CHARACTERIZATION OF TUMOR-DERIVED EXOSOMES AND THEIR ROLE IN IMMUNE REGULATION

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Tumor cells usually express specific antigens that are potentially immunogenic; however, established tumors primarily induce immune tolerance. In the last decade, a population of small membrane vesicles, termed “exosomes”, has gained increasing attention for their potential role in tumor immune regulation. Exosomes are formed in the late endocytic compartments and are released upon their fusion with the plasma membrane. They are secreted by various cell types, especially tumor cells and cells in the hematopoietic system. Although tumor-derived exosomes usually contain tumor antigens, they have been shown to exert diverse immunosuppressive effects. However, the ability of tumor-derived exosomes to induce antigen-specific immunosuppression has not been well examined. Also, the immunoregulatory effect of exosome-like vesicles in the blood circulation of tumor-bearing hosts remains unclear.

In this thesis, we first investigate the role of tumor-derived exosomes in mediating antigen-specific immune suppression using ovalbumin (OVA) as a model tumor antigen. We demonstrate that exosomes derived from OVA-expressing tumor cell lines potently suppress OVA-specific delayed-type hypersensitivity (DTH) response. We also show that exosomes are mostly taken up by dendritic cells (DCs) after local administration, and the mRNA levels of TGF-β1 and IL-4 in the draining lymph node were significantly elevated in correlation with

suppression of the DTH response. Furthermore, tumor-derived exosomes affect the function of DCs *in vitro* by inhibiting their maturation and inducing TGF-β1 production. These results suggest that tumor-derived exosomes are able to confer antigen-specific immune suppression possibly by DC-mediated mechanism.

We further investigate the immunoregulatory effect of plasma-derived exosomes and demonstrate that plasma-derived exosomes isolated from mice bearing OVA-expressing tumors are able to suppress the OVA-specific DTH response. However, enrichment of tumor-derived exosomes in blood plasma was not identified and the suppressive effect is partially mediated by the MHC class II+ vesicle portion. The third part of the thesis discusses the B cell stimulatory and the consequent T cell inhibitory effect of exosomes derived from tumor cells with mycoplasma infection.

The work presented in this thesis increases our understanding of the immunoregulatory role of tumor-derived exosomes, circulating exosomes in tumor-bearing hosts as well as exosomes derived from pathogen-infected tumor cells.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

AICD: activation-induced cell death

AIDS: Acquired Immune Deficiency Syndrome

APC: antigen-presenting cell

ATCC: American Type Culture Collection

BALF: bronchoalveolar fluid

BMDC: bone marrow-derived dendritic cell

BSA: bovine serum albumin

CD: cluster of differentiation

cDNA: complementary deoxyribonucleic acid

CFA: complete Freund's adjuvant

CIA: collagen-induced arthritis

CTL: cytotoxic T lymphocyte

CTLA: cytotoxic T lymphocyte antigen

DC: dendritic cell

DTH: delayed-type hypersensitivity

EAE: experimental autoimmune encephalomyelitis

EBV: Epstein - Barr virus

ELISA: enzyme-linked immunosorbent assay

FACS: fluorescence-activated cell sorting

FasL: Fas ligand

FBS: fetal bovine serum

FoxP3: forkhead box P3

GM-CSF: granulocyte/macrophage colony-stimulating factor

GPL: glycopeptidolipid

HA: hemoglutanin

HIV: human immunodeficiency virus

HMGB1: high mobility group box chromosomal protein 1

HSP: heat shock protein

i.d.: intradermal(ly)

i.p.: intraperitoneal(ly)

i.v.: intravenous(ly)

ICAM-1: intercellular adhesion molecule 1

IDO: indoleamine 2,3-dioxygenase

IFA: incomplete Freund’s adjuvant

IFN: interferon

Ig: immunoglobulin

IL: interleukin

iNOS: inducible nitric oxide synthase

kDa (kD): kiloDalton

KLH: keyhole limpet hemocyanin

LFA1: lymphocyte function-associated antigen 1

LPS: lipopolysaccharide

MACS: magnetic-activated cell sorting

MDSC: myeloid-derived suppressor cells

MHC: major histocompatibility complex

MIIC: MHC-class II-rich compartment

miRNA: microRNA

mRNA: messenger RNA

MVB: multivesicular bodies

NK cell: nature killer cell

NOS2: nitric-oxide synthase 2

OVA: chicken egg ovalbumin

PAMPs: pathogen-associated molecular patterns

PBS: phosphate-buffered saline

PD-L1, PD-1: programmed death ligand-1, programmed death-1

PS: Phosphatidylserine

qRT-PCR: quantitative reverse transcription-polymerase chain reaction

s.c.: subcutaneous(ly)

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAA: tumor-associated antigen

TCR: T cell receptor

TGF-β: transforming growth factor-β

Th cells: T helper cells

TIM: T cell immunoglobulin domain and mucin domain-containing protein

TLR: Toll-like receptor

TNF: tumor necrosis factor

Tr-1: T regulatory-type 1 cells

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand

Treg cells: T regulatory cells

TSA: tumor-specific antigen

TsAP6: transmembrane protein tumor suppressor-activated pathway 6

Tsg101: tumor susceptibility gene 101

VEGF: vascular endothelial growth factor

PREFACE

I would first like to express my deep gratitude to my advisor, Dr. Paul Robbins, for his guidance throughout my graduate study, and being always supportive and encouraging when I was in the face of adversity. I would also like to thank my committee members: Dr. Adrian Morelli, Dr. Walter Storkus, Dr. Christine Milcarek, Dr. Russell Salter, and Dr. Martin Schmidt for their valuable advices and suggestions on my research projects.

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1.0 INTRODUCTION

1.1 TUMOR IMMUNOLOGY

1.1.1 Tumor antigens

Genetic instability is a hallmark of tumor cells compared with normal cells, leading to the generation of new antigens, or the abnormal expression of endogenous proteins. Tumor antigens were initially classified into two categories based on their expression pattern. Tumor-specific antigens (TSA) refer to the antigens uniquely expressed by a given type of tumor cells (unique antigen), which usually result from genetic mutations or chromosomal translocations. Tumor-associated antigens (TAA) are expressed by both tumor cells and normal cells (shared antigen), however due to the changes in epigenetic controls these proteins are over-expressed in tumor cells with enhanced antigenicity (1, 2).

Modern classification further specifies tumor antigens based on their molecular structure and origin, such as antigens resulting from mutated oncogenes/tumor suppressor genes, antigens resulting from other mutated genes, oncogenic viral antigens, as well as altered cell surface glycolipids and glycoproteins. Particularly, TAAs are divided into four major categories: cancer-testis antigens (oncospermatogonal antigens) which are predominantly expressed in gametogenic tissues and cancer (e.g. MAGE, NY-ESO-1), cancer differentiation antigens which are associated

with the differentiation of a specific cell type (e.g. MART-1, gp100), over-expressed antigens which are aberrantly up-regulated in cancer cells (e.g. Her2/Neu, EGFR), and oncofetal antigens which are typically present only during fetal development but are also found in certain types of cancer (e.g. carcinoembryonic antigen) (3, 4).

Tumor antigens can be used for identification of tumor cells and more importantly, they are critical for triggering anti-tumor immune responses and therefore can be used in cancer immunotherapy.

1.1.2 Tumor immunosurveillance

The hypothesis of "Tumor Immunosurveillance" was first proposed by Burnet and Thomas in the 1950s (5, 6). It suggested that tumor antigens could provoke effective immune responses and that the host immune system was able to recognize and eliminate transformed cells, therefore protecting the host against the development of cancer. Original evidence supporting this idea includes the finding that in animal models chemically-induced tumors were rejected when transplanted into syngeneic hosts, whereas transplants of normal tissues were accepted (7, 8). It was also found that patients with genetic immunodeficiency exhibited increased incidence of cancers of viral origin or cancers related to bacterial infection (9-11), highlighting the protective effect of the immune system against pathogen-associated cancers. However, it was not until the development of knockout mouse models in tumor studies that the theory of immunosurveillance was further confirmed and became widely accepted. Several representative experiments demonstrated that the RAG-/- mice (lacking T cells and B cells), γ-IFN-R-/- and STAT-1-/- mice (with compromised innate and adaptive immunity) were more sensitive to carcinogens, and also had higher incidence in developing spontaneous tumors, which can be rejected in

immunocompetent mice (12-14). In humans, the observations that patients with Acquired Immune Deficiency Syndrome (AIDS) are more cancer-prone (15), immunosuppressed transplant recipients display higher incidence of non-viral tumors (16), and cancer patients can develop tumor-specific adaptive immunity (17) further supported a pivotal role of the immune system in defeating cancer.

In 2004, Schreiber and colleagues extended the concept of cancer immunosurveillance to "Cancer Immunoediting", in which the interactions between tumor cells and the immune system throughout tumor development are viewed as a dynamic process comprised of three phases: elimination, equilibrium and escape. In the elimination phase, innate and adaptive immune cells composing the immunosurveillance network patrol the body for the detection and eradication of tumor cells. Tumor cells surviving this process will then co-exist with the immune system in an equilibrium state without growing or being attacked. The last phase occurs when tumor cells are immunologically sculpted and eventually become capable of evading the immune system for their growth, resulting in cancer (18).

1.1.3 The immune system and tumors

The host immune defense is composed of both innate and adaptive immunity. The innate immune system responds to pathogens and foreign antigens in a rapid fashion; however their recognition repertoire is generic and limited. Cells in the innate immune system include mast cells, phagocytes (macrophages, neutrophils and dendritic cells), basophiles, eosinophils and natural killer cells (NK cells). Compared with the non-specific innate immunity, adaptive immunity represents a more sophisticated system which has the ability to direct antigen-specific immune responses and confer long-term memory. Cells in the adaptive immune system include

CD4+ T-helper cells, CD8+ cytotoxic T lymphocytes and B cells. Killing of tumor cells by killer T cells (and/or NK cells), usually with the assistance of helper T cells, is the ultimate action of an effective immune response against tumors (19, 20). In general, induction of an effective anti-tumor response requires three steps: (a) appropriate presentation of tumor antigens; (b) selection and activation of antigen-specific T cells as well as non-specific effector cells; (c) recruiting antigen-specific T cells to the tumor site for effective elimination of malignant cells (21). The function and regulation of the key players mediating anti-tumor responses are further discussed below.

1.1.3.1 Dendritic cells (DCs)

DCs are professional antigen-presenting cells (APCs) with the unique ability to induce primary immune responses and establish immunological memory, serving as key instructors of antigen-specific adaptive immunity (22, 23). Originating from DC progenitors in the bone marrow, immature DCs circulate in the blood and also reside in tissues that are in contact with the external environment, mainly the skin (where there is the specialized DC type called Langerhans cells) and the inner lining of intestines, stomach, nose and lungs. Immature DCs have high phagocytic capacity. Upon tissue damage or antigen challenge, they capture antigens and migrate to lymphoid organs, during which time they mature and subsequently activate T cells and B cells to initiate antigen-specific immune response (23).

In most tissues, DCs are present in an immature state. Immature DCs have abundant intracellular MHC class II-rich compartments (MIICs). Exogenously acquired protein antigens are typically processed and presented in the context of MHC class II molecules. In addition, it has been found that exogenous antigens can also be processed and presented in the MHC class I pathway via “cross-presentation” (24). Immature DCs are low in or absent of adhesive and co-

stimulatory molecules (e.g. ICAM-1, LFA-3, CD40, CD80, and CD86) and are incapable of T cell priming. In order to differentiate into mature DCs with T cell stimulatory ability, immature DCs require maturation-inducing stimuli, including pathogen-related molecules such as lipopolysaccaride (LPS) (25), apoptotic bodies, and proinflammatory mediators such as tumor necrosis factor α (TNF-α), IL-1 and IL-6 (23). Mature DCs are characterized by abundant expression of molecules involved in T cell binding and co-stimulation, abundant IL-12 production, and high MHC class I and II expression for antigen presentation to either CD8+ CTLs or CD4+ T helper cells (26).

Expression of the costimulatory molecules CD80 and/or CD86 on DCs and their interaction with CD28 on T cells are essential for the effective activation of T cells and IL-2 production (27). Absence of co-stimulatory signals at the time of antigen recognition by the TCR may lead to T cell anergy (28). Alternatively, CTLA-4 is another CD80/CD86 ligand on activated T cells and their interaction leads to regulatory/suppressive effect on T cell function (29, 30).

While DCs are well known for their ability to induce strong immune response, a growing body of evidence has demonstrated that they also have regulatory (suppressive) activity and play an important role in the maintenance of tolerance. It seems that DCs have functional plasticity and keep tailoring their responses according to the environmental signal. With signals that hamper DC maturation, antigen-presenting DCs will be maintained in the immature state and become “tolerogenic”, resulting in poor stimulation of naïve T cells. Moreover, those DCs are able to exert regulatory functions by: (a) inducing antigen-specific T cell anergy or deletion (31); (b) inducing regulatory T cells, including the natural-occurring CD4+CD25+ Tregs (32), IL-10-producing T regulatory-type 1 cells (Tr-1 cells) (33) as well as CD8+ T regulatory cells (34); and

(c) polarizing T cells away from a Th1 or Th17 type response and toward a Th2 type response (35).

Although it was shown that DCs induce protective immunity against tumor challenge and the density of DCs at the tumor site correlates with better prognosis (36, 37), it has also been found that tumor-associated DCs or DCs isolated from cancer patients often display changed morphology and compromised function. It has been well-documented now that, in the tumor microenvironment, the differentiation, maturation, activation, and longevity of DCs are largely suppressed by tumor-derived factors, representing one of the pathophysiological mechanisms by which tumors survive immunological recognition and elimination (38).

1.1.3.2 CD4+ T-helper cells

CD4+ T cells are termed T-helper cells (Th cells) for their capacity to help the activation of CTLs and B cells and to recruit cells of the innate immune system by producing distinct pattern of cytokines and chemokines (39). Th cells usually exert anti-tumor activity indirectly through the activation of CTLs and macrophages, which are able to kill tumor cells or pathogen-infected cells directly. There are two predominant Th cell subtypes, designated as Th1 cells and Th2 cells. Th1 cells are characterized by the production of IFN-γ, IL-2, and TNF-α and the expression of the transcription factor T-bet, which drive cell-mediated immune responses for the eradication of intracellular pathogens. Th1 cells can also enhance the uptake of tumor cells by APCs, and induce apoptosis of tumor cells via release of cytokines that activate the death receptors on the surface of tumor cells (40). Th2 cells are characterized by the secretion of IL-4, IL-5, IL-10 and IL-13 and the expression of the transcription factor GATA-3, which activate B cells and favor humoral or antibody-mediated immune response. Naïve CD4+ T cells activation and their further

proliferation and differentiation into effector and memory T subsets require two signals: (a) TCR recognition of antigen in the context of MHC class II molecules (Signal 1); (b) co-stimulation of CD28 co-receptor on T cells by APC-expressed CD80/CD86 (Signal 2). In addition, the polarizing cytokines (e.g. IL-12 vs. IL-4) present in the extracellular milieu dictates their commitments to either Th1 or Th2 type (Signal 3). Besides Th1 and Th2, CD4+ T cells can also differentiate into another two subsets, regulatory T cells which promote tumor immune tolerance (41) and Th17 cells which have both pro- and anti-tumor effects (42).

1.1.3.3 CD8+ cytotoxic T cells

CD8+ T cells are commonly referred as cytotoxic T cells (CTLs) because of their potent killing ability. CTL activation requires TCR recognition of antigen-peptide presented in MHC class I molecules, co-stimulatory signals and is supported by the pro-inflammatory cytokines (such as IL-12 and IFN-γ) provided by Th cells. Activated effector CTLs can traffic into tumor sites and destroy tumor cells or infected cells upon cell-cell contact by releasing cytotoxins (e.g. perforin and granulysin) and granzymes, or by expressing death receptors (e.g. Fas receptor and TRAIL) which induce target cell apoptosis. Memory CTLs home to the lymphoid tissues and can efficiently respond to tumor antigens cross-presented by APCs and expand rapidly. CTLs are considered the key effector cells involved in eliminating tumor cells *in vivo*. It has been shown that depletion of CD8+ T cells expedite tumor growth and progression (13, 43), and CD8+ T cell infiltration in primary tumor sites or lymph nodes is associated with a better clinical prognosis (44, 45). However, although CD8+ T cells play a major role in cell-mediated anti-tumor response, their optimal priming and memory induction require help from CD4+ T cells and is tightly controlled by the cytokine profile produced by Th cells; thus CTL alone may not be sufficient for effective cancer prevention and rejection (46).

1.1.3.4 Regulatory T cells

Regulatory T cells (Treg cells) play a pivotal role in mediating peripheral tolerance and preventing autoimmune diseases such as type-1 diabetes (47). They are also beneficial for limiting chronic inflammatory diseases, such as inflammatory bowel disease (IBD) and asthma (48, 49). However in cancer, Treg cells play a negative role and actively suppress the anti-tumor immunity. CD4+ T cell subsets with regulatory properties include naturally occurring CD4+CD25+ Treg cells and induced Treg cells (e.g. Tr1 and Th3 cells). In mice the transcription factor forkhead box P3 (FoxP3) is a unique marker for Treg cells.

In murine tumor models, it has been demonstrated that highly suppressive Treg cells were induced within a relatively short time span after tumor challenge following the initial arousal of protective responses (50). Treg cells have been shown to suppress tumor-specific CTL function and abrogate CTL-mediated tumor rejection (51, 52). Selective accumulation of Foxp3+ Treg cells and the predominance of Treg cells in tumor-infiltrating lymphocytes (TILs) were found in late stages of tumor progression, suggesting that the tumor microenviroment promotes the generation of Treg cells (53). Reciprocally, depletion of Treg cells leads to the restoration of antitumor immunity (54, 55). In humans, increased percentage of CD4+CD25+ Treg cells in TILs was found in cancer, and poor prognosis or decreased survival rates were correlated with higher Treg cell frequencies (56).

Treg cells potently suppress T effector cell proliferation and functions, predominantly in a contact-dependent manner (57). The basic suppression mechanisms include: (a) self-production of inhibitory cytokines, such as IL-10, TGF-β and IL-35, or the enticement of effector T cells to produce these cytokines; (b) cytolysis of CD8+ CTL and NK cells via granzyme-dependent and perforin-dependent pathways; (c) metabolic disruption by IL-2 deprivation (via expression of

high affinity CD25, the IL-2 receptor), or adenosine receptor 2A- or cyclic AMP-mediated suppression; (d) modulation of DC maturation and function by lymphocyte-activation gene 3 – MHC class II mediated suppression of DC maturation and CTL4-CD80/CD86 induced production of the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO) by DCs (58).

In induced Treg cell populations, Tr1 cells are IL-10 producing cells that exist abundantly in the intestine and make the host tolerant to food antigens. They do not express high levels of CD25 or Foxp3, require IL-10 for their formation and secrete both IL-10 and TGF-β upon maturation. Th3 cells are also prevalent in the intestine, contribute to the generation of "oral tolerance", and mainly produce TGF-β. In addition, a minority population of CD8+ Treg cells with the ability to target activated T cells and complement the suppression provided by CD4+Foxp3+Tregs has also been characterized (59).

1.1.4 Tumor immune evasion

The immunosurveillance theory hypothesizes that anti-tumor immune responses are elicited *in vivo* and the immune system can be effective in eliminating cancerous cells before they become harmful. However, once tumor cells escape and grow, spontaneous eradication of tumors is rarely observed. It is thus proposed that there is a strong selective pressure favoring tumor strains that are easily neglected by the immune system or tumor cells evolved with diverse immune evasion strategies.

Poor anti-tumor immunity can be a result of insufficient antigenic recognition of tumor cells, due to either sequestration of tumors in tissues that are inaccessible to the immune system (e.g. the central nervous system), loss of surface antigen, or shielding/embedding of TAA in

tumor stroma in their early stages. In addition, down-regulation of the antigen-processing machinery, especially MHC class I molecules on tumor cells, has been observed in a wide variety of tumor types (60, 61). Of note, other than “hiding” in a passive fashion, tumors are also found to be able to actively induce immune tolerance.

Many tumor cells express immunosuppressive factors on their surface, the two most well-identified molecules being Fas ligand (FasL) and Programmed death ligand 1 (PD-L1, also known as B7-H1). Upon direct contact, FasL triggers the apoptosis of activated T and B lymphocytes which express up-regulated levels of Fas receptor. Expression of FasL has been found on a number of cancer cells, including melanoma, breast cancer, ovarian cancer, liver cancer and colon cancer (62). Similarly, activated T cells usually express the PD-L1 receptor PD-1, and PD-L1:PD1 interaction inhibits T cell activation and proliferation (63). Abundant expression of PD-L1 is found on tumor cells and tissues and its expression has been shown to reciprocally correlate with the number of tumor-infiltrating lymphocytes (64).

Moreover, tumor-mediated interference with DC maturation and function represents another pathway by which tumors escape host immunosurveillance via inhibiting the activity of DCs. It has been recognized that tumor cells are capable of creating an immunosuppressive microenvironment by an active process of “tolerization” (65). Such a microenvironment usually lacks the molecules and cytokines promoting DC maturation and functions (e.g. GM-CSF, IL-12, IL-18, IFN-γ and IL-4) and those inducing Th1 type response (e.g. IL-12, IL-18 and IFN-γ), but is abundant in those suppressing DC maturation and function (e.g. TGF-β, IL-10, IL-6, IDO, VEGF and NOS), which are largely produced by tumor cells, stromal cells and tumor-associated macrophages and Treg cells (65). Correspondingly, there are few functionally mature DCs versus abundant immunosuppressive or “tolerogenic” DCs in the tumor microenvironment.

"Tolerogenic" DCs produce reduced levels of type 1 polarizing cytokines (e.g. IL-12 family members) and increased level of immunosuppressive cytokines (e.g. IL-10, TGF-β and VEGF). They may also express high levels of the tryptophan catabolizing enzyme IDO, which degrades free tryptophan and "starves" responder T cells of the essential amino acid, resulting in increased T cell apoptosis (66). In addition, there is also an imbalance of a few angiogenic-inhibitory DCs/myeloid DCs and abundant vascular DCs/plasmacytoid DCs that promote tumor vascularization (65).

Other than inhibiting DC maturation, tumor cells are also able to redirect or re-polarize the differentiation of hematopoietic precursors from the conventional DC lineage to myeloid-derived suppressor cells (MDSCs) or macrophages/monocytes (38). MDSCs are immunosuppressive and support mutagenesis, neoplastic growth, angiogenesis and metastasis in the tumor microenvironment (67). It was also described that upon transition of normal cells/tissues to malignancy the expression of DC chemoattractants is usually lost or down-regulated, thus facilitating the avoidance of tumor cells from DC recruitment and immunological recognition (38).

In cancer patients as well as in mouse models, Treg cells were observed to be specifically recruited and expanded, and to prevent the eradication of immunogenic tumors (68, 69). Elimination of Tregs triggers antitumor responses leading to tumor rejection (54, 70, 71). Interestingly, tumor immune evasion often displays antigen-specificity. In other words, tumor cells are able to induce tolerance specific to their antigens. Such tolerance seems to occur predominantly at the level of T cells, via either unresponsiveness or active suppression. The evidence for antigen-specific tumor immune tolerance is further discussed below.

1.1.5 Antigen-specific unresponsiveness and immune suppression

Deletion and/or anergy of antigen-specific T cells were frequently observed in tumor-bearing hosts. In general, initial activation of tumor antigen-specific T cells is observed, but is usually not sustained. In transgenic mice with T cell receptor (TCR) specific for an immunoglobulin (Ig) antigen, injection of large numbers of plasmacytoma cells secreting that particular Ig leads to deletion of antigen-specific CD4+ T cells in the periphery and in the tumor, as well as generation of unresponsive functional T cells, and such phenomenon became more profound as the tumor burden increases (72, 73). In another mouse model, TCR-transgenic CD4+ T cells specific for influenza hemoglutanin (HA) adoptively transferred into mice bearing HA-expressing lymphoma or HA-expressing renal carcinoma were found to have diminished antigen response and were unable to be primed *in vivo* (74). It has also been mentioned that induction of tolerance to certain tumor antigens requires T cell recognition of tumor antigens cross-presented by APCs, but not by tumor cells themselves (75).

In addition, tumor-specific CD4+Foxp3+ Treg cells were identified in human metastatic melanoma (76), demonstrating the existence of naturally occurring Treg cells with specificity to tumor antigens (76). Similarly, TAA-specific Tregs were found frequently present in the peripheral blood of colorectal cancer patients (77). These observations indicate that tumor-specific Treg cells exist or evolve in tumor-bearing individuals and these Treg cells can respond and suppress anti-tumor response in an antigen-specific way.

Overall, tumorigenesis is a slow process similar to chronic infection (65). The lack of an acute phase causes inefficient activation of both innate and adaptive immunity. Tumor immune evasion is a complicated network involving the functional change of various immune cells in the tumor microenvironment, ultimately leading to the imbalance of insufficient effector cells and

excessive regulatory cells, and the formation of tumor immune tolerance. Multiple mechanisms have been identified to contribute to tumor immune evasion, and antigen-specific immune suppression is usually associated with tumor growth. In the recent decade, a population of small vesicular membrane organelles released by tumor cells, known as “exosomes”, has been intensively studied for their role in tumor immune regulation. These subcellular mediators seem to help convey and probably amplify the immunoregulatory effect of tumor cells.

1.2 EXOSOMES

1.2.1 Exosome history

The term “exosomes” was first proposed in 1981 to describe exfoliated membrane vesicles, comprising a mixture of large vesicles with an average diameter of 500 to 1000 nm and a second population of vesicles about 40 nm in diameter with 5’-nucleotidase activity shed by various normal and neoplastic cell lines (78). In 1985, Jonestone *et al* observed externalization of the transferrin receptor in vesicular form in sheep reticulocytes (79), and later reported microvesicle release of endocytic origin of around 50 nm in diameter, which they called “exosomes” (80, 81). By that time they suggested exosome secretion as a mechanism to discard unnecessary membrane proteins during the maturation of reticulocytes. It was not until 1996, when Raposo *et al* adopted the term to describe the secretion of internal MHC class II-containing vesicles from the late endocytic MIIC compartment by B lymphoblastoid cells and reported their T cell stimulatory ability (82), that these particular membrane structures began to be widely studied. Since then, many cell types, especially cells in the immune system and tumor cells, have been

found to release “exosome-like” vesicles and different functionalities of these vesicles have been identified. It is now commonly accepted that exosome release represents an alternative way of intercellular communication.

1.2.2 Exosome definition

Different types of membrane vesicles are secreted by cells (83). They are either formed at the surface of a blebbing plasma membrane, or are formed inside of the internal compartments. Thus a clear definition of “exosome” is necessary for their biological study. Based on the morphological and physical characteristics, there are three major criteria to define a membrane vesicle as an exosomes: a size of 30-100 nm in diameter, a density of 1.13-1.19g/ml in a sucrose gradient and an endocytic origin (83, 84). They typically show “cup-shaped” or “saucer-like” morphology under electron microscope, and are limited by a lipid bi-layer. The main protein markers of exosomes include tetraspanins and proteins in the MVB pathway (e.g. Alix and Tsg101) (83).

1.2.3 Exosome biogenesis, cellular origin and modulation of secretion

Exosomes contain cytosolic proteins and expose the extracellular domains of transmembrane proteins. Many of the cytosolic proteins identified in exosomes have been found in the endocytic pathway (e.g. annexin II, Rab5/Rab7 and Tsg101) (84, 85). These facts support an endosomal origin of exosomes and an “inward budding” mode of exosome formation. Indeed, enrichment of exosomes in the MIICs and their release upon fusion of MIIC with the plasma membrane was observed by electron microscopy in B lymphocytes (82). It is now believed that exosomes are

formed by “inward/reverse budding” of the limiting membrane of the multivesicular bodies (MVBs) in the late endocytic compartment and are released upon fusion of MVB with the plasma membrane (86, 87).

In contrast to apoptotic bodies, exosome secretion is a naturally occurring process of most live cells. A wide variety of cell types have been shown to release exosomes, especially cells in the hematopoietic system, including reticulocytes (79, 81, 88, 89), DCs (90), B and T lymphocytes (82, 91-93), platelets (94), mast cells (95, 96), and macrophages (97). In addition, most tumor cell lines actively produce exosomes both *in vitro* and *in vivo*. Exosomes are also secreted by epithelial cells (98), fibroblasts (99), astrocytes and neurons (100).

Exosome secretion can be modulated in different cell types by either ligand cognition or stress conditions. For example, exosome secretion by reticulocytes (79, 81, 88, 89), resting B cells (101-104), T cells (92, 93) and mastocytes (96) is trigged only upon maturation or activation of certain cell surface receptor. Tumor cell lines and DCs constitutively secrete exosomes *in vitro*, whereas radiation or senescence signals are able to increase the level of secretion (99). It is reported that exosome secretion is up-regulated upon p53 activation and the subsequent increased expression of the transmembrane protein tumor suppressor-activated pathway 6 (TsAP6) (105). In addition, it has been reported that diacylglycerol kinase α regulates the secretion of exosomes by T lymphocytes (106) and brefeldin A-inhibited guanine-nucleotide exchange protein 2 (BIG2) regulates the exosomal release of the type 1 tumor necrosis factor receptor (TNFR1) from human vascular endothelial cells (107). Exosome secretion by DCs is also affected by their maturation status, as immature DCs secrete more exosomes than mature DCs (108-110).

1.2.4 Exosome molecular composition

1.2.4.1 Protein composition

The protein composition of exosomes is largely dependent on their cellular origin while typically enriched in certain molecules. Proteins commonly found on most exosomes include targeting/adhesion molecules (e.g. tetraspanins, lactadherin and intergrins), membrane trafficking molecules (e.g. annexins and Rab proteins), cytoskeleton molecules (e.g. actin and tubulin), proteins involved in MVB formation (e.g. Alix, Tsg101 and clathrin), chaperones (e.g. Hsp70 and Hsp90), as well as signal transduction proteins (e.g. protein kinases, 14-3-3, and heterotrimeric G proteins) and cytoplasmic enzymes (e.g. GAPDH, peroxidases, and pyruvate kinases) (83, 84, 111). Exosomes secreted by APCs (DC and B cells) are also enriched in antigen-presenting molecules including MHC class I and class II complexes and costimulatory molecules (112). Many tumor-derived exosomes contain tumor antigens (113). Proteomic analysis reveals that the protein composition of exosomes is distinct from microvesicles produced by apoptotic cells (114).

1.2.4.2 Lipid composition

Although the conserved lipid composition of exosomes is not yet clear, the presence of various lipids have been reported in different exosomes. B cell-derived exosomes contain lyso-bis-phosphatidic acid (LBPA), which is enriched in the late endocytic compartments. DC-derived and platelet-derived exosomes possess low levels of phosphatidylserine (PS), which is normally present at the cytosolic side of the plasma membrane, on their surface. Exosomes derived from EBV-transformed B cells are enriched in cholesterol, similar to the “lipid rafts” present on the plasma membrane (84). Exosomes derived from DCs and mast cells are also enriched in

sphingomyelin, lysophosphatidylcholine (LPC) and saturated fatty acid, while relatively poor in phosphatidylcholine and diacylglycerol (115).

1.2.4.3 mRNA and microRNA

Recently, functional RNA molecules, including mRNA and microRNA, were identified on exosomes derived from cells under normal and pathological conditions. In normal cells, it has been reported that exosomes derived from mouse and human mast cell lines, as well as primary bone marrow-derived mouse mast cells, contain RNA. Moreover, mouse exosomes are able to transfer exosomal RNA to human mast cells and new mouse proteins were translated in the recipient cells, indicating genetic material exchange as a new level of exosome-mediated communication between cells (116). In malignant cells, it was reported that glioblastoma microvesicles transport RNA and proteins that promote tumor growth (117). In addition, the microRNA profiles of circulating exosomes in cancer patients were found significantly distinct from that observed in benign diseases, highlighting their potential use as diagnostic biomarkers at both protein and microRNA levels (118). These functional RNA populations in exosomes are called “exosomal shuttle RNA” (116).

Collectively, the basic molecular composition of an exosome is shown in Figure 1.

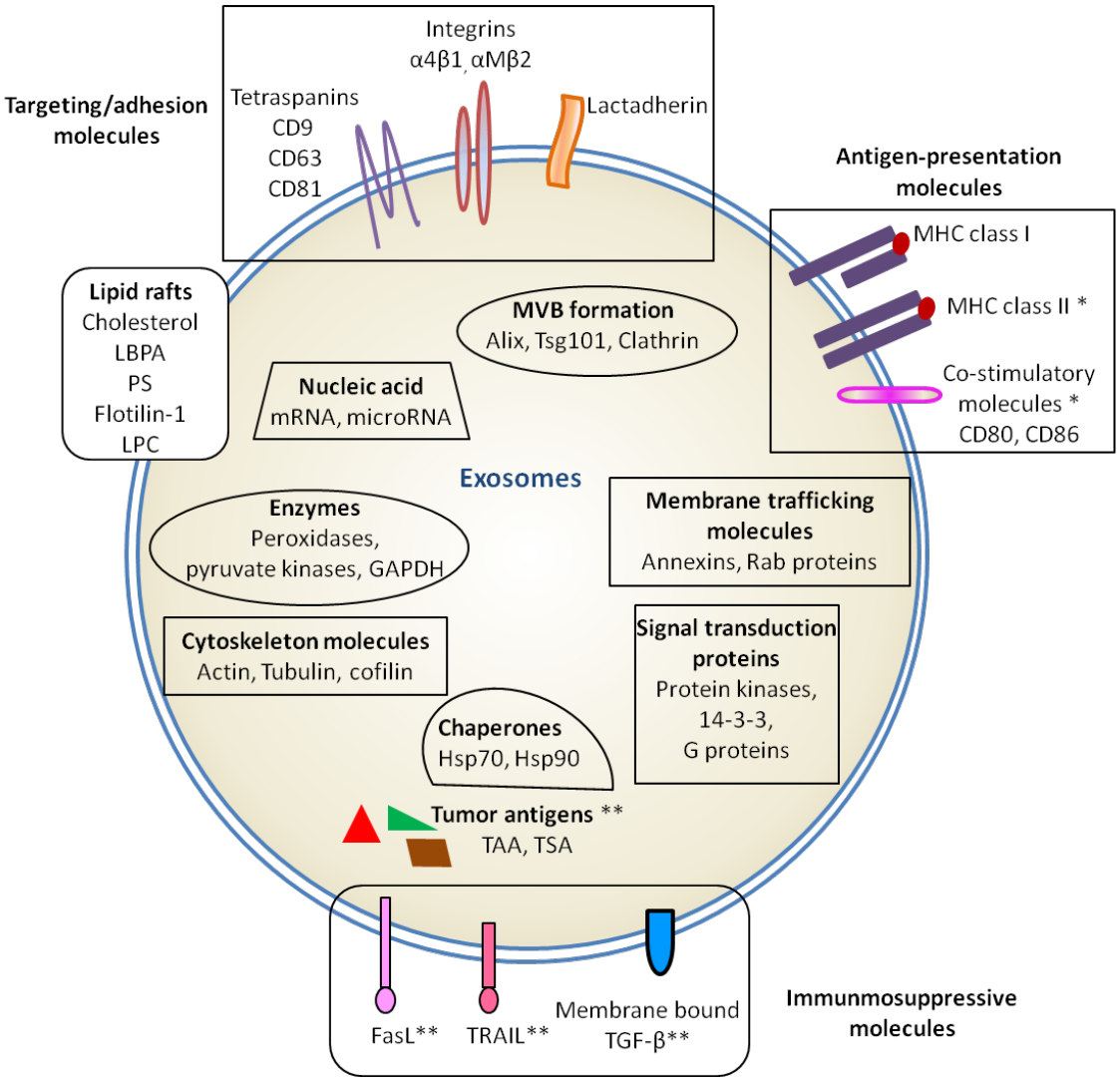


Figure 1: Schematic image of the molecular composition of a typical exosome.

*: MHC class II molecules and co-stimulatory molecules are presented on APC-derived exosomes. **: Tumor-derived exosomes usually contain tumor antigens. Certain immunosuppressive molecules (e.g. FasL, TRAIL and TGF-β) have also been identified on exosomes derived from specific tumor cells. Figure created according to (83, 84, 114).

1.2.4.4 Lactadherin and exosome display technology

Lactadherin (also called milk fat globule-EGF factor 8 protein, MFG-E8) is a major protein component identified on exosomes derived from immature DCs and tumor cells (108). Lactadherin is a secreted protein with a lipid-binding domain called C1C2 domain, which has been found to mediate the secretion of Lactadherin in association with exosomes. Its N-terminal secretion signal is needed to direct Lactadherin to the exosomal compartment. Lactadherin also contains a PS-binding domain that binds to integrins, and mediates the phagocytosis of apoptotic bodies by macrophages.

Delcayre *et al* developed the "Exosome Display Technology" based on the identification of the addressing domains required for targeting specific proteins to the exosome surface (119). By inserting the coding sequence of the target protein between the leader sequence (LS) and C1C2 domains of Lactadherin, they successfully generate chimeric proteins that bind to exosome surface with biological activities (Fig. 2). Expression of the full length HIV protein Nef and IL-2 cytokine was both demonstrated. This technology enables the anchorage and display of native full-length protein on exosome surface, with multiple potential uses such as biomarker detection, targeted delivery of vesicles with ligands or antibodies to specific tissues and exosome targeted DNA vaccines (119).

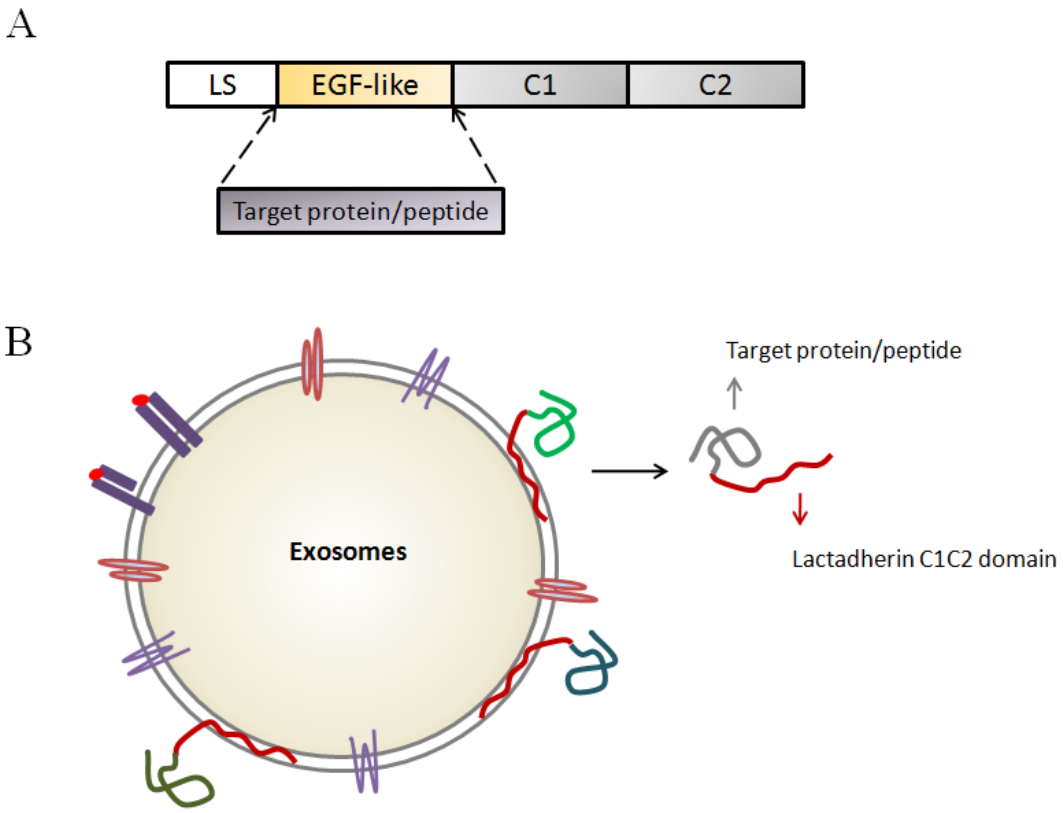


Figure 2: Schematic diagram of exosome display strategy using the C1C2 domain of Lactadherin.

