close the gap in knowledge concerning lymphoma TME immunosuppression, we have treated normal human PBLs with PKH67-labeled lymphoma exosomes and monitored uptake by measuring fluorescence at different time points using flow cytometry and fluorescent microscopy. We expected to observe a disparity in exosome uptake between blood cells of lymphoid and myeloid lineages that we hypothesized was perhaps due to myeloid derived cells such as monocytes and macrophages, being better equipped for exosome uptake than lymphocytes such as B cells, T cells, and NK cells. However, this was not what we observed with B cells being the most effective and efficient at TEX uptake.

Our data is not consistent with previous studies, which show a disparity in uptake between myeloid derived cells and lymphocytes(Zech *et al.*, 2012, Danesh *et al.*, 2014). We demonstrated that NK and T cells have lower levels of internalization than monocytes and B cells. In our study model, B cells showed a high level of uptake, possibly due to the B cell origin of the lymphoma exosomes, supporting the idea of uptake specificity given the exosomes were collected primarily from cells originating from B cell lymphoma disease. These findings are in line with those of Hazan-Halevy et al.(Hazan-Halevy *et al.*, 2015) and Gutzeit et al.(Gutzeit *et al.*, 2014) concerning B cells and Riches et al. in their work with breast tissue(Riches *et al.*, 2014). In this study, we

observed that DLCL2 exosomes were taken up rapidly and preferentially into CD19+ B lymphocytes and CD14+ monocytes. Only a small percentage of T cells and NK cells showed internalization or binding to exosomes, even after 24 hour incubations. We further investigated if the origin of the exosome would influence the preference or rate of uptake on the peripheral cell (**Figure 3**). Exosomes were taken from two lymphoma cell lines (DLCL2 and FSCCL) and from the cervical cancer cell line HeLa. In our hands there was little difference recorded in exosomal uptake. This may indicate that the uptake is controlled by something specific to the exosome rather than the cell of origin.

The mechanisms of uptake into B lymphocytes remain to be elucidated, whether it be caveolin, clathrin, cholesterol, lipid-raft, or receptor-mediated endocytosis, or something completely novel and not proposed. In this work we have demonstrated a natural preference of TEXs to B cells, further supporting the concept of targeting therapy to this lymphocyte population. However, to fully appreciate and dissect the mechanism, many more cell line-derived as well as patient procured exosomes, from varying pathologies will need to be investigated, a process that has only just begun in our laboratory. By understanding the structure and marker/receptor profiles on the exosome, the cell of origin as well as the recipient cell's membranes as well as the protein, RNA and DNA contents from within the exosome will further the ability to regulate the role of the TEX in the pathobiology of hematologic malignancies and to identify novel therapeutic approaches.

In addition to the indirect effects of exosomes through interactions with APCs, and the limited ability of NK cells and T cells for exosome uptake, these cell populations seem to have a wide variety of responses to direct exposure to exosomes. Whether these

responses are the result of surface interactions rather than uptake, or due to secondary effects from other cells which more readily internalize exosomes is not always apparent. There has been evidence for both possibilities, and it is likely that exosomes interacting with lymphocytes deliver signals by direct surface contact more frequently than internalization(Clayton *et al.*, 2004, Hwang & Ki, 2011). The surface interactions between exosomes and recipient cells can occur via membrane-bound activating or inhibitory proteins which directly signal through relevant receptors and initiating downstream pathways. TEXs are enriched in proteins specialized for surface interactions, such as integrins, MHC class I and II molecules, co-stimulatory molecules (CD40, CD86), various growth factor receptors, such as EGFR (epidermal growth factor receptor) or HER-2 (human epidermal growth factor receptor 2), death receptor ligands such as FasL (Fas ligand), TRAIL (tumor-necrosis-factor-related apoptosis-inducing ligand) or PD-L1 (programmed cell death ligand 1) and inhibitory factors such as PGE2 (prostaglandin E2). Therefore uptake is not necessary for a cell to be altered by exosomes, and may likely be the case for changes induced by exosomes in T cells and NK cells. In fact, much of the immune suppression mediated by TEX occur through surface molecules such as FasL, TGF-β1, and IL-10(Szajnik *et al.*, 2010, Szczepanski *et al.*, 2011, Whiteside, 2013).

Perhaps an equally important aspect could be that exosome uptake have downstream direct and indirect effects on PBLs. Through producing cytokines like TNF-α, presenting antigen on MHC I and II, and providing co-stimulatory signals via CD80/CD86 and CD40(Skokos *et al.*, 2003, Danesh *et al.*, 2014), many of the exosomal effects observed in T and NK cells may be an indirect result from primary changes

induced in macrophages, monocytes, B cells, and DCs which actually internalize the vesicles. Uptake of exosomes induce monocytes to produce TNF-α(Danesh *et al.*, 2014), macrophages and DCs capture and present antigen to T cells(Skokos *et al.*, 2003), and B cells can be activated by antigen-carrying exosomes with subsequent Th1 cell stimulation(Qazi *et al.*, 2009). Antigen-bearing TEX seem to require uptake and processing by DCs before they can efficiently stimulate a specific CTL response(Wolfers *et al.*, 2001, Dai *et al.*, 2005). The exosomes from DCs carry MHC I, MHC II, CD80 and CD86, and are therefore equipped to elicit T cell activation through surface interactions(Viaud *et al.*, 2010).

To summarize, in the present study, we characterized DLCL2 cell line derived exosomes on different PBL populations showing that there is preference of targeted uptake. However, it is still not clear whether exosomes are still inducing cellular signaling pathways in the T cell and NK cells through direct cell to cell contact. From these findings, we hope a better understanding of tumor cell/TME communication may result, further leading to increased knowledge of how the tumor cells communicates with and manipulates the TME. Through understanding these signaling pathways, we may better prepare therapeutic modalities to enhance immune cell surveillance and killing of these tumors which up to now seem immune.

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## Declaration of Interest

The authors report no conflicts of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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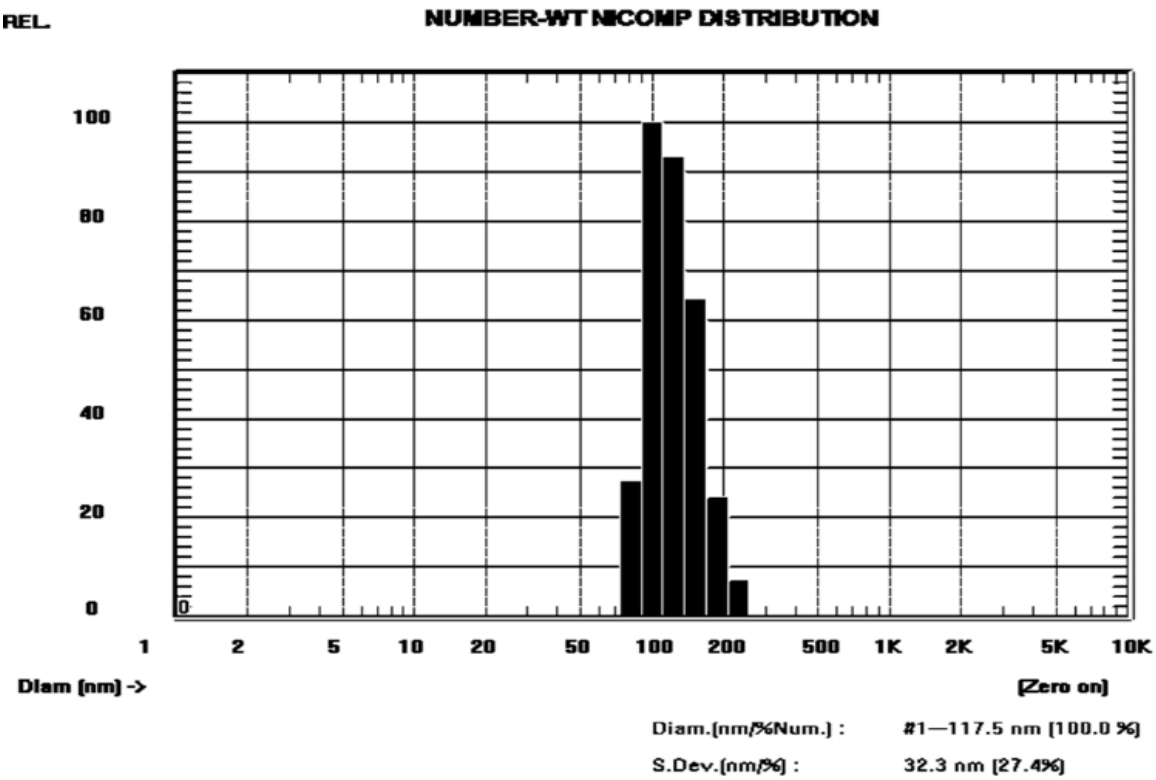
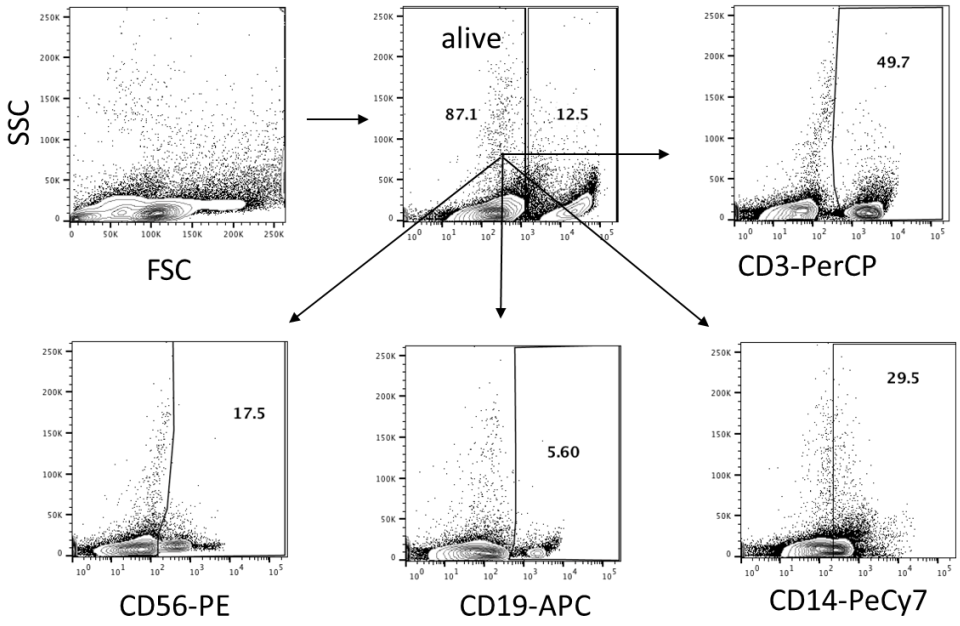
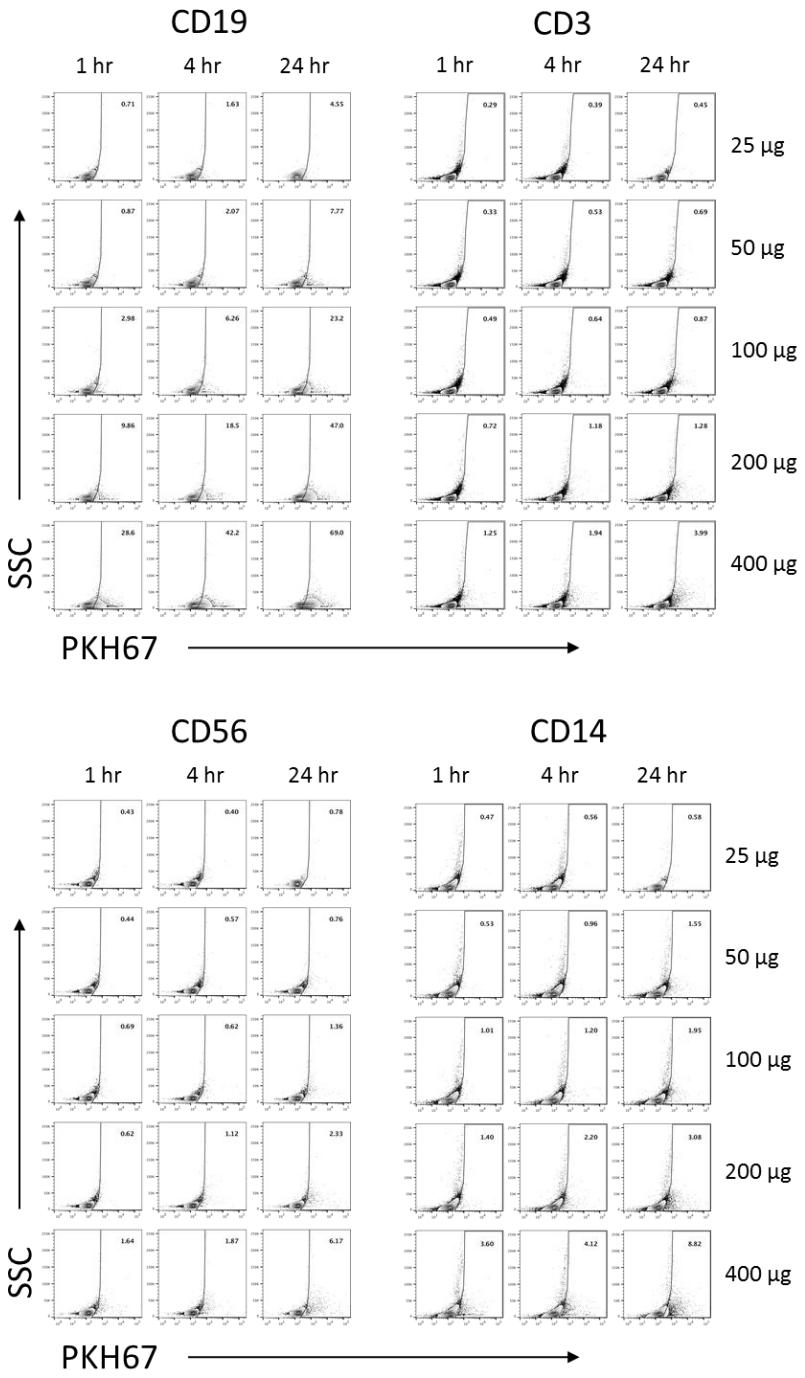



