Regulation of Anti-tumor Immunity in Colorectal Cancer by Interaction of Exosome Immune Complexes with Fc gamma Receptors

by

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ABSTRACT

Targeted therapy is difficult for colorectal cancer (CRC) due to lack of conserved tumor associated antigens (TAA). Immunotherapies for CRC directed at TAA that can overcome intestinal tolerance need to be developed to improve survival of CRC patients. Exosomes are extracellular vesicles secreted by CRC cells that carry TAAs and display unique surface phospholipids (PPL). Although exosomes represent an ideal delivery vehicle for TAA into the immune system, most evidence indicates that they are immunosuppressive. However, the surface PPL on exosomes can be bound by PPL-specific immunoglobulin G (IgG) to generate immune complexes (IC). We hypothesized that engagement of the Fc region of the IC with activating IgG-binding Fc receptors on dendritic cells (DC) and macrophages could prime sufficient $CD8^+$ T-cell anti-tumor immunity and release of immune-enhancing cytokines to overcome immunosuppression. To test this, DC or macrophages were stimulated with exosomes from MC38 murine CRC cells complexed or not with anti-PPL IgG. Greater of activation of STAT1 along with expression of inflammatory genes IL-1B, IL-6, COX-2 and IL-12 were observed in DC and macrophages stimulated with IC than with exosomes alone. To verify that this was Fc receptor-dependent, we co-cultured $CD8^+$ T cells with DC exposed to IC or exosomes alone in the presence or absence of Fc receptor-blocking antibodies. IC led to greater T cell activation than exosomes alone only when they could bind Fc receptors. In mice bearing an MC38 tumor transfected with OVA and given OVA-specific OT-I T cells, splenic DC upregulated the activation marker CD86 when treated with IC but not exosomes alone. No difference in IFNγ expression by tumor infiltrating T cells was detected in these mice, however splenic OVA-specific T cells exhibited greater expression of IFNγ following IC treatment. This suggests that exosome IC can prime $CD8^+$ T cells under ideal conditions but not in the suppressive CRC environment. Further investigation of anti-PPL will allow us to understand whether this strategy could help increase anti-tumor immunity and survival in CRC patients.