(A). Constructs of chimeric proteins with the coding sequence of target protein/peptide inserted between the leader sequence (LS) and the C1C2 domain of Lactadherin. (B). Lactadherin C1C2 domain binds strongly to the lipid membrane of exosomes and targets fused protein/peptide to the outer surface of exosomes. Cells expressing the chimeric proteins produce exosomes that display the target protein/peptide. Figure created according to (119).

1.2.5 Exosome purification

The current exosome purification protocol commonly relies on differential ultracentrifugation. To isolate exosomes from cell conditioned media, culture supernatants are usually subjected to several rounds of centrifugation with increasing speed up to a maximum of 10,000 × g to remove cells and cellular debris. The exosome fraction can be pelleted down by ultracentrifugation at 100,000 × g, usually followed by a PBS wash step and another round of ultracentrifugation to remove contaminating proteins. Alternatively, a single filtration step using a 0.22 μm filter can also eliminate cells and large cellular debris while retaining small membrane vesicles for further purification (120). Combining the differential centrifugation and filtration steps will help reduce the contamination of exosome preparations with larger vesicles that are shed from the plasma membrane. Of note, since exosomes are abundantly present in serum, the fetal bovine serum (FBS) used in culture media needs to be pre-cleared by ultracentrifugation, or replaced with necessary supplement/bovine serum albumin (BSA) to exclude pre-existing serum vesicles (98). Purification of exosomes from body fluids (e.g. plasma, serum, tumor ascites, urine, broncho-alveolar lavage) shares the same principle as when starting from culture media, while a dilution step may be necessary to reduce the viscosity of certain fluids (120). For some applications, an extra purification step of ultracentrifugation on D_2O/sucrose density cushion or sucrose gradient can be added to achieve higher purity of exosome preparations (120, 121). In addition, another exosome isolation method using magnetic beads coated with antibodies against proteins exposed on exosome membranes has been reported (122, 123). Exosomes are relatively stable at 4°C and can be long-term stored at -80°C.

1.2.6 Interaction of exosomes with immune cells

It is now believed that exosomes / membrane microvesicle are important mediators of cell-to-cell communication (124). However, how these vesicles interact with the cells in the immune system is still quite an enigma. Capture of exosome by DCs has been observed and co-incubation of exosomes and DCs with blocking antibodies specific for various integrins, adhesion molecules or tetraspanins reduced exosome capture by DCs (125). Direct interaction between DC-derived exosomes and T cells through lymphocyte function-associated antigen 1 (LFA-1) / intercellular adhesion molecule 1 (ICAM-1) has been reported (126). In a recent review by Thery *et al*, several types of interactions were proposed based on indirect evidence and *in vitro* studies, including binding/adhesion of vesicles to the surface of a recipient cell through exosomal adhesion molecules (e.g. integrins, ICAM-1, lactadherin), or phosphatidylserine/lysophosphatidylcholine and cellular receptors (e.g. LFA1, T cell immunoglobulin domain and mucin domain protein 1 (TIM1), TIM4); direct fusion of vesicles with recipient plasma membrane after adhesion; or internalization of vesicles into endocytic compartments through receptor-mediated endocytosis or phagocytosis (83). It was also suggested that the exosome membranes have symmetrical phatidylethanolamine (PE) repartitions, which may facilitate their absorption, but not fusion with target cells such as DCs (127).

1.2.7 Tumor-derived exosomes and immune modulation

1.2.7.1 Immunogenic properties of tumor-derived exosomes and tumor exosome-based cancer vaccines

We have mentioned that the protein composition of exosomes largely reflects that of their parental cells, which means that cell-type specific differences exist in exosomal protein expression. In tumor-derived exosomes, one of the most significant specificities is the expression of tumor antigens. Striking enrichment of tumor antigens was often found in tumor-derived exosomes when compared with whole cell lysates (113), such as melan-A (128), Silv (129), carcinoembryonic antigen (130) and mesothelin (131). The initial observation that most tumor cells sort tumor-specific antigens into exosomes for secretion has led to the investigation of exosome-based cancer vaccines. Pioneer studies using tumor-derived exosomes as a source of tumor rejection antigens to pulse DCs showed that exosomes could transfer tumor antigens to DCs and that those DCs were able to induce CD8+ T cell-dependent anti-tumor effects in mice (129). In a similar human *ex vivo* model system, DCs pulsed with exosomes derived from malignant effusions expressing tumor antigens cross-presented the antigens to antigen-specific CTLs (128). Direct application of tumor-derived exosomes for the enhancement of anti-tumor immunity was also investigated. It has been reported that tumor-derived exosomes can induce specific anti-tumor response when the parental tumor cells were genetically modified to express the pro-inflammatory factor IL-18, IL-12 or IL-2 (132-134), or when the parental tumor cells, which have an APC origin, were heat-shocked to increase the expression of MHC molecules and co-stimulatory molecules on exosomes (135). These promising results promoted several phase I clinical trials using tumor-derived exosomes or exosome pulsed DCs as cancer vaccines (136, 137), with efficacies yet to be determined. It can be noted, however, that effective anti-tumor

immune responses were mostly achieved when tumor-derived exosomes were loaded onto matured APCs or were modified to express pro-inflammatory factors or stress proteins (129, 132-135), all of which favor the development of an active immune response. In addition, surface targeting of antigens to vesicle membranes also seems to enhance the immunogenicity of tumor-derived exosomes, as surface anchorage of the superantigen SEA (138) or membrane targeting of chicken egg ovalbumin (OVA) fused with Lactadherin (139) resulted in enhanced CTL activity and delayed tumor growth.

It is still unclear, however, whether tumor-derived exosomes naturally stimulate efficient anti-tumor immune responses *in vivo.* Paradoxically, literature is indeed flourishing with examples proving the role of tumor-derived exosomes in immune suppression in the past few years.

1.2.7.2 Immunosuppressive and pro-tumorigenic properties of tumor-derived exosomes

Studies in the 1980s showing that membrane vesicles shed from murine melanoma cell lines inhibited the expression of the immune response region-associated (Ia) antigen by macrophages provided early evidence suggesting that tumor-derived membrane vesicles is a possible mechanism whereby tumor-bearing hosts become immunocompromised (140). In the past few years, the immunoregulatory functions of tumor-derived exosomes have been intensively studied and diverse suppressive effects affecting various components in the immune system have been identified.

First, tumor-derived exosomes can directly suppress the activity of effector cells. Certain tumor cell lines were reported to produce exosomes expressing FasL or TRAIL, both of which trigger the apoptotic death of activated T cells (141, 142). Also, Epstein-Barr Virus (EBV)-associated nasopharyngeal carcinoma (NPC) was shown to release exosomes containing high

amounts of galectin-9, which is able to induce apoptosis of mature Th1 lymphocytes when interacting with the membrane receptor Tim-3. These exosomes protect galectin-9 from being proteolytically cleaved and induce massive apoptosis of EBV-specific CD4+ cells (143). In addition, ovarian tumor-derived exosomes were found to down modulate CD3-ζ chain expression and impair TCR signaling (144), suggesting that tumor-derived exosomes can also down-regulate T cell function besides direct killing. Moreover, NKG2D-dependent cytotoxicity of NK cells and CD8+ T cells was inhibited by NKG2D ligands-expressing exosomes derived from human breast cancer and mesothelioma cell lines (145, 146). Similarly, murine mammary carcinoma exosomes were shown to promote tumor growth in vivo by suppressing NK cell function (147). These reports revealed the capacity of tumor-derived exosomes to negatively regulate the effector arm of the immune system.

Evidence is also available that suggests exosomes can target cells in the antigen presentation arm. It has been shown that the differentiation of murine BMDC was inhibited in the presence of tumor-derived exosomes, largely due to the induction of IL-6 in these precursor cells (148). Furthermore, human tumor exosomes were found to skew monocyte differentiation into DCs toward the generation of MDSCs and exert TGF-β mediated suppressive activity on T cells *in vitro* (149). Interestingly, significant expansion of CD14+HLA-DR-/low and TGF β-secreting cells were also found in the peripheral blood of melanoma patients with advanced disease, and high levels of these cells is usually associated with poor response to tumor vaccines (149).

Moreover, tumor-derived exosomes seem to support the function of cells in the regulatory arm. A representative finding is that human tumor-derived exosomes selectively impaired IL-2 response to cytotoxic effector cells while supporting Treg cell activities through a

TGF-β-dependent mechanism (131). Recently it was reported that tumor-derived microvesicles induce, expand and up-regulate suppressor functions of human Treg cells as well as enhance their resistance to apoptosis via a TGF-β- and IL-10- dependent mechanism (150).

In addition to attenuating different branches of the anti-tumor immunity, the role of tumor-derived exosomes in stimulating angiogenesis was also implicated (151). Proteomic analysis of mesothelioma cell-derived exosomes detected the presence of developmental endothelial locus-1 (DEL-1), which is a strong angiogenic factor that can increase the vascular development in the neighborhood of the tumor (152). By dynamic light scattering and fluorescent exosome labeling, melanoma-derived exosomes were observed to stimulate paracrine endothelial signaling, which could contribute to endothelial angiogenesis and tumor metastasis (153). All these evidence supports a pro-tumorigenic role of tumor-derived exosomes via pleiotropic mechanisms (Fig. 3). It is still unclear, however, whether exosomes from a given tumor will have the sufficient complexity to possess multiple suppressive functions (154). It is likely that the predominant regulatory role(s) of exosomes depends on their molecular phenotypes and may exhibit cell specificity.

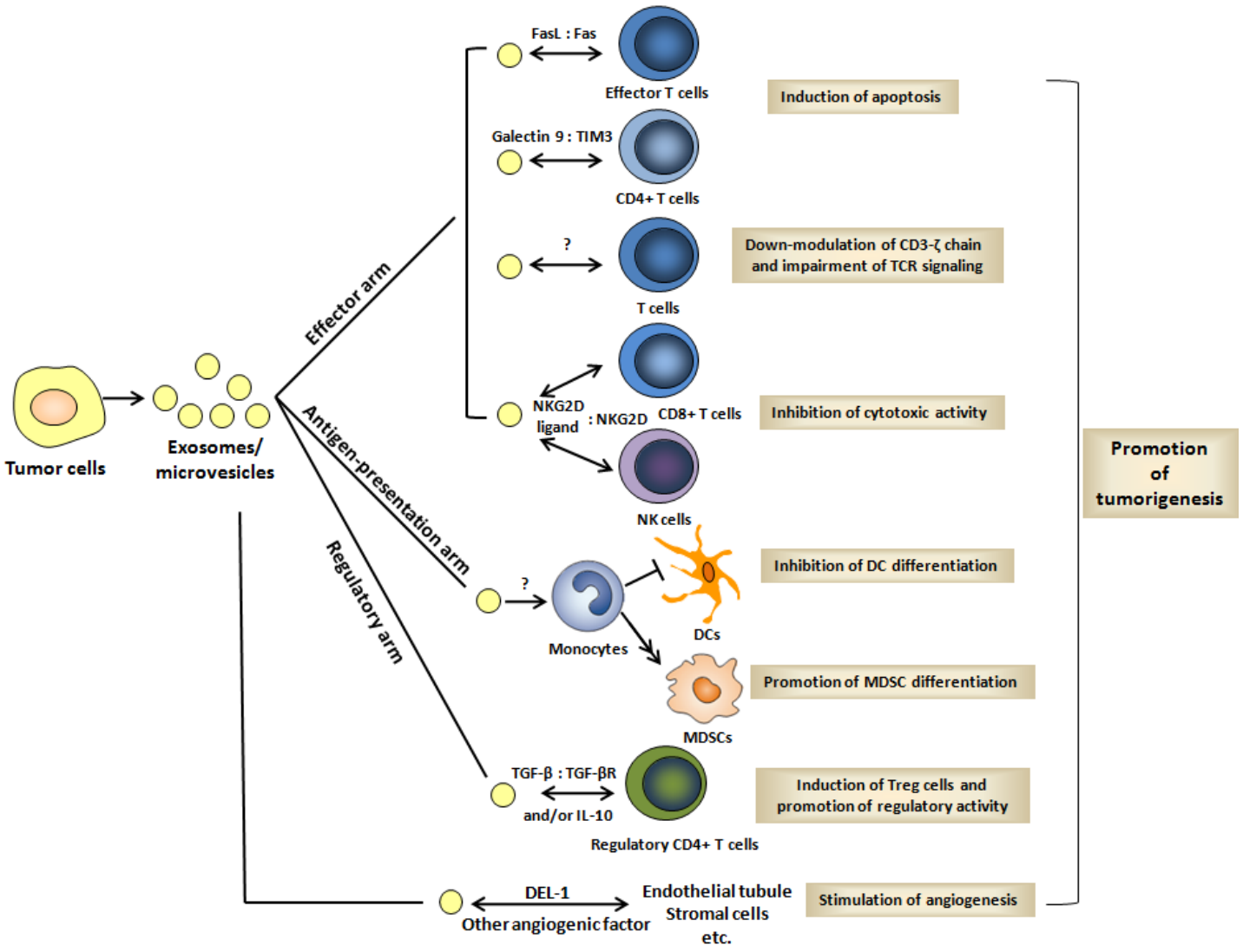


Figure 3: The pro-tumorigenic role of tumor-derived exosomes.

Tumor-derived exosomes have been shown to exhibit diverse immunosuppressive properties: attenuate the function of effector cells; skew APC differentiation for the acquisition of a suppressive phenotype; and promote the activity of regulatory T cells. Tumor-derived exosomes were also found to stimulate angiogenesis which supports tumor growth. Figure created according to (83).

1.2.8 Tolerogenic/immunosuppressive exosomes of other cellular origin

Other than tumor-derived exosomes, exosomes derived from immune cells, epithelial cells and specific tissues also have immunosuppressive properties. For DC-derived exosomes, the maturation state of DCs largely determines their immunogenicity. Therefore, while exosomes derived from mature DCs can prime and activate T cells (usually after capture by recipient DCs) (110, 155), exosomes derived from immature DCs tend to induce immune tolerance. For example, MHC alloantigen expressed on immature DC-derived exosomes induces donor-specific T cell tolerance and prolong allograft survival (156). In addition, BMDCs treated with IL-10, or genetically modified to express FasL or IL-4 produce anti-inflammatory exosomes which are able to suppress murine delayed-type hypersensitivity (DTH) and collagen-induced arthritis (CIA) (157, 158). Similar to some tumor-derived exosomes, activated T cells secrete FasL-bearing exosomes, which induce apoptosis of bystander T cells for activation-induced cell death (AICD) (159). Intestinal epithelial cells were found to produce exosome-like and MHC class II-bearing “tolerosomes”, which, after *in vitro* antigen pulse, are capable of inducing antigen-specific tolerance in naïve recipient animals and are thus believed to participate in the development of oral tolerance (160). Recently it was found that human placenta secretes NKG2D ligands via exosomes that down-regulate the cognate receptor expression (161), suggesting that placenta-derived exosomes may contribute to the prevention of fetal rejection.

1.2.9 Tolerogenic/immunosuppressive exosomes derived from body fluids

Besides *in vitro* cell culture, exosome-like vesicles can also be isolated from different body fluids, such as plasma (162), serum (118, 163), bronchoalveolar fluid (BALF) (164, 165), urine

(166), ascites (136), malignant effusions (128, 167), epididymal fluid (168), amniotic fluid (169) and human breast milk (170). Interestingly, most of these vesicles have been shown to be tolerance-inducing. Especially, exosomes isolated from blood plasma or serum of animals exposed to or immunized with particular antigens exhibit potent antigen-specific suppressive effect. For example, plasma-derived exosomes isolated from mice immunized with keyhole limpet hemocyannin (KLH) were able to suppress KLH-specific DTH response when administrated locally (171); and "tolerosomes" isolated from serum shortly after OVA antigen feeding induce OVA-specific tolerance in naïve recipient animals (172). Interestingly, in these two different experimental settings, exosome-mediated antigen-specific immunosuppressions are both dependent on the expression of MHC class II molecules, either on exosomes or on recipient cells. In addition, exosomes isolated from maternal peripheral circulation were reported to have placenta markers and were able to induce T cell signaling defects by suppressing T cell expression of CD3ζ and JAK3, potentially attenuating any immune response against the fetus (173). Notably, it has been described that tumor-derived exosomes present in the serum of patients with tumors and the amount increases as the disease progress (118). Moreover, exosomes isolated from sera and malignant ascites of ovarian cancer patients were reported to have tumor-growth promoting effect (174). It is important to note, however, that exosomes isolated from blood circulation have heterogeneous cellular origin, and while enrichment of vesicles secreted by certain cell types may occur, the composition of vesicle populations remains vague and is potentially dynamic. Therefore further investigation is needed to determine the functional portions under different scenarios.

Immunosuppressive exosomes obtained from other body fluids include those isolated from BALF of mice respiratory exposed to olive pollen allergen, which can prevent antigen-

specific allergic reaction (165); and those isolated from human breast milk and colostrum, which can increase the number of Treg cells and inhibit T cell activation *in vitro* (166).

1.2.10 Exosomes derived from pathogen infected cells

The MVB pathway for exosome formation and secretion is also shared by the endocytic protein processing machinery. In APCs, the MVB compartments appear to be MIICs in which exogenously acquired antigens are being processed. Therefore when these cells are infected with pathogens, pathogenic components can be incorporated into exosomes for secretion during the invagination of the limiting membrane, and, in many cases, these components seem to endow exosomes with special immunogenic properties. It has been reported that bacterially infected macrophage secreted exosomes carrying bacterial coat components such as glycopeptidolipids (GPLs) which bind to the pathogen-associated molecular pattern (PAMP) receptor, stimulating bystander macrophages and neutrophils to secrete pro-inflammatory mediators, including TNF-α, RANTES (also known as CCL5), and inducible nitric oxide synthase (iNOS) (97, 175, 176).

In addition, it was suggested that some viruses such as retrovirus (e.g. HIV) exploit the machinery that generates the internal membranes of MVB to coordinate their own assembly and release. Therefore, these viruses and host released exosomes may have biochemical similarities, and it was proposed that these retroviruses can be regarded as “Trojan exosomes”. Nevertheless, it seems to be more suitable to consider enveloped viruses budding from the internal compartments as another type of secreted membrane vesicles rather than exosomes discussed in this thesis (177-179). In fact, a recent report just showed that HIV-1 budding from CD4+ T lymphocytes is independent of exosomes (180).

Notably, it was found that exosome fractions prepared from long-term cultured DCs infected with mycoplasma induce polyclonal B cell proliferation, although whether exosomes with incorporated mycoplasma components or mycoplasmas co-purified with exosomes are responsible for the effect is not determined (181). Mycoplasmas are the smallest bacteria without a cell wall and live as parasites because of their inability to biosynthesize. They cause different diseases and may also act as opportunistic pathogens that colonize a host with a weak immune system. Laboratory maintained cell cultures can also be easily infected with mycoplasmas. Therefore, this finding on one hand raises the caution for exosome investigators in discriminating mycoplasma-related immune response from intrinsic immunomodulatory effects of exosomes, while on the other hand reveals the possibility that exosomes released by mycoplasma-infected cells can be endowed with mycoplasma-related immunogenicity and exert altered functions.

1.3 DELAYED-TYPE HYPERSENSITIVITY

Hypersensitivity refers to excessive reactions produced by a normal immune system with undesirable outcomes (discomfort, tissue damage or sometimes fatality). Hypersensitivity reactions require a pre-sensitized state of the host (182). Based on the mechanisms involved and time taken for the reaction, hypersensitivity reactions can be divided into four types: type I, type II, type III and type IV. A comparative summary of different types of hypersensitivity is presented in Table 1 below.

Table 1: Coombs and Gell classification of hypersensitivity reactions.

	Alternative names	Antigen	Mediator	Effector cells	Response time	Examples
Type I	Immediate hypersensitivity	Exogenous (allergens)	IgE	Mast cell, basophils	15-30 minutes	Allergic asthma, allergic rhinitis
Type II	Cytotoxic hypersensitivity	Endogenous, or exogenous chemicals (haptens) attached to cell surface	IgG, IgM and complement	phagocytes, killer cells	Minutes-hours	Drug-induced hemolytic anemia, erythroblastosis fetails
Type III	Immune complex hypersensitivity	Soluble , not attached, exogenous or endogenous	IgG, IgM and complement	Platelets, neutrophils	3-10 hours	Arthus reaction, systemic lupus erythematosus
Type IV	Cell-mediated or delayed-type hypersensitivity	Soluble proteins, haptens, heavy metals tissues & organs	Th1 cells, CD8+ T cells	T cells, macrophages, monocytes	24-72 hours (granuloma: 21-28 days)	Tuberculin test, contact dermatitis, granuloma

* Table created according to (182, 183).

Type I, II, III hypersensitivities are antibody-mediated immune responses. Type IV hypersensitivity (also called delayed-type hypersensitivity, DTH) reactions are antibody-independent responses mediated by antigen-specific effector T cells. Depending on the type of antigen and the effector T cells mediating the response, type IV hypersensitivity can be further divided into the classic DTH reaction initiated by Th1 CD4+ T cells (e.g. tuberculin skin test) and direct cell cytotoxicity mediated by CD8+ T cells (e.g. poison ivy). DTH reaction includes two phases: sensitization and elicitation. During the sensitization phase, cutaneous APCs (primarily Langerhans cells) take up and process antigens, and activate T cells in the regional lymph nodes. Memory T cells subsequently develop and reside in the dermis. In the elicitation

phase of a classic DTH reaction, protein antigens injected into the subcutaneous tissue are processed by Langerhans cells and presented on MHC class II molecules. Antigen-specific Th1 cells recognize the antigen at the site of injection and release inflammatory cytokines (e.g. IFN-γ, TNF-α, TNF-β, IL-1, IL-6, IL-3 and GM-CSF) and chemokines (e.g. CXCL8, CXCL11, CXCL10 and CXCL9), which recruit other effector cells (mainly monocytes and macrophages, also NK cells) and more T cells to the site of antigen administration for antigen elimination. These inflammatory mediators stimulate the expression of adhesion molecules on endothelium and increase local blood vessel permeability for the extravasation of plasma and cells, resulting in visible swelling. Fully developed response usually appears 24-48 hours after challenge, and the magnitude of DTH response is typically measured as the extent of swelling at the site of challenge (183-185).

DTH response can be regulated by multiple approaches at both sensitization and elicitation phase. Promoted by Th1 cells and Th1-type cytokines, DTH response is down-regulated by Th2 cells and Th2-type cytokines. Treatment with neutralizing antibody to either IFN-γ or IL-4 by at the time of immunization resulted in the significant suppression or striking increase of DTH responses, respectively (186). Adoptive transfer of antigen-specific Th2 cells before immunization provided an immunological environment rich in IL-4 and led to inhibition of DTH response, which could be restored by IL-4 neutralization just before cell transfer (187). The effect of IL-4 in modulating DTH response is also implicated in the difference of immune response between genders (188). In addition to IL-4, virally delivered IL-10 was effective in suppressing DTH response in both sensitization stage (injected 24 hr before immunization) and effector stage (24 hr before challenge) when injected locally (189). Similarly, IL-10-secreting Peyper's patch cells from antigen-fed mice were shown to suppress antigen-specific DTH

response induced in footpads when injected i.v. before antigen challenge (190). Furthermore, the regulatory cytokine TGF-β1 has been shown to inhibit murine DTH, and it can significantly inhibit the elicitation of DTH when injected up to 8 hr after antigen challenge (191).

T regulatory cells have been reported to mediate DTH suppression. Human CD4+CD25low adaptive T regulatory cells mediated the suppression of donor-specific DTH response in tolerant organ transplant recipients. These T regulatory cells were CD4+ TGF-β1+ and appeared functionally and phenotypically distinct from the CD4+CD25+FoxP3+ natural Treg cells (192). Moreover, suppression of DTH by antigen-specific CD8+ regulatory T cells has also been reported. These CD8+ T regulatory cells were not canonical cytotoxic T cells and expression of FasL was dispensable for the effect, whereas IFN-γ receptor stimulation was required for their suppressor function (193). Such evidence suggests that antigen-specific DTH response can be limited by Th2 polarization, regulatory cytokines as well as the activation of T regulatory cell populations.

1.4 B CELLS AND B CELL-DERIVED IL-10

B cells are the major players in humoral immunity. The principal function of B cells is to produce antibodies, which bind to specific antigenic targets, neutralizing their function or facilitating their destruction by other immune cells. B cells also perform the role of APCs. With regard to tumor immunity, activated B cells can secret neutralizing antibodies that would aid the phagocytosis of tumor cells by APCs or the recognition of tumor cells by killer cells for destruction, although in comparison to T cells their role in anti-tumor immunity is less important.

IL-10 is a key cytokine that inhibits cell-mediated immunity while promoting humoral responses. It plays an important role in restraining the intensity of inflammatory responses. IL-10 is mainly produced by monocytes, Th2 cells, T regulatory cells, as well as mast cells. In fact, emerging reports suggest an underappreciated cellular source of IL-10, B cells. It was found that EBV infection induces purified tonsil B lymphocytes to produce high levels of IL-10, which acts as an autocrine growth factor for EBV infected B lymphocytes (194). It was also revealed that B cells regulate experimental autoimmune encephalomyelitis (EAE) in mice through provision of IL-10 and can be essential to restrain unwanted autoaggressive T cell responses (195). B cells were recently reported to be the predominant IL-10-producing cells in peripheral lymphoid tissues at baseline and during diverse immunological challenge *in vivo,* and, in a murine model of cytomegalovirus infection, B cell-derived IL-10 nonredundantly decreased virus-specific CD8+ T cell responses and plasma cell expansion. The dominance of B cells in IL-10 production is mostly in lymphoid tissue, while myeloid cells were dominant in blood and liver (196). A hypothetical concept was proposed that Toll-like receptor (TLR) stimulation controls the production of IL-10 and the subsequent immune suppression mediated by B cells, and thus B cells are able to translate exposure of certain microbial infections into protection from chronic inflammatory disease (197). It has been further proposed that depending on the stimulatory signals B cells receive, IL-10 can be produced by both naive and memory B cells. While naïve B cell IL-10 production functions primarily in the maintenance of immune homeostasis and prevention of autoimmune diseases, memory B cell IL-10 production functions primarily to down-regulate excessive pathogenic immune responses and resolve active disease exacerbation (198). Therefore, B cell-derived IL-10 seems to be a mechanism evolved for the limitation of

overly aggressive immune responses in both pathogen-specific responses and autoimmune diseases.

SCOPE OF THIS THESIS

Since exosomes became the object of increasing interest from immunologists in the late 90s, the research on tumor-derived exosomes basically falls into two directions: explore their potential use as a new source of tumor rejection antigens for cancer vaccines, or investigate their role in tumor immune evasion. While pioneer studies revealed promising efficacy in stimulating anti-tumor responses by utilizing tumor-derived exosomes that express tumor antigens, mounting reports are showing paradoxical-looking results of the multiple mechanisms by which tumor-derived exosomes suppress anti-tumor responses.

We started our study based on the hypothesis that tumor cells release exosomes not to enhance the immune response against them, but as a strategy to instruct the host immune system to accommodate their survival and growth. We also hypothesize that tumor antigens present in the form of exosomes will help the induction of antigen-specific immunosuppression/tolerance. Our lab has previously demonstrated the anti-inflammatory and immunosuppressive effects of exosomes derived from DCs genetically modified to express IL-10, IL-4 or FasL using murine footpad DTH or CIA models (157, 158, 171). DTH response represents a type of Th1-dominant cell-mediated response which can be used to assess antigen-specific immune suppression. Therefore, we tested the ability of exosomes derived from OVA-expressing tumor cells to down-regulate the OVA-specific DTH response. We observed that these exosomes were able to confer immune suppression on the OVA-specific DTH response, an effect possibly mediated by

exosome-conditioned DCs based on the results of experiments investigating exosome *in vivo* trafficking and their effect on cultured DCs. These findings support a role of exosomal tumor antigen in inducing antigen-specific suppression, which has not been reported in the large number of previous studies examining exosome-mediated immune suppression. We believe exosome-mediated antigen-specific immune suppression represents a novel mechanism of tumor immune evasion. This study also highlights the possibility of utilizing tumor-derived exosomes containing certain antigen to suppress antigen-specific inflammatory response.

Meanwhile, studies on exosome-like vesicles present in various body fluids including peripheral blood also began flourishing. Our lab has previously shown that plasma-derived exosomes isolated from antigen-sensitized mice were able to suppress antigen-specific DTH response, which effect was mediated by MHC class II+ vesicles (171). Therefore we were quite interested in evaluating whether plasma-derived exosomes isolated from tumor-bearing mice could suppress tumor-specific response. In a similar DTH experiment setting, we found that plasma-derived exosomes from mice bearing OVA-expressing tumor were also effective in suppressing OVA-specific DTH response. This interesting observation made us ask two questions: 1) are tumor-derived exosomes enriched in the blood plasma of tumor-bearing mice; and 2) what are the major cellular origins of plasma-derived exosomes and which vesicle portion(s) primarily mediate the suppressive effect. Unexpectedly, tumor-derived exosomes were not detected in a murine melanoma model using a FLAG-tagging method, whereas the suppressive effect was found to be at least partially dependent on the MHC class II+ vesicle portion. These results suggest that exosomes derived from circulating immune cells which have sampled tumor antigens may help deliver systemic suppression of the anti-tumor response.

The third part of this thesis presents the effect of tumor-derived exosomes on splenic lymphocytes when the parental tumor cells are infected with mycoplasma pathogen. While exosomes derived from healthy tumor cell lines showed no stimulatory effect on spleen cells, exosomes derived from mycoplasma infected tumor cell lines induced T cell-independent B cell activation and B cell-dependent cytokine production, including the regulatory cytokine IL-10. Interestingly, B cell activation and IL-10 production was accompanied by subsequent inhibition of T cell proliferation and TCR signaling. This part of the study started from an accidental observation, but led to interesting and revelatory results. We dissected the response of B and T lymphocytes to tumor-derived exosomes carrying mycoplasma components and revealed the potential antagonizing effect of B cell mitogenisis to T cell activation. It will also help us better understand the impact of components from co-existing opportunistic pathogens released in the form of tumor-derived exosomes on host immune modulation.

Immune regulation by tumor-derived exosomes was an exciting, but poorly-known field when I first started this thesis work. The studies presented in this thesis also involve the initial efforts in optimizing exosome purification strategies to ensure both yield and quality, in improving exosome imaging techniques, as well as in developing methods to elucidate their function as immune regulators. We are thrilled to find that many important and valuable works are reported or are being done in the meantime of our research. We are also glad that our work may contribute to a better understanding of tumor-derived exosomes and their role in immune regulation.

2.0 TUMOR-DERIVED EXOSOMES CONFER IMMUNE SUPPRESSION IN AN ANTIGEN-SPECIFIC MANNER

2.1 ABSTRACT

Tumor cells usually express specific antigens that are potentially immunogenic; however, established tumors primarily induce immune tolerance. Tumor cells actively release a population of small membrane vesicles, termed “exosomes,” whose immunostimulatory effect and immuosuppressive effect have been both reported. However, whether tumor-derived exosomes carrying tumor-specific antigen are able to mediate antigen-specific immune suppression is unclear. In this study, we tested the immunosuppressive effect of tumor-derived exosomes in an antigen-specific murine delayed-type hypersensitivity (DTH) model. We demonstrate that exosomes derived from tumor cells expressing the model antigen ovalbumin (OVA) potently suppress OVA-induced DTH response, while exosomes derived from their OVA-negative parental tumor cell lines showed little suppressive effect. Interestingly, these exosomes were not able to suppress DTH response elicited by an irrelevant antigen, keyhole limpet hemocyanin (KLH). Trafficking studies showed that following local injection, tumor-derived exosomes were mostly internalized by dendritic cells (DCs) and transported to the draining lymph node (LN). Analysis of the cytokine expression profile in the draining LN reveals significant increases in the mRNA levels of TGF-β and IL-4 in exosome-mediated suppression. Moreover, these tumor-

derived exosomes were able to down-regulate the maturation of bone marrow-derived DC and induce TGF-β production. These results suggest a novel role of tumor-derived exosomes in mediating antigen-specific immune suppression, possibly by providing exosomal antigen while at the meantime modulating the way of antigen presentation. We also demonstrate the possibility of utilizing tumor-derived exosomes containing certain antigen to suppress antigen-specific inflammatory response.

2.2 INTRODUCTION

Tumorigenesis is usually associated with a large number of genetic and epigenetic changes, leading to the expression of tumor-specific antigens (TSA) or tumor-associated antigens (TAA) that are potentially immunogenic. Although tumor-specific immune responses can be elicited *in vivo* (1), they are usually insufficient to eradicate the tumor and established tumors tend to induce immune tolerance (65). Effective anti-tumor response requires efficient T cell priming by professional antigen-presenting cells (APCs), in particular dendritic cells (DCs), which process and present cellular tumor antigens. Tumor antigens are also found present in tumor-derived exosomes (129, 131, 199), a population of small membrane vesicles actively secreted by tumor cells both *in vivo* and *in vitro*. These vesicles are formed by reverse budding of the multivesicular bodies (MVBs) in the late endosomes and released upon fusion of MVB with plasma membrane, with a lipid bilayer structure of 30-100 nm in diameter (84, 86, 125). Tumor-derived exosomes usually express tumor antigens and a panel of selected proteins partially reflecting the protein composition of the parental cells.

Tumor-derived exosomes were originally viewed as a new source of tumor rejection antigens and potential cell-free cancer vaccines. However, research on tumor-derived exosome-based vaccines showed that the efficacies of these vaccines were mostly achieved when tumor-derived exosomes were processed by mature APCs or were modified to express pro-inflammatory factors or stress proteins (129, 132-135). Targeting of antigens to the exosome membrane surface may also enhance their ability to induce anti-tumor responses (138, 139). It is still unclear, however, whether tumor-derived exosomes stimulate efficient anti-tumor immune responses naturally. It was reported that exosomes expressing tumor markers abundantly accumulated in the malignant effusions of tumors in advanced stages, at which time point anti-tumor immunity is usually overwhelmed (128). Moreover, pre-treatment of unmodified tumor-derived exosomes promoted tumor growth in several murine tumor models (147, 174).

Indeed, diverse immunosuppressive effects of tumor-derived exosomes have been reported. These effects affect various components in the immune system, including inhibiting the differentiation of DCs, promoting the generation of myeloid suppressor cells (148, 149), impairing the proliferation of cytotoxic effector cells while supporting the activity of regulatory T cells (Treg) (131), and down-modulating the killing function of natural killer cells (NK cells) and CD8+ T cells (145-147). Direct killing of T cells and impairment of T cell receptor (TCR) signaling have also been reported (144, 167, 200). These findings suggest that instead of stimulating anti-tumor response, tumor-derived exosomes may help convey and even amplify the immunoregulatory effect of tumor cells (83, 124). However, most of these effects described are antigen-independent and it remains unclear whether exosomal tumor antigens play a role in exosome-mediated immune suppression.

Antigen-specific immune suppression has been observed in mice with tumors and also in oral tolerance models (201, 202). Interestingly, exosome-like vesicles purified from biological fluids of animals sampled with specific antigens were found to induce antigen-specific immune suppression. For example, “tolerosomes” isolated from serum shortly after antigen feeding or from an *in vitro* pulsed intestinal epithelial cell line induced antigen-specific tolerance in naïve recipient animals (160, 172). We previously demonstrated that exosomes derived from the plasma of antigen-sensitized mice suppressed the DTH response induced by that specific antigen (171). Also, exosomes isolated from bronchoalveolar fluid of mice exposed to an allergen prevented the specific allergic reaction (165). Besides these exosomes of potentially heterogeneous cellular origin, it was also reported that exosomes derived from immature DCs induced allograft tolerance in a rat cardiac transplantation model, with mismatched MHC molecules serving as an alloantigen (156). Since in tumor-bearing mice and during human cancer development, antigen-specific immune suppression and activation of antigen-specific regulatory T cell subsets have been observed (201, 203, 204), it is of big interest to determine whether tumor-derived exosomes induce immune suppression in an antigen-specific manner.

In this study, we investigated the ability of exosomes derived from OVA-expressing tumor cells to modulate antigen-specific immune response in a murine footpad DTH model. DTH response is a cell-mediated, antigen-specific immune response. We show that local administration of these exosomes potently suppresses OVA-specific DTH response, and the suppressive effect is dependent on the presence of OVA antigen in tumor-derived exosomes. Analysis of the potential suppression mechanism suggests that tumor-derived exosomes can target the function of DCs. Our findings suggest that suppression of the immune response

specific to TSA/TAA may be a novel suppressive mechanism conferred by tumor-derived exosomes.

2.3 MATERIALS AND METHODS

2.3.1 Cell lines

The human breast cancer cell line MCF-7 and SK-BR-3, the murine (C57BL/6 background) fibrosarcoma cell line MCA205, B lymphoma line TA3, thymoma cell line EL4 and melanoma cell line B16-F0 (B16) were purchased from American Type Culture Collection (ATCC). The EL4-OVA (EG7) and B16-OVA (MO5) cell lines were generously provided by Dr. Walter Storkus (University of Pittsburgh). The human melanoma cell line 82.55 was kindly provided by Dr. John Kirkwood (University of Pittsburgh Cancer Institute). The DC2.4 cell line, the OVA-specific T cell hybridoma line 33.70 (H-2K^b restricted) (205) were generously provided by Dr. Louis Falo (University of Pittsburgh). Cells were tested to be free of pathogens. Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2mM of L-glutamine, 0.1mM of non-essential amino acids, 1 mM of sodium pyruvate, 10mM of HEPES, Antibiotic-Antimicotic (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B, GIBCO), and 50 μM of 2-ME. The EG7 and MO5 cell lines were under G418 selection (0.8 mg/ml and 1.5 mg/ml, respectively).

2.3.2 Mice

Female C57BL/6 mice (H-2K^b) were purchased at 6-8wk of age from The Jackson Laboratory. Animals were maintained in a pathogen-free animal facility at University of Pittsburgh Biotechnology Center. All animal experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee.

2.3.3 Exosome purification

Exosomes were isolated from cell culture supernatant by serial centrifugation and filtration. FBS used in culture media for exosome isolation was pre-cleared by ultracentrifugation at 100,000 × g for 3 hr at 4^{0}C. 48 hr culture supernatants were centrifuged at 1000 × g for 10 min and 10,000 × g for 30 min to remove cells and membrane debris, filtered through 0.22 μm sterilizing filter (Corning), concentrated using Centricon Plus-70 (100 kDa cutoff) filter units (Millipore), followed by ultracentrifugation at 100,000 × g for 1.5 hr. Exosome pellets were washed with sterile PBS, ultracentrifuged again and resuspended in sterile PBS. Exosome quantification was done by Bradford protein assay (Bio-Rad). In our experience, approximately 0.4-0.5 μg of exosomes were obtained per million cells (end count). Exosome preparations were stored at -80^{0}C (for biochemical analysis) or 4^{0}C (for immediate use in biological assay).