***Supplementary Figure 1.*** Size Distribution of WSU-DLCL2 Exosomes. Number-weight distribution of EV size using dynamic light scattering (DLS) analysis with Nicomp BZ3000 instrument. Sample was read every minute for 30 minutes, with a calculated average diameter of 117 nm. One representative diameter histogram is shown.




***Supplementary Figure 2.*** Gating Strategy to Assess Binding of PKH67-labeled Exosomes to PBL Populations. Antibodies specific to human T cells (CD3+), NK cells (CD56+), B cells (CD19+), and monocytes (CD14+) were used to identify populations within the peripheral blood leukocytes (PBLs). One representative dot plot is shown from two independent experiments




***Supplementary Figure 3.*** Internalization of PKH67-labeled exosomes by healthy PBL. Leukocytes were assayed by flow cytometry using specific antibodies to B Lymphocytes (APC-anti-CD19), T lymphocytes (PerCP-anti-CD3), NK cells (PE-anti-CD56) and monocytes (PeCy7-anti-CD14). Representative dot plots are shown for each cell population.

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# CHAPTER THREE

# EFFECT OF SURVIVIN AND LYMPHOMA-DERIVED EXOSOMES ON NATURAL KILLER CELLS

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**Abstract**

Natural killer (NK) cells have inherent abilities to kill target cells and are among the first responders in recognizing and destroying infected or transformed cells. However, many types of cancers inhibit the surveillance and cytotoxic abilities of NK cells by releasing exosomes, vesicles that can modulate the tumor microenvironment and intercellular communication for the purpose of enhancing tumor malignancy. Recently, cancer cell exosomes have been found to contain Survivin, an inhibitor of apoptosis (IAP) protein that prevents cell death and decreases immune response of lymphocytes. The purpose of this study is to explore the effect of Survivin and lymphoma-derived exosomes, which contain Survivin, on the immune functions of NK cells, such as the production and release of cytokines and cytotoxic granules. NK cells were obtained from the peripheral blood of healthy donors, and treated with pure Survivin protein, or exosomes from two lymphoma cell lines, DLCL2 and FSCCL. RNA was isolated from NK cell samples for measurement by PCR, and intracellular flow cytometry was used to determine protein expression. Degranulation capacity, cytotoxicity, and levels of NK cell activating receptor NKG2D were also assessed. Lymphoma exosomes were examined for size and protein content. This study established these lymphoma exosomes contained Survivin and FasL, but were negative for MICA/B and TGF-β. Treatment with exosomes did not significantly alter NK cell functionality, but extracellular Survivin was seen to decrease NKG2D levels and the intracellular protein levels of perforin, Granzyme B, TNF-α and IFN-γ.

## Introduction

The active role of the tumor microenvironment (TME) in tumor pathogenesis has recently been gaining intense scrutiny as it can be responsible for delivering signals for clonal expansion, drug resistance, metastatic migration, and immune modulation. Cancer development is not an autonomous process but depends on surrounding non-malignant components such as fibroblasts, microvasculature, stroma, mesenchymal cells, and immune cells. Interaction between tumors and their surrounding environment occurs via direct contact, soluble factors, and material exchange. One method that has received much interest in the preceding decade is small bilayer lipid vesicles known as exosomes, or more generally, as extracellular vesicles (EVs). These endosomally derived 30-150 nm vesicles contain proteins, lipids, and nucleic acids like miRNA, mRNA (Ratajczak *et al.*, 2006, Valadi *et al.*, 2007), and even DNA (Cai *et al.*, 2014, Kahlert *et al.*, 2014) that can be sent into the extracellular environment and delivered to recipient cells either locally or systemically. Most tissue and immune cell types have been shown to produce exosomes, but tumor cells seem to produce increased numbers (Kim *et al.*, 2005, Taylor & Gercel-Taylor, 2008, Ohno *et al.*, 2013, Turay *et al.*, 2016). These tumor-derived exosomes (TEX) have a myriad of effects within the TME and beyond, with resulting enhanced malignant potential, niche preparation for metastasis, and angiogenesis (Peinado *et al.*, 2012, Rak & Guha, 2012). Importantly, TEX can have many influences on immune cells, resulting in both activation and suppression of tumor immunity (Clayton *et al.*, 2007, Clayton *et al.*, 2008, Bu *et al.*, 2011, Taylor & Gercel-Taylor, 2011, Filipazzi *et al.*, 2012).

Natural killer (NK) cells are important in the surveillance and eradication of virally infected and malignantly transformed cells, and are susceptible to suppression in

the TME (Fernández-Messina *et al.*, 2010, Wada *et al.*, 2010). TEX carrying ligands such as FasL, TGF-β, IL-10 and NKG2DL are implicated in the downregulation of T cell and NK cell activity (Andreola *et al.*, 2002, Raffaghello *et al.*, 2004, Clayton & Tabi, 2005, Huber *et al.*, 2005, Bianco *et al.*, 2007, Yoshimura & Muto, 2011). As NK cells form the first line of defense and are ready to kill without prior activation, their function is critical to controlling spontaneous tumorigenesis of epithelial and lymphoid malignancies (Smyth *et al.*, 2000, Guerra *et al.*, 2008). NK cell activation is based on a complex interplay of their inhibitory and activating receptors. Healthy cells display MHC class I proteins on their surface which bind to inhibitory KIRs (Killer-cell Immunoglobulin-like receptor) on NK cells to prevent activation. Stressed cells often lose MHC class I and upregulate ligands such as MICA/B (MHC class I-related chains [MIC] A/B) and ULBPs (UL16 binding proteins) which bind to activating receptor NKG2D (Natural Killer Group 2D receptor) (Lanier, 2008, Brenner *et al.*, 2010, Belting *et al.*, 2015). Ligand binding stimulates downstream signaling to activate cytokine production of IFN-γ and TNF-α, and degranulation to release cytolytic proteins such as perforin and Granzyme B. These proteins initiate apoptosis in the target cell by opening pores in the target cell membranes and cleaving cellular proteins. The cytolytic functionality of NK cells can be inhibited by TEX through decreased release of perforin (Liu *et al.*, 2006) and overexposure to soluble MICA/B with subsequent downregulation of the NKG2D receptor (Clayton & Tabi, 2005, Oppenheim *et al.*, 2005, Clayton *et al.*, 2008, Chitadze *et al.*, 2013, Reiners *et al.*, 2013).

Exosomes as a vehicle for immune suppression can act through multiple modalities, such as surface ligands like FasL, PD-L1, and TGF-β, and content such as

miRNAs and proteins (Whiteside, 2017). In our lab, we discovered tumor-derived exosomes contain Survivin (Khan *et al.*, 2011, Valenzuela *et al.*, 2015), an anti-apoptotic protein that is generally found in the cytoplasm, mitochondria and nucleus of cancer cells (Fortugno *et al.*, 2002). When this cancer-specific member of the Inhibitor of Apoptosis (IAP) family of proteins is found in the extracellular compartment, we have shown evidence of increased resistance to treatment, invasive potential, and proliferative capacity in surrounding cells (Khan *et al.*, 2009). Additionally, extracellular Survivin can act upon T cells to skew their behavior towards Th2 and decrease CD8+ T cell activity (Jutzy *et al.*, 2013). It is unclear whether other immune cell populations, such as NK cells, are also able to be affected by Survivin or exosomes from lymphoma cells. In this study, we investigate the influence of exosomes from lymphoma cell lines and Survivin on NK cell function. Our results indicate that conditioning of NK cells by extracellular Survivin-containing exosomes had little effect on NKG2D receptor levels, degranulation capacity, or cytolytic potential. However, while mRNA levels of NK cell proteins were not consistently affected, protein levels of IFN-γ, TNF-α, Perforin and Granzyme B were decreased as a result of treatment with recombinant Survivin protein.