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TABLE OF CONTENTS

LIST OF RECURRING ABBREVIATIONS

β2GPI – beta 2 glycoprotein I

β2M – beta-2 microglobulin

AA – arachidonic acid

ACK – ammonium chloride potassium

ADCC – antibody-dependent cellular cytotoxicity

AKT – protein kinase B

APC – antigen presenting cell

AV – annexin V

B6 – C57BL/6 wildtype mice

BCA – bicinchoninic acid assay

BMDC – bone marrow derived dendritic cells

BMDM – bone marrow derived macrophages

BSA – bovine serum albumin

CAF – cancer associated fibroblast

CCL – C-C motif ligand

CD – cluster of differentiation

cDC – conventional dendritic cell

CFSE – carboxyfluorescein diacetate succinimidyl ester

CIN – chromosomal instability

CMV – cytomegalovirus

COX – cyclooxygenase

CRC – colorectal cancer

CRISPR – clustered regularly interspaced short palindromic repeats

CT – computed tomography

CTL – cytotoxic T lymphocyte

CTLA-4 – cytotoxic t-lymphocyte protein 4

CXCL – CXC motif ligand

DAMP – damage associated molecular pattern

DC – dendritic cell

DHA – docosahexaenoic acid

DMEM – Dulbecco's modified eagle medium

EDTA – Ethylenediaminetetraacetic acid

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

ELISA – enzyme linked immunosorbent assay

ERK – extracellular signal-regulated kinase

EV – extracellular vesicle

Exo – exosome

$F(ab')_2$ – antibody fragment devoid of the Fc region

FA – fatty acid

FBS – fetal bovine serum

Fc – fragment crystallizable region

FcγR – fragment crystallizable gamma receptor

FDA – Food and Drug Administration

FOLFIRI – folinic acid, 5-flurorouracil and irinotecan protocol

FOLFOX protocol – folinic acid, 5-flurorouracil and oxaliplatin protocol

FOXP3 – forkhead box P3

GCSF – granulocyte colony stimulating factor

GI – gastrointestinal

GM-CSF – granulocyte macrophage colony stimulating factor

H-2Kb – murine major histocompatibility complex I alloantigen

HER2 – human epidermal growth factor receptor 2

HIV – human immunodeficiency virus

HLA – human leukocyte antigen

HRP – horseradish peroxidase

HSP70 – heat shock protein 70

I-Ab – murine major histocompatibility class II alloantigen

IC – immune complex

ICAM-1 – intercellular adhesion molecule 1

IEL – intraepithelial lymphocyte

IFN – interferon

IFNγ – interferon gamma

Ig – immunoglobulin

IL – interleukin

IT – intratumoral

ITAM – immunoreceptor tyrosine-based activation motif

ITIM - immunoreceptor tyrosine-based inhibitory motif

IV – intravenous

JAK – Janus kinase

KRAS – Kirsten rat sarcoma

LAS – large angle scatter

LFA-1 – Lymphocyte function-associated antigen 1

LOH – loss of heterozygosity

LOHHLA – loss of heterozygosity in human leukocyte antigen

LOX – lipoxygenase

LPS – lipopolysaccharide

LT – leukotriene

MAPK – mitogen-activated protein kinase

MDA5 – melanoma differentiation-associated gene 5

MDSC – myeloid derived suppressor cell

MFI – mean fluorescent intensity

MHC – major histocompatibility complex

MLH1 – MutL homolog 1

MMR – mismatch repair

mPS – monoclonal phosphatidylserine antibody

MRI – magnetic resonance imaging

MSH – MutS homolog

MSI – microsatellite instability

MSS – microsatellite stable

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

n-3 – omega-3

n-6 – omega 6

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NK – natural killer

NOTAM – novel γ-chain signaling deficient mouse model

NSCLC – non-small cell lung cancer

OVA – ovalbumin

PBS – phosphate buffered saline

PD-1 – programmed cell death protein-1

PD-L1 – programmed death-ligand 1

PD-L2 – pro programmed death-ligand 2

pDC – plasmacytoid dendritic cell

PG – prostaglandin

PI3K – phosphoinositide 3-kinase

PLCγ – phospholipase C gamma

pLN – popliteal lymph node

PMA – phorbol 12-myristate 13-acetate

PMS2 – postmeiotic segregation increased S. Cerevisiae 2

POLδ – polymerase delta

POLε – polymerase epsilon

Poly I:C – polyinosine-polycytidylic acid

PPL – phospholipid

pPS – polyclonal phosphatidylserine antibody

PS – phosphatidylserine

PUFA – polyunsaturated fatty acid

qPCR – quantitative polymerase chain reaction

qRT-PCR – quantitative real time polymerase chain reaction

RA – retinoic acid

RBC – red blood cell

RIG-I – retinoic acid-inducible gene-I

RPMI – Roswell Park Memorial Institute medium

sExo – serum exosome

SLE – systemic lupus erythematosus

sOVA – soluble ovalbumin

SRC – proto-oncogene tyrosine-protein kinase

SSC – side scatter

STAT – signal transducer and activator of transcription proteins

SYK – spleen tyrosine kinase

TAA – tumor associated antigen

TAP – transporter associated with antigen processing

TCGA – The Cancer Genome Atlas

TCR – T cell receptor

TGFβ - transforming growth factor beta

Th1 – T helper 1

Th2 – T helper 2

TIL – tumor infiltrating lymphocyte

TLR – toll-like receptor

TNF – tumor necrosis factor

TP53 – tumor protein 53

Treg – T regulatory cell ($FoxP3^{+}CD4^{+}$)

TRIM21 – tripartite motif-containing protein 21

VEGF – vascular endothelial growth factor

Wnt-β-catenin – wingless-integrated beta catenin

WT – wildype

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Colorectal cancer as a disease

Ranking second to cardiovascular disease, malignant neoplasms are the second most common cause of mortality in the developed world[1]. Cancer is a heterogeneous disease than can arise in any tissue within the body. Genetic and environmental factors have been identified to play a synergistic role in development of these neoplasms. Despite early work to identify these specific genetic and environmental factors, much more work is needed to uncover the intricacies of cancer development. New light has recently been shed on the importance of the interaction of tumors with the immune system. This has led to the development of new immunotherapies that have successfully extended the lives of cancer patients with advanced cancer[2]. Although these immunotherapies remain an attractive avenue for disease treatment and future research, the efficacy of these therapies remains limited and their cost remains high[3,4,5]. Thus, they are currently not available to all cancer patients. This highlights the fact that cancer remains both an economic and social burden to the healthcare system and families presented with cancer. Nevertheless, development of new immune-based therapies continues such that monotherapies consisting of a cocktail of chemotherapeutic drugs will soon no longer be the proposed first-line therapy for cancer. It is increasingly recognized that multiple angles of therapy, including the combination of traditional therapies with immunotherapy, provides new avenues that will offer cancer patients the most promising care leading to tumor eradication with as few side effects as possible[6,7]. This goal will only be achieved through further studies on the intricacies of the interaction between the tumor and different arms of the immune system.

CRC is the third most common cancer worldwide with 1.8 million new cases diagnosed in 2018[8]. Following behind lung cancer, CRC is the second most deadly cancer, accounting for 862 000 deaths as determined by the World Health Organization as of 2018[8]. This data is followed by a similar trend observed in Canada where CRC is the second and third most commonly diagnosed cancer in men and women, respectively[9]. Despite the colorectum being an organ commonly affected by cancer, in Canada rates of CRC are decreasing[9]. Age standardized data from 1988 to 2010 shows a decrease in disease incident rate of 11.3% for males and 14.5% for females[10].