2.3.4 Transmission electron microscopy

Purified exosome preparations (5 μl of ~0.1-0.5 μg/μl) were loaded on Formvar/carbon-coated grids and negatively stained with 1% uranylacetate. Photos were taken on a JEM-1011 transmission electron microscope with Advanced Microscopy Techniques (AMT) software.

2.3.5 Western blotting and immunoprecipitation

Cell pellets were lysed in NP-40 lysis buffer in the presence of protease inhibitor (Sigma-Aldrich). Cell lysates or exosomes (10 μg of proteins) were separated on 12% or 10% SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore), blocked with 5% milk-PBST and incubated with different primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies (Santa-Cruz). Protein bands were visualized using an enhanced chemiluminescence detection kit (PerkinElmer Life Science). The primary antibodies used were: MART-1 (Ab-3) from Lab Vision; erbB2 (ab2428) from Abcam; Alix (3A9) from Biolegend; Tsg101 (C-2), HSC70 (B-6) and HSP90α/β (H-114) from Santa-Cruz; HMGB1 from GeneTex; and β-actin from Abcam. For OVA immunoprecipitation (IP), cell lysates (200 μg for EL4 and EG7 cells and 400 μg for B16 and MO5 cells) or exosomes (300 μg, pre-lysed) were incubated with anti-OVA (Chemicon, 1:50) at 4^0C for overnight. 40 μl of 50% Protein A–Sepharose bead slurry was added and incubated for 4 hr at 4°C. The beads were washed and the antibody-bound complexes were eluted by boiling the beads in SDS loading buffer for 5 min. Proteins were resolved by 10% SDS-PAGE and detected by Western blot first using anti-OVA (Abcam) then stripped and re-blotted with anti-OVA (OVA-14, Sigma).

2.3.6 Flow cytometry

For exosome surface staining, exosomes (150 μg) were incubated with aldehyde/sulfate latex beads (1% solids, 10 μl for each staining, Invitrogen) at 4 ^{0}C for overnight. The reaction was stopped with 100mM Glycine. Beads were washed twice in flow buffer (1% FBS in PBS) and stained with PE-conjugated antibodies specific for I-A^{b} (AF6-120.1, BD) or CD81 (Eat2, BD), or biotin-H-2K^{b} (AF6-88.5.5.3, eBioscience) followed by streptavidin-PE (eBioscience). Tumor cells were stained with the same antibodies. For surface staining of BMDCs, cells were washed and stained with FITC-CD11c (N418, eBioscience) and PE-CD86 (GL1, BD), or PE-CD11c (N418, eBioscience) and FITC-I-A^{b} (AF6-120.1, BD). To examine exosome *in vitro* binding to T cells, T cells were washed and stained with PE-CD3 (500A2, eBioscience) and PE-Cy5-CD4 (RM4-5, eBioscience), or PE-CD3 (500A2, eBioscience) and PE-Cy5-CD8a (53-6.7, eBioscience), or PE-Cy5-CD4 (RM4-5, eBioscience) and PE-Foxp3 (FJK-16s, eBioscience). To examine T cell activation after exosome treatment, T cells were stained with PE-CD69 (H1.2F3), PE-CD44 (IM7), or PE-CD62L (MEL-14) from eBioscience. Beads and cells were analyzed on BD FACScan™ flow cytometer. Results were analyzed using Flowjo software.

2.3.7 Induction of DTH response and exosome treatment

Mice at 8-9 wk of age were immunized by intradermal (i.d.) injection of 150 μg of OVA (grade V, Sigma) 1:1 emulsified in Freund's complete adjuvant (CFA, Pierce) at tail base. Fourteen days later, mice were boosted by 50 μg of OVA (grade V, Sigma) 1:1 emulsified in Freund's incomplete adjuvant (IFA, Pierce). Seven days later, the right hind paw was injected i.d. with 10 μg of exosomes along with 30 μg of OVA (grade II, Sigma) in a total volume of 50 μl of PBS.

The left hind footpad was injected with OVA antigen alone. Footpad thickness was measured using a spring-loaded caliper (Dyer) before, 24 and 48 hr post-challenge. Paw swelling was determined by the increase in footpad thickness. The DTH response to keyhole limpet hemocyanin (KLH) was induced by immunizing the mice with 100 μg of KLH (Sigma) 1:1 emulsified in CFA, and challenging the mice in the footpad with 20 μg of KLH 14 days post-immunization. Each set of experiment was performed with 5 mice per group and repeated at least twice.

2.3.8 Analysis of exosome in vivo trafficking by immunofluorescence

Exosome labeling with the green fluorescent linker PKH67 (Sigma) was done according to the manufacturer's guidelines. 50 μg of labeled MO5 exosomes were injected into the right hind paw of OVA-immunized mice along with OVA antigen. Mice were euthanized 24 or 48 hour post-injection. Footpad and popliteal lymph nodes (LNs) were isolated and fixed in 2% paraformaldehyde followed by 30% sucrose. Fixed tissues were cryo-sectioned and stained with anti-mouse CD11c or CD3 (BD), followed by GaH-Cy3. Nuclei were stained with DAPI (Molecular Probes). TUNEL staining was performed using a Terminal Transferase kit plus Biotin-16-dUTP and Cy3-streptavidin (Roche). Photos were taken on an Olympus Provis fluorescence microscope with MagnaFire software. Exosome trafficking to spleen after systemic injection was examined by injecting 50 μg of labeled MO5 exosomes i.p.. Mice were sacrificed 6 hr or 48 hr post-injection and spleens were fixed, cryo-sectioned, treated with ABC kit (Vectabond™, Vector Labs) and stained with biotinylated anti-CD11c or anti-CD3 (eBioscience), followed by streptavidin-Cy3 (Jackson ImmunoResearch).

2.3.9 Quantitative reverse transcription-PCR

Popliteal LNs were isolated and snap frozen in liquid nitrogen. Total RNA was purified using the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen), and treated with DNase I (Ambion). RNA quality and quantity were measured on a NanoDrop micro-volume spectrophotometer (Thermo Scientific). Reverse transcription was done using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Quantitative PCR was performed on an iCycler (Bio-Rad) using SYBR® GreenER™ qPCR SuperMix for iCycler® (Invitrogen). The primers used include: TGF-β forward 5'-TGAGTGGCTGTCTTTTGACG-3' and reverse 5'-AGCCCTGTATTCCGTCTCCT-3'; IL-4 forward 5'-ACAGGAGAAGGGACGCCA-3' and reverse 5'-GAAGCCCTACAGACGAGCTCA-3'; IL-10 forward 5'-AAGGACCAGCTGGACAACAT-3' and reverse 5'-TCATTTCCGATAAGGCTTGG-3'; IFN-γ forward 5'-GCGTCATTGAATCACACCTG-3' and reverse 5'-TGAGCTCATTGAATGCTTGG-3'; Foxp3 forward 5'-TCTTGCCAAGCTGGAAGACT-3' and reverse 5'-GGGGTTCAAGGAAGAAGAGG-3'; and β-actin forward 5'-GACGGCCAGGTCATCACTAT-3' and reverse 5'-AAGGAAGGCTGGAAAAGAGC-3'. Data were analyzed by iCycler iQ analysis software (Bio-Rad). Relative mRNA expression was normalized to the level of β-actin mRNA and calculated using the $\Delta\Delta C_T$ method.

2.3.10 Generation of BMDCs and exosome treatment

Bone marrow (BM) cells were flushed from tibias and femurs of 10-12 wk old mice and a single cell suspension was prepared. Erythrocytes were depleted with ACK cell lysing buffer (Biowhittaker). Cells were cultured in complete RPMI-1640 media with 20 ng/ml of GM-CSF

and IL-4 (PeproTech) in 6-well-plate at 2×10^6 cells/5ml/well. For every 3-4 days, each well was replenished with 2 ml of fresh media with GM-CSF and IL-4. Cells were left in bulk culture until day 8, when suspended and semi-adherent cells were collected and the purity of CD11c+ cells was checked by flow cytometry. Cells were then cultured in 12-well-plate at 1×10^6 cells/2ml/well and treated with 10 μg/ml of exosomes or left untreated for 3 days. 1 μg/ml of LPS (Sigma) was added to untreated cells for the last 24 hr as a DC maturation control. Cells were then harvested and analyzed by flow cytometry.

2.3.11 T cell purification, exosome binding and T cell hybridoma assay

CD4+ or CD8+ T cells were purified from splenocytes and LN cells of OVA-immunized mice using mouse CD4 and CD8 negative isolation kit (Dynal Biotech). Cells were co-incubated with PKH67-labeled EG7 or EL4 exosomes (8 μg exosomes/0.5×10^6 cells/well in 96-well-plate) for 18 hr, collected, washed with PBS, cytospined onto slides, fixed, treated with ABC kit, stained with biotin-CD4 (GK1.5, eBioscience) or biotin-CD8a (53-6.7, eBioscience), followed by streptavidin-Cy3 (Jackson ImmunoResearch), and then examined under fluorescent microscope. Alternatively, cells were treated with exosomes for 48 hr in the presence of 30 U/ml recombinant murine IL-2 (Biolegend). Cells were collected and expression of CD69, CD44, and CD62L were analyzed by flow cytometry. For T cell hybridoma assay, 1×10^5 33.70 cells were cultured with 20 μg/ml of exosomes in 200 μl media per well in 96-well-plate for 48 hr. As an activation control, 1×10^5 DC2.4 cells and 1×10^5 33.70 cells were co-cultured with 50 ng/ml of SIINFEKL peptide for 24 hr. Culture supernatants were collected and IL-2 levels were measured by ELISA.

2.3.12 ELISA

TGF-β1 levels in culture supernatants and exosome preparations were measured using mouse TGF-β1 ELISA kit (eBioscience) upon acidification. IL-2 levels in culture supernatants were measured using mouse IL-2 ELISA MAX^{TM} Deluxe kit (Biolegend).

2.3.13 Statistics

DTH results were analyzed by one-way ANOVA with Fisher's post-hoc test. qRT-PCR results were analyzed by Mann-Whitney *U* test. A value of $p < 0.05$ was considered statistically significant. All tests were conducted using the SPSS statistical software (SPSS).

2.4 RESULTS

2.4.1 Tumor cells produce exosomes that contain TAA/TSA

Exosomes can be isolated from the culture supernatants of different tumor cell lines, showing a typical vesicular structures ranging from 30 to 120 nm in diameter (Fig. 4A). We found that tumor-derived exosomes usually express TAA. For example, exosomes produced by a MART-1 positive human melanoma cell line express MART-1, and exosomes produced by the erbB2 (Her2/Neu) positive human breast cancer cell line SK-BR-3 express erbB2 (Fig. 4B). Exosomes derived from two matching pairs of murine tumor cell lines, differing only in the expression of the model antigen OVA, were specifically characterized: the murine thymoma cell line EL4 and

its OVA-expressing derivative cell line EG7 (206), and the murine melanoma cell line B16 and its OVA-expressing derivative cell line MO5 (207). Exosomes were purified from culture supernatants and were examined by electron microscopy (Fig. 5A). Western blot analysis showed that exosome preparations were more enriched in the MVB markers Alix and Tsg101 compared with cell lysates. Expression of HSC70, HSP90, β-actin and high mobility group box-1 (HMGB1) were also found in exosome preparations (Fig. 5B). Full-length OVA protein was detected as a doublet form of around 40 to 45 kDa by IP analysis in both EG7 and MO5 cell lysates, but not in EL4 or B16 cell lysates. Similarly, OVA expression was detected in EG7 and MO5 exosomes, but was absent in EL4 and B16 exosomes (Fig. 5C).

In addition, surface expression of MHC molecules on both cells and exosomes were examined. FACS analysis showed that EL4 and EG7 cells express high levels of MHC class I molecule (H-2K^b), while B16 and MO5 cells express relatively low levels of MHC class I molecules. Expression of MHC class II molecules (I-A^b) was not detected on these tumor cells. Interestingly, exosomes derived from all four tumor cell lines express only marginal to undetectable levels of MHC class I molecules and no MHC class II molecules on their surface. The tetraspanin CD81 was expressed on B16 and MO5 exosomes, but was very low on EL4 and EG7 exosomes (Fig. 5D). These results show that exosomes derived from tumor cells stably transfected to express OVA contain full-length OVA protein, but lack the expression of MHC molecules, and that exosomes derived from each pair of tumor cell lines are similar except for the presence of OVA.

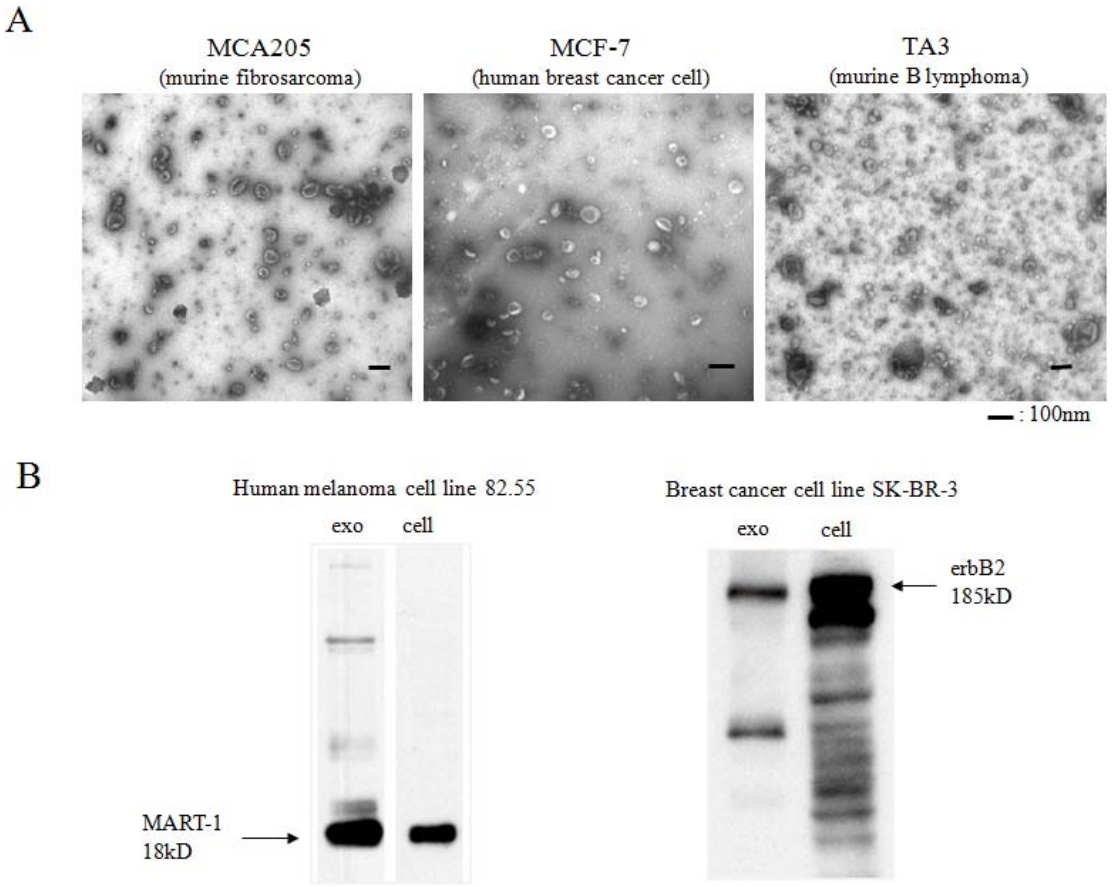
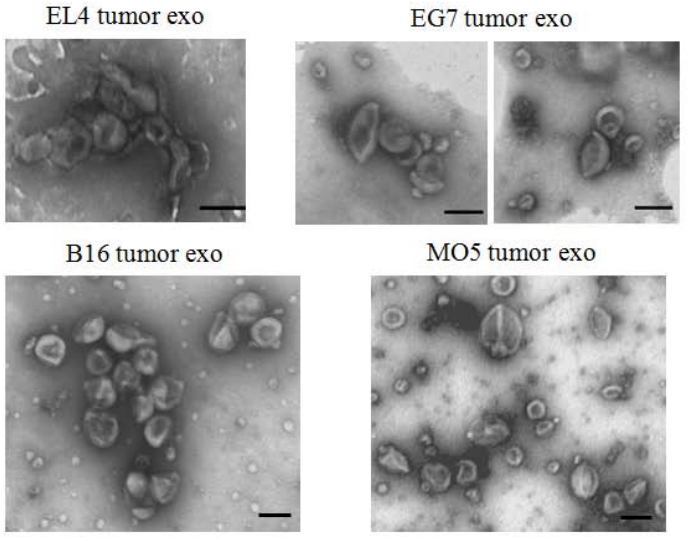


Figure 4: Tumor cells produce exosomes and tumor-derived exosomes contain TAA.

(A). Electron microscopy of exosomes derived from MCA205, MCF-7 and TA3 cell culture supernatants. Scale bar: 100 nm. (B). Expression of MART-1 on exosomes derived from 82.55 melanoma cells and expression of erbB2 on exosomes derived from SK-BR-3 cells were detected by Western blotting. 10 μg of proteins were loaded each lane.

A



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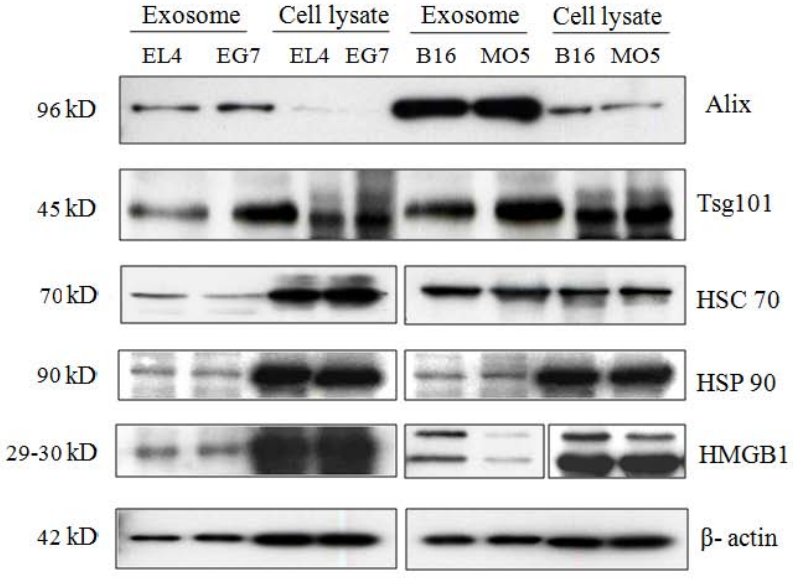
B



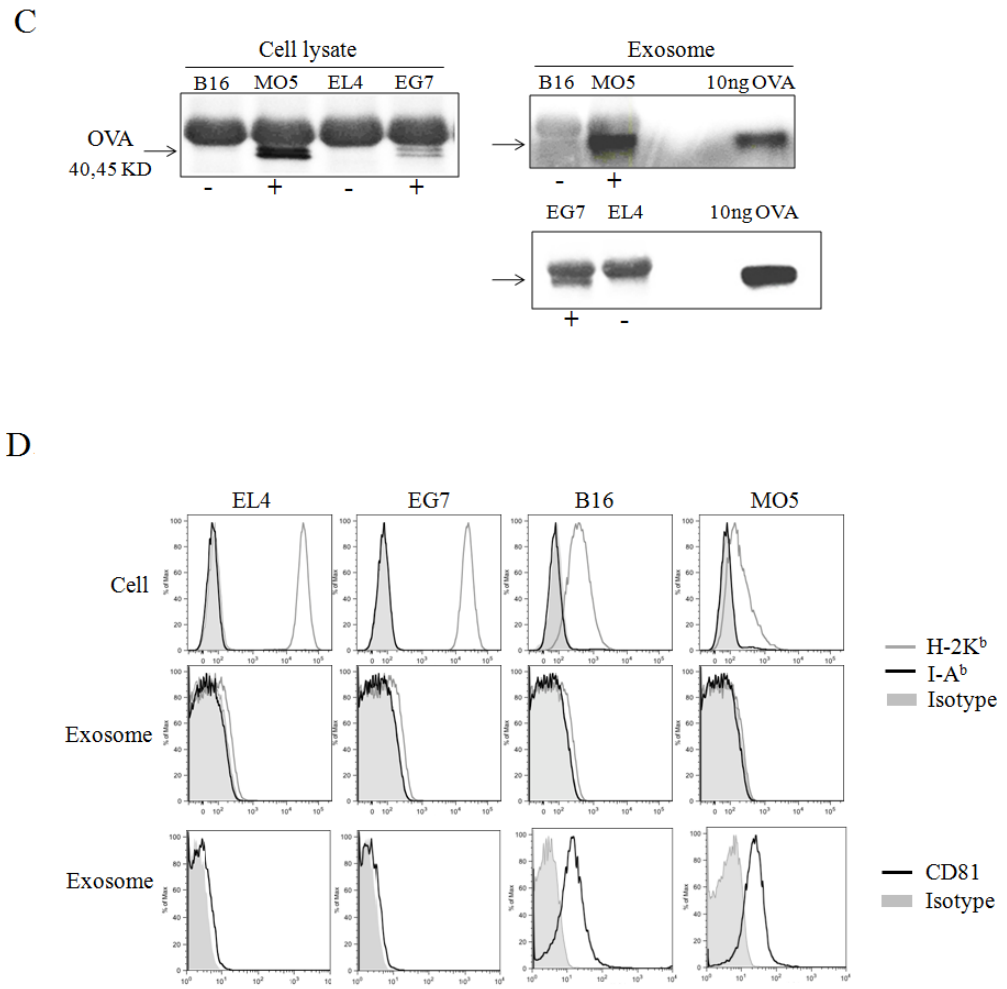


Figure 5: Characterization of exosomes derived from OVA-expressing tumor cell lines and their parental cell lines.

(A). Electron microscopy of exosomes derived from EL4, EG7, B16 and MO5 cell lines. (B). Western blot analysis of protein expression on cell lysates and exosomes. 10 µg of proteins were loaded each lane. (C). IP detection of full-length OVA protein (40~45 kDa) in EG7 and MO5 cell lysates and their exosomes. (D). FACS analysis of MHC class I, MHC class II and CD81 expression on cells and exosomes.

2.4.2 Exosomes derived from OVA-expressing tumor cells suppress OVA-specific DTH response

To examine if tumor-derived exosomes are able to regulate the immune response specific to the antigen they carry, we tested if exosomes containing OVA antigen can modulate OVA-specific DTH response in a murine footpad model. Briefly, C57BL/6 mice pre-sensitized with soluble OVA protein were injected with 10 μg of exosomes in the footpads of the right hind paws, with the contralateral footpads receiving a PBS injection, by the time of OVA antigen challenge in both hind paws. The magnitude of the DTH response was monitored by measuring footpad swelling 24 and 48 hr post-challenge. As shown in Figure 5, local administration of EG7 exosomes significantly reduced paw swelling by more than 50% compared with mice treated with EL4 exosomes or PBS (Fig. 6A). Similarly, treatment with MO5 exosomes was also able to decrease inflammation by around 50% compared with treatment with B16 exosomes or PBS (Fig. 6B). Interestingly, the reduction of swelling in the exosome-treated paws was always accompanied by a comparable reduction in the contralateral, untreated paw. Similar "contralateral effect" has been previously described in both rabbit arthritis and mouse DTH models, with the mechanism remains unclear but is likely to be a systemic effect mediated by functionally modified endogenous APCs (158, 208). These results demonstrate the suppressive effect of EG7 and MO5 exosomes on OVA-specific immune response in this particular model. The ineffectiveness of EL4 and B16 exosomes indicates a critical role of exosomal OVA antigen played in conferring the suppressive effect. Also, similar suppressive effect was observed using exosomes derived from two different types of tumor cell lines, suggesting that this is not a cell line-specific effect.

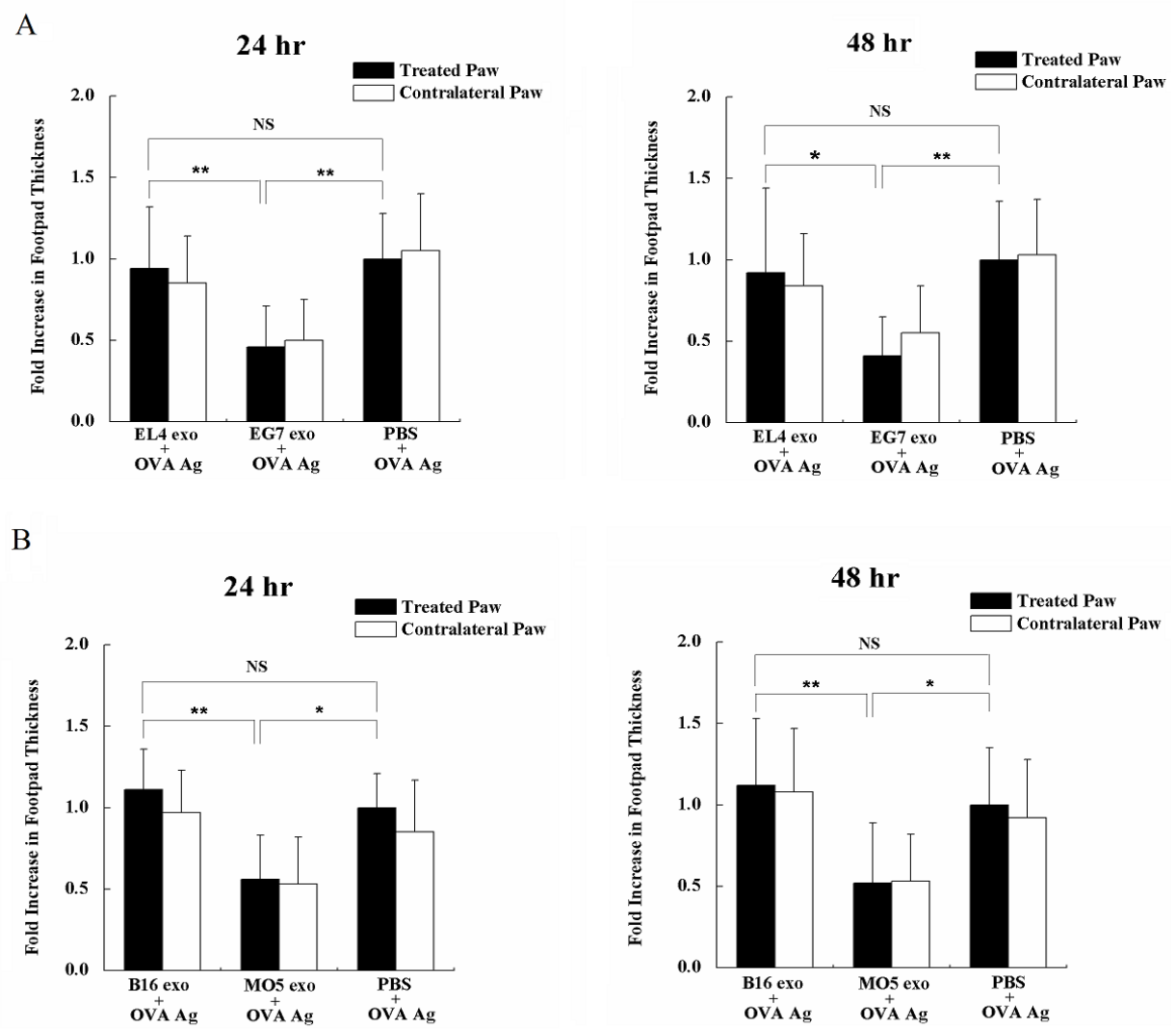


Figure 6: Suppression of OVA-specific DTH response by exosomes derived from OVA-expressing tumor cells.

Mice immunized with OVA were treated with different tumor-derived exosomes or PBS in their right paws by the time of OVA antigen challenge. Paw swelling was measured 24 and 48 hr post-injection. The mean increase of footpad thickness of the treated paws (right paws) in PBS treated group at each time point was set to 1, and the increases of footpad thickness in other groups were normalized as fold increase. Data represent the pooled results of two independent experiments and are the means ± SD with 10 mice per group. Footpad administration of EG7 exosomes (A) and MO5 exosomes (B) significantly suppressed the OVA-specific DTH response. Significance at: **, $P<0.01$; *, $P<0.05$; NS, not significant.

2.4.3 Exosomes derived from OVA-expressing tumor cells have no suppressive effect on KLH-specific DTH response

Given that EG7 and MO5 exosomes show superior suppressive effect on OVA-induced DTH response than their OVA-negative counterparts, we further investigated if they were also able to suppress the immune response induced by an irrelevant antigen, KLH. Mice pre-sensitized with KLH were treated with different exosomes in a similar experiment setting at the time of antigen challenge. In contrast, neither EG7 exosomes nor MO5 exosomes were effective in reducing footpad swelling and the magnitude of KLH-specific DTH response was comparable to mice treated with EL4 exosomes or B16 exosomes or PBS control (Fig. 7). This result strongly suggests that local administration of EG7 and MO5 exosomes were only effective against the immune response induced by OVA antigen and they suppress inflammation in an antigen-specific manner.

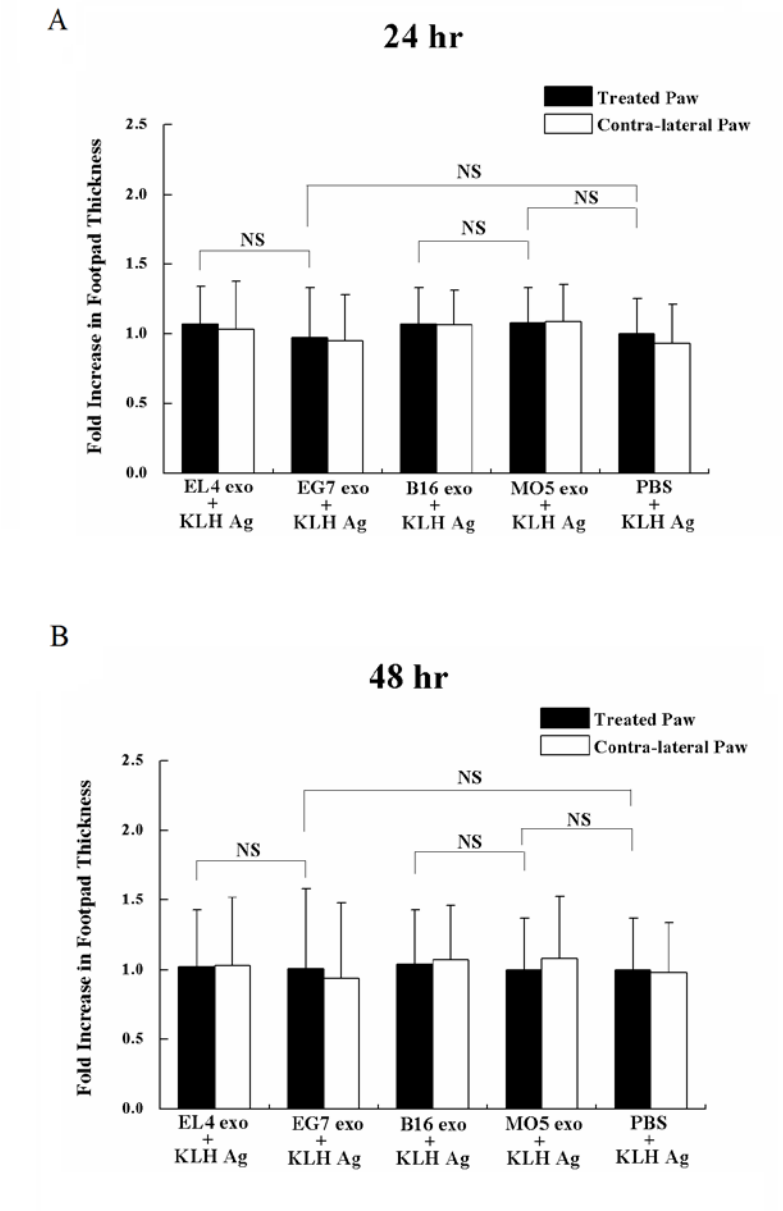
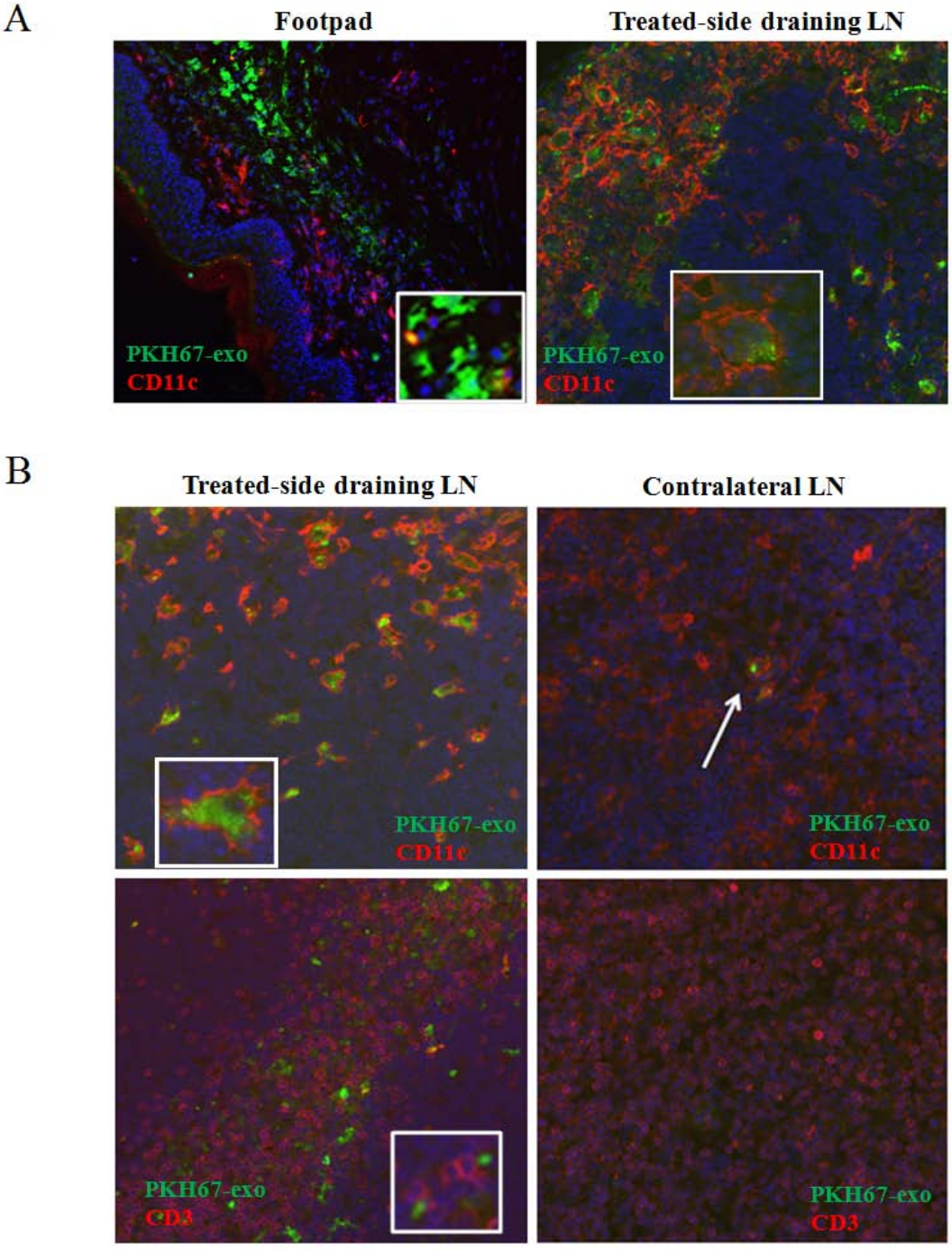


Figure 7: Exosomes derived from OVA-expressing tumor cells were unable to suppress KLH-specific DTH response.

Mice immunized with KLH antigen were treated with different tumor-derived exosomes or PBS in their right paws at the time of antigen challenge. Paw swelling was measured 24 and 48 hr later. The increases of footpad thickness in different treatment groups are normalized to the mean increase of treated paws in PBS treated group. Compared with PBS control group, exosome treatments showed no suppressive effect on KLH-induced immune response. Data represent the pooled results of two independent experiments and are the means ± SD with 10 mice per group. NS: not significant.

2.4.4 Tumor-derived exosomes are internalized by DCs and traffic to draining lymph node after local injection

Cutaneous DTH reactions are initiated when skin APCs, predominantly Langerhans cells, present antigens to CD4+ memory T cells, which then release inflammatory mediators to recruit effector cells from lymphoid tissue to the site of antigen challenge. To better understand the mechanism of exosome-mediated immune suppression, we investigated the trafficking of tumor-derived exosomes and their interaction with immune cells after footpad injection. MO5 exosomes labeled with the green fluorescent linker, PKH67, were injected into the right hind paws of OVA-immunized mice along with OVA antigen. Mice were euthanized 24 or 48 hr post-injection. Footpads and the draining popliteal LNs were collected, cryo-sectioned and stained for either DCs (CD11c+) or T cells (CD3+). Co-localization of exosomes and dermal DCs were observed in footpad tissue and a large amount of DCs with internalized exosomes were found in the treated-side draining LN 24 hr post-injection (Fig. 8A). At 48 hr post-injection, more DCs with internalized exosomes were found in the treated-side LN. Very few exosomes were detected in the contralateral LN (Fig. 8B). Meanwhile, green exosomes were mostly localized in the T cell area and the interaction between exosomes and T cells seems to be either direct membrane contact or via exosome-containing DCs (Fig. 8B). Unlike systemic injection (i.v. or i.p., Fig. 9), significant trafficking of exosomes to spleens was not observed following footpad injection (data not shown). Furthermore, exosome treatment did not increase the number of apoptotic cells in LNs compared with PBS treatment, as determined by TUNEL staining (Fig. 8C), suggesting that here exosomes do not induce apoptosis of lymphocytes and that alternative mechanisms must contribute to the suppressive effect of these exosomes.