## Materials and Methods

### *Cell Culture*

Human lymphoma cell lines WSU-DLCL2 and WSU-FSCCL were a kind gift from Dr. Ayad Al-Katib at Wayne State University (Detroit, MI)(Al-Katib *et al.*, 2009). Cell lines were grown in RPMI 1640 media supplemented with 10% USDA-sourced heat-inactivated fetal bovine serum (FBS) (Mediatech, Manassas, VA), 4 mM L-

glutamine, 0.1 mg/ml streptomycin, and 100 units/ml penicillin and incubated at 37°C and 5% $CO_2$. Trypan blue staining was used to measure cell density (confluent at $1X10^6$/ml) and viability (above 90%).

### *NK Cell Isolation and Culture*

Natural killer (NK) cells were prepared from freshly collected plasma apheresis peripheral blood obtained from healthy donors at the Life Stream Blood Bank (San Bernardino, CA), in accordance to LLU IRB protocols. Peripheral blood was prepared as described previously (Ferguson Bennit *et al.*, 2017). NK cells were obtained from the total lymphocyte fraction by negative immunomagnetic cell separation using either EasySep or RosetteSep negative selection kit (STEMCELL, Vancouver, BC) or MACS magnetic NK Cell Isolation kit (Miltenyi Biotec, Auburn, CA). The purity of the NK cell population was determined by flow cytometric analysis and ranged from 85-95%, with all kits showing comparable performance in obtaining CD3-/CD56+ NK cells (greater than 90% purity and viability). NK cells were cultured at a high density of $5 \times 10^6$ cells/well in 6-well non-pyrogenic polystyrene culture plates overnight in RPMI 1640 media (Mediatech Inc. Manassas, VA, USA), supplemented with 10% FBS (Hyclone, Logan, UT), 1 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 U/ml human recombinant IL-2 before use in experiments.

### *Exosome Isolation*

Lymphoma cells were cultured for 24-48 hours in media depleted of exosomes from fetal bovine serum (FBS, Hyclone, Logan, UT) by overnight ultracentrifugation at

100,000 x g. Exosomes were isolated using previously described methods (Valenzuela *et al.*, 2015) from conditioned medium after serial centrifugation with commercially available ExoQuick-TC™ (System Biosciences, Mountain View, CA). Exosome pellets were resuspended in 40-70 µl PBS and either used immediately for experiments or stored at −80˚C. The quantity of exosomal protein was determined by BCA protein assay (Pierce/Thermo Scientific, Rockford, IL). The size and concentration of exosomes isolated using ExoQuick was determined using nanoparticle tracking analysis (NTA) with a NanoSight NS300 instrument (NanoSight, Malvern Instruments Ltd, Malvern, UK). Samples were diluted 1:100 in PBS. Five sequential measurements of 60 seconds each were recorded at a flow rate of 20 µl/minute. The instrument was set to a detection threshold of 8 and 25 frames per second. Analysis was performed on software NTA 3.2 Dev Build 3.2.16.

### *Flow Cytometry Antibodies*

Antibodies and other reagents used were: Intracellular Fixation and Permeabilization Buffer kit, fixable viability dye e-Fluor 780, CD56 PerCP-eFluor 710, MICA/B Alexa Fluor 488, CD69 PE-Cy7 (eBioscience, Grand Island, NY); CD16 PE, CD56 PE (BD Biosciences, San Jose, CA); CD3 FITC, IFN-γ PE-Vio770, TNF-α PE-Vio770, CD107a FITC (Miltenyi Biotec, Auburn, CA); CD3 PE-Cy7, CD3 FITC, CD56 APC (MY31) (Tonbo Bioscience, San Diego, CA); Perforin Pacific Blue, Granzyme B Pacific Blue, NKG2D APC (BioLegend, San Diego, CA); Survivin Alexa Fluor 647 (Cell Signaling Technology, Danvers, MA). Data was collected on a Miltenyi MACSQuant

Analyzer flow cytometer (Miltenyi Biotec, Auburn, CA) and analyzed using FlowJo v10.0.8p software (Tree Star, Ashland, OR).

### *Flow Cytometry Analysis of Exosomes*

Purified exosomes (200 μg) were incubated with 10 μl of 4-μm-diameter aldehyde/sulfate latex beads (Invitrogen, Eugene, OR) in PBS for one hour at room temperature with gentle agitation. After washing with PBS, samples were blocked with 200 mM glycine for 30 minutes, and washed again. Beads were incubated with the following antibodies for 30 minutes: anti–CD63 (Millipore, Billerica, MA), anti-CD9 (BD Biosciences, San Jose, CA), anti-HLA-A,B,C (BioLegend, San Diego, CA), anti-TSG101 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HSP70 (Novus Biologicals, Littleton, CO), anti-LAMP-1 (Miltenyi Biotec, Auburn, CA), anti-MICA/B (eBioscience, Grand Island, NY), anti-CD81 (ProSci, Fort Collins, CO), anti-TGF-β (Peprotech, Rocky Hill, NJ), anti-FasL (Boster, Pleasanton, CA). Staining with secondary antibodies for 30 minutes followed, using goat-anti-rabbit Alexa Fluor 488 (Invitrogen, Euguene, OR) and goat-anti-mouse FITC (Becton Dickinson, Franklin Lakes, NJ). A "bead-only" control, as well as isotype-matched antibody controls were also prepared. Samples were washed twice, fixed with 1% paraformaldehyde and analyzed using a MACSQuant Analyzer and FlowJo software. Single beads were gated for fluorescence analysis.

### *Degranulation Assay*

NK cell degranulation was evaluated by staining for CD107a (LAMP-1) during NK cell stimulation. Briefly, peripheral blood mononuclear cells (PBMCs) were treated

for 24 hours either with or without IL-2 (100 U/ml) and exposed to either 0, 0.1, or 1.0 µg/ml of recombinant Survivin (Abcam, Cambridge, MA). Cells were then incubated in 96-well round bottom plates for 6 hours with PMA (10 ng/well) and Ionomycin (50 ng/well) as a positive control for activation, K562 cells ($5X10^5$/well) for targeted stimulation, or media as a negative control. Monensin (3 µM, Sigma Aldrich, St. Louis, MO) was added after the first hour to inhibit Golgi transport and prevent both release of cytokines (for intracellular detection) and to prevent acidification of endocytosed vesicles containing CD107a antibody. The anti-CD107a-FITC antibody (Miltenyi Biotec, Auburn, CA) or isotype control were added before NK cells were stimulated to allow binding to the degranulation marker as granules were exocytosed. Surface and intracellular staining was performed prior to flow cytometry, and analysis performed on the CD3-/CD56+ gated population.

### *NKG2D Receptor Expression*

PBMCs obtained from healthy volunteers were co-incubated with isolated exosomes or Survivin to determine the percentage of NK cells (CD3-/CD56+) expressing the natural cytotoxicity receptor NKG2D on the cell surface, as previously described (Hong 2014). Briefly, PBMCs were activated with IL-2 alone or with additional stimulation of PMA/Ionomycin or K562 cells and co-incubated with exosomes (10 µg protein/ml) or Survivin (0-1 µg/ml) for 24 h at 37°C. Cells were then stained with APC-conjugated NKG2D antibody (BioLegend, San Diego, CA) and run on a MACSQuant flow cytometer (Miltenyi Biotec, Auburn, CA). The frequency of NKG2D+ cells were measured from the gated CD3-/CD56+ NK cells. The data are expressed as %NKG2D+

cells/total CD3-CD56+ cells. The mean fluorescence intensity (MFI) of NKG2D on NK cells was also recorded. PBMC incubated in medium without exosomes were used as controls.

### *Cytotoxicity Assay*

To determine cell-specific killing capacity of NK cells, the release of cytoplasmic lactate dehydrogenase (LDH) from lysed target K562 (erythroleukemia) cells was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Fitchburg, WI), according to the manufacturer's instructions. Briefly, NK cells isolated from healthy donors were incubated overnight at $5X10^6$/ml in complete RPMI1640 media with 100 IU/ml IL-2. NK cells were then treated for 24 hour with either 10 µg/ml Survivin protein (Abcam, Cambridge, MA), exosomes from lymphoma cells (10-50 µg /ml), or media for control. A range of effector to target ratios from 20:1, 10:1, 5:1, 2.5:1, 1.25:1, 0.625:1 were used, and the target K562 cells were added at 10,000 cells/well. After four hours of incubation at 37°C, the LDH containing media was transferred to a separate plate and absorbance read at 490 nm with a Bio-Tek µQuant microplate reader. The percentage of specific lysis was calculated according to a standard equation, specific lysis (%) = (experimental release – effector spontaneous release – target spontaneous release)/(target maximum release – target spontaneous release) X 100.

### *Western Blot*

Western blotting was performed as previously described (Valenzuela *et al.*, 2015). Membranes were immunostained using the following antibodies: rabbit polyclonal anti-

Survivin (1:500-1000, Novus Biologicals, Littleton, CO); rabbit monoclonal anti-XIAP, anti-cIAP1, anti-cIAP2, rabbit polyclonal anti-β-actin (1:500-1000, Cell Signaling Technology, Danvers, MA); mouse polyclonal anti-LAMP-1 (1:500, BioLegend, San Diego, CA); mouse monoclonal anti-Granzyme B (OriGene, Rockville, MD); and rabbit polyclonal anti-Perforin (Boster, Pleasanton, CA). Goat anti-mouse and goat anti-rabbit Dylight 800 secondary antibodies (Thermo Scientific, Waltham, MA) were used at a 1:10,000 dilution. Immunoreactive bands were detected using the Odyssey imaging system (LI-COR Biosciences, Lincoln, NE). β-actin or LAMP-1 were used as loading controls for either cell lysates or exosomal protein, respectively.

***PCR***

Perforin, Granzyme B, TNF-α and IFN-γ mRNA expression was evaluated by PCR from magnetically sorted NK cells treated either with Survivin protein or lymphoma-derived exosomes or an untreated control. Cells were resuspended in 500 µL TRI Reagent Solution (Ambion, Grand Island, NY) and RNA was extracted according to the manufacturer's protocol. RNA concentration was measured using Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA). Single-strand cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. PCR amplification was performed with Thermo Scientific Maxima Hot Start PCR Master Mix (2X) (Thermo Scientific, Grand Island, NY) using 200 ng of cDNA. PCR thermal cycles were performed with Eppendorf Mastercycler gradient thermal cycler using the following conditions: 2 min at 94°C, followed by 34-38 cycles of 30 s at 95°C, 45 s at annealing Tm, and 1 min at 72°C,

followed by a final extension time of 10 min at 72°C. The primer pairs were designed with Primer-BLAST (NCBI) and synthesized by IDT (Integrated DNA Technologies, San Diego, CA). Sequences for each primer pair are listed in **Table 1**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as control. The PCR products were analyzed on a 1% agarose gel by electrophoresis in the presence of ethidium bromide and visualized with UV light.