A
Footpad
Treated-side draining LN
PKH67-exo
CD11c
PKH67-exo
CD11c
B
Treated-side draining LN
Contralateral LN
PKH67-exo
CD11c
PKH67-exo
CD11c
PKH67-exo
CD3
PKH67-exo
CD3

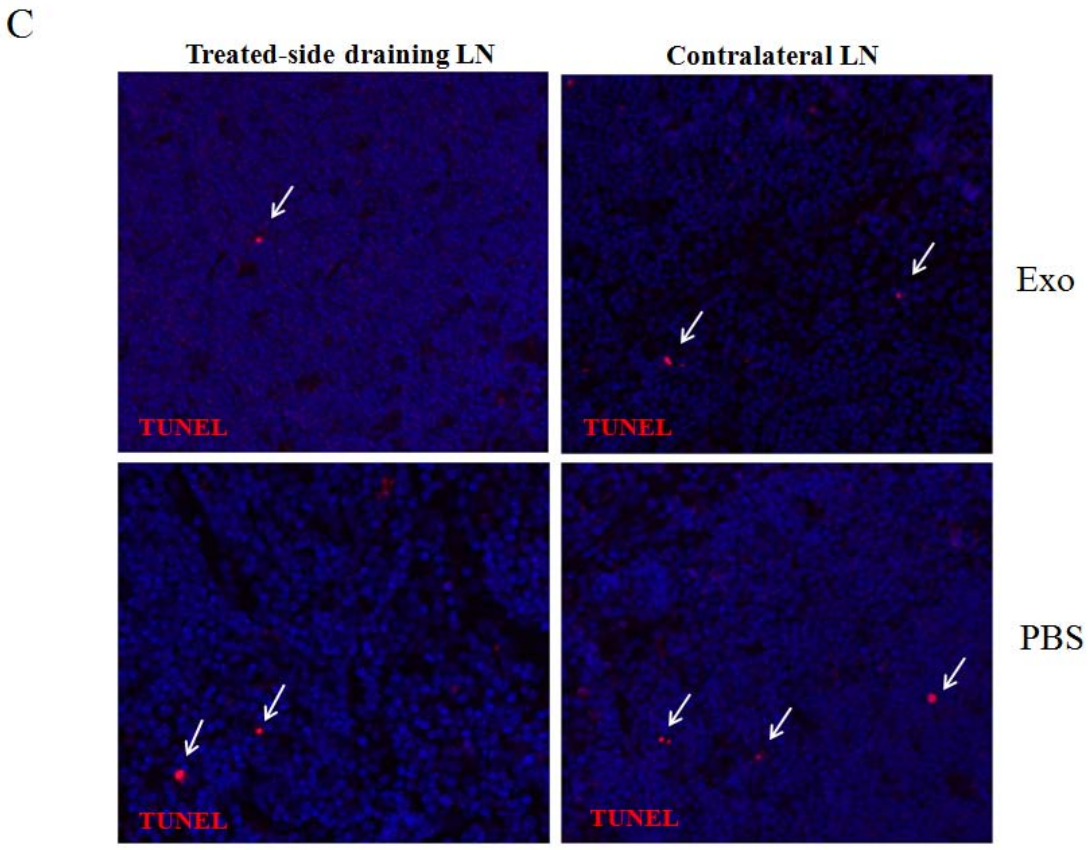


Figure 8: Exosome trafficking after footpad administration.

PKH67-labeled MO5 exosomes were injected along with OVA antigen in the right footpad of OVA-immunized mice. Footpad and the popliteal LNs were harvested, cryo-sectioned and examined by immunofluorescence. (A). 24 hr post-injection, exosomes (green) were captured by dermal CD11c+ DCs (red) in footpads and transported to treated-side LN. (B). 48 hr post-injection, large numbers of exosome-internalized DCs appear in the treated-side LN. Most exosomes (or exosome-containing DCs) were physically adjacent to CD3+ T cells (red, lower left panel). Very few exosomes were observed in the contralateral LN. (C) TUNEL staining for apoptotic cells (red) in treated-side and contralateral LNs 48 hr post-injection. Magnification: 20×.

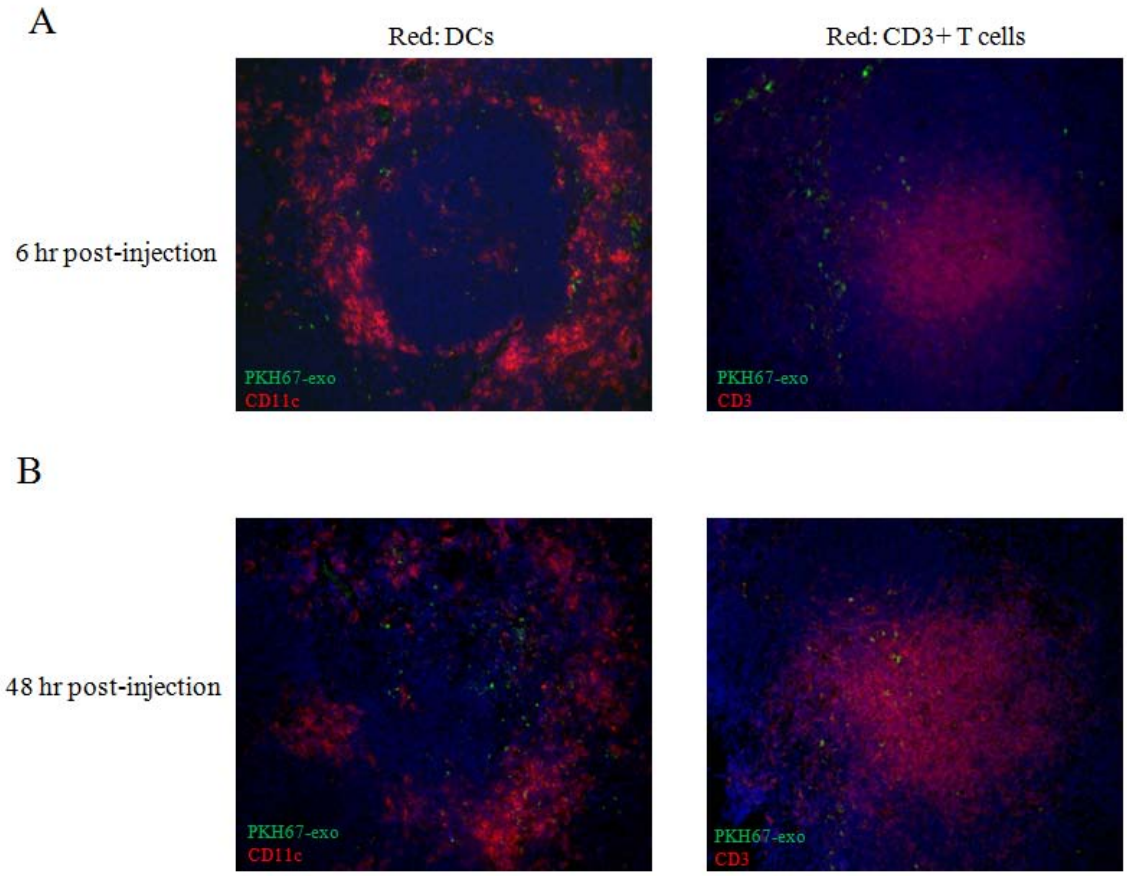


Figure 9: Exosome trafficking after i.p. injection.

PHK67 labeled MO5 exosomes were injected i.p.. Mice were sacrificed 6 hr or 48 hr post-injection and spleen sections were examined by immunofluorescence. (A). Exosomes (green) were found predominantly in splenic DC area at 6 hr post-injection. (B). Exosomes traffic to T cell area at 48 hr post-injection. Magnification: 20×.

2.4.5 Suppression of DTH response is associated with increased TGF-β and IL-4 mRNA expression

Next we wanted to determine if suppression of OVA-specific DTH response was associated with the production of regulatory cytokines. To test this, 48 hr after exosome treatment (EL4 or EG7 exosomes) and OVA antigen challenge, treated-side popliteal LNs were isolated and the mRNA levels of different cytokines were analyzed by qRT-PCR. We found that TGF-β and IL-4 mRNA levels were both significantly increased in mice treated with EG7 exosomes, compared with mice treated with EL4 exosomes or PBS (Fig. 10A-B). IL-10 mRNA expression was also significantly increased by EG7 exosome treatment compared with PBS treatment (Fig. 10C). Moreover, there was a clear tendency of decreased IFN-γ mRNA in the EG7 exosome treated group compared with PBS treated group ($p=0.07$) (Fig. 10D). Interestingly, increased IL-10 mRNA was also found in EL4 exosome treated group, although the DTH response was not effectively suppressed. Furthermore, Foxp3 mRNA levels were also examined and were comparable between all treatment groups (Fig. 10E), indicating that the suppression may not directly result from the expansion of FoxP3+ Tregs.

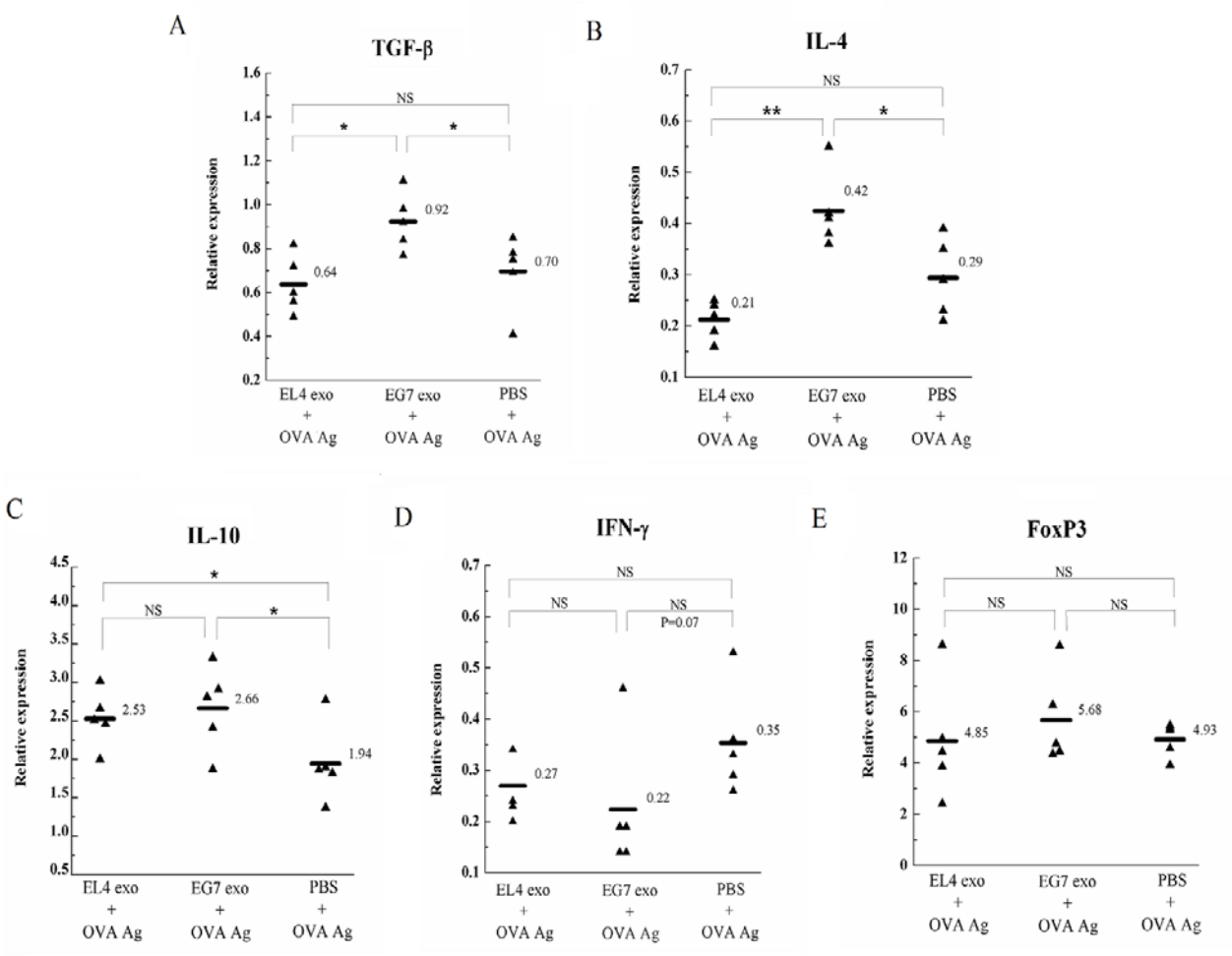


Figure 10: qRT-PCR analysis of cytokines and FoxP3 mRNA expression in draining LN after exosome treatment in DTH model.

Panels show the relative mRNA levels of TGF-β (A), IL-4 (B), IL-10 (C), IFN-γ (D) and FoxP3 (E) normalized to β-actin mRNA level in the treated-side popliteal LNs of mice 48 hr post-treatment with EL4 exosomes, EG7 exosomes or PBS. Significance at: **, $P<0.01$; *, $P<0.05$; NS, not significant.

2.4.6 Tumor-derived exosomes down-regulate BMDC maturation markers and induce TGF-β production *in vitro*

DCs play an essential role in antigen-presentation and the initiation of antigen-specific immune responses. It was reported that tumor-derived exosomes can inhibit the differentiation of myeloid precursors into bone marrow-derived DCs (BMDCs) (148). Given that tumor-derived exosomes were mostly internalized by DCs after local injection in our DTH model, we asked whether these vesicles could also affect the function of differentiated DCs. Therefore the effect of exosomes on cultured BMDCs was examined. Briefly, day 8 BMDCs were treated with 10 μg/ml of exosomes for 3 days and the expressions of MHC class II molecules and CD86 were determined by FACS analysis. Interestingly, treatment with different exosomes all resulted in down-regulation of MHC class II molecule ($I\text{-}A^b$) and the co-stimulatory molecule CD86 on DCs (Fig. 11A). Furthermore, exosome treatment induced TGF-β1 production in DC culture (Fig. 11B). TGF-β1 levels in exosome preparations were also measured and were around 10-15 pg per 10 μg of exosomes (Fig. 11C), which was significantly less than the total amount increased in DC culture supernatant, indicating that TGF-β1 is produced by DCs in response to exosome treatment. These results demonstrate the inhibitory effect of tumor-derived exosomes on DC maturation *in vitro*, and such effect was observed on exosomes derived from both OVA-expressing and OVA-negative tumor cells.

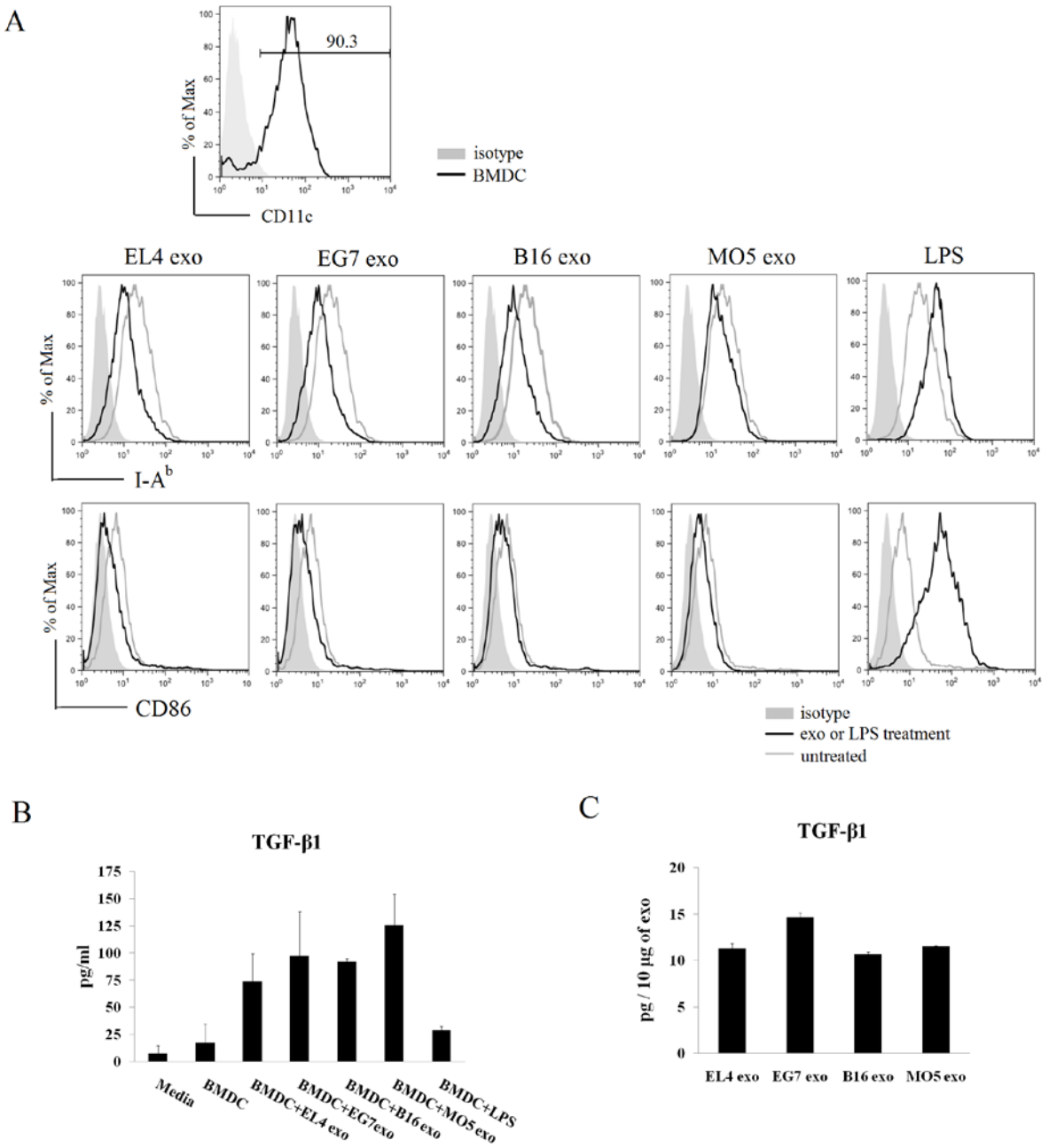


Figure 11: Tumor-derived exosomes inhibit BMDC maturation and induce TGF-β1 production.

(A). Day 8 BMDCs (purity > 90%) were treated with 10 µg/ml of different exosomes or left untreated for 3 days. The expression of I-A^b and CD86 were analyzed by FACS. LPS treatment for 24 hr (1 µg/ml) was used as a DC maturation control. (B). TGF-β1 protein levels in BMDC culture supernatants after exosome treatment. Data show the mean values of two independent experiments ± SD. (C). TGF-β1 protein contents in exosome preparations (per 10 µg of exosomes). For each exosome sample, data show the mean values of three preparations ± SD.

2.4.7 Tumor-derived exosomes alone do not activate T cells in vitro

Besides DC internalization, exosomes may also have the opportunity to interact directly with T cells after local injection, thus we tested the T cell response to direct exosome treatment *in vitro*. CD4+ or CD8+ T cells were purified from the splenocytes and LN cells of OVA-immunized mice and treated with either EL4 exosomes or EG7 exosomes or left untreated. Direct binding of PKH67 labeled exosomes to both CD4+ and CD8+ T cells were observed by fluorescent microscopy and FACS analysis (Fig. 12). Treatment with exosomes for 48 hr showed no effect on the expression of T cell activation markers CD69, CD44 and CD62L, compared with untreated control (Fig. 13A). In addition, to examine if exosomal OVA antigen can directly activate antigen-specific T cells, the MHC class I-restricted OVA-specific T cell hybridoma 33.70 cells were treated with exosomes for 48 hr and were found unresponsive to either EG7 exosomes or MO5 exosomes, while they were well stimulated by DC2.4 cells in the presence of SIINFEKL peptide (Fig. 13B). These results show that here T cells do not directly respond to tumor-derived exosome treatment *in vitro,* and suggest that exosomes may affect T cell activity and exert suppressive effect via DCs *in vivo.*

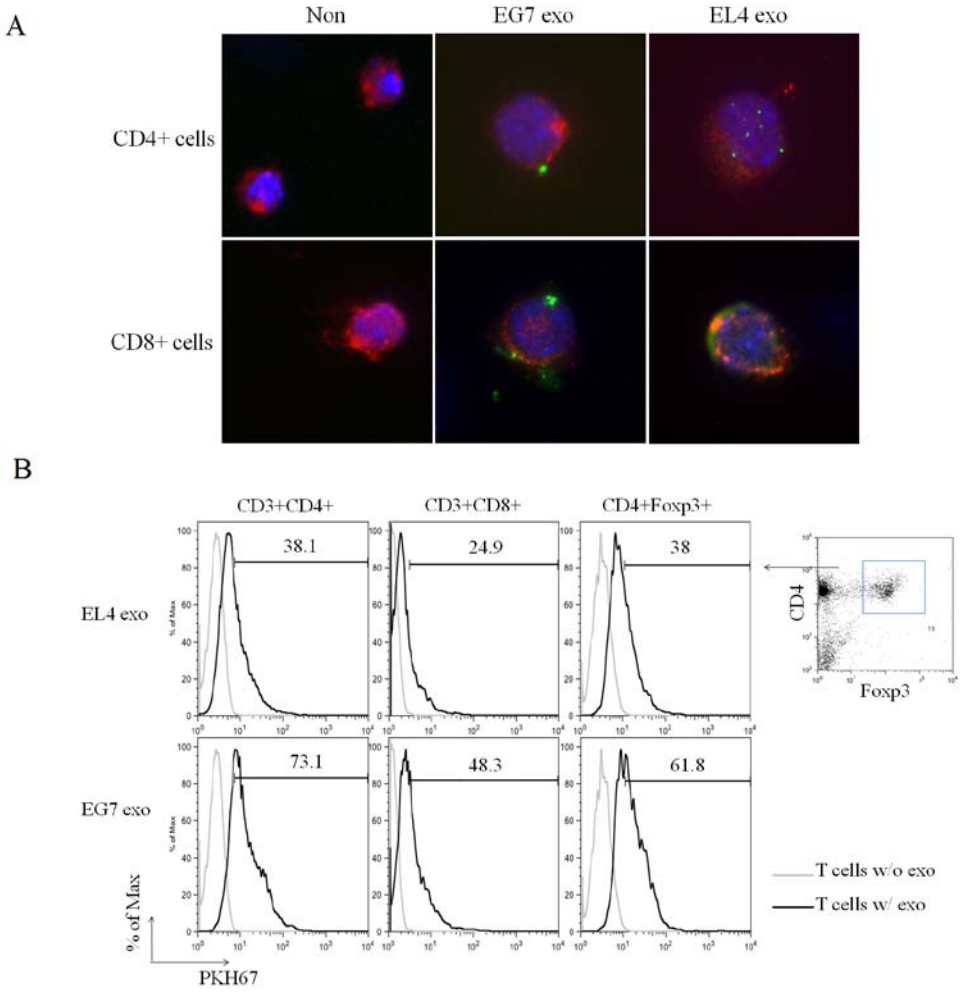


Figure 12: Direct binding of exosomes to T cells *in vitro*.

CD4+ or CD8+ T cells purified from the splenoctyes and LN cells of OVA-immunized mice were co-incubated with PKH67-labeled EG7 or EL4 exosomes for 18 hours. (A). Cells were washed, cytospined, and stained for CD4 or CD8 (red). Exosome (green) binding was examined under fluorescent microscope. Magnification: 100×. (B). Cells were washed and the percentage of PKH67+ cells in CD3+CD4+ T cells, CD3+CD8+ T cells and CD4+Foxp3+ Treg cells were analyzed by FACS.

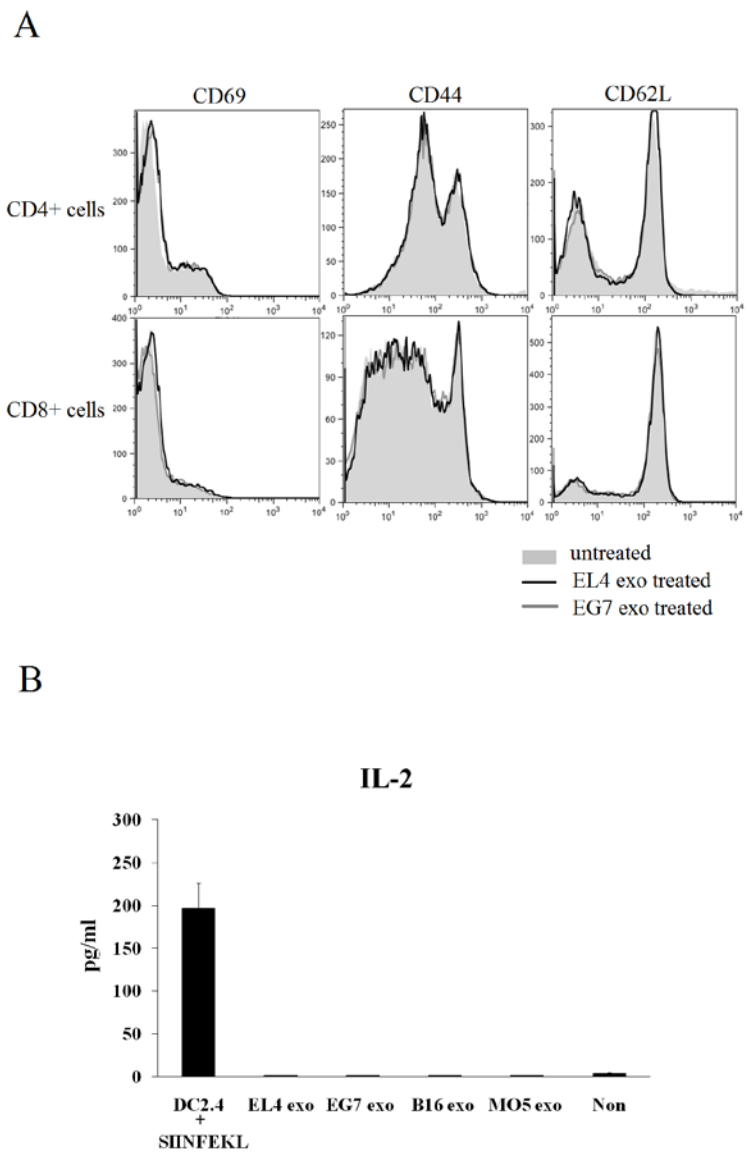


Figure 13: Direct treatment with tumor-derived exosomes has no effect on T cell activation *in vitro*.

(A). CD4+ and CD8+ T cells were purified from the splenocytes and LN cells from OVA-immunized mice, and treated with EL4 exosomes, EG7 exosomes or left untreated for 48 hr. The expression of CD69, CD44 and CD62L on T cell subsets were analyzed by FACS. (B). OVA-specific T cell hybridoma 33.70 (H-2K^b restricted) were co-cultured with different tumor-derived exosomes for 48 hr or stimulated with DC2.4 cells with SIINFEKL peptide for 24 hr. Activation of 33.70 cells were determined by measuring the IL-2 production in culture supernatants.

2.5 DISCUSSION

Whether tumor-derived exosomes carrying tumor antigen are immunostimulatory or immunosuppressive can be controversial according to existing research reports (129, 132-135) (131, 146-149). Although increasing evidence is showing that tumor-derived exosomes down-regulate anti-tumor immunity by a variety of mechanisms, the role of tumor antigens secreted in the form of exosomes in exosome-mediated-immunoregulation is still unclear. Whether tumor-derived exosomes induce antigen-specific immune suppression also has not been well-documented. In the present study, we demonstrated that exosomes derived from tumor cells stably expressing a model antigen were able to suppress the inflammatory response induced by that antigen. The effective suppression seems to require exosomal antigen and is specific to the immune response induced by that particular antigen.

DTH response is an important manifestation of cell-mediated immunity and allows for rapid assessment of antigen-specific immune responses and immune suppression. We have previously demonstrated the suppressive effect of exosomes derived from genetically modified DCs expressing exogenous IL-10, IL-4 or FasL in DTH and CIA model, showing that DC-derived exosomes can be potent therapeutic agents to suppress unwanted inflammatory response (157, 158, 209). Here we tested the immunosuppressive effect of tumor-derived exosomes in a similar DTH model. In the two pairs of tumor cell lines used for exosome production, the EG7 and MO5 cells stably express OVA antigen and secrete full-length OVA protein into exosomes. Interestingly, we observed down-regulation of OVA-specific DTH response when introducing EG7 or MO5 exosomes in the elicitation phase, whereas KLH-specific DTH response was not suppressed by these two exosomes.

To better understand the underlying mechanism of DTH suppression induced by tumor-derived exosomes, we examined the fate of exosomes after injection and their interaction with immune cells by trafficking analysis. We found that exosomes injected locally were predominantly internalized by skin DCs and migrated to draining LN, where further interaction with other immune cells especially T cells would occur. We also examined the cytokine expression profile in the draining LN and found that the suppressive effect is associated with the up-regulation of TGF-β and IL-4 mRNA synthesis. TGF-β is known to block the activation of lymphocytes and monocytes, and can convert effector T cells into Tregs (210). It also has been shown to inhibit the elicitation of murine DTH response when injected up to 8 hr after antigen challenge (191). TGF-β was also shown to be responsible for antigen-specific down-regulation of Th1-type responses and the generation of a suppressive Th2-type response (211). The Th2 cytokine IL-4 can suppress Th1-type response and we have previously reported that it plays an important role in suppressing autoimmune disease in murine models of DTH, arthritis and type I diabetes (209, 212, 213). IL-4 also has been shown to support the differentiation of TGF-β-producing cells (214). Our result shows that elevated IL-4 and TGF-β mRNA expression was correlated with the suppression of OVA DTH response. Although the exact cell type(s) producing these cytokines remains to be determined, these cells seem to be activated only by OVA-containing exosomes, but not by OVA negative exosomes.

Based on the observation that exosomes were mostly taken up by DCs, we further examined the effect of exosomes on DCs *in vitro*. Indeed, we found that treatment with tumor-derived exosomes could down-regulate the expression of MHC class II molecules and CD86 on BMDCs and induce TGF-β production (Fig. 11). Inhibition of DC differentiation from BM precursors by tumor-derived exosomes has been reported (148), our findings further suggest that

spontaneous maturation of differentiated CD11c+ DCs can also be inhibited by tumor-derived exosomes and they may be able to predispose DCs to acquire a potentially suppressive or tolerogenic phenotype *in vivo*. Therefore, we propose DCs play an important role in exosome-induced DTH suppression. One possible mechanism is that DCs preferentially acquire exosomal OVA antigen for reprocessing and present antigen in favor of the activation of antigen-specific Tregs. Although no significant increase in Foxp3 mRNA was observed after EG7 exosome treatment (Fig. 10), the percentage of CD4+Foxp3+ Treg cells usually increases in both splenocytes and LN cells after OVA immunization (data not shown), and it is possible that the existing OVA-specific Tregs were better activated by DCs presenting exosomal OVA antigen. It is also possible that regulatory cells other than Fox3+ Tregs were involved (193).

Interestingly, although EL4 and B16 exosomes showed similar effects on DCs *in vitro*, they were not as effective in suppressing the OVA-specific DTH response as EG7 and MO5 exosomes. This may be due to the different immunogenicity between the recall OVA antigen and exosomal OVA. The crude OVA protein (grade II) used for DTH elicitation is highly immunogenic (215) (and our observation). Whereas soluble OVA in the form of exosomes could be easily acquired by DCs while exosomes condition DCs toward a suppressive phenotype at the same time. This is also reflected by the ineffectiveness of both sets of exosomes in suppressing KLH-specific response, which seems to further suggest that exosomal antigen is responsible for suppression. From this point of view, our results provide an indirect evidence that exosomal tumor antigen could be more tolerance-inducing.

A direct effect of tumor-derived exosomes on primed T cells or antigen-specific T cell hybridoma was not observed in our experiment setting (Fig. 13). Although the two pairs of tumor cell lines differ in their immunogenicity, their exosomes were found all express low to

undetectable levels of MHC molecules, and therefore should lack the ability to present the OVA epitope in the MHC context on their surface (Fig. 5). In fact, even for DC-derived exosomes which usually express MHC-antigen complex, they need recipient DCs to efficiently stimulate naïve or specific T cells (155, 216). Thus in our DTH model, although exosomes may have the opportunity to directly encounter memory T cells after local injection, exosomes alone may not be able to induce their activation. In the DTH experiment, EG7 and MO5 exosomes treatment did not completely block the DTH response but partially reduced the reaction magnitude (Fig. 5), suggesting that memory CD4+ T cells were still activated and began recruiting effector cells after antigen challenge, and the partial inhibition of the response is probably due to more efficient activation of regulatory cells and/or Th2 polarization in mice received OVA-containing exosomes.

The immune regulation associated with tumor growth is a complex, long-term process and we do not want to conclude that the behavior of tumor-derived exosomes in tumor-bearing hosts is exactly reflected in this DTH model. As a unique “non-self” antigen, OVA is more relevant to TSAs that arise from mutations of endogenous proteins. Actually, local injection of enriched exosomes may facilitate their effective interaction with immune cells. However, it may reflect a particular stage of tumor progression when tumor-derived exosomes have abundantly accumulated, and tumor-specific memory T cells and Treg cells co-exist in the tumor microenvironment. It is possible that APCs sampling exosomal tumor antigens would preferentially activate tumor-specific Treg cells rather than effector T cells. Indeed, tumor-derived exosomes may represent a key source of tumor antigens, especially when there is no extensive tumor cell death caused by therapeutic intervention. The presence of exosomal tumor

antigens in a tolerogenic microenvironment, which can be created by both tumor cells themselves and immunosuppressive vesicles, could lead to tumor-specific immune suppression.

Our results also demonstrate the possibility of utilizing tumor-derived exosomes containing certain antigen to suppress antigen-specific inflammatory response. However, it is important to note that the nature of antigen and the way how it is presented on the vesicles may affect their immunogenicity, as it was reported that tumor-derived exosomes engineered to express membrane-exposed antigen are more immunogenic than those bearing a soluble form of the same antigen (139). Finally, further studies addressing the protein or protein complex expressed by tumor-derived exosomes that contribute to their suppressive effect will be of great importance.

Taken together, our studies demonstrate that local administration of tumor-derived exosomes bearing a particular model antigen can reduce antigen-specific inflammatory response, possibly via DC-mediated mechanism. This finding suggests a novel role of tumor antigens released in the form of tumor-derived exosomes in mediating tumor-specific immunosuppression. The fact that tumor-derived exosomes appear to work in an antigen-specific manner could also make them attractive therapeutic agents comparing to the approaches that generate global immune suppression.

3.0 CHARACTERIZATION OF PLASMA-DERIVED EXOSOMES FROM TUMOR-BEARING MICE AND THEIR IMMUNOSUPPRESSIVE EFFECTS

3.1 ABSTRACT

Exosomes are small membrane vesicles that are formed within the late endocytic compartment and are secreted by a wide variety of cell types, including hematopoietic cells, endothelial cells as well as tumor cells. In recent years, the role of exosomes as potential mediators of cell-to-cell communication was being recognized. In this study, we examined exosome-like vesicles isolated from the blood plasma of tumor-bearing mice or naïve mice and investigated their ability to regulate immune responses. We demonstrated that plasma-derived exosomes from mice bearing OVA-expressing tumor, but not from naïve mice or mice bearing OVA-negative tumor, were able to suppress the OVA-specific DTH response. To determine if tumor-derived exosomes are accumulated in the circulating blood of tumor-bearing mice, we developed an exosome-tagging method by fusion of FLAG epitope with the Lactadherin C1C2 domain, and generated stable tumor cell lines that produce FLAG-tagged exosomes. However, in mice bearing either subcutaneous or metastatic FLAG+ melanoma cells, enrichment of FLAG+ tumor-derived exosomes in plasma exosome fractions was not observed. Meanwhile, expression of MHC class I and class II molecules, CD11b, B220, CD22 and CD9 were found on plasma-derived exosomes, suggesting that they are possibly released by hematopoietic immune cells. Finally, depletion of

the MHC class II+ vesicle portion in plasma-derived exosomes resulted in partial abrogation of the suppressive effect. We hypothesize that exosomes in the plasma of tumor-bearing hosts serve to dampen tumor-specific immune responses.

3.2 INTRODUCTION

Cells communicate with each other by different mechanisms, including direct cell contact mediated by adhesion molecules, secretion of protein factors (e.g. cytokines, chemokines and growth factors) and small molecules (e.g. nucleotides, nitric oxide), and nanotubular transporting of intercellular organelles (217-219). In addition, the release of small membrane vesicles represents another important, but underappreciated way of cell-to-cell communication (124).

Circulating microparticles with membrane structures include apoptotic bodies, microvesicles shed from the surface of a blebbing plasma membrane, and exosomes secreted from the intracellular endocytic compartment. Studies have shown that microparticles in blood circulation are of pathophysiological relevance, and vesicles of particular cellular origin are elevated in different diseases such as cancer (leukocyte-derived microparticles) (220), congestive heart failure (endothelial microparticles) (221), and thrombosis (platelet-derived microparticles) (222). Pregnant women have increased numbers of placenta-derived microparticles, and in the situation of preeclampsia, the number of monocyte-derived microparticles increases, possibly reflecting systemic inflammation (223, 224).

Among the different types of microparticles, exosomes represent a population of vesicles with relatively small size (30-120 nm in diameter) and their effects on immune modulation have been extensively studied. Exosomes are formed in the intracellular multivesicular bodies

(MVBs) and are released into the extracellular milieu upon the fusion of MVBs with the plasma membrane. They express selective intracellular proteins and surface markers of the parental cells, and contain mRNA and microRNA. Exosomes are secreted by most hematopoietic cells, including dendritic cells (DCs), B cells, T cells, and platelets. They are also released from tissue sources such as endothelium and placenta. Exosomal-like vesicles are found present in the blood plasma/serum of normal human individuals (162, 225). These plasma vesicles are enriched in the tetraspanin molecules CD9, CD81 and CD63 as well as MHC molecules; however, their exact biological activity and the composition of cells secreting these vesicles remain vague. Exosome-like vesicles are also found present in various biological fluids such as ascites (226), bronchoalveolar fluid (164, 165), urine (227, 228), saliva (229), epididymal fluid (168), amniotic fluid (169) and breast milk (170).

Circulating plasma-derived exosomes represent an easy and abundant source of exosomes that are systemically present *in vivo*. One of the major research focuses of plasma-derived exosomes is to use them as cancer diagnostic markers. Investigation of the application of plasma-derived exosomes in tumor diagnosis is based on the findings that plasma-derived exosomes from certain cancer patients express tumor-associated antigens, and/or have distinct microRNA profiles comparing to those of normal individuals. For example, ovarian cancer-associated expression of claudin proteins can be detected in the peripheral circulation of a majority of ovarian cancer patients (230); in breast cancer patients increasing levels of circulating particles expressing carcinoembryonic antigen and the cancer antigen 15-3 correlated with increasing size of tumors (220); exosomal microRNA from ovarian cancer patients exhibit significantly distinct profiles from that observed in benign ovarian disease (118); and in glioblastoma patients, mRNA

variants and microRNAs characteristic of gliomas could be detected in serum microvesicles (117).

Several studies have suggested a tolerogenic role of plasma-derived exosomes. Early reports showed that a transferable tolerogenic factor can be isolated by ultracentrifugation from rat serum after antigen feeding, and this factor was later found to have exosome characteristics and mediate oral tolerance (231, 232). In mice, serum exosomes isolated after antigen feeding were able to transfer antigen-specific tolerance when injected into naive recipients and suppress a Th1-dominated DTH response (160). Such exosome-mediated tolerance is MHC class II-dependent and requires an intact immune system in the fed donor (172). Moreover, it was also shown that serum-derived exosomes from antigen-fed mice abrogated allergic sensitization in a model of allergic asthma when exosomes were administered i.p. to naïve recipient mice, indicating their ability in suppressing Th2-type responses (233). It is suggested that intestinal epithelial cells are responsible for producing these tolerogenic exosomes, and antigen-specific regulatory T cells are induced by exosomes in the recipients (160, 231, 232). Furthermore, we have previously demonstrated that MHC class II+ plasma-derived exosomes isolated from antigen-sensitized mice induce antigen-specific suppression in a murine DTH model (171).

Plasma/serum-derived exosomes with suppressive functions were also found in cancer patients. Exosome-like microvesicles isolated from the sera of patients with oral cancer express FasL and induce apoptosis of activated T lymphocytes (234). Similarly, microvesicles derived from the sera of head and neck cancer patients, but not from the sera of normal controls, induce apoptosis of a cultured T cell line; and higher FasL expression levels were found on vesicles derived from patients with active diseases than vesicles derived from patients with no evident disease or normal controls (235). However, whether plasma-derived exosomes play a role in

tolerizing tumor antigens in tumor-bearing hosts is unclear. Moreover, although many reports view these suppressive plasma vesicles as circulating tumor-derived exosomes, their exact cellular origin(s) and functional vesicle portion(s) remain an unresolved question.