### *Real-time qRT-PCR Analysis*

NK cell RNA was prepared as described previously. Target genes perforin, granzyme B, IFN-γ and TNF-α were quantified by real-time PCR using the CFX96 system (Bio-Rad Laboratories, Hercules, CA). GAPDH was used as the reference gene for normalization. Reactions were performed in triplicate with a 25-µL mixture containing cDNA samples, primers, and iQ Sybr Green supermix (Bio-Rad Laboratories, Hercules, CA). The relative amount of mRNA in experimental cells was calculated using $2^{-\Delta\Delta CT}$ method and CFX manager software (Bio-Rad Laboratories, Hercules, CA).

### *Statistical Analysis*

Data are expressed as the mean ± SE (standard error) and statistical analysis were conducted using GraphPad Prism software v.5.01 for Windows (San Diego, CA). Comparisons between groups were performed using student's t test and one-way ANOVA with Dunnett's multiple comparison test, post hoc. Probability of $p < 0.05$ was considered to indicate a statistically significant difference. Each experiment was repeated at least three times to assess the level of reproducibility.

**Table 1**. PCR primer sequences for cytokine and cytotoxic granule proteins.

| Target gene | NCBI Reference Sequence | Primer sequence | Tm (°C) | Amplicon size (bp) |
|---|---|---|---|---|
| IFN-γ | NM_000619.2 | Sense 5'-CTGTTACTGCCAGGACCCAT-3'<br>Anti-sense 5'-GCATCTGACTCCTTTTTCGC-3' | 59 | 412 |
| TNF-α | NM_000594.3 | Sense 5'-GTCCTCTTCAAGGGCCAAGG-3'<br>Anti-sense 5'-CAGACTCGGCAAAGTCGAGA-3' | 57 | 258 |
| Perforin | NM_005041 | Sense 5'-TGGTGGACTACACCCTGGAA-3'<br>Anti-sense 5'-CACCTGGCATGATAGCGGAA-3' | 57 | 561 |
| Granzyme B | NM_004131.4 | Sense 5'-GGCAGATGCAGGGGAGATCA-3'<br>Anti-sense 5'-TACAGCGGGGGCTTAGTTTG-3' | 59 | 729 |
| GAPDH | NM_002046.5 | Sense 5'-ACGGATTTGGTCGTATTGGGCG-3'<br>Anti-sense 5'-CTCCTGGAAGATGGTGATGG-3' | 60 | 212 |

Base pairs (bp), Melting temperature (Tm).

## Results

Isolated exosomes were evaluated for size using nanoparticle tracking analysis (NTA) with a NanoSight NS300 (Malvern Instruments Ltd, Malvern, UK). Mean DLCL2 vesicle diameter was 137 nm (+/- 56 nm), with a mode of 114 nm. Mean FSCCL vesicle size was 156 nm (+/- 63 nm), with a mode of 128 nm (**Figure 1A**). We also evaluated the lymphoma exosomes for several protein markers using flow cytometry and Western blotting. Our data showed these lymphoma vesicles did not strongly express CD63, LAMP-1, CD9, or TSG101. However, there was detection of MHC class I and CD81. As a control, protein expression from exosomes derived from normal human plasma were used. These plasma vesicles were positive for CD63, TSG101, HSP70, and MHC class I (**Figure 1B and C**).

It was previously established that exosomes from an aggressive diffuse large cell lymphoma (WSU-DLCL2) cell line contained Survivin (Valenzuela *et al.*, 2015). We wanted to confirm the presence of exosomal Survivin in WSU-FSCCL, an indolent follicular small cleaved cell lymphoma cell line. Western blot confirmed these B cell lymphoma cell lines express cellular and exosomal Survivin, in addition to other IAPs (**Figure 2A**). It was also determined that sublethal amounts of stress due to treatment with etoposide (0.1 μM) did not alter IAP expression levels. PCR results were inconsistent in what IAPs were detectable, and often negative. Housekeeping genes β-actin and GAPDH were used as loading controls (**Figure 2B**).

Survivin in the extracellular space has be previously shown to be taken up by T cells and elicit effects such as decreased proliferation, decreased cytolytic capabilities of

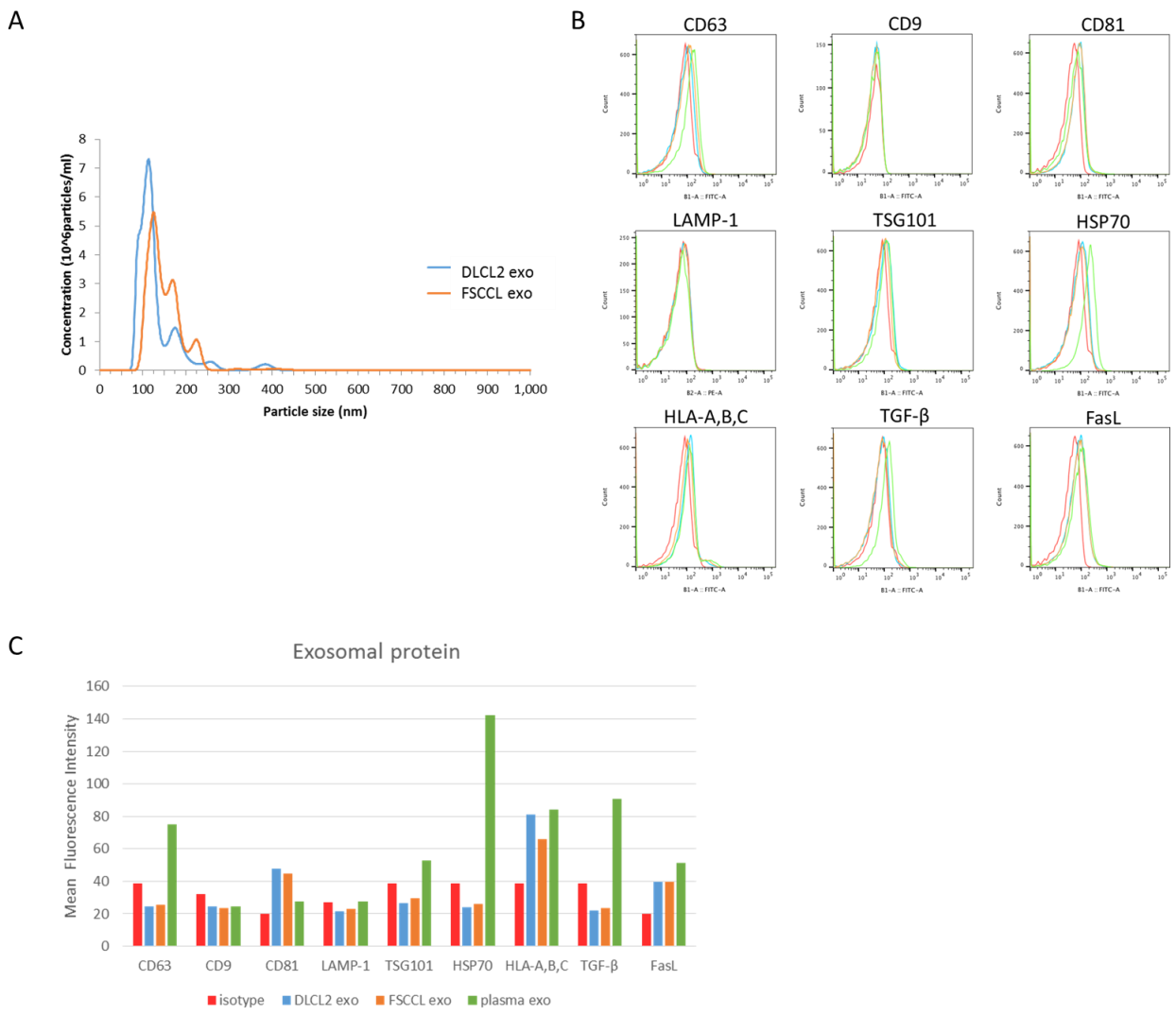


***Figure 1***. Size and Protein Markers of Exosomes Derived from WSU-DLCL2 and WSU-FSCCL Lymphoma Cell Lines. **(A)**. Sizing analysis of lymphoma exosomes was performed with the NanoSight NS300. Mean size for exosomes was 137 nm (+/- 56 nm) for DLCL2 and 156 nm (+/- 63 nm) for FSCCL. **(B)** For analysis of expression of surface molecules, 200 µg of exosomes were incubated with 10 µl of 4-µm-diameter aldehyde/sulfate latex beads and stained with antibodies. Only the population containing single beads was gated and analyzed. Red = isotype, green = plasma exosomes, blue = DLCL2 exosomes and orange = FSCCL exosomes. **(C)** Mean fluorescence intensity (MFI) of exosome staining was compared with exosomes stained with an isotype control and exosomes from plasma.