In this study, we investigate the ability of plasma-derived exosomes from tumor-bearing mice in regulating the immune response specific to the model tumor antigen OVA, and examined the presence of tumor-derived exosomes in plasma exosome preparations using a FLAG-tagging system. We demonstrate that plasma-derived exosomes from mice bearing OVA-expressing tumors can induce immune suppression of OVA-specific DTH response. We also demonstrate that enrichment of tumor-derived exosomes in blood circulation is not observed in a murine melanoma model and may not be necessary for the suppressive effect, which is at least in part, dependent on the presence of MHC class II+ vesicles.

3.3 MATERIALS AND METHODS

3.3.1 Cell lines

The C57BL/6 background thymoma cell line EL4 and melanoma cell line B16-F0 (B16) were obtained from American Type Culture Collection. The EL4-OVA (EG7) and B16-OVA (MO5) cell lines were generously provided by Dr. Walter Storkus (University of Pittsburgh). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2mM of L-glutamine, 0.1mM of non-essential amino acids, 1 mM of sodium pyruvate, 10mM of HEPES, Antibiotic-Antimicotic (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B,

GIBCO), and 50 μM of 2-ME. The EG7 and MO5 cell lines were under G418 selection (0.8 mg/ml and 1.5 mg/ml, respectively).

3.3.2 Mice

Female C57BL/6 mice (H-2K^b) were purchased at 6-8wk of age from The Jackson Laboratory. Animals were maintained in a pathogen-free animal facility at University of Pittsburgh Biotechnology Center. All animal experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee.

3.3.3 Tumor inoculation and blood collection

Tumor cells for inoculation were tested to be free of pathogens. Cells were prepared by washing with PBS and counted using trypan blue exclusion. Tumor cells (2.5×10^5 or 5×10^5 cells in a total volume of 100 μl of PBS) were injected subcutaneously (s.c.) in mice abdomen or otherwise stated. Tumor size was measured using a metric caliper (Fisher Scientific) and tumor volume (in mm^3) was calculated as: $V= 0.5\times a\times b^2$ (a: long diameter; b: short diameter). Mice were sacrificed when they have excessive tumor size (> 20 mm in diameter) or tumor ulceration. Blood was collected at indicated time points. Approximately 0.5 - 0.8 ml of whole blood was collected from each mouse by submandibular bleeding (236). Briefly, mice were first anesthetized by i.p. injection of 200-250 μl of 2.5% Avertin (Sigma-Aldrich), and the cheek was poked with a lancet (Goldenrod 5 mm animal lancet, MEDIpoint) at the position where the retro-orbital and submandibular veins join at the origin of the jugular vein. The drops of blood exude from the point of penetration were collected into a microtube with 150 μl of 0.05% of EDTA as

anticoagulant. Blood was also obtained from naïve mice of a similar age. Blood plasma was separated from blood cells by centrifugation in tabletop micro-centrifuge at 10,000 × g for 20 min at 4°C. Because of the small volume of blood plasma (~ 200 - 400 ul) that can be obtained from a single mouse, blood plasma obtained from mice in the same group were pooled together for exosome isolation.

3.3.4 Exosome purification from blood plasma or culture supernatant

To isolate plasma-derived exosomes, blood plasma was centrifuged at 10,000 × g for 20 min at 4°C for 3 times and any sediment was discarded. Plasma from mice inoculated with the same tumor cells were pooled and subjected to ultracentrifugation at 100,000 × g for 1.5-2 hr. Pellets were washed with sterile PBS and centrifuged again at 100,000 × g for 1.5 hr. Exosome pellets were then resuspended in sterile PBS. To isolate exosomes from culture supernatant, FBS used in culture media was pre-cleared by ultracentrifugation at 100,000 × g for 3 hr at 4^0C. 48 hr culture supernatants were centrifuged at 1000 × g for 10 min and 10,000 × g for 30 min to remove cells and cellular debris, filtered through 0.22 μm sterilizing filter (Corning), concentrated using Centricon Plus-70 (100 kDa cutoff) filter units (Millipore), and subjected to ultracentrifugation at 100,000 × g for 1.5 hr. Exosome pellets were washed with sterile PBS, ultracentrifuged again and resuspended in sterile PBS. Protein concentration was quantified by Bradford protein assay (Bio-Rad).

3.3.5 Electron Microscopy

Exosome preparations (5 μl of ~0.1-0.5 μg/μl) were loaded on Formvar/carbon-coated grids and negatively stained with 1% uranylacetate. Photos were taken on a JEM-1011 transmission electron microscope with Advanced Microscopy Techniques (AMT) software.

3.3.6 Induction of OVA-specific DTH response and exosome treatment

8-9 wk old mice were immunized with 150 μg of ovalbumin (OVA, grade V, Sigma) 1:1 emulsified in Freund's complete adjuvant (CFA, Pierce) by intradermal (i.d.) injection at the tail base. 14 days later, mice were boost immunized with 50 μg of OVA (grade V, Sigma) 1:1 emulsified in Freund's incomplete adjuvant (IFA, Pierce). After 7 days, the right hind paw was injected i.d. with 10 μg of plasma-derived exosomes along with 30 μg of OVA (grade II, Sigma) in a total volume of 50 μl of PBS. The left hind footpad was injected with 30 μg of OVA antigen alone. Footpad thickness was measured using a spring-loaded caliper (Dyer) before, 24 and 48 hr after challenge. Paw swelling was determined by the increase in footpad thickness. Each set of experiments was performed with 5 mice per group and repeated at least twice.

3.3.7 Histology

48 hr post-antigen challenge, the right hind paws (treated paws) were removed and fixed in Shandon Glyo-Fixx (Thermo-Fisher Scientific) for at least 48 hr. The fixed tissues were placed in Shandon TBD-2 decalcifier, formic acid 26%, for 72 hr to decalcify the bones. Tissue was then processed in the Shandon Citadel 1000 Tissue Processor for overnight according a standard

protocol. The paraffin-embedded samples were sectioned (5 μm), deparaffinized, stained with hemotoxylin and eosin, and examined for infiltrating mononuclear cells under the microscope. Pictures were taken on an Olympus Provis microscope with the MagnaFire software.

3.3.8 Lactadherin-FLAG construct and retroviral packaging

The cloning vector p6mLSC1C2 was a generous gift from Dr. Alain Delcayre (ExoThera L.L.C.). It contains the mouse Lactadherin leader-sequence (LS) and C1C2-coding sequence between the Hind III and BstB I sites of pcDNA6-Myc/His (Invitrogen). A BsmB I cloning site is used to insert genes between the LS and C1C2. BsmB I digestion produces non-complementary overhangs for gene insertion (119). For FLAG epitope (DYKDDDDK) insertion, the plasmid was digested with BsmB I, treated with Antarctic phosphatase and then enzymatic reaction cleanup (ERC) kit (Qiagen). The annealed oligos 5'-GTCTATGGATTACAAGGATGACGACGATAAG-3' and 5'-CGGTCTTATCGTCGTCATCCTTGTAATCCAT-3' were ligated into the digested plasmid. The ligation products were transformed into DH5α competent cells (Invitrogen) and selected on LB/Amp plates, and the plasmid DNA were purified by MiniPrep (Qiagen). Successful incorporation of the FLAG oligos were confirmed by BsmB I digestion for the elimination of the BsmB I site. Linear Lactadherin-FLAG construct were cloned by PCR of the MaxiPrep (Qiagen) product of the inserted plasmid using the following primer: forward 5'-TAGCGAATTCATGCAGGTCTCCCGTGTGCTG-3' and reverse 5'-TAGCGTCGACTTAACAGCCCAGCAGCTCCAG-3', which also added 5' EcoRI site and 3' SalI site. Sequencing was performed to confirm the insertion orientation of FLAG oligo as well as the accuracy of PCR replication. The PCR products were cleaned by ERC kit, digested with

EcoRI and SalI separately, and cleaned again. Meanwhile, the pBABE-Y-puro vector was digested with EcoRI and SalI, treated with Antarctic phosphatase and cleaned with ERC kit. The Lactadherin-FLAG construct were then ligated into the vector, transformed into DH5α competent cells, select and expanded in LB/Amp media. For retroviral packaging, HEK293T cells were co-transfected with pAmpho and pBABE-puro with the Lactadherin-FLAG insertion using the Lipofectamine 2000 protocol. The retroviral supernatants were collected 48 hr post-transfection, centrifuged twice at 300 × g for 5min to remove cellular debris, and stored at -80°C.

3.3.9 Infection of tumor cells with retroviruses

B16 and MO5 cells growing in 10 cm dishes at around 30% confluence were infected with 2 ml of retroviral supernatants in the presence of 4 μg/ml of polybrene. 24 hr post-infection, the old media were replaced with fresh media. Puromycin selection began at 48 hr post-infection. Cells were first selected with 1.5 μg/ml of puromycin for 2 days and stably infected cells were then maintained in media with 1 μg/ml of puromycin.

3.3.10 Western Blotting

Exosomes (10 μg of proteins) were denatured in Laemmli sample buffer with 2-ME, separated on 12% or 10% SDS-PAGE, transferred onto PVDF membranes (Millipore), blocked with 5% milk-PBST and incubated with different primary antibodies, followed by washing and the application of HRP-conjugated secondary antibodies. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection kit (PerkinElmer Life Science). The primary antibodies used include purified anti-mouse CD11b, anti-mouse CD22, anti-mouse B220 and

anti-mouse H-2K^b/H-2D^d from Biolegend, and anti-I-A^b from BD. The secondary antibodies used include HRP-linked anti-mouse IgG (Santa-Cruz) and HRP-linked anti-rat IgG (Cell Signaling). For FLAG detection, the HRP-linked monoclonal anti-FLAG M2 antibody (Sigma) was used and the blots were developed by ECL kit directly.

3.3.11 ELISA

For FLAG detection, exosome samples were incubated in Anti-FLAG M2 coated plate (Sigma) (100 µl/well) at 4^{0}C for overnight. Anti-FLAG-BioM2 (Sigma, 2-3 µg/ml) was used as detection antibody, followed by Avidin-HRP and TMB substrate solution (eBioscience). The reactions were stopped by 2N H_2SO_4, and the plate was read at 450 nm. FLAG+ tumor-derived exosomes diluted into different concentrations were used as standard. For the detection of MHC class II molecules, anti-mouse I-A^b (KH74, Pharmingen, 4 µg/ml) was used as capture antibody, exosome samples (10 µg in 50 µl) was incubated at 4^{0}C for overnight, and biotin-anti-mouse I-A^b (Biolegend, 2 µg/ml) was used as detection antibody.

3.3.12 Exosome MACS depletion

200 µg of plasma-derived exosomes were incubated with 50 µl anti-MHC class II MACS beads, anti-CD11b MACS beads or goat anti-mouse IgG MACS beads (Miltenyi Biotec) in a total volume of 500 µl for 1 hr at 4^{0}C with rotation. Samples were then applied on MS column and washed with 500 µl PBS for 5 times. The flow-through portions were collected and exosomes were retrieved by ultracentrifugation at 100,000 × g for 2 hr.

3.3.13 Statistics

DTH results with three or more groups were analyzed by one-way ANOVA with Fisher's post-hoc test using the SPSS statistical software (SPSS). Results between two groups were analyzed by Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

3.4 RESULTS

3.4.1 Isolation of exosome-like vesicles from mouse blood plasma

To investigate the immunoregulatory effect of blood borne exosome-like vesicles in tumor-bearing hosts, exosomes-like vesicles were isolated from the blood plasma of tumor-bearing mice. Four groups of tumor-bearing mice were generated by s.c. inoculation of different tumor cells in mice abdomen: the thymoma cell line EL4, the melanoma cell line B16, and their respective OVA-expressing derivative cell lines EG7 and MO5. Blood was collected 3 weeks after tumor inoculation or from naïve control mice at a similar age, and plasma-derived exosomes were isolated by ultracentrifugation (Fig. 14A). The recovered vesicle fractions were examined by electron microscopy. Plasma-derived exosomes from either tumor-bearing mice or naïve mice showed typical flattened membrane vesicular structure of 50-100 nm in diameter (Fig. 14B).

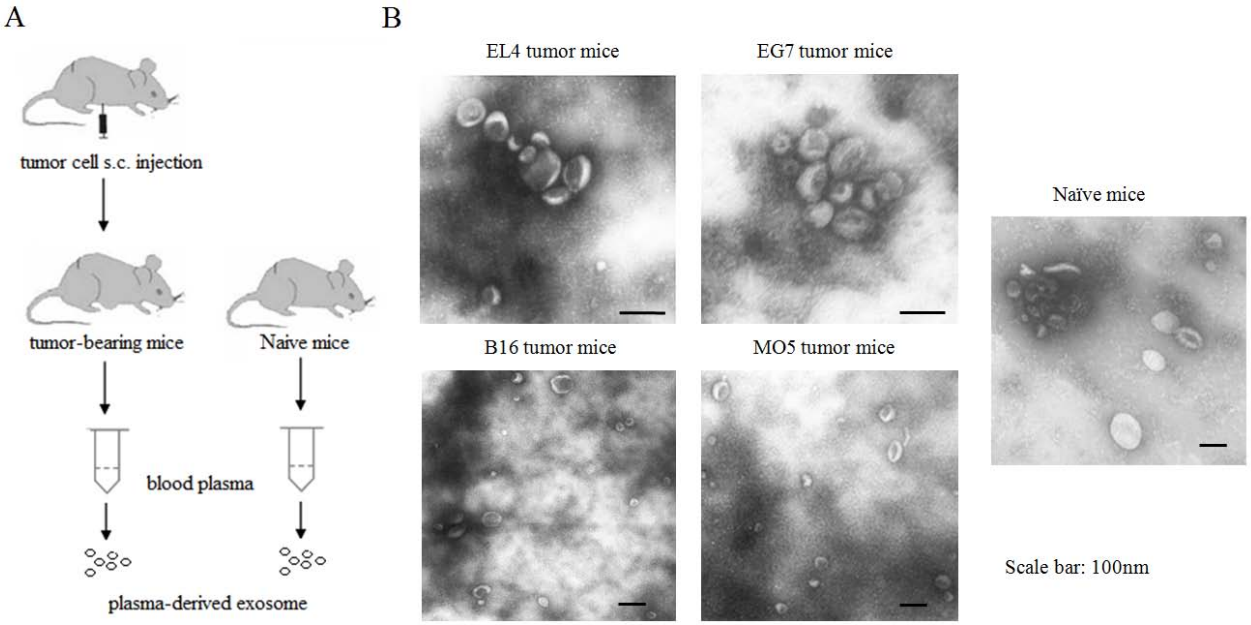


Figure 14: Plasma-derived exosomes isolated from tumor-bearing mice and naïve mice.

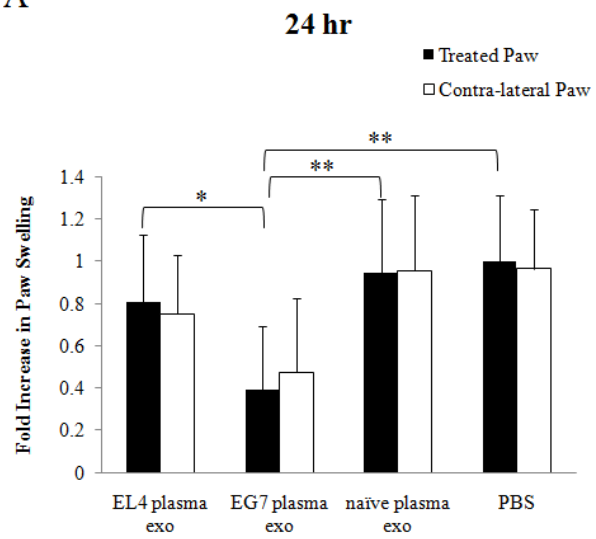
(A). Schematic diagram of plasma exosome isolation. (B). Electron microscopy of plasma-derived exosomes isolated from naïve mice or mice bearing subcutaneous EL4, EG7, B16, or MO5 tumor.

3.4.2 Plasma-derived exosomes from mice bearing OVA-expressing tumor can suppress OVA-specific DTH response

To determine whether plasma-derived exosomes from tumor-bearing mice can regulate anti-tumor immune responses, their ability in suppressing the immune response specific to a tumor antigen was tested in a mouse footpad DTH model. Mice pre-sensitized with OVA antigen were

injected with 10 μg of plasma-derived exosomes into the right hind paw by the time of OVA challenge at both hind paws. The magnitude of the DTH response was determined by measuring footpad swelling 24 and 48 hr post-challenge. Interestingly, treatment with plasma-derived exosomes isolated from EG7 or MO5 tumor-bearing mice significantly reduced paw swelling, indicating effective suppression of OVA-specific DTH response. In contrast, treatment with plasma-derived exosomes isolated from EL4 or B16 tumor-bearing mice, or naïve mice were not effective in suppression compared with PBS control (Fig. 15A-B). Comparable reductions of swelling in the untreated, contralateral paws were also observed in mice treated with EG7 or MO5 plasma exosomes. Correspondingly, much less mononuclear cell infiltration was observed in the footpad tissue of mice received EG7 or MO5 plasma exosome treatment 48 hr post-challenge, compared with those received EL4, B16 or naïve plasma exosome, or PBS treatment (Fig. 15C). These results demonstrate that local administration of plasma-derived exosomes isolated from mice bearing tumor cells expressing OVA antigen are able to suppress OVA-specific DTH response. Suppression was not conferred by plasma-derived exosomes isolated from naive mice or mice bearing OVA-negative tumors, suggesting that the suppressive effect has antigen specificity. Overall, blood-borne exosome-like vesicles seem to confer suppression of the immune responses against the antigen expressed by the *in vivo* growing tumor, an effect quite similar to that of tumor-derived exosomes as described in Chapter 2.

A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EG7 plasma exo
naïve plasma exo
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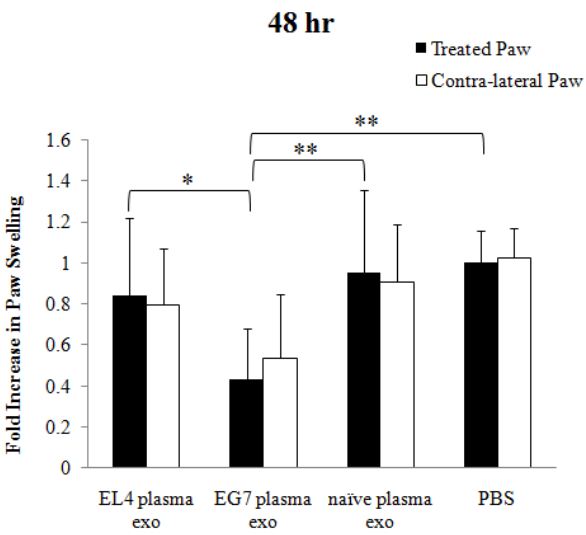
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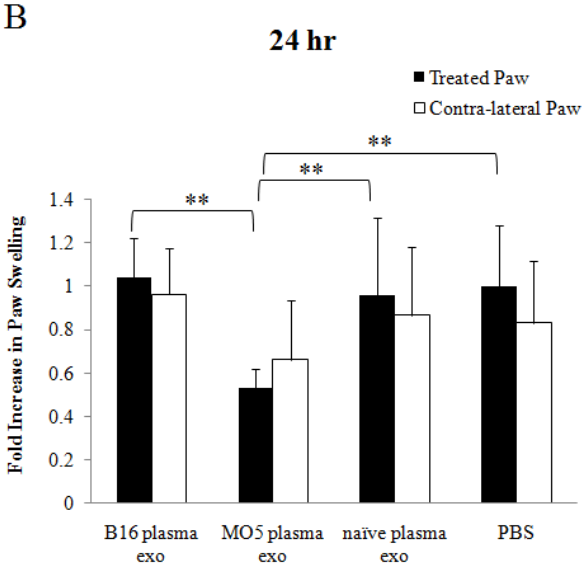
EL4 plasma exo
EG7 plasma exo
naïve plasma exo
PBS

B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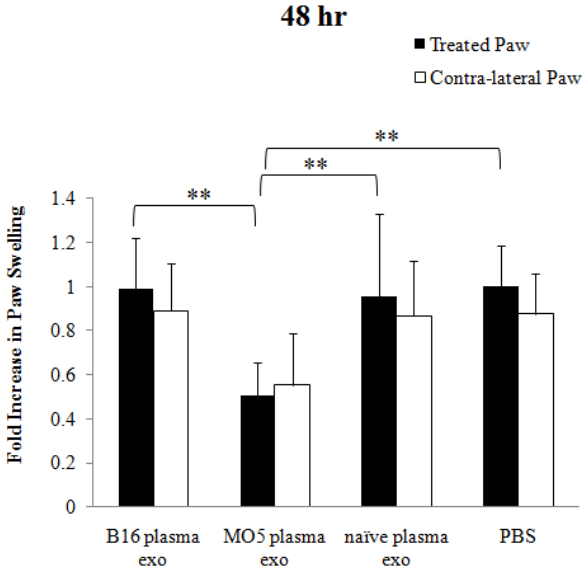
B16 plasma exo
MO5 plasma exo
naïve plasma exo
PBS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MO5 plasma exo
naïve plasma exo
PBS

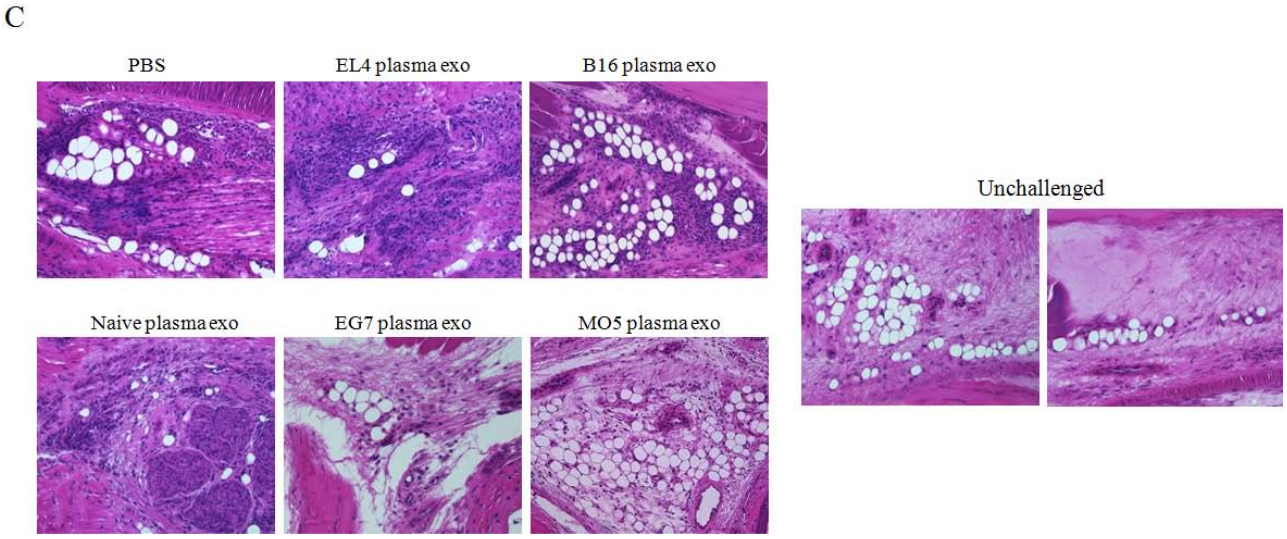


Figure 15: OVA-specific DTH response was suppressed by local administration of plasma-derived exosomes from mice bearing EG7 or MO5 tumor, but not by plasma-derived exosomes from naïve mice or mice bearing EL4 or B16 tumor.

OVA-immunized mice were injected with 10 μg of plasma-derived exosomes or PBS in their right hind paws at the time of OVA antigen challenge. Paw swelling was measured 24 and 48 hr post-challenge. The mean increase of footpad thickness of the treated paws (right paws) in PBS treated group at each time point was set to 1, and the increases of footpad thickness in other groups were normalized as fold increase. Data represent the pooled results of two independent experiments and are the means ± SD with 9-11 mice per group. Compared with PBS treated control, EG7 plasma exosomes and MO5 plasma exosomes significantly suppressed the OVA-specific DTH response (A-B). Significance at: **, $P<0.01$; *, $P<0.05$. (C). Histological analysis by H&E staining of the footpad tissue taken at 48 hr post-challenge. Mice treated with PBS, naïve, EL4 or B16 plasma exosomes show severe infiltration of mononuclear cells, whereas mice treated with EG7 or MO5 plasma exosomes show only mild to few mononuclear cell infiltration. Unchallenged footpad tissue was included as a control. Magnification: 20×.

3.4.3 Exosomes released by tumor cells were undetectable in plasma-derived exosomes isolated from mice bearing B16 or MO5 tumor

Exosomes in blood circulation comprise vesicles released by different cell types. Based on the similar suppressive effects of plasma-derived exosomes from tumor-bearing mice and tumor-derived exosomes as we previously observed, we asked whether tumor-derived exosomes are enriched in plasma exosome preparations. To facilitate the identification of tumor-derived exosomes, a FLAG tag was targeted to the membrane surface of tumor-derived exosome using the "exosome display" method (119). Briefly, a single FLAG tag was inserted between the coding sequence of Lactadherin LS and C1C2 domain, and the chimeric protein sequence was stably incorporated into B16 or MO5 tumor cells by retroviral infection (Fig. 16A). Infected B16 or MO5 tumor cells (B16-FLAG cells and MO5-FLAG cells) produce exosomes with FLAG-tagged Lactadherin expressed on their membrane surface. As shown by Western blotting, exosomes purified from the culture supernatant of B16-FLAG cells and MO5-FLAG cells express FLAG-lactadherin fusion protein at around 40-45 kDa (Fig. 16B). Strong FLAG signal was also detected on these exosomes by ELISA in a dose-dependent manner (Fig. 16C).

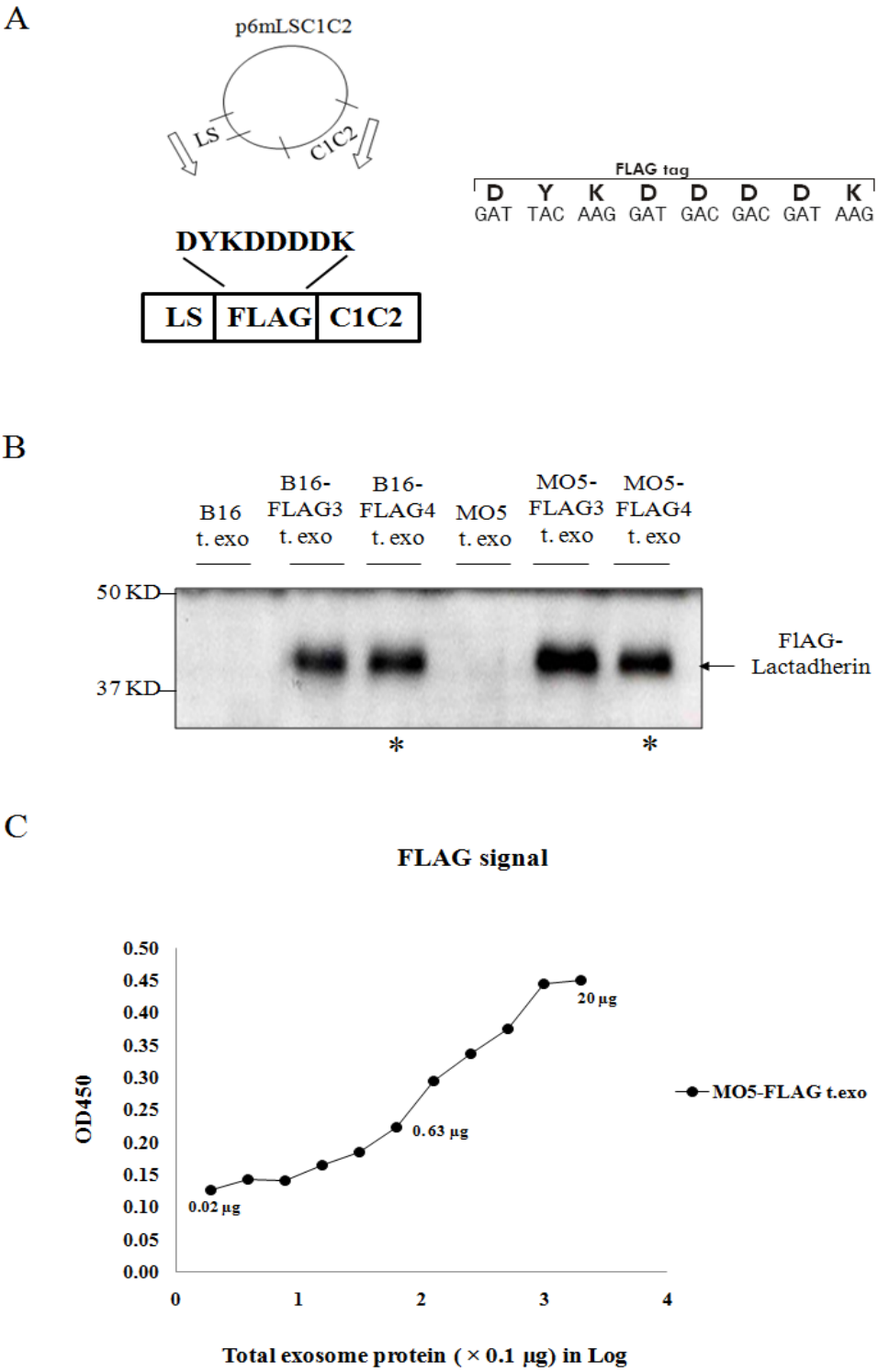


Figure 16: Generation of stable tumor cell lines producing FLAG-tagged exosomes.

(A). Schematic diagram of FLAG-Lactadherin construct. (B). Retroviral infection of B16 and MO5 tumor cell lines generated stable cell lines that constantly release FLAG-tagged exosomes. B16 and MO5 cells lines were both infected with two FLAG-Lactadherin retrovirus preparations

(FLAG3 and FLAG4, retroviruses packaged with two individual colonies). Western blotting for FLAG epitope shows the expression of FLAG-Lactadherin fusion protein on exosomes isolated from the culture supernatant of infected tumor cell lines. Exosomes derived from uninfected B16 and MO5 tumor cells were included as negative controls. 10 μg proteins were loaded in each lane. *: B16-FLAG4 and MO5-FLAG4 cell lines and exosomes will be used in the following experiments. (C). ELISA detection of FLAG epitope on exosomes derived from MO5-FLAG cells. MO5-FLAG tumor-derived exosomes were serially diluted by 2-fold (20 μg-0.02 μg) and the FLAG signals were shown as the value of OD450.

Next, to determine if tumor-derived exosomes accumulate in the peripheral circulation as tumor progresses, the presence of tumor-derived exosomes in plasma exosomes isolated from tumor-bearing mice at different tumor growth stages were examined. In a subcutaneous melanoma model, mice were inoculated with 5×10^5 B16-FLAG cells or B16 cells s.c. at the abdomen. Blood were obtained 5 days (when mice develop palpable tumors), 11 days (when averaged longer diameter of tumors reaches 5-6 cm) and 18 days post-inoculation (when averaged longer diameter of tumors exceeds 10 cm), and plasma exosomes were isolated respectively. However, Western blotting did not detect specific FLAG signal in B16-FLAG plasma exosomes compared with B16 plasma exosomes at all these time points (Fig. 17A). To examine if mice with metastatic melanoma have more enriched tumor-derived exosomes in circulation, mice were systemically inoculated with B16-FLAG cells or B16 cells by i.v. injection and plasma exosomes were isolated at the same time points. Similarly, specific FLAG signal was not detected in B16-FLAG plasma exosomes (Fig. 17B). In both cases, the presence of a non-specific protein band with a molecular weight similar to FLAG-Lactadherin interfered with FLAG detection. We then tested the detection limit of Western blot analysis in identifying

FLAG+ exosomes. B16-FLAG tumor-derived exosomes were serially diluted with either B16 tumor-derived exosomes or B16 plasma-derived exosomes (50%, 10%, 1% and 0.1% in 10 µg of total proteins). Western blotting was able to detect specific FLAG-Lactadherin band when FLAG+ exosomes were present at 10% or higher percentages in a total of 10 µg proteins. The signals obtained at 1% or 0.1% were considered non-specific (Fig. 17C). Therefore, the result of Western blot analysis suggests that in plasma-derived exosomes from mice bearing subcutaneous or metastatic B16 melanoma, the presence of tumor-derived exosomes is undetectable or the percentage is lower than 10%, even when the tumors progress to the late stage. To further confirm this result, plasma exosomes were isolated from mice bearing subcutaneous MO5-FLAG tumor in a similar experiment setting and the FLAG signal was examined by ELISA. MO5-FLAG tumor-derived exosomes mixed with naïve plasma exosomes at different percentages (10 µg, 5 µg, 2.5 µg and 1.25 µg in a total of 15 µg proteins) were used as positive controls. The result shows that MO5-FLAG plasma exosomes obtained on day 7, 14 and 21 after tumor inoculation give similar signals as naïve plasma exosomes (Fig. 17D). Taken together, these results show that in our tumor model, FLAG+ exosomes were undetectable in the plasma-derived exosomes isolated from mice bearing FLAG+ melanomas, indicating that tumor-derived exosomes may not be enriched in the blood circulation of mice bearing certain types of tumor such as melanoma.

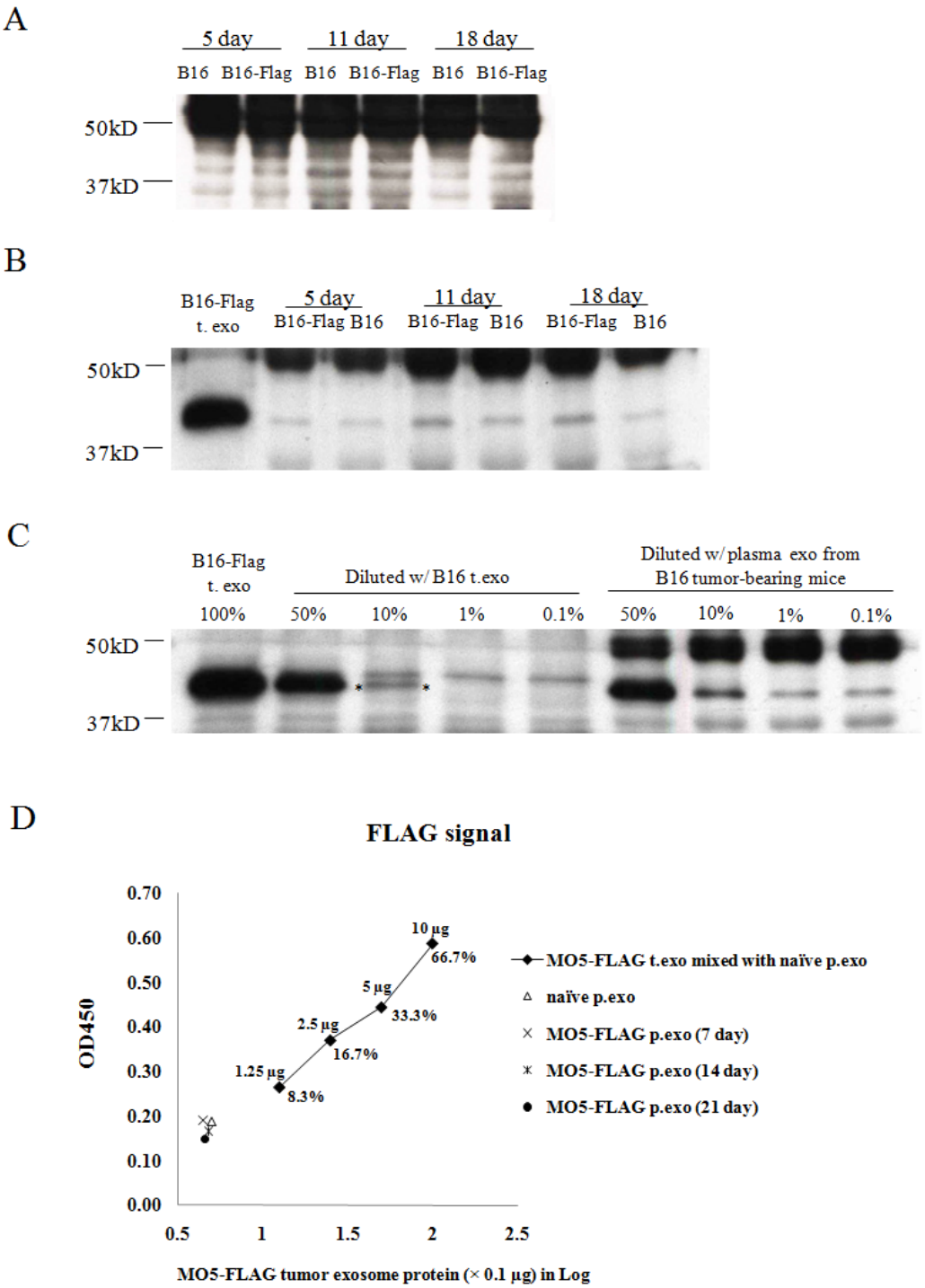


Figure 17: Detection of FLAG+ tumor-derived exosomes in plasma-derived exosomes isolated from tumor-bearing mice.

(A). Western blot analysis of FLAG-Lactadherin expression on exosomes isolated from the blood plasma of mice bearing subcutaneous B16 or B16-FLAG tumor. Blood were collected 5

days, 11 days and 18 days after s.c. inoculation of 5×10^5 tumor cells in the abdomen, and exosomes were isolated respectively. 10 μg of proteins were loaded per lane. (B). Western blot analysis of FLAG-Lactadherin expression on plasma-derived exosomes isolated from mice i.v. inoculated with B16-FLAG or B16 cells. B16-FLAG tumor-derived exosomes (t.exo) were loaded as a positive control. 10 μg of proteins were loaded per lane. (C). Detection limit of Western blot analysis for FLAG+ exosomes. B16-FLAG t.exo were diluted into decreasing concentrations (50%, 10%, 1% and 0.1% in 10 μg of total protein) by mixing with B16 t.exo or plasma exosomes from B16 tumor-bearing mice. Specific FLAG signal can be detected when B16-FLAG t.exo are present at 10% or higher percentages in both cases. B16-FLAG t.exo was distinguishable at 10% when mixing with B16 t.exo (marked by "**"). When B16-FLAG t.exo were present at low concentrations in B16 plasma exosomes (1% and 0.1%), FLAG detection seems to be interfered by a non-specific protein band with a molecular weight similar to FLAG-Lactadherin. (D). Detection of FLAG+ exosomes in plasma exosomes isolated from mice bearing subcutaneous MO5-FLAG tumor (MO5-FLAG p.exo) by ELISA. MO5-FLAG p.exo were isolated 7, 14 and 21 days after tumor inoculation. 15 μg of total protein were used in each well. Positive controls were made by mixing MO5-FLAG t.exo with naïve p.exo into decreasing percentages (10 μg, 5 μg, 2.5 μg and 1.25 μg in a total of 15 μg proteins, respective percentages as 66.7%, 33.3%, 16.7% and 8.3%). Naïve p. exo was included as a negative control.