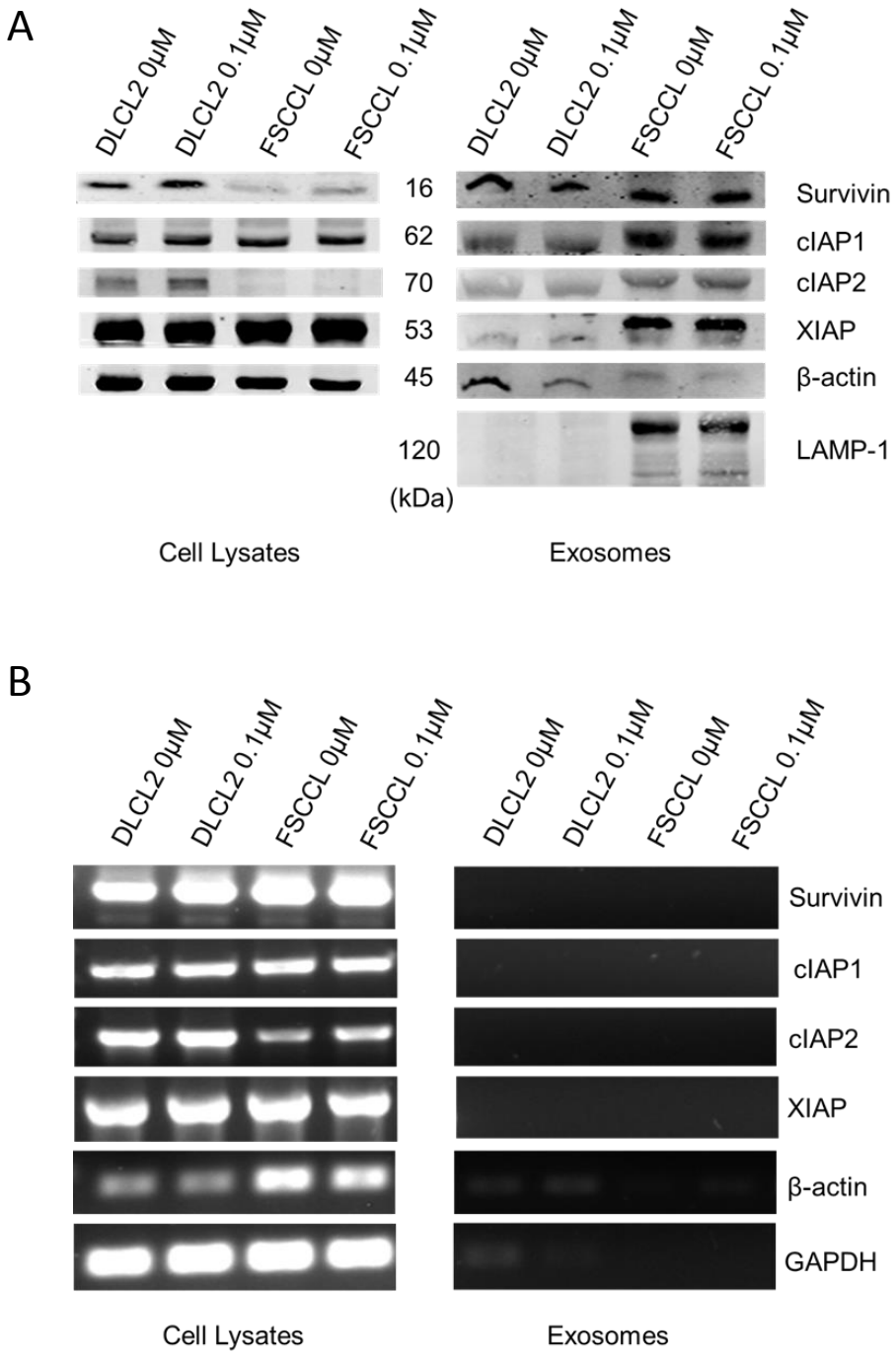


***Figure 2***. Lymphoma cells and exosomes contain Survivin and other IAP proteins. **(A)** Western blot showing IAPs contained in cell lysates and exosomes. Cells were treated with sublethal amounts of etoposide (0.1 μM) for 24 hours. GAPDH and β-actin were used as controls. **(B)** RT-PCR analysis of mRNA content in cells and exosomes.

CD8+ T cells, and a skewing of the cytokine profile to that of a Th2 population (Jutzy *et al.*, 2013). As NK cells are frequently found to be inhibited in the TME, we investigated whether extracellular Survivin, or lymphoma exosomes containing Survivin, would also modify NK cell function.

A well-recognized mechanism by which NK cells are inhibited in the TME is through interference with the activating receptor NKG2D. Soluble and exosomal release of the NKG2D ligand MICA/B has been shown to reduce NKG2D expression on NK cells (Hedlund *et al.*, 2011). We therefore examined whether lymphoma exosomes carried MICA/B (**Figure 3**), and if exposure to these exosomes or extracellular Survivin protein would alter NKG2D levels. Analysis of exosome-coated aldehyde beads by flow cytometry did not detect expression of MICA/B, although the parent lymphoma cells did express this NKG2D ligand (**Figure 3A**). Investigation of NKG2D levels on NK cells did not find significant changes after exposure to exosomes or Survivin, except in the NK cell group activated by a classical target, erythroleukemia cell line K562 (Zamai *et al.*, 1998). In this group there was a noticeable decrease in NKG2D levels after treatment with Survivin protein compared to media control (**Figure 3B**), although the mechanism by which this occurs is yet to be determined.

To evaluate whether mechanisms other than NKG2D downregulation might be contributing to inhibition of NK cell function, we tested degranulation levels of NK cells after conditioning with Survivin or lymphoma exosomes. Activated NK cells mobilize cytosolic granules containing perforin and Granzyme B to the plasma membrane for release, or degranulation. This process exposes the lysosomal membrane glycoproteins CD107a, also known as LAMP-1, which can be used as a measurement of NK cell

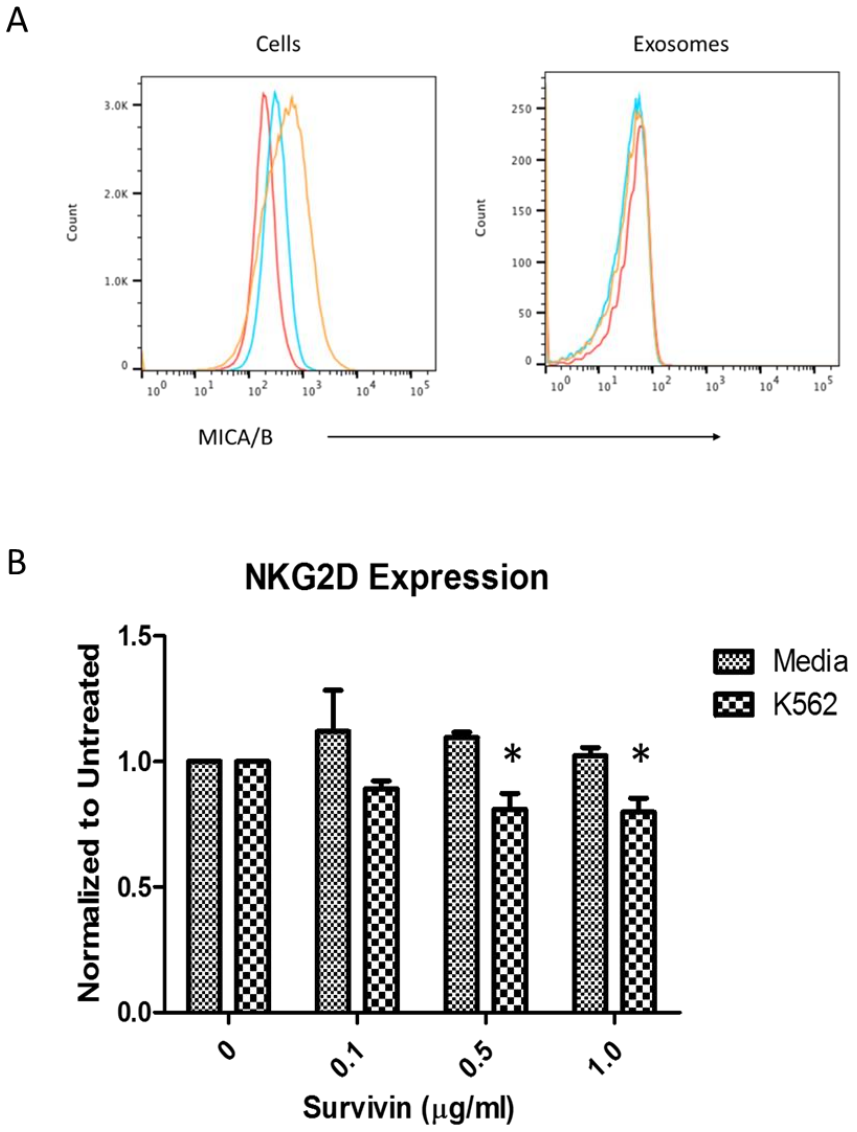


***Figure 3***. NKG2DL are present on lymphoma cells but not exosomes, and treatment with Survivin protein decreases NKG2D expression. **(A)** Lymphoma cells express MICA/B (an NKG2DL), but exosomes from these two lymphoma cell lines did not. Red = isotype, blue = DLCL2, orange = FSCCL. **(B)** NKG2D expression on NK cells (CD56+) after treatment with Survivin (0-1.0 μg/ml). NK cells were activated by IL-2 (100 U/ml) and K562 target cells, and treated with Survivin for 24 hours. The receptor expression was assessed by mean fluorescence intensity (MFI) measured by fluorescent staining with NKG2D-APC antibody. MFI was normalized to an untreated group and data are presented as mean (± SEM) of three independent experiments (* is $p<0.05$), Survivin treatment versus untreated control.

activation or inhibition (Alter *et al.*, 2004). As expected, there were higher levels of degranulation in the NK cell group activated with IL-2, and increased degranulation in NK cells that were activated with PMA/Ionomycin and K562 cells. However, no significant changes were seen upon treatment with Survivin (**Figure 4**) or exosomes (data not shown).

NK cell function can also be affected by the levels of its cytotoxic granule proteins and cytokines, as seen for example, in the TME of multiple myeloma where inhibition of NK cell activity occurred via lower levels of perforin and Granzyme B, despite unchanged degranulation (Sarkar *et al.*, 2013). Therefore, we next determined whether levels of perforin, Granzyme B, or cytokines TNF-α or IFN-γ would be affected by exposure of NK cells to lymphoma exosomes or Survivin protein. We treated NK cells with either exosomes (10 or 50 μg/ml) or Survivin protein and investigated protein levels by intracellular flow cytometry (**Figure 5A**). Protein levels were not affected by activation with PMA/Ionomycin or K562 activation (data not shown). Exosomes were not able to exert a noticeable effect upon intracellular protein levels. However, treatment with extracellular Survivin protein consistently decreased protein amounts. We also used Western blot analysis to compare protein levels of perforin and Granzyme B in NK cells treated with Survivin protein (**Figure 5B**).

In addition to measuring protein levels, we also investigated mRNA expression of perforin, Granzyme B, TNF-α and IFN-γ. We found that treatment of NK cells with lymphoma exosomes or extracellular Survivin for 24 hours did not result in consistent alterations to levels of RNA by either block RT-PCR (**Figure 6A**) or real time qRT-PCR (**Figure 6C**).

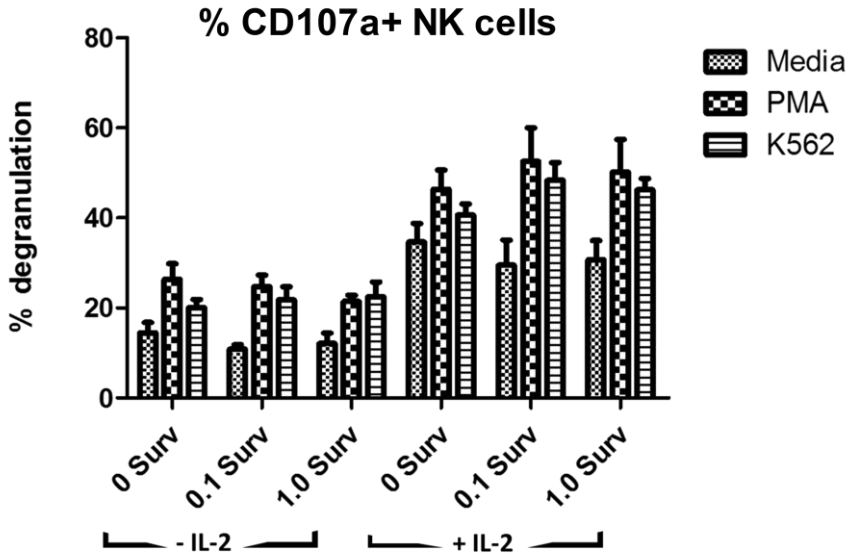


***Figure 4***. Degranulation of NK cells affected by IL-2 and activation by PMA and K562, but not by Survivin. PBMCs from healthy donors (n=7) were treated with recombinant Survivin protein (0-1.0 μg/ml) with or without IL-2 for 36 hours. Degranulation activity of the gated CD56+ NK cell population was measured after 6 hour stimulation by PMA and Ionomycin, K562 target cells, or media control using flow cytometry. Data presented as % of total gated NK cells that were CD107a-FITC +.