3.4.4 Cellular marker expression on plasma-derived exosomes

Given that tumor-derived exosomes are not enriched in plasma-derived exosomes, we performed Western blot analysis on plasma exosomes isolated from mice bearing MO5 tumor to examine possible cellular markers. As shown in Figure 18, plasma-derived exosomes express both MHC class I and class II molecules. In addition, expression of CD11b, B220, CD22 and CD9 were also detected, suggesting that plasma-derived exosomes possibly comprise vesicles secreted by cells that are MHC class II+, CD11b+, B220+ and/or CD22+. The protein expression pattern of MO5 plasma exosomes isolated at different time points and naïve plasma exosomes were similar.

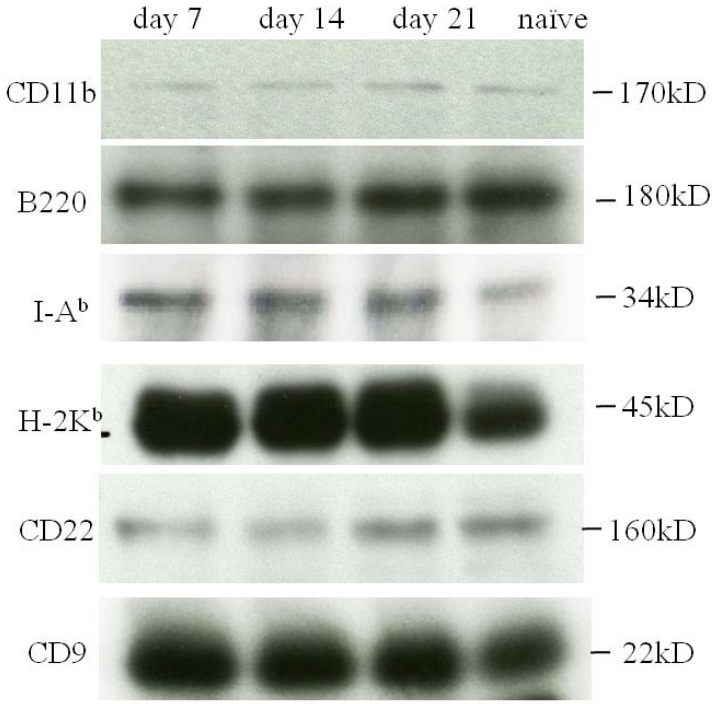


Figure 18: Protein expression on plasma-derived exosomes from MO5 tumor-bearing mice.

Mice were inoculated with 5×10^5 MO5 cells s.c. in the abdomen and blood were collected on day 7, 14 and 21 (average tumor size: 9.06, 163.14 and 1184.58 mm^3 out of 5) after tumor inoculation or from naïve mice. Western blot analysis for different cellular makers was performed.

3.4.5 Suppressive effect of plasma-derived exosomes isolated from mice bearing OVA-expressing tumor is partially dependent on MHC class II+ vesicles

We tested the suppressive effect of plasma-derived exosomes isolated from mice bearing MO5-FLAG tumor in OVA DTH model, and found that these exosomes were equally effective in

suppressing OVA-specific DTH response, suggesting that the FLAG tag does not affect the ability of plasma-derived exosomes in suppressing antigen-specific immune response (Fig. 19).

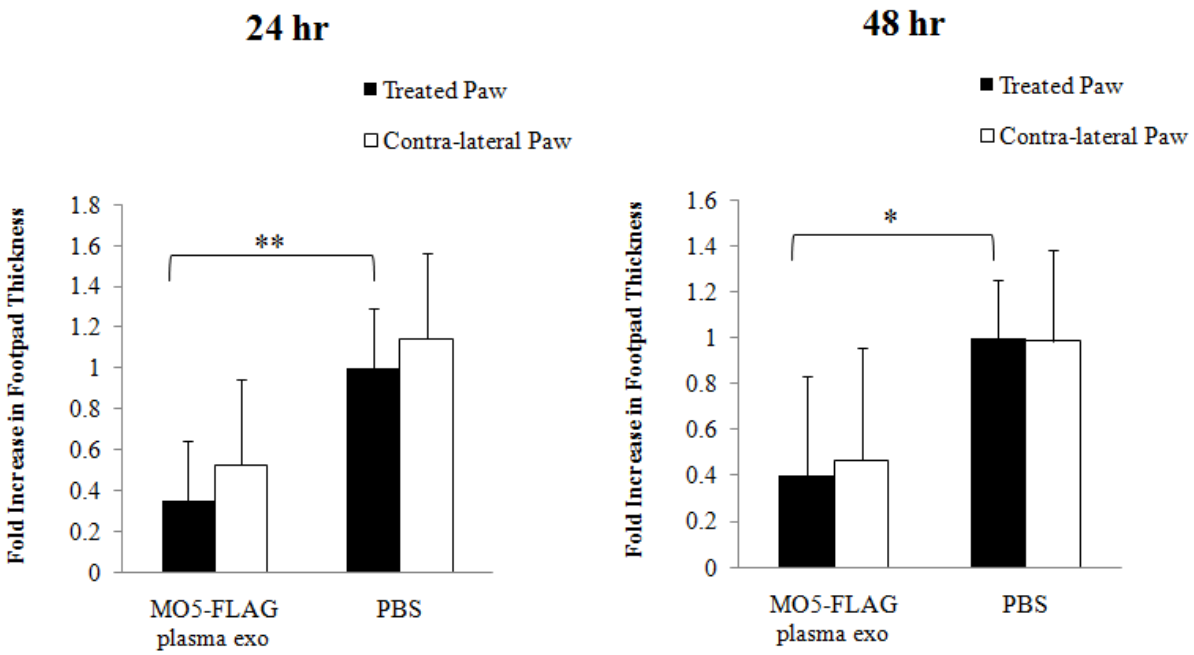
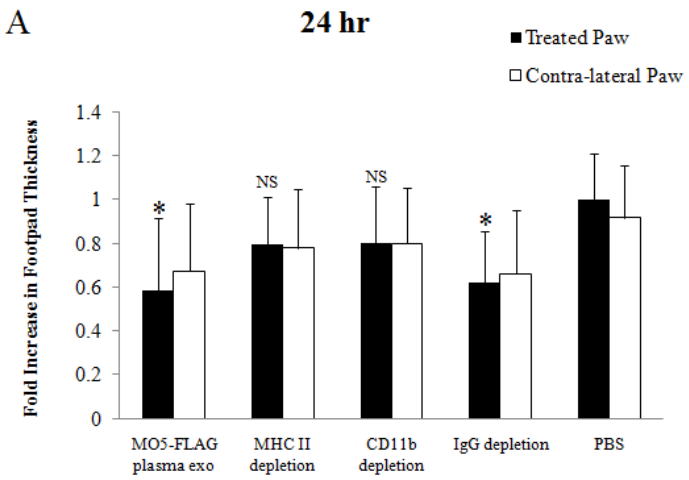


Figure 19: Plasma-derived exosomes from mice bearing MO5-FLAG tumor retain their ability in suppressing OVA-specific DTH response.

MO5-FLAG tumor cells were inoculated s.c. in the mouse abdomen and blood was collected 3 weeks post-inoculation (averaged tumor size: 834.79 mm^3 out of 10 mice). 10 µg of plasma exosomes or PBS were injected into the right hind paws of OVA immunized mice at the time of OVA challenge. Paw swelling was measured 24 and 48 hr post-challenge. Compared with PBS control, MO5-FLAG plasma exosomes significantly suppressed the magnitude of OVA-specific DTH response. n=5 in each group. Significance at: **, $P<0.01$; *, $P<0.05$.

Based on our previous observation that MHC class II+ exosomes in plasma suppress inflammation in an antigen-specific manner (171), we examined which vesicle portions in plasma exosomes were responsible for the suppressive effect. MHC class II+ vesicles or CD11b+ vesicles were depleted from MO5-FLAG plasma exosomes using magnetic beads, and 10 μg of non-depleted, MHC class II-depleted, CD11b-depleted, or IgG control beads-depleted plasma exosomes were administrated into mice of the OVA DTH model. Compared with undepleted exosomes and IgG-depletion, MHC class II-depletion partially abrogated the suppressive effect of MO5-FLAG plasma exosomes. CD11b-depletion co-depleted a quite amount of MHC class II+ vesicles and also resulted in partial abrogation of the suppressive effect (Fig. 20A-D). In a similar experiment setting, MHC class II-depletion also partially abolished the suppressive effect of EG7 plasma exosomes. Although compared with PBS group, MHC class II-depleted EG7 plasma exosomes showed a certain level of suppression at 48 hr time point, the extent of suppression was significantly less than undepleted exosomes (Fig. 20E-F). These results indicate that the suppressive effect of plasma-derived exosomes isolated from tumor-bearing mice was, at least partially, dependent on the MHC class II+ vesicle portion.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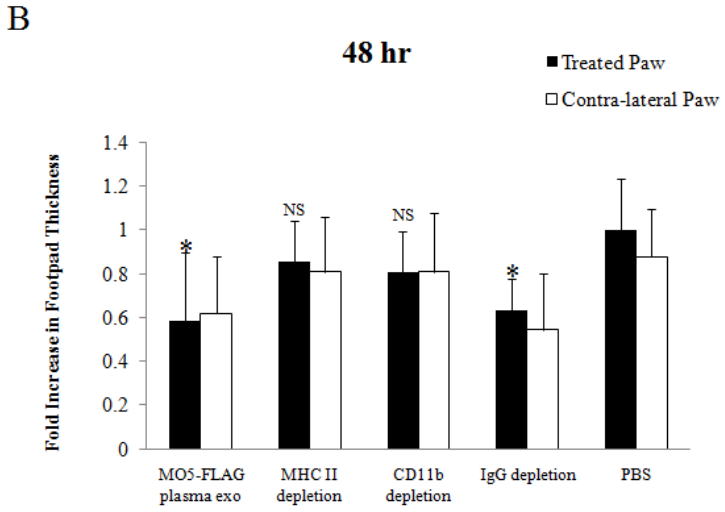
MO5-FLAG plasma exo
MHC II depletion
CD11b depletion
IgG depletion
PBS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CD11b depletion
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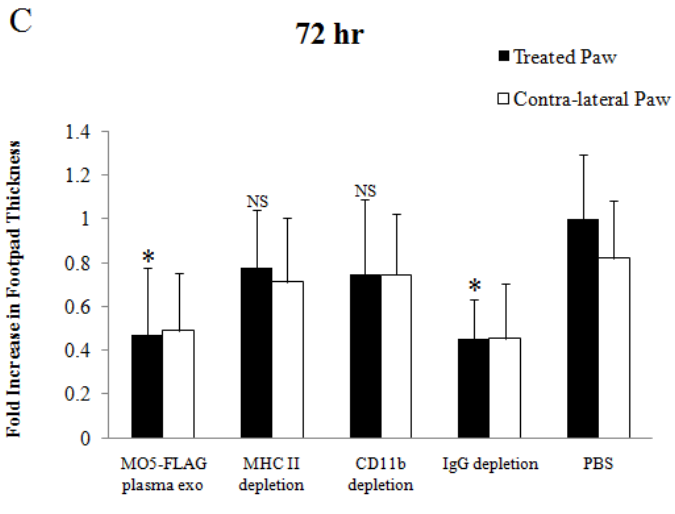
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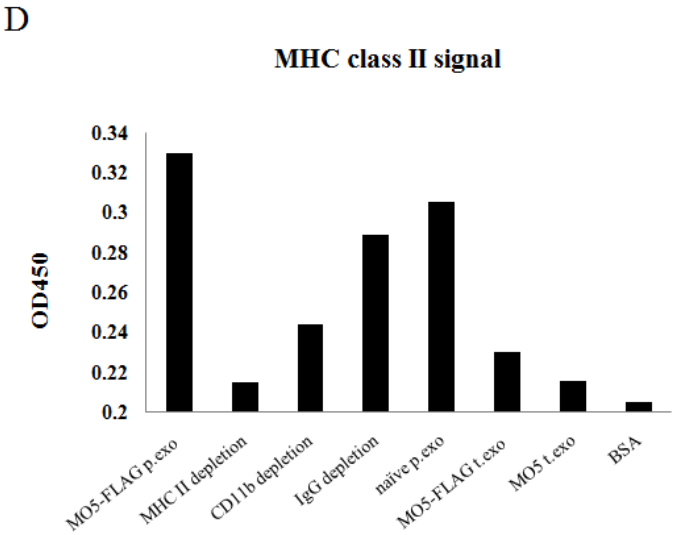
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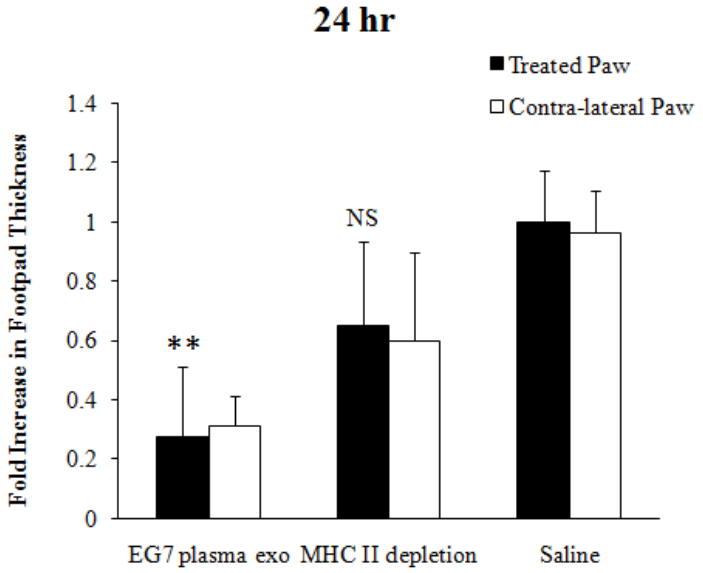

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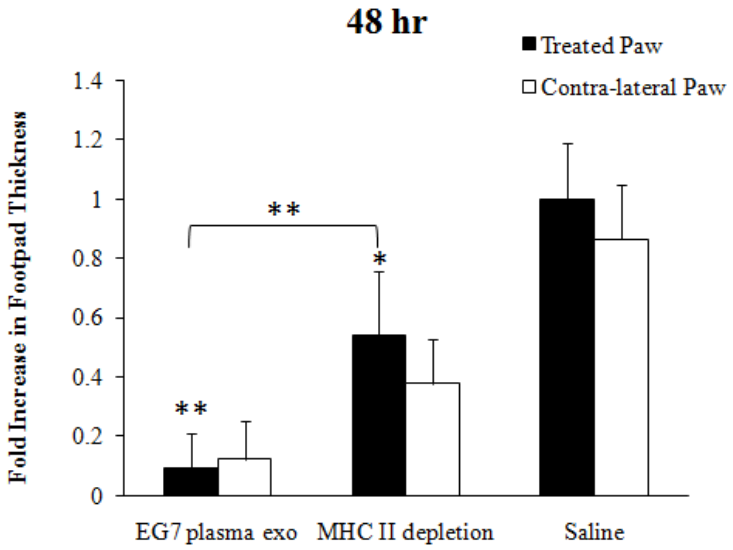

Figure 20: The suppressive effect of plasma-derived exosomes in the DTH model is partially dependent on MHC class II+ vesicles.

(A-C) Plasma-derived exosomes were prepared from mice bearing MO5-FLAG tumors 3 wks after tumor inoculation, and depleted with different vesicle portions using MHC class II, CD11b or IgG MACS beads. Exosomes were retrieved from the flow-through portion. 10 µg of undepleted or depleted exosomes were applied on OVA-immunized mice and the magnitudes of DTH responses were measured. n=5 in each group except the IgG group (n=4). (D) MHC class II expression on different exosome preparations detected by ELISA. 10 µg of exosomes (50 µl/well)

were tested and 10 μg of BSA were used as a negative control. MO5 tumor-derived exosomes (MO5 t.exo) were previously shown to be MHC class II negative. (E-F) Plasma-derived exosomes were prepared from mice bearing EG7 tumor 3 wks after tumor inoculation, and depleted with MHC class II+ vesicles using MACS beads. 10 μg of undepleted or depleted exosomes were applied on OVA-immunized mice and the magnitudes of DTH responses were measured. n=5 in each group. Significance at: **, $P<0.01$; *, $P<0.05$.

3.5 DISCUSSION

Identification of vesicles with characteristics similar to exosomes in the blood plasma suggests that peripheral circulation could be an efficient mechanism by which these subcellular mediators gain access to their targets in distant milieu. Therefore, exosomes with the ability to transfer and exchange information between cells could serve the purpose of conveying systemic effect for their parental cells. In the current study, our central hypothesis was that the circulating exosomes in tumor-bearing hosts possess immunoregulatory effect which helps to down-regulate anti-tumor immune responses. Our data provide novel information that plasma-derived exosomes from mice bearing OVA-expressing tumor have the ability to suppress OVA-specific DTH response. Such effect seems to be antigen specific since suppression was only conferred by plasma-derived exosomes from the mice with tumors expressing that particular antigen.

Previous reports have shown that oral administration of OVA antigen leads to the production of exosome-like “tolerosomes” which can be purified from serum and are capable of

inducing antigen-specific tolerance in naïve recipient mice. These tolerosomes were found to carry MHC class II with bound antigenic peptides sampled from the gut, and it was suggested that they convey tolerance possibly by efficient presentation of food antigen to the immune system and induction of regulatory T cells (160). Similarly, we previously observed that sensitizing mice with KLH antigen by tail base immunization give rise to plasma exosomes that can suppress KLH-specific DTH response, an effect which was not conferred by plasma exosomes from mice sensitized with the irrelevant antigen OVA. Here we demonstrate that exosomes with immunosuppressive properties are also present in the blood plasma of tumor-bearing mice and can suppress the immune responses specific to the model tumor antigen, suggesting that exosomes in circulation may also contribute to the generation of tumor immune tolerance.

Because the suppressive effect of plasma-derived exosomes is similar to the effect conferred by tumor-derived exosomes (as described in the previous chapter), we speculate that it is mediated by exosomes released by cells that carry or have sampled tumor antigens. Thus we first sought to identify the content of tumor-derived exosomes in plasma exosomes isolated from tumor-bearing mice. Our approach of adding FLAG tag between Lactadherin LS and C1C2 domain renders the FLAG epitope expressed outside of the exosome membrane, thus exosomes released by tumor cells can be easily detected by both Western blotting and ELISA (Fig. 16). However, when melanoma cells producing FLAG+ exosomes were introduced either subcutaneously or systemically into mice, the augmentation of tumor mass did not correlate with strengthened FLAG signal in plasma-derived exosomes (Fig. 17). Despite that technique limitation could possibly hamper the detection of FLAG+ exosomes at low amounts, our results suggest that at least in our murine melanoma model, tumor-derived exosomes were present at a

level significantly less than 8%, if any, of the total proteins in plasma-derived exosome, even when the tumors have progressed to late stages (Fig. 17D). This finding does not support our initial assumption that tumor-derived exosomes accumulate systemically as the tumor progresses, although in theory more tumor-derived exosomes would be released when the tumor size increases.

It was reported that exosomes expressing tumor markers can be isolated from the sera of ovarian cancer patients and the amount increases along with tumor progression (118). However, it was also mentioned that not in all cases tumor-derived exosomes were present in the blood circulation, for reasons presently unknown (174). In a study on serum exosomes of glioblastoma patients, tumor-specific EGFRvIII was detected in serum exosomes from 7 out of 25 patients (117). Combined with our results, it seems to suggest that different types of tumor and possibly different tumor growth pattern could both affect the accumulation of tumor-derived exosomes in peripheral circulation. Presumably, tumors that generate large amounts of malignant effusions such as ascites and pleural effusions tend to result in more tumor-derived exosomes in circulation (226, 237). Further analysis will be needed to explore on larger patient groups or animal models to determine its pathological relevance. Nevertheless, our findings indicate that caution is needed when using plasma/serum-derived exosomes as cancer diagnostic markers and when interpreting the detection results, since false negative results are likely to be obtained.

The fate of exosomes released by tumor cells *in vivo* remains to be investigated. it is however, likely that tumor-derived exosomes are actively taken up by APCs (e.g. DCs, macrophages) within the local tumor microenvironment before they have access to the blood stream, especially in the case of localized solid tumors. Uptake by cells is important for the functional activity of exosomes, which can then move to the draining site where they further

affect or interfere with the functions of lymphocytes. It was suggested that the presence of tumor-derived exosomes in the blood can be due to a disturbance in the homeostasis of exosome secretion and uptake (i.e. excessive production or inefficient clearance) (174).

Since tumor-derived exosomes are not the major components of plasma-derived exosomes in our tumor model, protein markers identified on plasma-derived exosomes becomes important for providing information on their cellular origins. It was reported that microvesicles from normal human blood are mostly derived from platelets and mononuclear phagocytes (225). We and others have previously shown that a significant portion of plasma-derived exosomes are positive for MHC class II molecules (162, 171), suggesting that they might be released by MHC class II-positive cells. Here we found that plasma-derived exosomes isolated from tumor-bearing mice express both MHC class I and class II molecules, as well as CD11b, B220, CD22 and CD9 (Fig. 18). We speculate that plasma-derived exosomes comprise vesicles secreted by immune cells that are systemically present, possibly monocytes, macrophages and B cells. Although plasma-derived exosomes seem to be predominantly CD11c negative ((171) and data not shown), it is not unlikely that that a portion of vesicles come from a specific DC lineage at a certain stage of maturation. In addition, although marked increase in the abundance of circulating microvesicles has been mentioned in human cancer patients (220), we found that the total protein amount of plasma-derived exosomes that can be isolated from naïve mice were only slightly lower than that from mice with advanced tumor, and no significant difference was found in the protein expression pattern between naïve plasma exosomes and those from tumor-bearing mice (data not shown).

Several lines of evidence suggested that tolerogenic plasma-derived exosomes exert suppressive effect through a MHC class II-dependent mechanism (171, 172). By depletion study,

we demonstrate that the suppressive effect of plasma exosomes isolated from tumor-bearing mice is at least partially dependent on the MHC class II+ vesicle portion. This result is consistent with our previous report demonstrating that MHC class II+ exosomes in the plasma of antigen-sensitized mice suppress inflammation in an antigen-specific manner when administrated locally (171). It is also consistent with the report that MHC class II+ tolerosomes isolated from the serum of antigen-fed mice transfer antigen-specific tolerance in recipient mice, and removal of the tolerosomes by absorption of MHC class II resulted in abrogated tolerance development (160). The effect of tolerosome was further determined to require MHC class II expression in the intestinal epithelial cells, be only effective in syngeneic recipients, and possibly function through the activation of CD4+ regulatory T cells (172). In our OVA DTH model, how the antigen-specific effect is conferred by plasma-derived exosome remains to be determined, however it is possibly mediated by exosomes carrying either OVA antigen or OVA-derived peptide in the context of MHC molecules. These vesicles may be reprocessed by APCs which preferentially present antigens to and activate specific regulatory T cells. In the scenario of tumor progression, it is possible that circulating immune cells especially MHC class II+ APCs sample tumor antigens by internalizing tumor-derived exosomes, apoptotic bodies, necrotic bodies or other cell fragments; then under the immunosuppressive signals in the tumor microenvironment, they in turn release tolerogenic exosome-like vesicles into the bloodstream and induce tumor-specific tolerance.

In summary, our studies demonstrate that exosome-like vesicles can be isolated from tumor-bearing mice, and these plasma-derived exosomes can induce suppression on antigen-specific, Th1-dominated DTH response. We also demonstrate in a murine melanoma model that plasma-derived exosomes comprise vesicles released from circulating immune cells rather than

tumor cells. Finally, the suppressive effect of plasma-derived exosomes was at least partially mediated by the MHC class II+ vesicle portion. These findings suggest that circulating exosomes can be potentially effective mediators in conveying systemic, antigen-specific immune regulation in tumor-bearing hosts.

4.0 EXOSOMES RELEASED FROM MYCOPLASMA INFECTED TUMOR CELLS INDUCE B CELL ACTIVATION AND INHIBITION OF T CELL PROLIFERATION IN SPLEEN CELLS

4.1 ABSTRACT

During the study of tumor-derived exosomes, we found certain immune responses elicited by tumor-derived exosomes were associated with mycoplasma infection. Exosomes purified from mycoplasma infected tumor cell cultures showed similar morphology as exosomes purified from uninfected tumor cells under electron microscope, and whole mycoplasma particles were not observed in exosome preparations. These exosomes strongly induced activation and expansion of splenic B cells, while only weakly activating a small portion of CD8+ T cells and had little impact on CD4+ T cells. In addition, robust cytokine production including IFN-γ, TNF-α and IL-10 was induced. Cytokine induction was significantly decreased in B cell-deficient μMT spleen cells, indicating that it was largely B-cell dependent. Intracellular staining revealed that B cells are the major IL-10 producers. In μMT spleen cells the induction of IFN-γ-producing CD8+ T cells was greatly increased. Furthermore, anti-CD3 stimulated T cell proliferation and T cell receptor signaling was inhibited in splenocytes treated with myco+ tumor exosomes. T cell proliferation was also inhibited when T cells were co-incubated with myco+ tumor exosome-treated B cells. The stimulatory effect of myco+ tumor exosomes was not affected by membrane

disruption procedures, but was completely abolished after treating parental cell cultures with mycoplasma removal reagent. Mass spectrometry identified potential mycoplasma proteins on myco+ tumor exosomes. This study characterizes the reactions of splenic B and T cells in response to tumor-derived exosomes carrying mycoplasmal components and revealed the potential antagonizing effect of B cell activation to T cell proliferation. These observations will help us better distinguish mycoplasma related immune responses in future exosome study, and provides us with new insights into the possible combinatory immune regulation by co-existing mycoplasma pathogens and tumor cells through exosome releasing.

4.2 INTRODUCTION

Tumor cells constantly secrete small membrane vesicles of 30-100 nm in diameter, termed "exosomes", both *in vivo* and *in vitro*. These small vesicles are formed by reverse budding of MVBs in the late endocytic compartments and are released upon the fusion of MVBs with plasma membrane (84, 86). Exosomal protein expression usually reflects that of the parental tumor cells, contains tumor specific antigens and is enriched in tetraspanin molecules (129). Tumor-derived exosomes were first explored for their potential to stimulate anti-tumor response as a new source of tumor rejection antigens. Studies to date suggest that activation of T cells requires tumor-derived exosomes to be taken up and processed by professional APCs, and over-expression of pro-inflammatory factors or stress proteins on exosomes enhances their stimulatory activities (129, 132, 135, 138). In recent years, mounting evidence shows that tumor-derived exosomes can be immunosuppressive. Exosomes derived from different tumor cell lines have been reported to exert diverse suppressive effects, such as negatively regulate the function of

APCs and effector cells (including natural killer cells and T cells), promote the generation of myeloid suppressor cells, and support the function of regulatory T cells (Treg cells) (131, 144, 146-149, 167). It is worth noting that studies on APC-derived exosomes revealed that intracellular pathogens can endow exosomes with special immunogenic properties. For example, exosomes secreted by bacterially infected macrophage contain bacterial molecules with pathogen-associated molecular patterns (PAMPs) and are pro-inflammatory (97, 176). Also, exosome preparations from mycoplasma infected long-term cultured dendritic cells (LTC-DCs) induce polyclonal B cell proliferation (181).

Mycoplasma is a distinct genus of bacteria for their minute size (0.2-0.4 μm) and the lack of a cell wall. These prokaryotic organisms live as parasites because of their inability to biosynthesize. Mycoplasmas cause numerous diseases and also act as opportunistic pathogens that colonize a host with a weak immune system (238, 239). They can infect different cell types by either attaching to the cell membrane or fusion with the host cells (240). Persistent mycoplasma infection induces chromosomal instability and malignant transformations of mammalian cells (241-247). Correspondingly, some tumor cell proteins were found to have mycoplasma origin (248). Mycoplasma infection of tumor cells was reported to increase tumor cell invasiveness (249). Mycoplasma can modulate the immune system and induce a wide range of immune responses. On one hand, many mycoplasma species activate monocytes and/or macrophages and induce the secretion of pro-inflammatory cytokines (250-252). On the other hand, mycoplasmas also induce immunosuppression, through various mechanisms including arginine depletion, cytotoxicity and induction of anti-inflammatory cytokines (240, 250, 253-255). In addition, temporary inhibition of cell-mediated or humoral immune responses by

mycoplasma infection were observed on different hosts with different mycoplasma species, through mechanisms yet to be identified (256-258).

The incidence of mycoplasma infection in established tumors *in vivo* is unclear yet. Nevertheless, different research groups have reported the detection of mycoplasma DNA in archived human cancer tissues, including ovarian cancer, gastric carcinoma, colon carcinoma, esophageal cancer, lung cancer, breast cancer and glioma, ranging from 40%-60% of the specimens tested (259, 260). These findings suggest the possible co-existence of mycoplasmas and tumor cells *in vivo*.

In vitro, mycoplasma infection is commonly found in laboratory cultured cell lines including tumor cell lines (261). During the study of tumor-derived exosomes, we found that certain immune responses elicited by exosomes were associated with mycoplasma infection. Here we report that exosomes derived from mycoplasma infected tumor cells preferentially activate B cells and induce robust cytokine production in spleen cell culture, including both proinflammatory and anti-inflammatory cytokines. However, T cell proliferation and TCR signaling is inhibited in this process. This study will help us better distinguish mycoplasma related immune responses in future exosome study, and also suggests the potential concurrent immune modulations by tumor cells and mycoplasma through exosome release *in vivo*, which may exacerbate the suppression of T cell activities.

4.3 MATERIALS AND METHODS

4.3.1 Cell lines and Mice

Murine melanoma cell line B16 and thymoma cell line EL4 were purchased from American Type Culture Collection. Cells were cultured in RPMI 1640 supplemented by 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, Antibiotic-Antimicotic (GIBCO), and 50 μM β-mercaptoethanol. Female wild type C57BL/6 (CD45.2+) mice were purchased at 6-7 wk of age from The Jackson Laboratory. Animals were maintained in a pathogen-free animal facility at University of Pittsburgh Biotechnology Center. Spleens from female C57BL/6 mice expressing the congenic marker CD45.1 (strain name: B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ) and from the B cell-deficient μMT mice were kindly provided by Dr. Geetha Chalasani (University of Pittsburgh).

4.3.2 Mycoplasma detection and elimination

Cell cultures were screened for mycoplasma using MycoAlert™ mycoplasma detection kit (Cambrex) and infections were confirmed using LookOut® Mycoplasma PCR detection kit and JumpStart™ *Taq* DNA polymerase (Sigma). DNA was separated in a 1.2% agarose gel and stained with ethidium bromide. For mycoplasma removal, infected cell lines were treated with Plasmocin™ (Invivogen) for 2 weeks and then cultured for another week before PCR test to ensure complete elimination.

4.3.3 Exosome preparation

Exosomes were isolated from cell culture supernatant by differential centrifugation and filtration. FBS used for culture media was pre-cleared by ultracentrifugation at 100,000 × g for 3 hr at 4°C. 48 hr culture supernatants were centrifuged at 1000 × g for 10 min and 10,000 × g for 30 min to remove cell and membrane debris, filtered through 0.22 μm sterilizing filter (Corning), and further concentrated using Centricon Plus-70 100kD cutoff filter units (Millipore). The concentrated supernatants were subjected to ultracentrifugation at 100,000 × g for 1 hr. Exosomes pellets were washed with sterile PBS, centrifuged at 100,000 × g for 1 hr, and resuspended in sterile PBS. Exosome quantification was done by Bradford protein assay (Bio-Rad).

4.3.4 Transmission electron microscopy

Exosome preparations were loaded on Formvar/carbon-coated grids and negatively stained with 1% uranyl acetate. Pictures were taken on a JEM-1011 transmission electron microscope with the Advanced Microscopy Techniques (AMT) software.

4.3.5 Spleen cell culture

Spleens were isolated from mice euthanized in CO_2 tank. Single cell suspensions were prepared by mincing the tissues through a 70 μm cell strainer after collagenase D digestion for 30 min. Erythrocytes were depleted using ACK cell lysing buffer (Biowhittaker). Spleen cells were

cultured in complete RPMI 1640 media, in the presence of 30 U/ml recombinant murine IL-2 (Biolegend).

4.3.6 ELISA

TNF-α and IL-10 levels in culture supernatants were detected using mouse TNF-α and IL-10 ELISA kit (eBioscience). IFN-γ ELISA was performed using purified anti-mouse IFN-γ as capture antibody and biotinylated anti-mouse IFN-γ as detection antibody (BD Pharmingen).

4.3.7 Flow cytometry

For surface staining, cells were washed in staining buffer (1% FBS in PBS) and stained with Ethidium monoazide (EMA) for dead cell exclusion. Cells were incubated at dark for 10 min and then exposed to intense light for 10 min at room temperature (RT). Cells were then washed and incubated with purified anti-mouse CD16/32 (Fc-block, eBioscience) for 10 min on ice, followed by incubation with fluorochrome-conjugated antibodies for 30 min on ice. When biotinylated antibodies were used, cells were further incubated with secondary reagent (streptavidin-fluorochrome) for 15 min on ice. For intracellular cytokine staining, cells were treated with Brefeldin A for the last 6 hrs in culture before being harvested. After surface staining, cells were fixed and permeabilized with Fix/Perm buffer (BD Biosciences) for 15 min in dark on ice, followed by washing with 0.1% saponin (Sigma). Cells were then stained with cytokine antibodies in 0.25% saponin with rocking for 1 hr at RT in dark. Antibodies used in surface marker characterization include: APC-eFluor780-B220, PacificBlue-CD19, PE-Cy7-CD25, PE-CD40, FITC-CD86, PE-CD80, PE-Cy7-CD23, FITC-CD19, eFluor450-IgD, PE-CD1d, PE-

CD43, PE-CD8, APC-eFluor780-CD4, FITC-CD69, APC-CD62L, eFluor450-CD44, PE-7-CD4, APC-CD8 and Biotin-CD5 from eBioscience, and FITC-IgM from BD Bioscience. Antibodies used in intracellular cytokine characterization include: FITC-CD19, FITC-B220, Biotin-CD4, PacificBlue-CD8, APC-IL-10 and APC-IFN-γ from eBioscience. Secondary reagents used include streptavidin-APC-eFluor780 and streptavidin-APC-Cy7 from eBioscience. Antibodies and secondary reagent used for T cell proliferation assays include: PE-Cy7-CD4, eFluor450-CD8, PE-CD45.1, APC-CD62L, Biotin-CD44 and streptavidin-APC-Cy7 from eBioscience. Flow acquisition was performed on LSRII analyzers (BD Biosciences), and data were analyzed using the Flowjo software (Tree star Corp.).

4.3.8 T cell proliferation assay

Splenic single cell suspension was prepared from C57BL/6 (CD45.2+) mice as mentioned above. For T cell depletion, splenocytes were first incubated with biotin-anti-mouse CD3 (10μl Ab/100 × 10^6 cells/1ml, eBioscience) at 4°C for 15 min, then with streptavidin MACS beads (100 μl/100 × 10^6 cells/1ml, Miltenyi) at 4°C for 15 min, followed by negative selection using autoMACS™ Pro Separator (Miltenyi). To purify B cells from total splenocytes, cells were first incubated with biotin anti-mouse CD3, CD11c, F4/80 and PDCA-1(each at 10μl Ab/100 × 10^6 cells/1ml, eBioscience), then with streptavidin MACS beads, followed by autoMACS negative selection. B cell purity was checked by FACS and the percentages of remaining Non-B-APCs are: CD11c+ cells <1.3 %; F4/80+ cells <0.1%; and PDCA-1+ cells <1 %. To purify T cells from CD45.1+ B6 splenocytes, cells were first incubated with biotin-anti-mouse CD19, B220, IgM, CD11c, F4/80, PDCA-1, IA/IE, and CD25 (eBioscience), then with streptavidin MACS beads, followed by autoMACS negative selection. T cell purity was checked by FACS and the percentage of CD4+

plus CD8+ T cells reached 90%. Purified T cells were labeled with 2 μM of CFSE. To assess anti-CD3 stimulated T cell proliferation, T cell-depleted splenocytes or purified B cells were cultured in 24-well-plate at a cell density of 2.5×10^6/1 ml media/well in the presence of 80U/ml IL-2, with or without treatment of 1 μg/ml B16 myco+ exosomes. On the following day, 0.5×10^6 of purified CD45.1+ T cells were added to each well and the media volume was brought up to 3 ml. 10 μg/ml of purified anti-mouse CD3e (BD pharmingen) was added for stimulation. Cells were harvested after 3 days and CFSE dilution of CD45.1+ T cells were analyzed by FACS.

4.3.9 CFSE labeling of cells

T cells purified from CD45.1+ splenocytes were labeled with CFSE using CellTrace™ CFSE cell proliferation kit (Molecular Probes, Invitrogen). Briefly, 2 μM CFSE working solution were prepared in PBS containing DMSO (10%), and mixed well with cell pellet at the ratio of 5×10^6 cells/1 ml of CFSE. Cells were incubated at 37^oC for 10 min before the reaction was quenched with complete media, and then washed in warm PBS.

4.3.10 Western blot

Splenocytes were collected after treatment and lysed in NP-40 lysis buffer in the presence of protease inhibitor (Sigma-Aldrich) and phosphotase inhibitor (1 mM Na_2VO_4). 10 μg of cell lysates was separated on 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membrane was blocked and incubated with phosphor-p44/42 MAP kinase antibody (1:1000, Cell Signaling), followed by horseradish peroxidase-conjugated anti-

rabbit secondary antibody (1:5000, Santa-Cruz). Protein bands were visualized using an enhanced chemiluminescence detection kit (PerkinElmer Life Science). For total ERK protein control, the same membrane was stripped in stripping buffer (Pierce), blocked, incubated with p44/42 MAP antibody (1:1000, Cell Signaling), and followed by anti-rabbit secondary antibody. Densitometric quantitations were done using the ImageJ software.

4.3.11 Mass Spectrometry

The LC-MS/MS and database searching were performed by the Mass Spectrometry Platform of Cancer Biomarkers Facility in University of Pittsburgh Cancer Institute and the following method information is provided by Dr. Brian Hood. Briefly, 10 µg of each protein sample was resolved by 1D-PAGE briefly to allow the protein to run into the stacking portion of the gel. The stacking gel portion of each sample was excised and subjected to in-gel digestion according to established protocols. Tryptic peptides were extracted from the gel pieces, lyophilized and resuspended in 0.1% trifluoroacetic acid prior to MS analysis. Nanoflow reversed-phase liquid chromatography (RPLC) was performed using a Dionex Ultimate 3000 LC system (Dionex Corporation, Sunnyvale, CA) coupled online to a linear ion trap (LIT) mass spectrometer (LTQ, ThermoFisher Scientific, San Jose, CA). The LIT-MS was operated in a data dependent MS/MS mode in which each full MS scan was followed by seven MS/MS scans where the seven most abundant peptide molecular ions are selected for collision-induced dissociation (CID). Tandem mass spectra were searched against a combined UniProt mouse protein database (03/2010) from the European Bioinformatics Institute (http://www.ebi.ac.uk/integr8) using SEQUEST (ThermoFisher Scientific). In addition, data were searched against two combined UniProt mycoplasma database (*M. hominis* and *A. laidlawii*; *M. agalactiae/ arthriditis/ pneumoniae/*

pulmonis). Results from both searches were further filtered using software developed in-house to determine unique peptides and proteins.

4.4 RESULTS

4.4.1 Exosomes derived from mycoplasma infected tumor cells induce cytokine production and B cell activation in spleen cell culture

Tumor cell lines can be infected with mycoplasma during long term culture, with no apparent alterations on cell growth and proliferation. Mycoplasma infection was detected in subcultures of the B16 and EL4 cell lines (Fig. 21A). Infected cell lines were isolated and exosomes produced by these cells were compared with exosomes produced by healthy cell lines. Similar amounts of proteins were obtained in exosomes prepared from infected or healthy cell lines. Electron microscopy (EM) showed that exosomes derived from infected cell lines (myco+ exosomes) display similar morphology as exosomes derived from healthy cell lines (myco- exosomes). Application of 0.22 μm filter on culture supernatants before ultracentrifugation theoretically excluded the co-sedimentation of mycoplasmas with exosomes, and whole mycoplasma organisms were not observed in myco+ exosome preparations by EM (Fig. 21B).

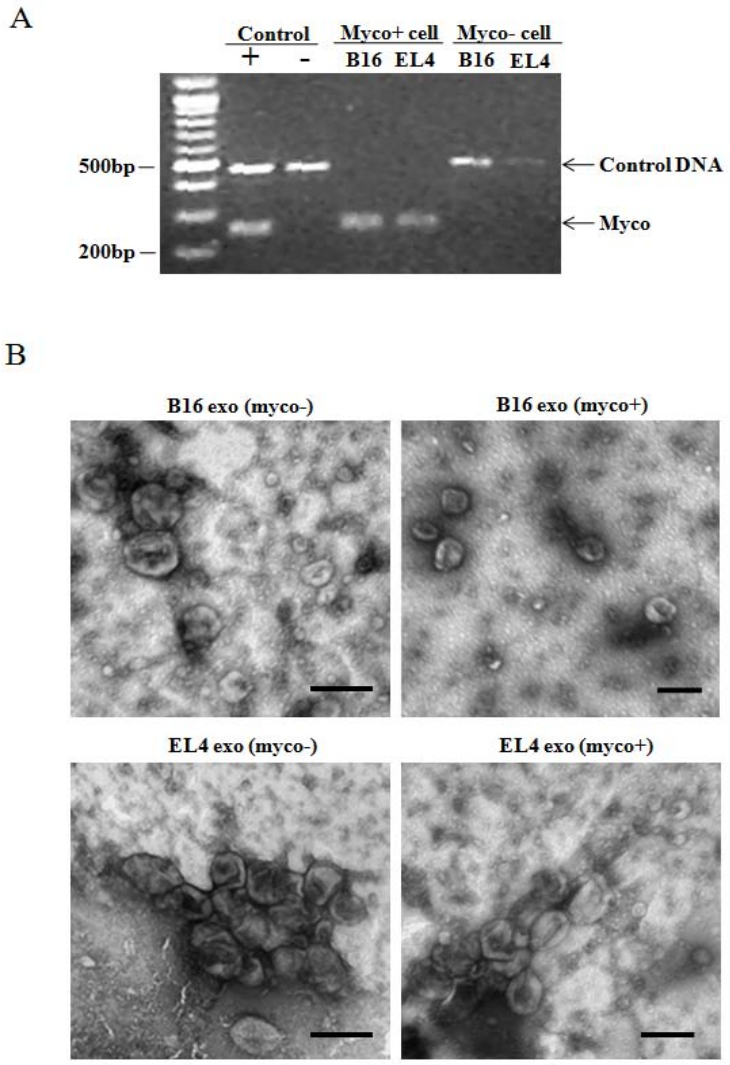

Figure 21: Mycoplasma detection and morphology of exosomes derived from healthy or mycoplasma-infected tumor cell lines.

(A). Detection of mycoplasma DNA in cell culture. Culture supernatants were tested by PCR using primer set specific to the highly conserved 16S rRNA coding region in the mycoplasma genome. Mycoplasma positive samples show bands in the range of 260 ± 8 bp. Positive and negative controls were both included. (B). Electron micrograph of exosomes prepared from healthy (myco-) and mycoplasma infected (myco+) tumor cell cultures. Scale bar: 100 nm.

Treatment with 1μg/ml of myco+ exosomes on spleen cell culture for 72 hr resulted in robust induction of the pro-inflammatory cytokine IFN-γ and TNF-α, as well as the anti-inflammatory cytokine IL-10. On the contrary, myco- exosomes did not stimulate cytokine production (Fig. 22A). Moreover, cytokine induction by myco+ exosomes was dose-dependent (Fig. 22B). Similar results were obtained using two different sets of tumor-derived exosomes.

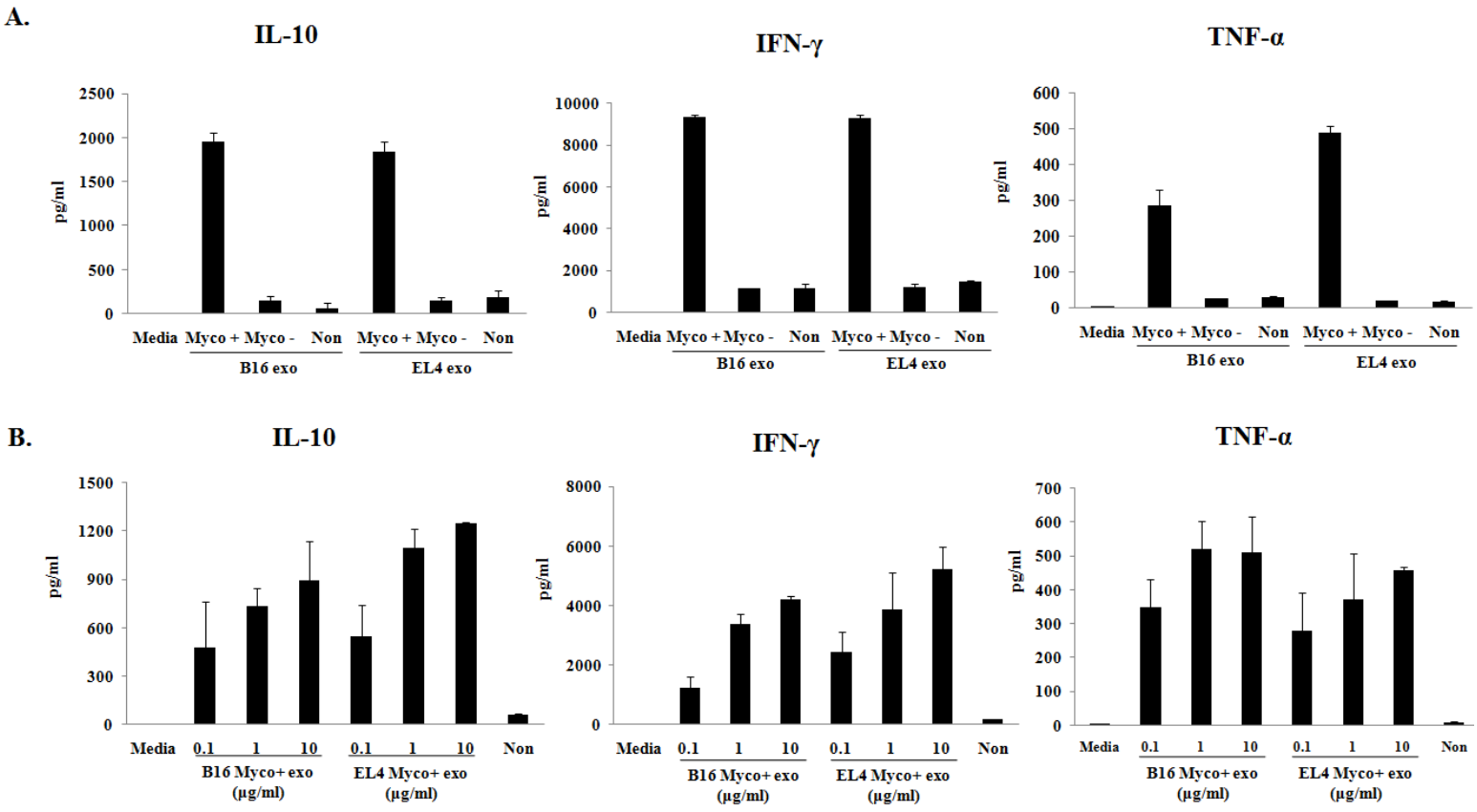

Figure 22: Cytokine induction in spleen cell culture by myco+ exosomes.

(A). Spleen cells from C57BL/6 mice were cultured in 24-well-plate at a density of 5×10^6 cells/1.5 ml media/well in the presence of 30 U/ml rmIL-2, and were treated with either myco+ exosomes or myco- exosomes or left untreated for 72 hr. IL-10, IFN-γ and TNF-α levels (pg/ml) in culture supernatants were measured by ELISA. Endogenous cytokine levels in culture media were also tested as controls. Each treatment was conducted in duplicates and similar results were obtained in two independent experiments. Data represent the averaged cytokine levels ± SD in

one representative experiment. (B). Dose-dependent induction of cytokines by myco+ exosomes. Spleen cells were treated with increasing dose of myco+ exosomes (0.1, 1 and 10 μg/ml) for 72 hr, and cytokine levels were measured by ELISA. Treatments were conducted in duplicates. Data represent the averaged cytokine levels ± SD in one representative experiment of two independent experiments with similar results.

FACS analysis shows that after myco+ exosome treatment, the expression of CD25, CD40, CD86 and CD80 were dramatically up-regulated on B220+CD19+ cells, suggesting that myco+ exosomes potently activated B cell populations. Meanwhile the expression of IgD and CD23 were both down-regulated, indicating that B cells were also undergoing maturation. IgM, CD1d, CD43 and CD5 were slightly increased. In contrast, myco- exosomes treatment had no effect on B cells (Fig. 23A). In addition, vigorous cell proliferation usually become visible in spleen cell culture around 48 hr after myco+ exosome treatment, and the percentage of B cells in total splenocytes was greatly increased (Fig. 23B). The effect of myco+ exosomes on T cell subsets was also examined. 72 hr treatment had little effect on CD4+ T cell activation while activated a small portion of CD8+ T cells, showed by the up-regulation of CD25, CD69 and CD44, as well as the down-regulation of CD62L. Myco- exosomes showed no effect on T cell activation compared with untreated control (Fig. 23C). Similar results were obtained with both B16 and EL4 exosomes (data show the representative results of B16 exosome treatment).

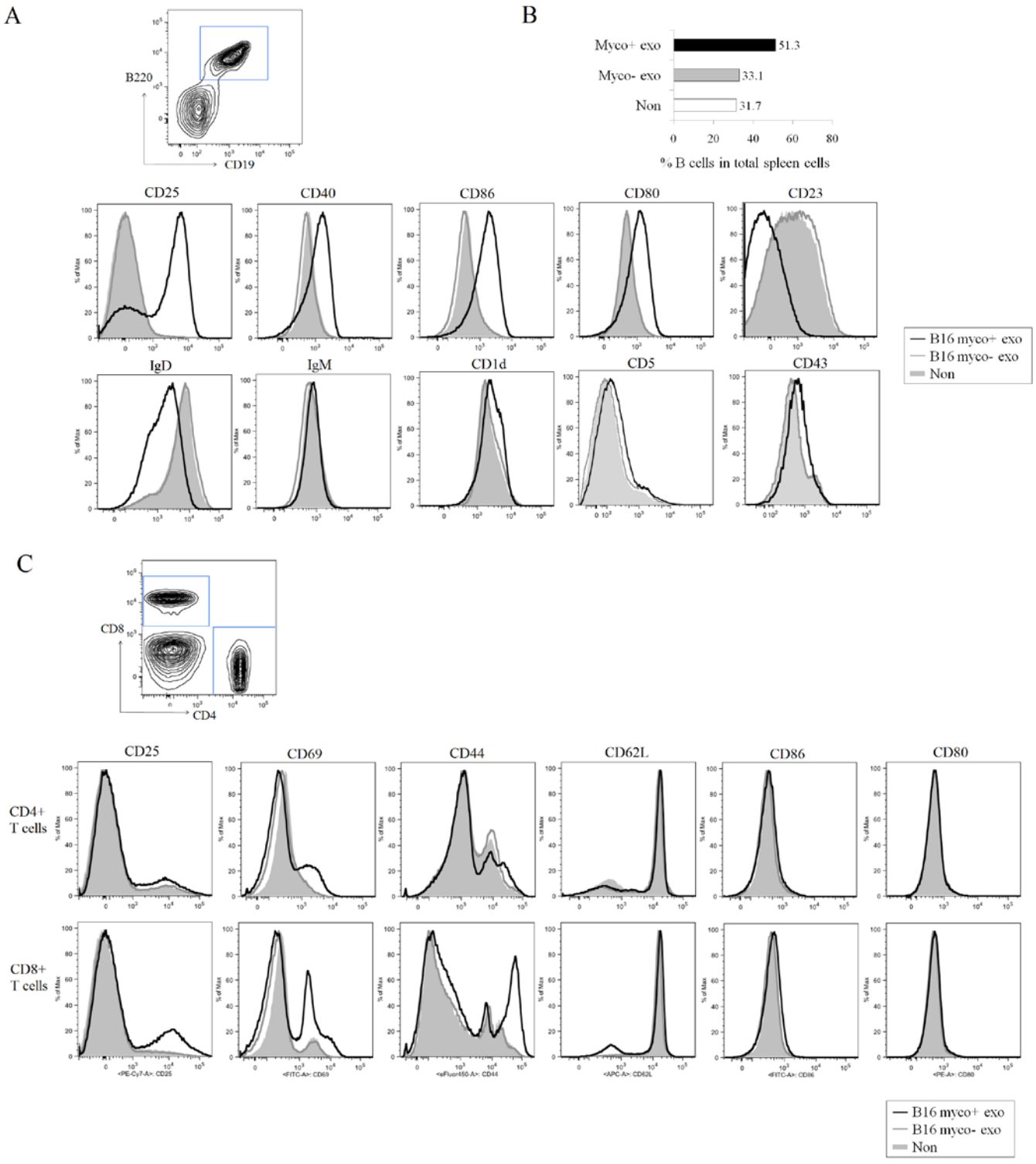

Figure 23: Myco+ tumor exosomes activate B cells and induce B cell expansion.

Spleen cells were cultured with 1 µg/ml of B16 myco+ exosomes, B16 myco- exosomes, or media alone for 72 hr. Cells were harvested and analyzed by FACS. (A). Expression of CD25,

CD40, CD86, CD80, CD23, IgD, IgM, CD1d, CD5 and CD43 in B cell gate (CD19+B220+). (B). Percentage of B cells in total splenocytes within live cell gate after exosome treatment. (C). Expression of CD25, CD69, CD44, CD62L, CD80 and CD86 in CD4+ and CD8+ T cell gates. Black line: B16 myco+ exosome; grey line: B16 myco- exosome; grey solid: untreated.

4.4.2 Cytokine productions are largely dependent on the presence of B cells

To determine if cytokine production correlates with B cell activation, we examined the cytokines produced by spleen cells from B cell deficient μMT mice upon exosome treatment. Spleen cells isolated from wide type (WT) mice or μMT mice were equally treated with 1 μg/ml of B16 myco+ exosomes or cultured untreated for 72 hr, and cytokine levels in culture supernatants were tested. Interestingly, there was a remarkable reduction in the amount of all the three cytokines produced by μMT spleen cells than that by WT spleen cells (Fig. 24), suggesting that cytokine induction by myco+ exosomes is largely B cell-dependent. Compared with untreated control, small amounts of cytokines were still being induced by B16 myco+ exosomes in μMT spleen cell culture, indicating that in the absence of B cells, other cell type(s) also respond to myco+ exosomes and produce cytokines, but to a much lower level.

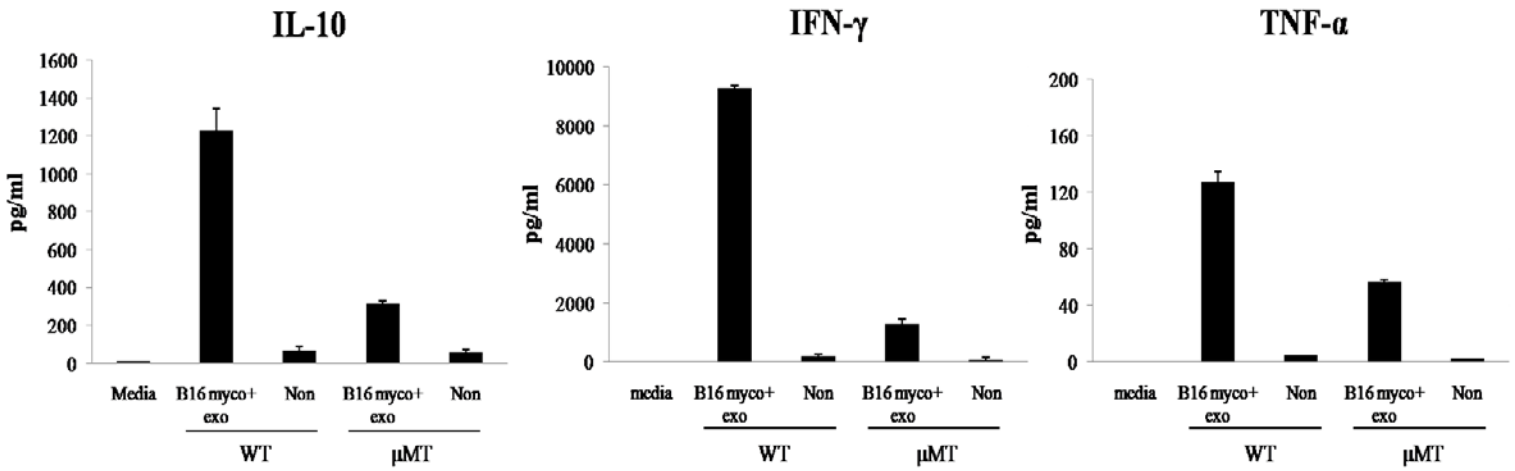

Figure 24: Cytokine induction by myco+ exosomes in WT and µMT spleen cell cultures.

Spleen cells from either WT mice or µMT mice were cultured in 24-well-plate at 5×10^6 cells/1.5 ml media/well with 30 U/ml of rmIL-2, and treated with 1µg/ml of B16 myco+ exosomes or left untreated for 72 hr. IL-10, IFN-γ and TNF-α levels in the culture supernatants were measured by ELISA. Each treatment was conducted in triplicates and data shows the averaged cytokine levels ± SD.

4.4.3 B cells are the major IL-10 producers

To identify the major cytokine-producing cells induced by myco+ exosomes, the percentage of IL-10+ cells and IFN-γ+ cells were analyzed in both B cell and T cell gates by intracellular staining at 48 hr time point of culture. The percentage of IL-10+ cells in B cell gate was increased by 3-fold after B16 myco+ exosome treatment (0.081%) compared with untreated control (0.0282%), while IL-10+ cells in CD4+ T cell gate was increased by less than 50% and that in CD8+ T cell gate remained almost unchanged (Fig. 25A). Meanwhile, the percentage of B cells in total splenocytes was greatly increased after B16 myco+ exosome treatment (56.6%) compared with untreated control (31.7%). The total cell number of cultured splenocytes was also increased after B16 myco+ exosome treatment (retrieved cell number per well: 3.375×10^6 compared with 2.475×10^6 in untreated control). Accordingly, the percentage of IL-10+ B cells in total splenocytes was increased by 5-fold and the number of IL-10+ B cells was increased by 7-fold, while the number of IL-10+ CD4+ T cells and IL-10+ CD8+ T cells were neither increased (Fig. 25B). The number of IFN-γ+ B cells, IFN-γ+ CD4+ T cells and IFN-γ+ CD8+ T cells were all found increased, with the increase of IFN-γ+ B cells being the most dramatic (5-fold) (Fig. 25B). These results show that myco+ exosomes specifically induce IL-10-producing B cells and B cells are the major IL-10 producers; whereas IFN-γ-producing cells were induced in both B cells and T cell subsets.

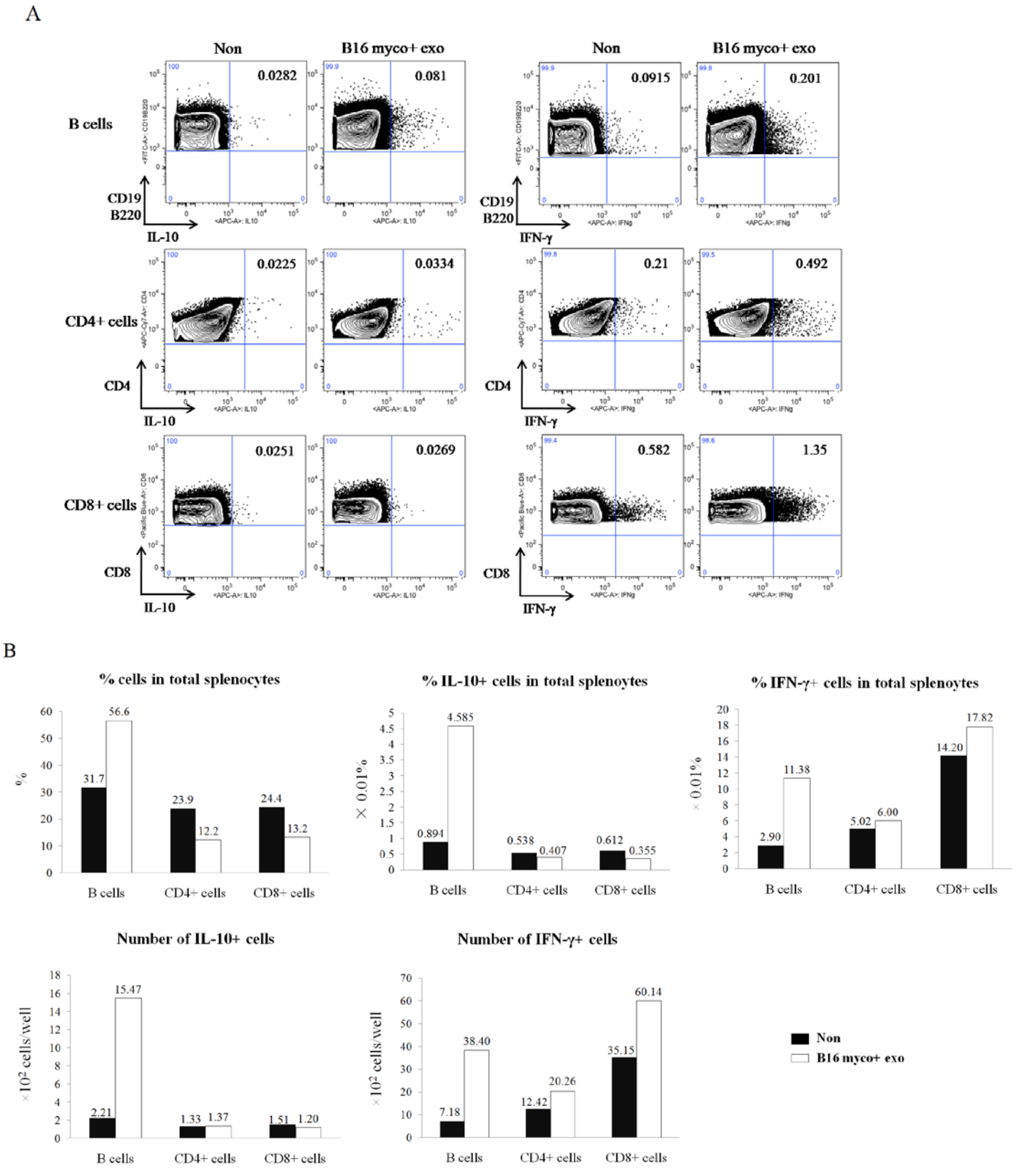

Figure 25: Intracellular staining of cytokine-producing cells induced by myco+ exosomes.

WT spleen cells were cultured with or without 1 μg/ml of B16 myco+ exosome for 48 hr. Brefeldin A was added to the culture for the last 6 hr before cells were harvested. Cells were first stained for surface markers CD19, B220, CD4 and CD8, fixed and permeablized, then stained for intracellular IL-10 and IFN-γ. (A). Percentage of IL-10+ or IFN-γ+ cells in B cell, CD4+ T cell and CD8+ T cell gates. Numbers in each plot represent % cells in outside gated region. (B). Plots showing that with (white bar) or without myco+ exosome treatment (black bar): the percentage of B cells, CD4+ T cells and CD8+ T cells in total splenocytes; the percentage of IL-10+ B cells, IL-10+CD4+ T cells and IL-10+CD8+ T cells in total splenocytes; the percentage of IFN-γ+ B cells, IFN-γ+CD4+ T cells and IFN-γ+CD8+ T cells in total splenocytes; number of IL-10+ B cells, IL-10+CD4+ T cells and IL-10+CD8+ T cells per well; and number of IFN-γ+ B cells, IFN-γ+CD4+ T cells and IFN-γ+CD8+ T cells per well. The number of cytokine-producing cells per well was calculated by multiplying the percentage of cytokine-producing cells in total splenocytes with the number of total cells harvested from each well (counted by trypan blue exclusion).

4.4.4 Induction of IFN-γ-producing CD8+ T cells by myco+ tumor exosomes was increased in the absence of B cells

We next compared the induction of IFN-γ-producing T cells by myco+ exosomes in μMT spleen cells with that in WT spleen cells. Surprisingly, a more striking increase in the percentage of IFN-γ+ cell in CD8+ T cell gate was induced in μMT spleen cells (> 3-fold) than in WT spleen cells (< 1-fold). The induction of IFN-γ+CD4+ T cells was much lower than that of IFN-γ+CD8+ T cells, and was comparable between WT and μMT spleen cells (Fig. 26). This indicates that the presence of B cells inhibits the induction of IFN-γ-producing CD8+ T cells by myco+ exosome treatment.

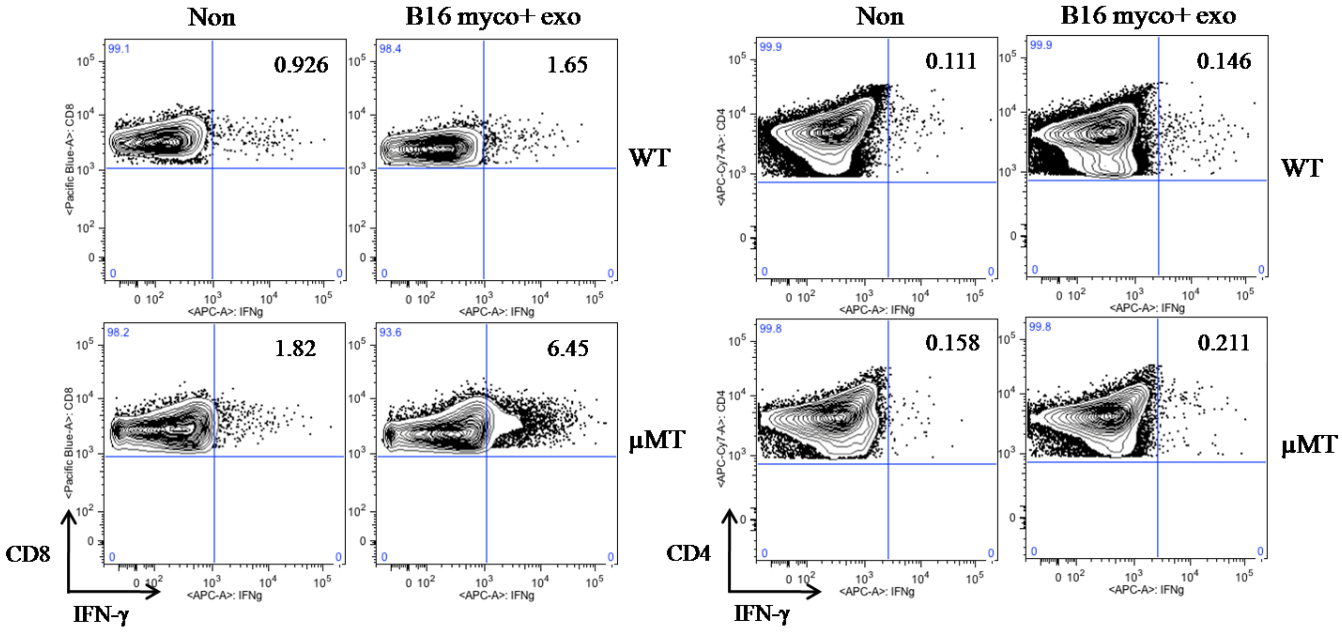

Figure 26: Percentage of IFN-γ-producing CD8+ T cells induced by myco+ exosomes increases in the absence of B cells.

WT or µMT spleen cells were cultured with or without 1µg/ml of B16 myco+ exosome for 48 hr and stained for intracellular IFN-γ. Induction of IFN-γ+CD8+ T cells and IFN-γ+CD4+ T cells were compared between WT and µMT spleen cell cultures. Numbers in each plot represent % cells in outside gated region.

4.4.5 T cell proliferation is inhibited in myco+ exosome-treated spleen cell culture or by myco+ exosome-treated B cells

Based on the observation that myco+ exosome induce B cell-derived IL-10 and the presence of B cells inhibit the induction of IFN-γ+CD8+ T cells, we sought to determine if T cell proliferation was affected in myco+ exosome-treated spleen cell culture. To better identify T cell populations,

T cells expressing the congenic marker CD45.1 were used. Total splenocytes (CD45.2+) were depleted with endogenous T cells by MACS beads, cultured with or without 1 μg/ml of B16 myco+ exosomes for 24 hr, before CFSE labeled CD45.1+ T cells were added. 10 μg/ml of anti-mouse CD3e was then added to the co-culture and T cells were allowed to proliferate for 3 days. CFSE dilution shows that when cultured in untreated splenocytes, both CD45.1+CD8+ T cells and CD45.1+CD4+ T cells proliferated upon anti-CD3 stimulation. However, T cell proliferation was almost completely inhibited when T cells were cultured in myco+ exosome-treated splenocytes. Proliferation of the activated T cell subsets with $CD44^{high}CD62L^{low}$ phenotype was also examined and similar inhibition was observed (Fig. 27A, B-1). Since B cells play a major role in IL-10 production, we tested if B cell alone, upon myco+ exosomes treatment, can inhibit T cell proliferation. B cells were purified from total splenocytes by MACS depletion of CD3+ T cells and Non-B-APCs (CD11c+, F4/80+ and PDCA-1+ cells). Purified B cells were cultured with or without 1 μg/ml of B16 myco+ exosomes for 24 hr before CFSE labeled CD45.1+ T cells were added to the culture. Cells were then stimulated with 10 μg/ml of anti-CD3e and T cells were allowed to proliferate for 3 days. CFSE dilution shows that myco+ exosome-treated B cells were equally capable of inhibiting the proliferation of CD8+ T cells and CD4+ T cells, as well as the $CD44^{high}CD62L^{low}$ T cell subsets (Fig. 27B-2).

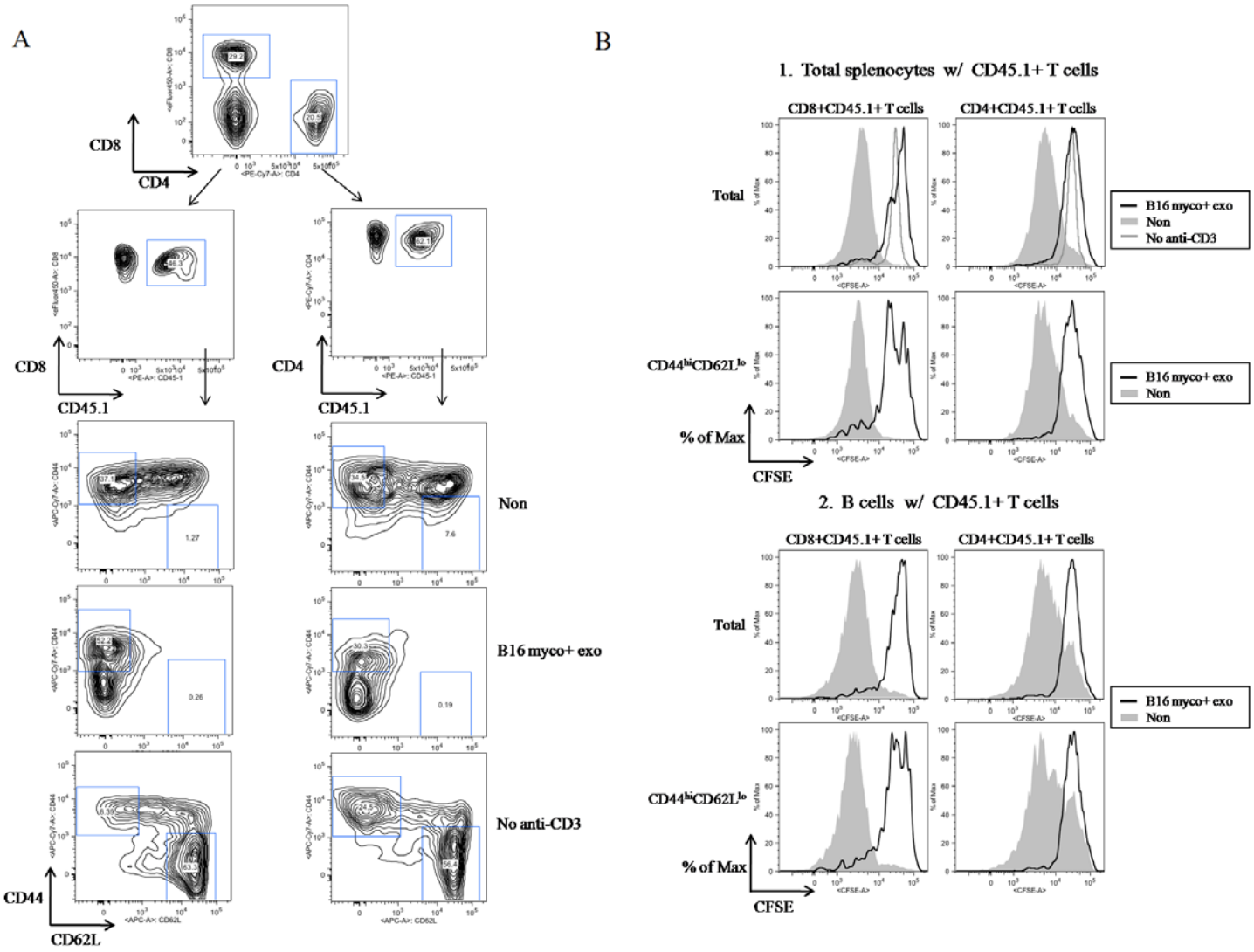

Figure 27: Myco+ exosome-treated splenocytes or B cells inhibit the proliferation of co-cultured T cells.

T-cell depleted splenocytes or purified splenic B cells were cultured in 24-well-plate at 2.5 × 10^6 cells/well with or without 1 μg/ml of B16 myco+ exosomes for 24 hr, before 0.5 × 10^6 of CFSE labeled T cells (CD45.1+) were added and stimulated with 10 μg/ml of anti-CD3e. Cells were co-cultured for another 3 days and T cell proliferation was analyzed by CFSE dilution. (A). Gating strategy to identify CD45.1+CD4+ T cells and CD45.1+CD8+ T cells. Expression of CD44 and CD62L were shown within each T cell gate in untreated and B16 myco+ exosome-treated co-cultures. Untreated cells without anti-CD3e were included as non-stimulated control. Activated T cell subsets ($CD44^{high}CD62L^{low}$) were further gated within T cell gates. (B). Proliferation of CD45.1+CD4+ T cells and CD45.1+CD8+T cells indicated by CFSE dilution. 1.

CD45.1+ T cells co-cultured with T cell-depleted total splenocytes. 2. CD45.1+ T cells co-cultured with purified B cells. Total: CFSE dilution of total CD45.1+CD4+ or CD45.1+CD8+ T cells. $CD44^{hi}CD62L^{lo}$: CFSE dilution of CD45.1+T cell subsets that are $CD44^{high}CD62L^{low}$. No anti-CD3 (gray line in 1): non-stimulated T cell proliferation control.

4.4.6 TCR signaling is impaired in myco+ exosome treated splenocytes

We next asked whether anti-CD3 stimulated TCR signaling was impaired in myco+ exosome-treated spleen cells. CD3 cross-linking triggers several downstream signal transduction pathways that lead to T cell activation and proliferation, including the MAP kinase pathway. Thus ERK phosphorylation, the last element in the MAP kinase cascade, was examined upon anti-CD3 stimulation. Spleen cells were treated with increasing doses of exosomes (0.1, 1 and 10 μg/ml) or cultured untreated for 48 hrs, and then stimulated with 1 μg/ml of anti-CD3e for 30 min before being harvested. Expression of phosphorylated ERK proteins (pERK1/2) and total ERK proteins (ERK1/2) were examined by Western blotting. Indeed, robust ERK phosphorylation was detected in untreated splenocytes and splenocytes treated with myco- exosome, whereas in splenocytes treated with myco+ exosomes, ERK phosphorylation was significantly inhibited in a dose-dependent manner (Fig. 28).

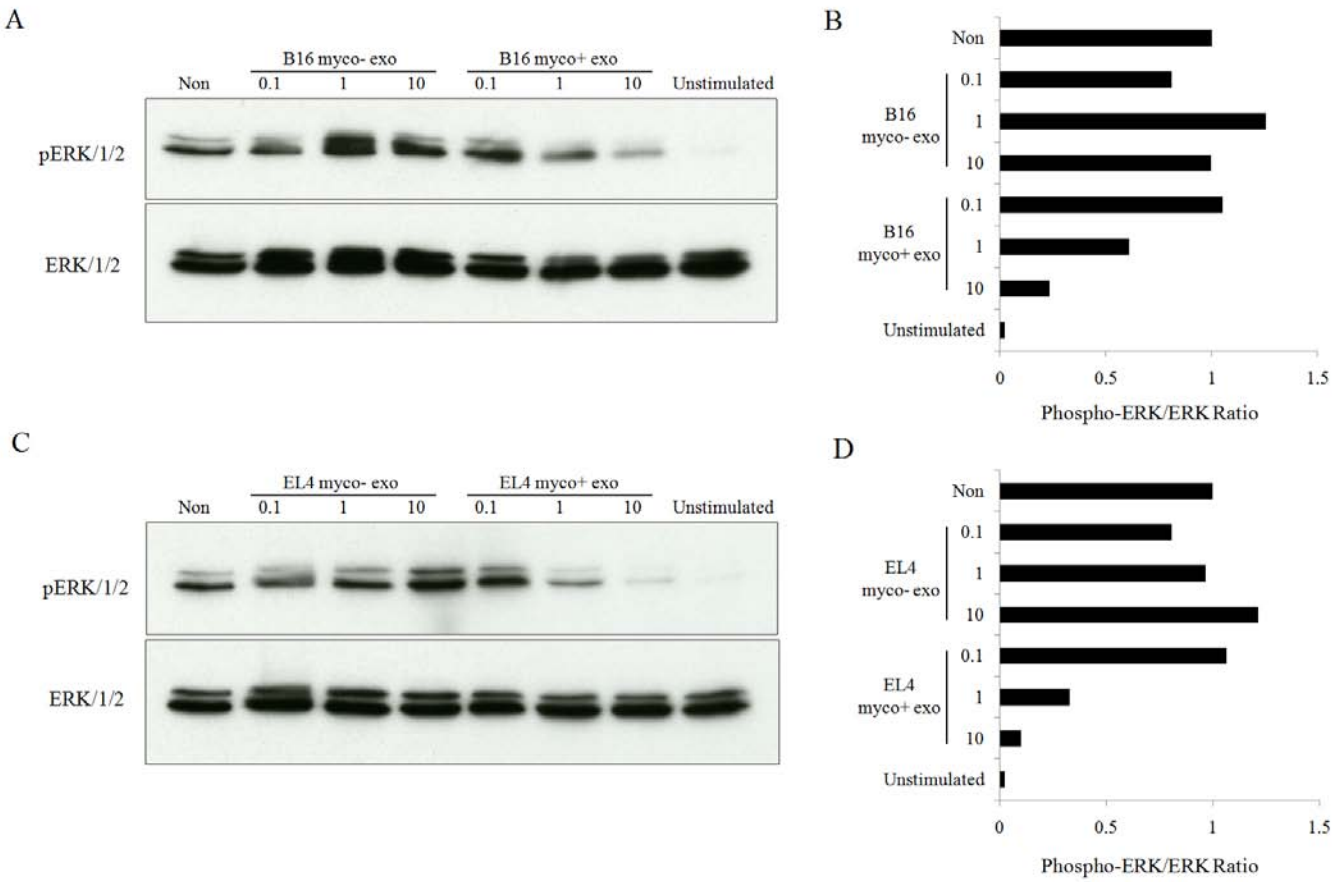

Figure 28: Myco+ exosome treatment inhibits anti-CD3e stimulated ERK phosphorylation.