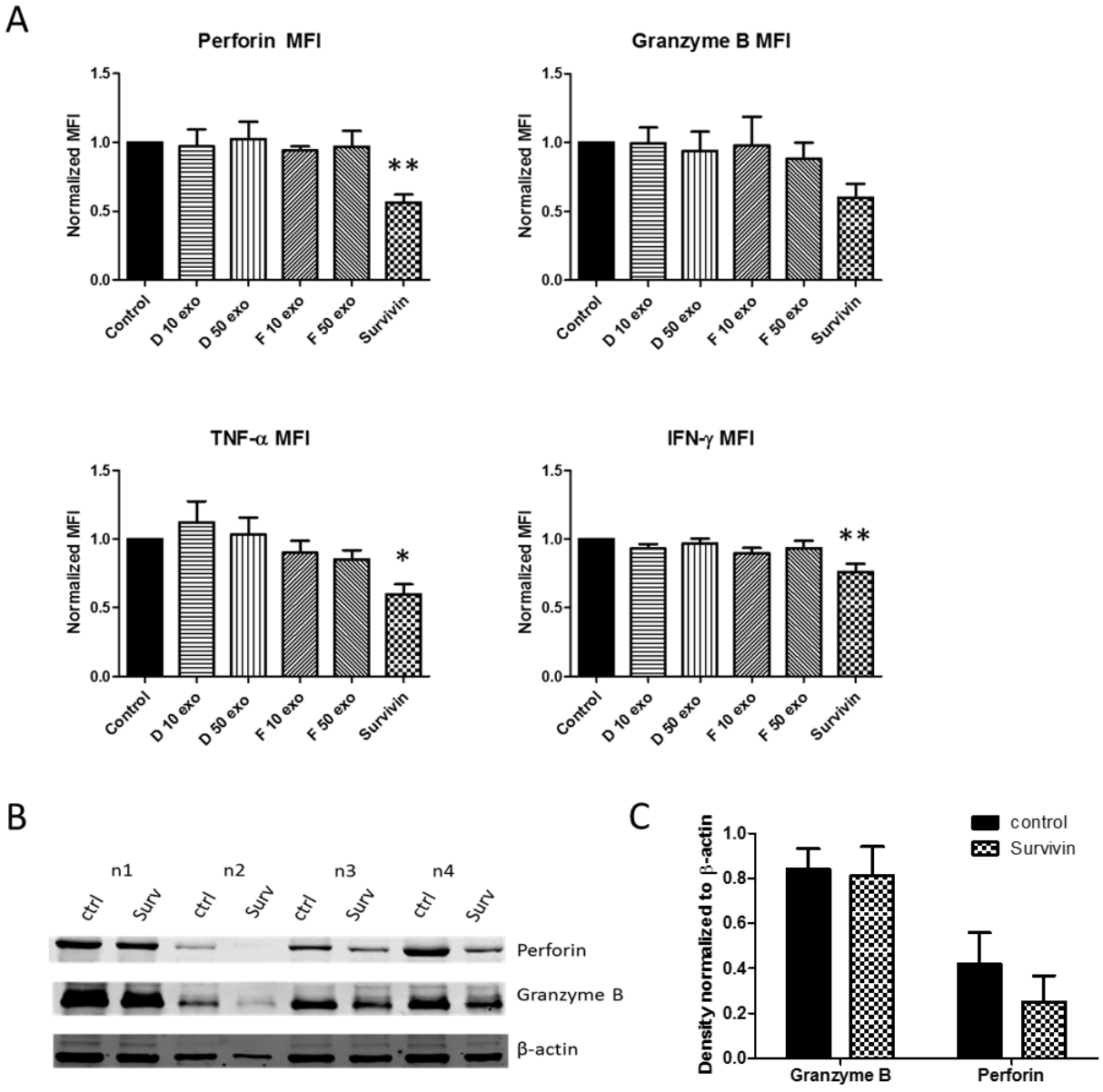


***Figure 5***. Survivin decreases NK cell intracellular protein levels of perforin, Granzyme B, TNF-α and IFN-γ. **(A)** CD3- CD56+ NK cells were treated with extracellular Survivin protein or lymphoma exosomes for 24 hours. Expression of perforin, Granzyme B, TNF-α and IFN-γ were measured by intracellular flow cytometry (n=5). Two concentrations of exosomes were investigated (10 μg and 50 μg). MFI was normalized to an untreated group and data are presented as mean (± SEM) of five independent experiments (* is $p<0.05$, ** is $p<0.01$), Survivin treatment versus untreated control.
**(B)** Protein levels of NK cells after treatment with Survivin by Western blot analysis. **(C)** Densitometry by ImageJ and normalized to β-actin (n=4).

As a test of functionality, we investigated the cytotoxic potential of NK cells by measuring their ability to lyse K562 target cells. NK cells were treated with 10 μg/ml recombinant Survivin for 24 hours prior to performing the lactate dehydrogenase release assay. NK effector cells were mixed with K562 target cells at various ratios, and the percentage of specific lysis was calculated. As shown in **Figure 7**, exposure to Survivin did not alter NK cell cytotoxic potential.

## Discussion

The ability of tumors to avoid immune attack is critical for cancer development and metastasis, but the mechanisms responsible have yet to be fully elucidated. Cancer exosomes have emerged as mediators of immune suppression facilitating tumor survival. In this work, we studied the influence of lymphoma-derived exosomes and extracellular Survivin on NK cells. We hypothesized that tumor-derived exosomes containing Survivin would affect NK functionality similarly to previous findings concerning T cell suppression by extracellular Survivin (Jutzy *et al.*, 2013). Although Survivin and other IAPs are present in lymphoma-derived exosomes, we found little observable difference in NK cells functionality after exposure to these exosomes. Conversely, extracellular Survivin treatment did decrease NK cell intracellular protein expression of perforin, Granzyme B, TNF-α and IFN-γ. This is perhaps due to a concentration effect, as the mixed contents of exosomes may dilute or overpower the influence of Survivin delivered exosomally as opposed to treatment by pure protein. While an effect on NK cell protein is seen with Survivin by itself, the effect is lost by dilution into exosomes. Therefore, Survivin in the

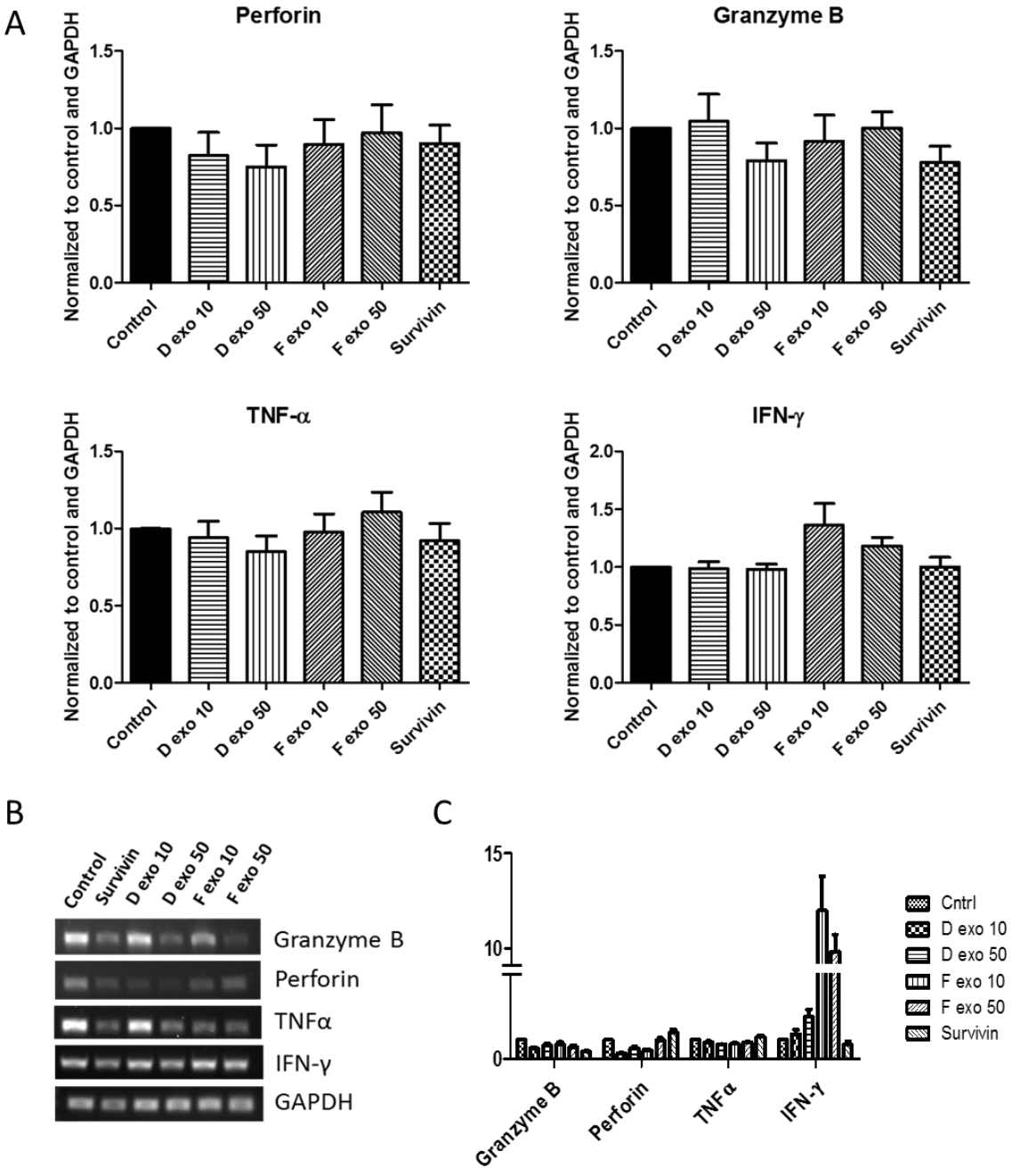


***Figure 6***. Lymphoma exosomes and Survivin had no consistent effect on mRNA levels of NK cell cytokines and granules. **(A)** ImageJ density analysis of block RT-PCR bands did not show statistical significance in mRNA levels after 24 hours treatment with lymphoma exosomes (10 μg and 50 μg) or Survivin (n=5). **(B)** Block RT-PCR amplicon products from NK cells from one donor run on 1% agarose gel. **(C)** Gene expression (ΔΔCq) was measured using real time qRT-PCR performed with a Bio-Rad CFX (n=5).

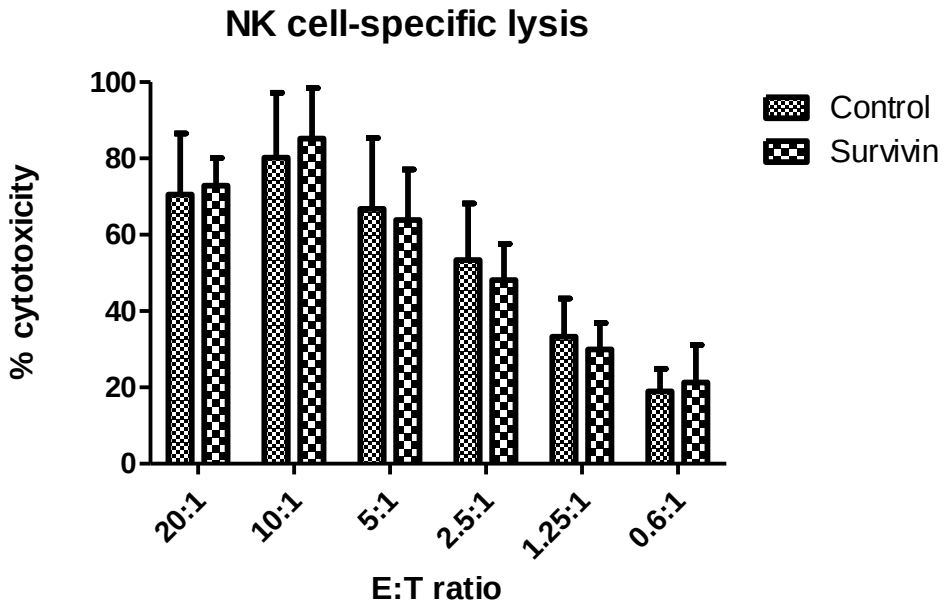


***Figure 7***. Survivin treatment had no effect on NK cell cytotoxicity. NK cell cytolytic capacity against target K562 cells was measured via lactate dehydrogenase (LDH) release at serial dilutions of the effector to target (E:T) ratio from 20:1 to 0.625:1. NK effector cells were activated overnight with IL-2 (100 U/ml) and treated with 10 μg/ml Survivin for 24 hours before being co-incubated with K562 cells for 4 hours.

TME sourced from necrosing tumor cells may show more effects than exosomal Survivin due either to concentration or uptake issues, as NK cells have not been shown to efficiently uptake exosomes (Ferguson Bennit *et al.*, 2017).

The lack of effects on NK cell functionality after exposure to lymphoma-derived exosomes could result from several factors. For example, lymphoma exosomes may not contain sufficient quantities of modulating proteins. Examination by Western blot and flow cytometry showed very low amounts of FasL, TGF-β, or MICA/B associated with these vesicles. There is literature support for NK cells being affected by tumor exosomes that harbor these proteins (Szczepanski *et al.*, 2011). Ovarian exosomes from cell lines and patients have shown variable surface expression of NKG2DL (MICA/B, ULBP1-3) with concomitant ability to downregulate NKG2D on PBMCs, decrease degranulation, and inhibit cytotoxicity (Labani-Motlagh *et al.*, 2016). Although leukemia and lymphoma-derived exosomes have been reported to immunosuppress cytotoxic activity of NK cells by binding NKG2D receptors (Hedlund *et al.*, 2011), it is possible the EVs obtained from the two cell lines examined in this work are lacking key modulatory proteins. Actual exosome protein expression is highly dependent on the cell of origin. While members of the tetraspanin family CD9, CD63 and CD81 are often presented as "exosome markers," this is not a binding condition. Early studies found CD63 to be low in exosomes compared to cells, while CD81 was enriched ten-fold (Escola *et al.*, 1998). CLL exosomes, for example, have been shown to be low in CD63 and CD81 (Paggetti *et al.*, 2015), but high in CD20, HLA-DR, Hsp72 and XIAP. A study examining several B cell lymphoma cell lines found no CD9 expression, and variable CD63 and CD81 levels (Oksvold *et al.*, 2014). Another common marker involved in exosome biogenesis is

TSG101, whose levels have been known to vary widely in exosomes from plasma of different donors, as well as to be in the lumen of exosomes (Kalra *et al.*, 2013, Muller *et al.*, 2014). These findings highlight the importance of choosing markers for EV phenotyping carefully, and recognizing the diversity in exosomes from different cellular origins. Characterization of the exosomes used in this study found their size to be within the accepted exosome range of 50-150 nm and in agreement with results from CLL exosomes noted to be 70-200 nm (Paggetti *et al.*, 2015). In this study, surface phenotyping revealed low CD63, CD9 and TSG101 expression, and moderate levels of CD81 and FasL. These lymphoma exosomes were also high in MHC class I markers, but had no detectable TGF-β and MICA/B. Low exosomal levels of TGF-β and MICA/B may partly explain the observed inability of these vesicles to decrease NKG2D expression on NK cells (Clayton *et al.*, 2008, Berchem *et al.*, 2016).

Another factor may be found in the uptake capabilities of NK cells for these exosomes. Previous studies have shown that NK cells have limited capacity for EV internalization (Hazan-Halevy *et al.*, 2015, Ferguson Bennit *et al.*, 2017). However, T cells also exhibit low uptake ability (Muller *et al.*, 2017) and yet are still affected by EVs in a variety of capacities including cytotoxicity, cytokine release, proliferation and activation (Whiteside, 2017). It should also be considered that K562 target cells are Fas negative, so if NK cells are being modulated in their FasL expression this would not elicit a change in the cytotoxicity assay (Yu *et al.*, 2009).

While Survivin did not have a significant effect on transcripts associated with NK cell activity, the functional proteins were decreased, perhaps through a post translational mechanism. This may have an inhibitory influence on the ability of cancer-associated NK

cells to eliminate tumor cells. The Survivin-induced decrease in perforin and Granzyme B may result in granules that are less effective, even if the degranulation process itself is unchanged.

Decreased cytokine levels after conditioning with extracellular Survivin may hinder NK cell ability to communicate with other cells in the TME. TNF-α is an important inflammatory cytokine for the activation of neutrophils, macrophages, and lymphocytes with roles in cell proliferation, differentiation, necrosis, apoptosis, and cytokine induction (van Horssen *et al.*, 2006). IFN-γ has a significant anti-tumor influence within the TME. It can slow the cell cycle of cancer cells, induce apoptosis, inhibit angiogenesis, and is a critical macrophage activating agent (Ikeda *et al.*, 2002, Schroder *et al.*, 2004). IFN-γ upregulates MHC class I molecules to promote antigen presentation and immune surveillance, promotes Th1 responses, and initiates a chemokine directed recruitment of other immune cells (Zaidi & Merlino, 2011). An essential role of NK cells in the early anti-tumor response is the rapid production of IFN-γ. Therefore, Survivin-induced reduction of IFN-γ may contribute to the suppression CTL- and NK-cell–mediated immune responses central to tumor immune escape. The immunomodulating effects of Survivin that reduce IFN-γ are in line with the previous T cell study which reported skewed immune responses away from Th1 cell-mediated cytotoxicity (Jutzy & Wall, 2017). Beyond its function in cell cycle and apoptosis, Survivin is establishing roles in the extracellular environment of enhancing tumor progression and immune suppression. This interaction with the immune system is complex and warrants further exploration.

The finding that Survivin can lower important proteins for NK cell function, while not being able to elicit a noticeable decrease in the actual killing ability of the NK cells against target cells implies that Survivin in the TME may be one component in a multifactorial inhibition of NK cells, with other entities also involved in the crippling of NK cell anti-tumor responses. Thus, anti-Survivin therapies and anti-TEX therapies may be useful as part of a combination attack against cancer. Continuing to dissect and understand the contribution of exosomes to immune modulation is crucial for prevention of tumor escape, harnessing the patients' own anti-tumor immune responses, and discovering novel therapies.

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# CHAPTER FOUR

# CONCLUSION

TEX play a key role in tumor progression, from promoting metastatic characteristics, angiogenesis, niche preparation, survival, and chemoresistance (Tickner *et al.*, 2014). They also exert a variety of detrimental effects on immune cells, including apoptosis of activated T cells, upregulation of Treg activity(Szajnik *et al.*, 2010), impairment of monocyte differentiation into dendritic cells (Iero *et al.*, 2008, Wieckowski *et al.*, 2009), and accumulation of myeloid-derived suppressor cells (Xiang *et al.*, 2009). Extracellular vesicles also contribute significantly to NK cell suppression via NKG2DL and TGF-β (Boissel *et al.*, 2006, Szczepanski *et al.*, 2011, Hilpert *et al.*, 2012).

NK cells are innate immune effector cells important in surveillance and destruction of cancer due to their ability to recognize and kill tumor cells in a direct way independent of prior sensitization and without MHC-restrictions. NKG2D is a critical activating receptor for anti-tumor immune surveillance (Smyth *et al.*, 2005), and its stress-inducible ligands MICA/B and ULBPs are often expressed on cancer cells. Tumors can evade the NKG2D ligand-receptor pathway by secretion of soluble and exosomal NKG2DL leading to receptor inhibition (Mincheva-Nilsson & Baranov, 2014).

NK cell suppression in the TME has been observed in many hematological malignancies like AML and CLL (Pierson & Miller, 1996, Costello *et al.*, 2002, Fauriat *et al.*, 2007). Studies on exosomes and B cell lymphoma are few, especially regarding interactions with NK cells. Therefore it was of interest to further explore the impact of exosomes in lymphoma, as a more complete understanding of the underlying processes may pave the way to more efficacious immunological therapies against cancer. In this

research work, we were particularly interested in the interactions of DLCL2 lymphoma exosomes with peripheral blood populations. Previous literature investigating exosome interactions with immune cells have reported that myeloid derived populations like neutrophils, monocytes, macrophages and DCs utilize multiple approaches for uptake, from phagocytosis to ligand binding. B cells have also been reported to be able to bind and internalize exosomes from DCs or malignant cells. However, T lymphocytes and NK cells have not been observed to have great ability to internalize exosomes, although there is extensive evidence for their being affected by exosomes. It is likely that these effector cells are susceptible to receptor-ligand binding induced alterations, especially by FasL, TGF-β, PD-L1, NKG2DL. We found that uptake was most efficient in B cells and myeloid derived cells, with over 40% of cells being positive for exosome internalization after 24 hours. In comparison, T cells and NK cells were less than 10% positive for uptake of exosomes. These findings align with other reports of exosomal internalization (Hazan-Halevy *et al.*, 2015, Muller *et al.*, 2017).