Spleen cells were treated with 0.1, 1 or 10 µg/ml of either myco+ exosomes or myco- exosomes for 48 hr, and 1 µg/ml of anti-CD3e were added for 30 min stimulation. Spleen cells were prepared for Western blot analyses using antibodies against phosphorylated ERK protein (pERK1/2) and total ERK protein (ERK1/2). (A). Spleen cells treated with B16 myco- exosomes or B16 myco+ exosomes. Non: untreated spleen cells with anti-CD3e stimulation. Unstimulated: untreated spleen cells without anti-CD3e stimulation. (B). Relative expression of phospho-ERK normalized to the expression of total ERK in (A). (C). Spleen cells treated with EL4 myco- exosomes or EL4 myco+ exosomes. (D). Relative expression of phospho-ERK normalized to the expression of total ERK in (C).

4.4.7 Cytokine induction effect of myco+ exosomes does not require exosome membrane integrity

Particles larger than 0.22μm were filtered out from culture supernatants for exosome preparation and whole mycoplasma organisms were not observed by electron microscopy. Therefore we speculate that the cytokine induction and B cell-stimulatory effects are caused by mycoplasma component(s) in association with exosome structures. It was reported that the trafficking inhibitor primaquine, which prevents the transport of molecules from MIIC to the plasma membrane and therefore has the potential to inhibit exosome release, can completely abolish a similar B cell mitogenic capacity of membrane vesicles derived from mycoplasma infected DCs (181), indicating that the functional vesicle populations were exosomes formed in the MVB pathway. To determine if intact exosome structure is required for the cytokine induction effect of myco+ exosomes we observed, myco+ exosomes were subjected to 5 cycles of freeze/thaw or repeated sonication, both of which have been shown to disrupt exosome membranes (157). Interestingly, membrane disruption had very little impact on the cytokine induction and B cell activation effects of myco+ exosomes (Fig. 29B and data not shown). In contrast, exosomes isolated from tumor cell cultures after treatment with mycoplasma removal reagent completely lost their stimulatory ability (Fig. 29A-B). These results suggest that the exosome-associated stimulant(s) is mycoplasma-derived, but does not require exosome membrane integrity for its stimulatory effect.

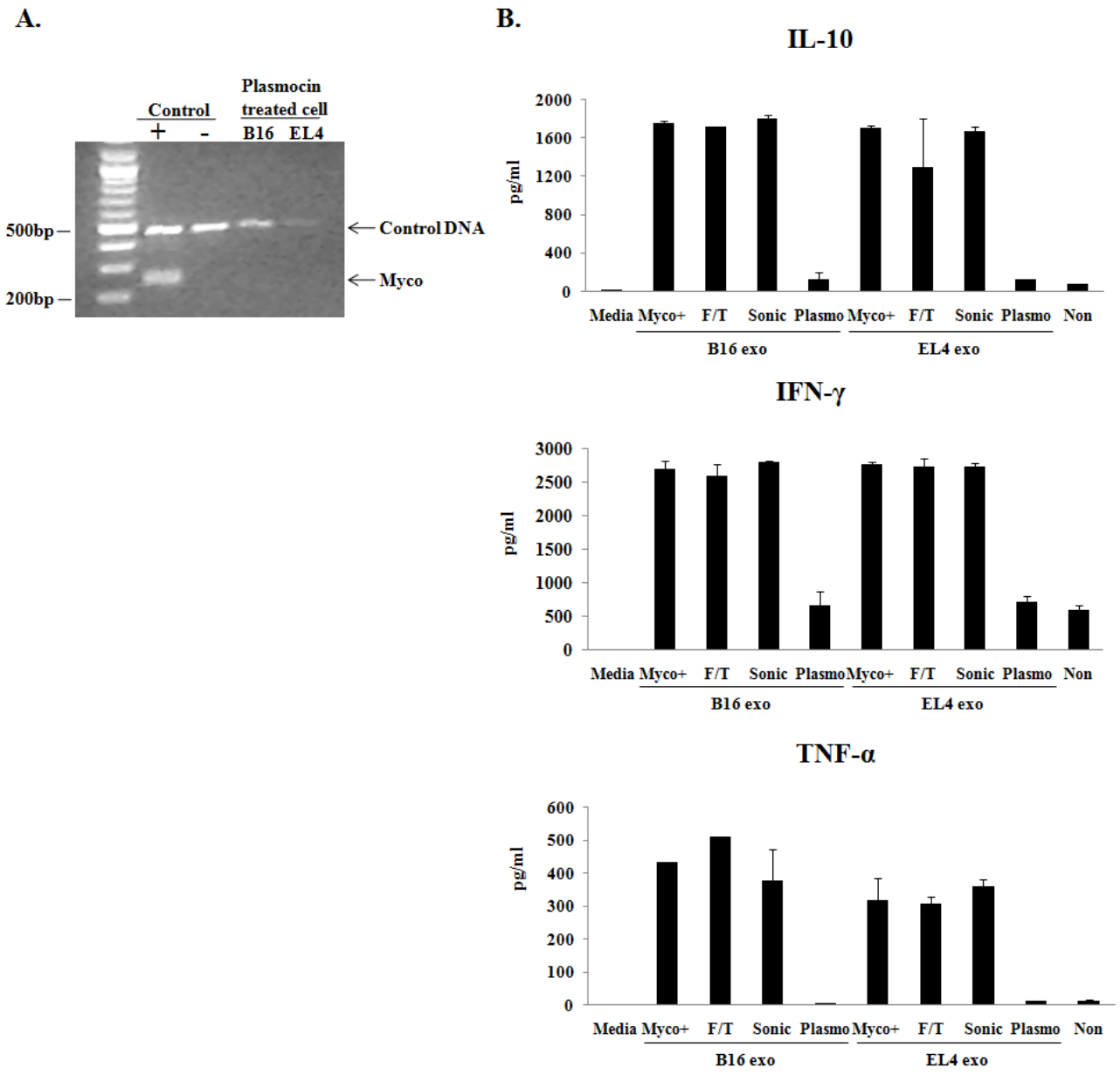

Figure 29: The cytokine-induction effect of myco+ exosomes is resistant to membrane disruption.

(A). Mycoplasma infected B16 and EL4 cell lines were treated with Plasmocin for 2 wk and tested to be mycoplasma-free. (B). myco+ exosomes were subjected to either 5 cycles of freeze/thaw (F/T) or sonication (Sonic). Spleen cells were treated with 1 μg/ml of myco+ exosomes, F/T exosomes, Sonic exosomes or exosomes derived from Plasmocin-treated cell cultures (Plasmo) for 72 hr, and cytokine productions were measured by ELISA.

4.4.8 Proteomic analysis of myco+ exosomes and myco- exosomes

To further investigate the exosome-associated mycoplasma components that possibly contribute to the B cell stimulatory effects, mass spectrometry (LS-MS/MS) analysis was performed on both B16 myco+ exosomes and B16 myco- exosomes. The initial database search identified certain Mycoplasma-related proteins specifically present in myco+ exosomes but not in myco- exosomes, including membrane-associated lipoproteins with potential immunostimulatory effects (Table 2). Multiple mycoplasma-associated enzymes and other cellular proteins were also indentified in myco+ exosomes. In addition, mycoplasma infection also seem to alter the endogenous protein composition of tumor-derived exosomes, as a variety of membrane proteins, enzymes, chaperons, nuclear proteins and structural proteins were found up-regulated in myco+ exosome (Table 3), while a repertoire of different proteins were found down-regulated in myco+ exosomes (Table 4). Although further confirmation is needed to validate the initial search results, identification of the putative mycoplasma proteins and the different endogenous protein expression pattern in myco+ exosomes will facilitate further effort to determine the exosome components responsible for the effects presented in this study.

Table 2: Mycoplasma protein hits specifically identified in myco+ B16 exosomes (selected).

Protein	Accession number	Peptides		Reported function
		number	% in total peptides	
Lipoproteins and membrane proteins				
Lmp3 protein	D1J7V1	3	0.67	Surface-located membrane lipoprotein
Integral membrane protein	A9NE35	2	0.45	
P37-like (*M.hyorhinis*) ABC transportersubstrate-binding lipoprotein	D1J8E9	1	0.22	P37 can increase the invasiveness and metastasis of cancer cells, and is an oncogenic marker (262)
P75 protein	D1J8G0	1	0.22	Induce TNF-α production in mouse macrophages (263)
Lipoprotein (*M.pulmonis*)	Q98PI7	5	1.3	
Lipoprotein (*M.pulmonis*)	Q98R04	2	0.52	
Lipoprotein (*M.pulmonis*)	Q98RF3	2	0.52	
Massive surface protein MspC (*M.arthritidis*)	B3PMQ9	2	0.52	
Massive surface protein MspH (*M.arthritidis*)	B3PN22	2	0.52	
Enzymes				
DNA methylase	A9NH63	6	1.34	
Glucose-6-phosphate 1-dehydrogenase	A9NGU2	5	1.12	
Peptidase M3B, oligoendopeptidase F	A9NH01	3	0.67	
DNA polymerase III, gamma and tau subunit	A9NE33	2	0.45	
Excinuclease ABC, subunit C	A9NEZ8	2	0.45	
V-type H+-transporting ATPase, subunit D	A9NHD6	2	0.45	
Acetyl-CoA carboxylase, biotin carboxylase	A9NFE7	2	0.45	
DNA polymerase III beta chain	D1J7E2	2	0.45	
NAD(+)-dependent DNA ligase	D1J7Y7	2	0.45	
DNA-directed RNA polymerase subunit alpha	Q98Q08	5	1.13	
ATP synthase epsilon chain	Q98QU6	4	1.04	
Glucose-6-phosphate isomerase	P78033	4	1.04	
Oligo-1,6-Glucosidase	Q98PT6	4	1.04	
Esterase/Lipase 1	Q98RH3	3	0.78	
Others				
50S ribosomal protein L16	A9NEE0	2	0.45	
ABC Transporter ATP-binding protein	D1J8A9	2	0.45	
Protein recA	A9NGM3	2	0.45	
Transcriptional regulator, AraC family	A9NHA0	2	0.45	
ABC-type transport system, permease component	A9NH78	2	0.45	
Transcriptional regulator, MarR family	A9NHK8	2	0.45	
Cell division protein ftsH	D1J8L1	1	0.22	
Cell division protein ftsZ	Q50318	3	0.78	
ABC Transporter ATP-binding protein	B3PM05	3	0.78	
Chromosome replication initiation and membrane attachment protein	B3PMZ9	3	0.78	
Segregation and condensation protein A	D3VRT6	3	0.78	
Cytadherence high molecular weight protein 2	P75471	3	0.78	

Dashed borders separate data obtained from two different searches against two combined Uniprot mycoplasma database (*M. hominis* and *A. laidlawii*; *M. agalactiae/ arthriditis/ pneumoniae/ pulmonis*).

Table 3: Endogenous murine proteins up-regulated in myco+ B16 exosomes (selected).

Protein	Accession number	No. of peptides identified		Normalized to total peptides (%)	
		Myco-exo	Myco+ exo	Myco-exo	Myco+ exo
Calpain-11	Q6J756	0	5	0	0.20
X-prolyl aminopeptidase (Aminopeptidase P) 2, membrane-bound	B1AVD2	0	4	0	0.16
Myotubularin related protein 2 (Fragment)	B8JJF3	0	4	0	0.16
Vomeronasal 1 receptor, F4	Q05A06	0	4	0	0.16
Slit homolog 3 protein	Q9WVB4	0	4	0	0.16
Diamine oxidase-like protein 2	Q6IMK7	0	3	0	0.12
Keratinocyte-associated transmembrane protein 2	Q8K201	0	3	0	0.12
DNA repair protein REV1	Q920Q2	0	3	0	0.12
Aconitate hydratase, mitochondrial	Q99KI0	0	3	0	0.12
Bifunctional purine biosynthesis protein PURH	Q9CWJ9	0	3	0	0.12
Fascin-3	Q9QXW4	0	3	0	0.12
Collagen alpha-4(VI) chain	A2AX52	0	2	0	0.08
Testis expressed gene 16 (Fragment)	B1AXV8	0	2	0	0.08
Ankrd11 protein	B2RY01	0	2	0	0.08
Bone morphogenetic protein receptor type-2	O35607	0	2	0	0.08
Poly [ADP-ribose] polymerase 2	O88554	0	2	0	0.08
Nuclear transition protein 2	P11378	0	2	0	0.08
Kallikrein 1-related peptidase b22	P15948	0	2	0	0.08
Isoform Mdm2-p90 of E3 ubiquitin-protein ligase Mdm2	P23804-1	0	2	0	0.08
Fibromodulin	P50608	0	2	0	0.08
Neurocan core protein	P55066	0	2	0	0.08
T-complex protein 1 subunit zeta	P80317	0	2	0	0.08
Tubulin beta-5 chain	P99024	0	2	0	0.08
Ras suppressor protein 1	Q01730	0	2	0	0.08
Cation-independent mannose-6-phosphate receptor	Q07113	0	2	0	0.08
Histone H1t	Q07133	0	2	0	0.08
BPAG1 isoform 3 (Fragment)	Q1KP04	0	2	0	0.08
Killer cell lectin-like receptor 2	Q60660	0	2	0	0.08
AKT-interacting protein	Q64362	0	2	0	0.08
All-trans-retinol 13,14-reductase	Q64FW2	0	2	0	0.08
Proline-rich protein 14	Q7TPN9	0	2	0	0.08
Nardilysin	Q8BHG1	0	2	0	0.08
Microtubule-associated protein 1S	Q8C052	0	2	0	0.08
Condensin complex subunit 2	Q8C156	0	2	0	0.08
Transcription factor SOX-30	Q8CGW4	0	2	0	0.08
Rootletin, isoform 1	Q8CJ40-1	0	2	0	0.08
Lactase-like protein, isoform 1	Q8K1F9-1	0	2	0	0.08
Ras-like protein family member 10A	Q8K5A4	0	2	0	0.08
Conserved oligomeric Golgi complex subunit 4	Q8R1U1	0	2	0	0.08
NEDD9-interacting protein with calponin homology and LIM domains	Q8VDP3	0	2	0	0.08

Transient receptor potential cation channel subfamily M member 2	Q91YD4	0	2	0	0.08
Prenylcysteine oxidase	Q9CQF9	0	2	0	0.08
Selenocysteine-specific elongation factor	Q9JHW4	0	2	0	0.08
72 kDa inositol polyphosphate 5-phosphatase	Q9JII1	0	2	0	0.08
Matrix metalloproteinase-16	Q9WTR0	0	2	0	0.08
Basement membrane-specific heparan sulfate proteoglycan core protein	Q05793	2	10	0.06	0.40
Transmembrane protein 63B	Q3TWI9	2	6	0.06	0.24

Data obtained from a search against a combined UniProt mouse protein database (03/2010) from the European Bioinformatics Institute (http://www.ebi.ac.uk/integr8).

Table 4: Endogenous murine proteins down-regulated in myco+ B16 exosomes (selected).

Protein	Accession number	No. of peptides identified		Normalized to total peptides (%)	
		Myco-exo	Myco+ exo	Myco-exo	Myco+ exo
Transmembrane glycoprotein NMB	Q99P91	27	6	0.85	0.24
5,6-dihydroxyindole-2-carboxylic acid oxidase	P07147	16	3	0.51	0.12
Multivesicular body subunit 12B	Q6KAU4	6	1	0.19	0.04
Sodium/potassium-transporting ATPase subunit alpha-1	Q8VDN2	30	5	0.95	0.20
Ras-related protein Rab-7a	P51150	7	1	0.22	0.04
Integrin beta-1	P09055	17	2	0.54	0.08
4F2 cell-surface antigen heavy chain	P10852	13	0	0.41	0
Melanocyte protein Pmel 17	Q60696	11	0	0.35	0
Histone H2B type 1-B	Q64475	11	0	0.35	0
High affinity cationic amino acid transporter 1	Q09143	10	0	0.32	0
Histone H2A type 1-F	Q8CGP5	10	0	0.32	0
Cofilin-1 OS=Mus musculus GN=Cfl1	P18760	7	0	0.22	0
Carbonic anhydrase 6	P18761	7	0	0.22	0
Solute carrier family 1 (Neutral amino acid transporter), member 5	Q5U647	7	0	0.22	0
EGF-like repeat and discoidin I-like domain-containing protein 3	O35474-1	6	0	0.19	0
Tubulin alpha-1B chain	P05213	6	0	0.19	0
Alpha-N-acetylglucosaminidase (Sanfilippo disease IIIB)	A2BFA6	5	0	0.16	0
Keratin, type II cytoskeletal 2 epidermal	Q3TTY5	5	0	0.16	0
Tetraspanin-7	Q62283	5	0	0.16	0
Solute carrier family 12 member 7, isoform 1	Q9WVL3-1	5	0	0.16	0
Basigin, isoform 1	P18572-1	4	0	0.13	0
Moesin	P26041	4	0	0.13	0
Synaptotagmin-4	P40749	4	0	0.13	0

M-phase inducer phosphatase 3	P48967	4	0	0.13	0
Monocarboxylate transporter 1	P53986	4	0	0.13	0
Histone H4	P62806	4	0	0.13	0
Adipocyte enhancer-binding protein 1, isoform 1	Q640N1-1	4	0	0.13	0
Centrosomal protein POC5, isoform 1	Q9DBS8-1	4	0	0.13	0
Guanine nucleotide-binding protein G(k) subunit alpha	Q9DC51	4	0	0.13	0
Clk2-Scamp3 protein	B2M0S2	3	0	0.09	0
Acetylcholine receptor subunit delta	P02716	3	0	0.09	0
Ras-related protein Rab-11A	P62492	3	0	0.09	0
Collagen alpha-2(V) chain	Q3U962	3	0	0.09	0
Sorting nexin-25	Q3ZT31	3	0	0.09	0
Nuclear receptor corepressor 1, isoform 1	Q60974-1	3	0	0.09	0
Leucine-rich repeat-containing protein 16A, isoform 1	Q6EDY6-1	3	0	0.09	0
Nipped-B-like protein, isoform 1	Q6KCD5-1	3	0	0.09	0
Cysteine-rich secretory protein LCCL domain-containing 1	Q8CGD2	3	0	0.09	0
Phosphofurin acidic cluster sorting protein 1	Q8K212	3	0	0.09	0
Putative uncharacterized protein	Q8R2W4	3	0	0.09	0
Vacuolar protein sorting-associated protein 28 homolog	Q9D1C8	3	0	0.09	0
6-phosphogluconate dehydrogenase, decarboxylating	Q9DCD0	3	0	0.09	0
Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1	Q9JJ06	3	0	0.09	0
Zinc finger homeobox protein 4	Q9JJN2	3	0	0.09	0
Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	Q9WUV2	3	0	0.09	0
Large neutral amino acids transporter small subunit 1	Q9Z127	3	0	0.09	0

Data obtained from a search against a combined UniProt mouse protein database (03/2010) from the European Bioinformatics Institute (http://www.ebi.ac.uk/integr8).

4.5 DISCUSSION

In this study, we demonstrate that tumor cells with mycoplasma infection release exosomes with B cell stimulatory and cytokine induction abilities, which were not observed in exosomes released from uninfected tumor cells. These effects of exosomes exclusively correlate with the infection status of their parental tumor cells, and can be completely abolished after treating the infected parental cells with mycoplasma removal reagent. Concerns about attributing mycoplasma-related immune responses to exosomes were raised in the study of DC-derived exosomes. It was reported that mycoplasma contaminants in DC exosome preparations induce polyclonal B cell mitogenisis, and the mitogenic capacity was gradually decreased after applying the culture supernatants on filters with reducing pore size (181). Thus whether whole, viable mycoplasmas are co-isolated with exosomes seems largely relate to the stringency of excluding mycoplasma-sized particles in the process of exosome purification. In our study, 0.22 μm pore filter was used and electron microscopy showed that whole mycoplasma organisms were absent in exosomes prepared from infected cell lines. Still, the seemingly “clean” exosome preparations induced splenic B cell activation, cytokine production, as well as cell proliferation typically seen after 48-72 hr of treatment with 1 μg/ml of exosomes. Furthermore, such effects were not affected by repeated freeze-thaw or sonication treatment, suggesting that intact exosome membranes are not necessary in initiating the immune responses. It has been reported that mycobacterial components actively traffic within infected macrophages with access to the MVB pathway and are released in exosome-like extracellular vesicles (176, 264). We speculated that

the stimulant(s) are mycoplasma components released in exosomes from infected tumor cells through a similar pathway.

Mycoplasma cell membrane contains abundant lipoproteins, many of which by themselves are immunogenic and/or mitogenic (265-267). Certain lipoproteins were found to induce T cell-independent B cell blastogenesis and secretion of proinflammatory cytokines (265), which resemble the effects of myco+ tumor exosomes we observed. Many mycoplasmal B cell mitogens function through a pathway distinct from that of lipopolysaccharide (LPS), as the effects were not abolished by polymyxin B and lymphocytes from mice that are poor responders to lipid A can also be stimulated (240, 265). In an effort to identify the potential mycoplasmal ligands in exosomes that are responsible for the effects, we performed proteomic comparison between myco+ exosomes and myco- exosomes. Within the mycoplasma protein hits that are specifically identified on myco+ exosomes, there was a group of membrane-associated proteins and lipoproteins with potential pro-inflammatory properties. Nevertheless, glycan moieties from mycoplasma were also found capable of inducing TNF-α production by human monocytes (252), therefore we cannot rule out the possibility that post-translational modifications and mycoplasma components other than proteins also contribute to the B cell stimulatory effect. The immune responses stimulated by those mycoplasma ligands are very likely to interfere with or even overwhelm the original immunomodulatory effect of exosomes released by host cells, and exosomes released from mycoplasma infected cells may stimulate similar immune responses regardless of the type of host cells.

Mycoplasma or its membrane lipoproteins are well-known for their ability to induce cytokine production, primarily the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, by human monocytes and macrophages (268). The anti-inflammatory cytokine IL-10 was also found

expressed in mycoplasma-associated human diseases (254). Here in murine spleen cell culture, we found that myco+ tumor-derived exosomes predominantly induce IL-10 in addition to IFN-γ and TNF-α, and the production of these cytokines was largely dependent on the presence of B cells. While IFN-γ-producing T cells especially CD8+ T cells were also induced besides B cells, IL-10-producing cells were mainly induced in the B cell population but not in the T cell population (Fig. 25). Our findings emphasize a role of B cells in producing cytokines, especially in producing IL-10, in response to exosomes containing mycoplasma components.

B cell-derived IL-10 can be produced by both naïve and memory B cells, as well as the regulatory B cell subsets with CD1d+CD5+ phenotype (198). Therefore more than one B cell subsets may contribute to IL-10 production. IL-10 sustains the growth of activated B cells, and acts as a hinge cytokine by suppressing cell-mediated immunity while promoting humoral immunity (194, 196). IL-10 production by B cell may function in the prevention of inflammatory responses in autoimmune diseases, as well as in the down-regulation of active disease exacerbation (198). Interestingly, we found that along with the dramatic decrease of IL-10 induction in μMT spleen cells, the percentage of IFN-γ-producing CD8+ T cells was significantly increased (Fig. 26), suggesting that B cells and B cell-derived IL-10 may play an important role in suppressing T cell activation. In myco+ exosome-treated WT spleen cells, we also observed significant increase in the percentage of IL-4-producing B cells and IL-4-producing CD4+ T cells by intracellular staining, however the total IL-4 level in culture supernatant was not increased (data not shown), presumably either the amounts of IL-4 produced by these cells were extremely low or IL-4 were rapidly consumed in this process.

Anti-CD3e stimulated proliferation of both CD4+ T cells and CD8+ T cells was strongly inhibited in myco+ exosome-treated splenocytes (Fig. 27). Correspondingly, the TCR signaling

in response to anti-CD3e was attenuated in a dose-dependent manner (Fig. 28). Such effect should not be a result of arginine depletion since it requires a functional arginine dihydrolase system in live mycoplasmas and affects B and T lymphocytes indiscriminately. Also, no induction of CD4+FoxP3+ T regulatory cells was found and no TGF-β was detected in this process (data not shown). In fact, B cell-derived IL-10 is highly likely to play an important role in T cell inhibition, and further studies using IL-10 neutralizing antibody as well as spleen cells derived from IL-10 deficient mice would clearly address the role of IL-10 in this process. Another possible reason for T cell inhibition is that up-regulation of CD25, the IL-2 receptor, on B cells may deprive IL-2 which is needed by T cells for their activation and proliferation. Although exogenous IL-2 was added (30-80 U/ml) to the culture throughout the assays, further study could increase the IL-2 amount and determine if T cell inhibition is caused by IL-2 deprivation.

Whether mycoplasma infection of cancer cells occur *in vivo* remains unclear, although certain archived human cancer tissue samples were reported to stain positive for mycoplasmas (259, 260). It has been reported that in hosts with deficient T cell activity, such as nude mice and cortisonized hamsters, mycoplasma infections reduced the tumorigenicity of tumor cells (269, 270). It can be speculated that in these hosts, mycoplasmas as well as exosomes derived from mycoplasma-infected tumor cells could both initiate B cell-dominated immune responses which aid in the eradication of infected tumor cells. However, based on our results, it is possible that in immune competent tumor-bearing hosts, once mycoplasmas colonize in the tumor tissue by opportunistic infection, the initial development of B cell responses may lead to the subsequent protective mechanisms such as the production of regulatory cytokines, resulting in the inhibition of T cell activity. In that point of view, exosomes derived from mycoplasma-infected tumor cells

may help jeopardize effective T cell responses in the anti-tumor immunity. We also observed that *in vitro*, myco+ exosomes were able to transfer mycoplasma infection to healthy cell lines at a very low protein level (0.1 μg/ml, data not shown), suggesting the ability of these exosomes to disseminate infections between cells, although we cannot completely rule out the possibility that mycoplasma organisms too few to be detected may still be present in exosome preparations. Overall, it will be quite interesting to investigate the role of exosomes in the development of combinatory immune responses induced by the co-existing mycoplasma pathogens and tumor cells *in vivo*.

In conclusion, our study characterized the splenic B cell and T cell responses to exosomes derived from mycoplasma-infected tumor cells. We demonstrated the preferential B cell activation and B cell-dependent cytokine induction by exosomes derived from infected tumor cells, and the consequent inhibition of T cell proliferation and TCR signaling. Our study for the first time dissected the reactions of different lymphocytes in response to tumor-derived exosomes carrying mycoplasma components and revealed the potential antagonizing effect of B cell activation to T cell activation. These observations will also help us better understand the impact of pathogenic components released in the form of exosomes on host immune modulation.

5.0 GENERAL DISCUSSION

Emerging evidence suggests that tumor-derived exosomes can play either immunostimulatory or immunosuppressive roles. Their immunostimulatory role is largely attributed to the presence of tumor antigens, which, when present in the form of exosomes, could be superior to other forms of antigens (e.g. cell lysates and soluble antigens), possibly due to more efficient delivery into APCs (113, 129, 139). In support of this idea, tumor-derived exosomes were reported to provide tumor-rejection antigens for DC cross-presentation and induce potent CD8+ T-cell-dependent antitumor effects *in vivo* (129). However, such an immunogenic role is not well reflected in advanced disease settings, in which tumor-derived exosomes are produced abundantly in the tumor microenvironment, yet induction of effective anti-tumor immunity is rarely observed. Indeed, the large number of reports stating that tumor-derived exosomes can display pleiotropic functions to subvert effective immune responses strengthened the idea that tumor cells may not produce exosomes for the purpose of evoking an anti-tumor immune response (131, 140, 144-149, 167, 174, 200, 271). Rather, as close replicas of the parental cells, tumor-derived exosomes may transmit the detrimental effects of tumor cells onto the immune system and facilitate tumor survival, growth and metastasis.

Tolerance to tumor antigens is usually developed during tumor progression (201, 203, 204). However, whether exosomal tumor antigens induce antigen-specific immunosuppression has not been discussed in the studies to date. The first part of this work was, therefore, based on

the hypothesis that tumor-derived exosomes and exosomal tumor antigens contribute to the induction of tumor-specific immunosuppression *in vivo*.

One of the many hurdles in studying the effect of exosomes in mediating antigen-specific immune suppression is the lack of an appropriate *in vitro* model that can fully recapitulate all the components participating in active immune suppression. Therefore we resorted to the DTH model, which is an important manifestation of antigen-specific cell-mediated immune response *in vivo.* Since cell-mediated immunity plays an essential role in anti-tumor immunity, and the DTH model has been previously utilized by the Robbins laboratory and others to assess antigen-specific immune suppression (157, 158, 160, 171, 172), we decided to evaluate the suppressive effect of tumor-derived exosomes using this model.

Using OVA as a surrogate tumor antigen, we initiated the characterization of exosomes derived from two OVA-expressing tumor cell lines (EG7 and MO5) and their parental cell lines (EL4 and B16). In fact, exosomes from each matching pair of tumor cells display similar morphology and protein expression patterns, with the exception of the presence of full length OVA protein. The observation that local administration of EG7 or MO5 exosomes, but not EL4 or B16 exosomes, was able to down-regulate the OVA-specific DTH response suggests a correlation between exosomal delivery of antigen and induction of suppression. Furthermore, we found that EG7 and MO5 exosomes were ineffective in suppressing KLH-specific DTH response, indicating that the suppressive effect is specific to the particular antigen they carry.

Encouraged by these results, we next sought to investigate the underlying mechanism. First, the trafficking of tumor-derived exosomes after footpad injection was tracked by fluorescent labeling of exosomes. We found that the majority of exosomes were taken up by dermal CD11c+ DCs and transported to the draining popliteal LN in less than 24 hours, and

exosome-internalized DCs were mostly present adjacent to CD3+ T cells. We also considered the possibility that exosomes interact directly with memory T cells in the dermis, as it was reported that DC-derived exosomes can be directly targeted to activated T cells via LFA-1 (126). However, we demonstrated *in vitro* that direct exosome treatment did not activate T cells from OVA sensitized mice within 48 hours. Additionally, OVA-specific T cell hybridoma cells were also irresponsive to EG7 or MO5 exosomes. In fact, direct T cell activation by tumor-derived exosomes has not been reported thus far; rather T cell stimulation requires exosomes to be processed by APCs (113). Therefore, we propose that DCs play an important role in mediating the suppressive effect of tumor-derived exosomes. Indeed, we found that tumor-derived exosomes inhibited the expression of MHC class II molecule and the co-stimulatory molecule CD86 on differentiated BMDCs. Moreover, under exosome treatment, BMDCs produce higher levels of regulatory TGF-β1. Interestingly, similar effects were reported when human DCs were cultured with human lung carcinoma cells (272). These results suggest that tumor-derived exosomes may be able to alter the normal function of DCs. In addition, we also detected increased mRNA levels of IL-4 and TGF-β1 in the draining LN in association with the reduced DTH response, suggesting a potential elevation in these Th2-type and regulatory cytokines. Although the exact mechanism of antigen-specific immune suppression remains to be determined, our findings suggest that better activation of antigen-specific regulatory T cells (which are usually induced after antigen sensitization) by DCs after taking up tumor-derived exosomes carrying that particular antigen could be responsible for the effect.

While this part of my thesis work revealed interesting immunoregulatory effects of tumor-derived exosomes, many questions remain. Future studies could include investigations of responder cells that produce IL-4 and TGF-β1, as well as on the correlation between exosome

treatment and regulatory T cell activation. Also, the molecular basis responsible for the suppressive effects of exosomes remains elusive, and to identify the contributing factor or factors will be a big challenge. Furthermore, tumor-derived exosomes bearing different tumor antigens should also be investigated in order to determine if induction of antigen-specific immune suppression is a common effect of tumor-derived exosomes.

How tumor-derived exosomes affect tumor immunity is a complex topic. Although somewhat controversial, existing reports may reflect the behavior of tumor-derived exosomes under different environmental conditions. It has been proposed that in the early phase of tumorigenesis, tumor cells and the exosomes they produce may have not yet acquired sufficient suppressive molecules and mechanisms, and the relative small amount of tumor-derived exosomes produced may serve to disseminate tumor rejection antigens to the immune system and elicit immune responses. In progressive diseases, however, increasing amount of exosomes possessing multiple mechanisms for attenuating different branches of the immune system would accumulate rapidly in the tumor microenvironment, and finally overwhelmed the anti-tumor immunity (113).

The second part of my thesis focused on the immunoregulatory effects of exosome-like vesicles systemically present in the blood plasma of mice with growing tumors. We demonstrated that plasma-derived exosomes isolated from mice bearing EG7 or MO5 tumors were also able to suppress OVA-specific DTH responses. This observation, which is consistent with previous reports showing that plasma-derived exosomes isolated from antigen-sensitized mice or antigen-fed animals can suppress an antigen-specific DTH response (160, 171, 172), further suggests that circulating exosomes can be a systemic factor transferring antigen-specific

tolerance. It has been shown that tumor-derived exosomes are present in the serum of cancer patients and that, in certain cases, the amount increases in patients with advanced cancers (118). However, this might be more often the situation with tumors that tend to generate large quantities of malignant effusions (226, 237). Using the FLAG-tagging system, we found that exosomes produced by tumor cells are undetectable in plasma in a murine melanoma model. Instead, the identification of several cellular makers (including MHC molecules, CD11b, B220, CD22 and CD9) indicates that they are possibly secreted by cells in the immune system. Our results suggest that exosomes released by tumors, especially localized solid tumors, may not have a half-life long enough to circulate in the blood stream before they are internalized by or bind to target cells. In fact, tumor-derived exosomes may exert immune regulation by a short-range mechanism, similar to what has reported for DC-derived exosomes (273); and while being dynamically produced and taken up, they gradually mould the tumor microenvironment to become immunosuppressive.

To better elucidate the fate of tumor-derived exosomes in tumor-bearing hosts, future work could be aimed at the identification of the cells that take up exosomes after they are released by tumor cells *in vivo*. Targeting GFP to exosome membrane using "exosome display technology" may help track the trafficking pattern of tumor-derived exosomes. To further identify the cells they interact with, one possible strategy is the use of mice with a *loxP*-flanked sequence controlling the expression of the downstream *GFP* gene. Upon generation of stable tumor cell lines that produce exosomes expressing Cre recombinase on their membrane surface, we can then inoculate mice with these tumor cells and visualize the cells/tissues that specifically internalize tumor-derived exosomes, as theoretically Cre can remove the control sequence and GFP will be expressed. The actual feasibility of this approach is still to be determined, as it is

unclear whether Cre can be functional after exosomes are internalized by APCs, since it can also be quickly degraded in the endocytic pathway.

Probably one of the biggest obstacles in understanding exosomes derived from body fluid is their heterogeneous cellular origin. Based on previous reports suggesting that MHC class II+ vesicles play an important role in tolerance induction (171, 172), we depleted MHC class II+ vesicles from plasma-derived exosomes from mice bearing OVA-expressing tumor and found that the suppressive effect was partially abrogated. Although these preliminary results did not completely answer the cell source of suppressive exosomes in plasma, it is very likely that these functional MHC class II+ vesicles are secreted by APCs that have sampled tumor-specific antigen.

Overall, what we have described in Chapter 2 and Chapter 3 is two separate, but closely related studies on the immunoregulatory effects of exosomes of defined tumor origin or exosomes retrieved from the blood plasma of tumor-bearing mice. We believe they may work concomitantly to induce tumor-specific immune suppression first within the tumor microenvironment and then cascade to amplify it into a systemic effect. Tumor-derived or circulating microvesicles were also reported to be enriched in factors that induce cancer metastasis and angiogenesis (151, 274, 275), or factors that have direct growth promoting effect on cancer cells (276), therefore together these vesicles can create a pro-tumorigenic network which allows tumor cells to survive and prosper. In another aspect, the antigen-specific suppressive effect of tumor-derived and plasma-derived exosomes could also make them attractive therapeutic agents to selectively dampen immune responses against a certain antigen.

In Chapter 4, we told a different story of tumor-derived exosomes when the parental cells have pathogenic infection. While typically no stimulatory effect was observed when treating splenic lymphocytes with tumor-derived exosomes, we found that exosomes prepared from a certain tumor cell subculture induced splenic B cell activation and proliferation. Such effect was then found to be associated with mycoplasma infection. Polyclonal B cell proliferation was reported to be induced by exosomes derived from mycoplasma-infected, long-term cultured DC cell lines (181). However, we reported for the first time that in addition to B cell proliferation, exosomes derived from mycoplasma-infected tumor cell line induce robust B cell-dependent cytokine production including the proinflammatory cytokines IFN-γ and TNF-α as well as the anti-inflammatory cytokine IL-10. Interestingly, B cells were found to be the major IL-10 producer. Moreover, T cell proliferation and TCR signaling induced by anti-CD3e were potently inhibited in myco+ exosome-treated spleen cell culture or by myco+ exosome-treated B cells alone. These results show that while myco+ tumor-derived exosomes stimulate T cell-independent B cell activation, they also induce consequent inhibition of T cell activation. Future study could be conducted to further elucidate the role of B cell-derived IL-10 in T cell inhibition, using IL-10 neutralizing antibody or IL-10 deficient mice. We believe that myco+ exosome treatment initiated one of the important mechanisms by which the immune system is instructed to take protective response to down-regulate cell-mediated response and avoid adverse reactions to microbial infections. Furthermore, the fact that several groups have identified the presence of mycoplasma in human cancer samples highlights the possibility that opportunistic pathogens may invade tumor-bearing hosts and co-exist with tumor cells. Therefore, exosome release by these tumor cells *in vivo* can be an alternative pathway which these pathogens exploit to disseminate their own components and influence the immune system. We speculate that the

initial B cell activation may lead to the inhibition of T cell-mediated response, thereby exacerbating the ineffectiveness of anti-tumor immunity.

Finally, we extended this part of work by demonstrating that the exosome-associated, mycoplasma-derived B cell stimulant is resistant to membrane disruption, and by reporting the preliminary mass spectrometry results of mycoplasma protein hits specifically found on myco+ exosomes. These data will aid further identification of proteins or other components that are responsible for the effects we have described.

In conclusion, tumor-associated immune suppression and tumor-specific tolerance is the result of complex interplays between different cells and environmental factors. My thesis projects have endeavored to investigate the role of tumor-derived exosomes and circulating exosomes in tumor-bearing hosts in immune regulation and especially in the generation of antigen-specific immune suppression. While this thesis has generated at least as many questions as it has answered, the data it presents provide us with a new perspective in understanding exosomes as effective contributors to tumor immune evasion, and will hopefully assist in the future development of strategies to break tolerance to tumor antigens and enhance anti-tumor immunity.

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