Analysis of lymphoma exosome protein content revealed an absence of TGF-β and MICA/B, and the presence of FasL and Survivin. Extracellular Survivin has been previously found to interact with T cell populations to promote a cancer permissive environment through inhibition of CD8+ T cell function and proliferation, as well skewing the cytokine profile to that of a Th2 surveillance response instead of a cytotoxic one (Jutzy *et al.*, 2013). Therefore, we investigated what contribution Survivin may have in the NK cell modifications frequently observed in tumor settings. Treatment with recombinant Survivin protein was able to decrease NKG2D receptor levels in NK cells stimulated with target cells, and decrease protein levels of TNF-α, IFN-γ, perforin, and

Granzyme B. Exposure of NK cells to lymphoma exosomes did not result in observable changes in degranulation, cytotoxic ability, or protein and mRNA levels of perforin, Granzyme B, and cytokines. The findings of this work contribute to a better knowledge of the repertoire of lymphoma exosomal contents and their potential immune modulating contributions. This may open new possibilities for the use of NK cells in immunotherapy against lymphoma and other hematological malignancies.

Although recent advances in cancer immunotherapies have ignited much enthusiasm and hope for patients and oncologists, therapeutic outcomes are often disappointing in many clinical trials. Cancer cell modulation of the TME is a critical factor in switching from a tumor-destructive environment to a tumor-promoting one that favors immunosuppressive immune cell populations such as MDSCs and Tregs. Immune cells capable of anti-tumor actions such as NK cells and T cells are frequently inhibited. TME mechanisms that impair NK cell anti-tumor function are detrimental to the success of monoclonal antibody immunotherapies that depend on NK-mediated killing strategies. As exosomes play a significant role in NK cell suppression, further research on the exosomes derived from B cell lymphoma was warranted. The findings of this work contribute to a better knowledge of the repertoire of exosomal contents and their potential immune modulating contributions. This will open new possibilities for the use of NK cells in immunotherapy against lymphoma and other hematological malignancies.

The future directions of this project include looking at changes in the proteome and transcriptome of NK cells upon conditioning with Survivin and lymphoma exosomes. These arrays would give a wider view of the extent of changes that occur when NK cells in the TME are exposed to lymphoma derived exosomes and extracellular Survivin, as

well as elucidate pathways and mechanisms utilized by Survivin to elicit the decreases in NK cell proteins. A further characterization of lymphoma exosomes from cell lines and patient samples might also reveal significant information as to the mechanism of immune suppression in this disease. As this study used NK cells from healthy donors, who had a wide degree of variability in their immune status and NK cell responses, it might also be helpful to repeat some experiments, particularly the mRNA investigations, with an NK cell line such as NK92. Additionally, we would like to explore the effects of extracellular Survivin on other cell populations in the TME, such as DCs and monocytes.

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## APPENDIX A

## BIOGRAPHICAL SKETCH

Heather R. Ferguson Bennit Ph.D. Candidate

24530 University Ave

Loma Linda, CA 92354

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### Education/Training

| | | | |
|---|---|---|---|
| Loma Linda University, Loma Linda, CA | MD | 2016-2020 | Medicine |
| Loma Linda University, Loma Linda, CA | Ph.D | 2010-2016 | Biochemistry |
| Walla Walla University, College Place, WA | BSc. | 2006-2010 | Bioengineering |

### Research Experience

September 2010 – 2016 PhD Candidate, Department of Biochemistry

Center for Health Disparities and Molecular Medicine, Loma Linda University

Research Advisor: Dr. Nathan R. Wall

Dissertation title: Effect of Extracellular Survivin and Lymphoma Exosomes on Natural Killer Cells

June – August 2009 NSF funded Undergraduate Research Experience, Department of Chemistry

Bourns College of Engineering, University of California, Riverside, CA

Research Advisor: Dr. Quan Cheng

Bioengineering research project developing novel surface interface for study of transmembrane proteins using surface plasmon resonance

April – June 2009 Undergraduate directed research, Department of Biology

Walla Walla University, College Place, WA

Research Advisor: Dr. Joan Redd

Investigated the effects oral administration of turmeric had on in vitro T cell proliferation rates of mice splenocytes, specifically T cells post stimulation with mitogen.

## Publications

### Manuscripts

Khan S, **Ferguson Bennit H**, Turay D, Perez M, Mirshahidi S, Yuan Y, Wall NR. Early diagnostic value of Survivin and its alternative splice variants in breast cancer. *BMC Cancer*. 2014;14:176.

Khan S, **Ferguson Bennit H**, Wall NR. The emerging role of exosomes in survivin secretion. *Histol Histopathol*. 2015 (30); 43-50. Review.

Khan S, **Ferguson Bennit H**, Valenzuela MMA, Turay D, Diaz Osterman CJ, Moyron RB, Esebanmen GE, Ashok A, Wall NR. Localization and up regulation of Survivin in cancer health disparities: a clinical perspective. *Biologics: Targets and Therapy*, 9:57-67, 2015. Review

Valenzuela MMA, **Ferguson Bennit H**, Gonda A, Diaz Osterman CJ, Hibma A, Khan S, Wall NR. Exosomes secreted from human cancer cell lines contain inhibitors of apoptosis (IAP). *Cancer Microenvironment*, 8(2):65-73, 2015.

Diaz Osterman CJ, Lynch JC, Leaf P, Gonda A, **Ferguson Bennit H**, Griffiths D, Wall NR. Curcumin Modulates pancreatic adenocarcinoma cell-derived exosomal function. *PLoS One*, 10(7), 2015.

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**Ferguson Bennit H**, Gonda A, Oppegard LJ, Chi DP, Khan S, Wall NR. Uptake of lymphoma-derived exosomes by peripheral blood leukocytes. *Blood and Lymphatic Cancer: Targets and Therapy*, 7:9-23, 2017.

### Abstracts

**Ferguson Bennit H**, Gonda A, Chi D, Oppegard L, Licero J, De Leon M, Khan S, Wall NR. Effects of Survivin and lymphoma cell-derived exosomes on natural killer cell function. AACR Special Conference: The Function of Tumor Microenvironment in Cancer Progression, San Diego, January 2016

**Ferguson Bennit H**, Valenzuela MMA, Jutzy JMS, Khan S, Wall NR. B-cell lymphoma derived exosomes are reservoirs of Inhibitors of Apoptosis. AACR, April 2014

**Ferguson Bennit H**, Valenzuela MMA, Wall NR. Survivin is released from B-cell lymphomas through exosomes. NSRF April 2012

Diaz-Osterman CJ, **Ferguson HR**, Valenzuela MMA, Khan S, Wall NR. Curcumin suppresses pancreatic cancer growth by targeting survivin. Gordon Conference, 2013.

Diaz-Osterman C, **Ferguson HR**, Valenzuela MMA, Khan S, Wall NR. 6-gingerol and curcumin suppress pancreatic cancer growth by targeting the inhibitors of apoptosis. AACR Conference on Pancreatic Cancer: Progress and Challenges, June 2012

Sigaran U, Diaz Osterman CJ, Stiff TR, **Ferguson HR,** Valenzuela MMA, Khan S, Wall NR. Curcumin induces pancreatic cancer cell death by targeting the inhibitor of apoptosis proteins. 13th Annual Health Disparities Research Symposium, August 7, 2013, Loma Linda University.

Diaz-Osterman C, **Ferguson H**, Valenzuela MMA, Khan S, Wall NR. 6-gingerol and curcumin suppress pancreatic cancer growth by targeting the inhibitors of apoptosis. Fifteenth Annual Basic Sciences Research Symposium, October 29, 2012, Loma Linda University.

Public Presentations

Poster presentation at AACR TME conference, San Diego 2016

Poster presentation at Southern California Flow Cytometry Summit, Irvine CA 2015

Oral presentation at CHDMM Chalk Talk, March 2014

Poster Presentation at AACR National meeting, San Diego (2014)

Poster presentation at Annual Postgraduate Conference, Loma Linda University (2013, 2015)

Poster presentation for Annual Basic Sciences Research Symposium, Loma Linda University (2011, 2012)

Poster presentation at National Student's Research Forum at UTMB, Galveston TX (2012)

Presented poster of research results for faculty and students at University of California, Riverside (2009)

Presented poster at Murdock Conference 2009 in Spokane, WA

Manuscripts in Preparation

**Ferguson Bennit HR**, Gonda A, McMullen JR, Kabagwira J, Wall NR. Peripheral blood cell interaction of cancer-derived exosomes affect immune function. (Review)

**Ferguson Bennit HR**, Gonda A, Oppegard L, Chi David, Licero J, Asuncion Valenzuela MM, Jutzy JMS, De Leon M, Khan S, Wall NR. Natural killer cell phenotype and functionality affected by exposure to extracellular Survivin and lymphoma-derived exosomes.

Nicholas D, Garcia-Perez D, Sorensen D, **Ferguson Bennit H**, Langridge W, De Leon M. Fatty acids, inflammation, and type 2 diabetes. Review

## Mentorship Experiences

**Research Mentor**

Laura Oppegard, 1st year Medical student (2015)

David Chi, 1st year Medical student (2015)

James McMullen, 1st year PhD student (2015)

Abby Hibma, 2nd year medical student (2013)

Amber Gonda, 2nd year PhD student (2013)

Ron Moyron, 1st year PhD student (2013)

Naomi Jackson, 1st year PhD student, Big Brother/Big Sister program 2013-2014

Sonia Whang, 1st year PhD student (2012)

## Professional Memberships

| | |
|---|---|
| 2012-2017 | American Association for Cancer Research (AACR) |
| 2010-2017 | American Physician Scientist Association (APSA) |
| 2011-2017 | Southern California Flow Cytometry Association |
| 2015-2017 | International Society for Extracellular Vesicles (ISEV) |
| 2014-2017 | American Society of Clinical Oncology (ASCO) |
| 2007-2010 | Engineers Without Borders (EWB) |
| 2006-2010 | Society of Women Engineers (SWE) |

## Volunteer and Leadership Experiences

| | |
|---|---|
| 2017-2018 | School of Medicine Class of 2020 Senator |
| 2016 | Interviewer for potential participants of ABC Summer Research Program |
| 2014-2015 | MD/PhD Representative for Basic Science Student Council |
| 2014 | Treasurer for St Baldrick's Foundation for Pediatric Cancer Event, Loma Linda University |
| 2013 | Basic Science Symposium Steering Committee, Student Representative |
| 2012-2014 | Academic Affairs Vice President for Basic Science Student Council |
| 2011-2012 | Secretary for Basic Sciences Student Council |
| 2006-2009 | Secretary of local chapter of Engineers Without Borders |

## Honors

| | |
|---|---|
| 2010-2015 | Graduate Student Stipend Award |
| 2013 | Dean's Award for Excellence for Best Written Comprehensive Exam |
| 2010 | Completed Bachelor of Science with summa cum laude honors |
| 2006-2009 | Dean's List of Distinguished Students (GPA over 3.75) every quarter |
| 2006 | James R. Hoffa Memorial Scholarship |
| 2006 | High School Valedictorian |