Although the incidence rate appears to be headed in right direction, this decrease in incidence rate is restricted to older adults compared to the younger adult population where the number of CRC cases are increasing. Four major risk factors have been identified for CRC that are also shared risk factors for other non-communicable diseases[10]. These include: tobacco, alcohol, unhealthy diets that lack fruit and vegetables and physical inactivity[11]. The Canadian Cancer Society also recognizes that specific dietary factors increase CRC risk including consumption of red meat, processed meat and low fiber intake. In addition, other diseases that strike Western society including diabetes, inflammatory bowel disease and cystic fibrosis also increase CRC[10]. Although there has been a decrease in CRC rates over time, this is not attributed to changes in lifestyle factors but is instead a result of the implementation of screening and early detection initiatives in Canada[10]. CRC is thought to be a largely preventable cancer through modification of lifestyle factors and the unique opportunity for minimally invasive-colorectal screening. Despite these programs, approximately 20% of CRC are still diagnosed at stage IV and most other cases are diagnosed at stage III[10]. Like many cancers, early detection and intervention is key for increasing survival rates. Five-year survival for stage I CRC is 92%, whereas it decreases to 11% for stage IV disease[10]. The increasing rate of CRC diagnoses in the young adult population presents an area of opportunity for health officials to promote healthy diet and lifestyle factors. Although more work is required to promote screening and early detection, much research is still also needed to uncover molecular changes and events that occur during CRC development before new CRC diagnosis rates can begin to approach zero.

Clinical and pathological aspects of CRC

The large intestine consists of the colon and rectum. Although cancers occurring along the large intestinal tract are all grouped under the term “CRC”, the frequency of tumors arising in different sections of the colon including the ascending, transverse, descending and rectum varies considerably due to local factors. Despite CRC being one of the most common cancers, lifetime risk for the general population is 5-6%[10,11]. This risk becomes increased following the inheritance of various genetic factors. While 20% of CRC are thought to have some degree of familial risk, only 5-10% of cases are thought to arise directly as a result of hereditary syndromes[12]. Individuals with these syndromes have very high rates of cancer and undergo early

routine endoscopy and colonoscopy to identify polyp growth and manage early stage disease starting in adolescence. In contrast, the identification of sporadic CRC is often because of various symptoms including blood in stool, anemia, abdominal pain, fatigue and unintended weight loss[13]. Colonoscopy is currently the gold standard for the diagnosis of colorectal tumors and additional imaging by CT or MRI is used for staging and to investigate potential metastatic spread[13]. In addition, carcinoembryonic antigen levels in the blood can be measured but its lack of specificity means it is most useful in determining the presence of liver metastasis and treatment response following resection in known CRC patients[13,14].

First-line treatment strategy for CRC varies depending on the stage of the tumor[13]. Non-metastasized primary tumors are removed by surgical resection followed by a chemotherapy regimen of leucovorin (folinic acid), 5-fluorouracil, and oxaliplatin (FOLFOX protocol)[13,15]. Addition of targeted therapies including monoclonal antibodies directed toward epidermal growth factor receptor (EGFR) or vascular endothelial growth factor (VEGF) have not increased survival in early stage CRC[15]. In contrast, patients with advanced metastasized CRC undergo neoadjuvant treatment to decrease tumor load and stage prior to surgical resection[13,16,17]. These patients then receive FOLFOX or the alternative combination of leucovorin, 5-fluorouracil and irinotecan (FOLFIRI) and anti-VEGF antibody therapy[13,16,17]. Adjuvant radiation therapy is only commonly used for cancers in the rectum, not other intestinal sites, with the aim of decreasing tumor load prior to surgical resection in order to spare rectal anatomy as well as decreasing the incidence of local recurrence[18,19,20]. For metastatic CRC, monoclonal antibody therapy directed towards VEGF and EGFR has been linked to increased median progression free survival[21]. However, the use of an EGFR inhibitor is limited since it has only demonstrated efficacy in patients resistant to FOLFOX/FOLFIRI therapy or who are chemotherapy naïve[22,23]. In addition, administration of targeted therapies requires an understanding of the molecular features of the tumor since EGFR blockade only demonstrates efficacy in tumors without mutations in *KRAS* or *NRAS*[22]. As with most cancers, many patients become refractory to therapy and management of recurrent cancer is often palliative. More recently, immunotherapy such as inhibitors of the programmed cell death protein 1 (PD-1) and PD-1 ligand (PD-L1) receptor-ligand interaction has shown promising results in a small subset of CRC[24]. Immunotherapy is thus an attractive avenue

and studies aimed at developing a better understanding of molecular events leading to its success or failure will allow us to make better use of this new therapeutic avenue.

Molecular aspects of CRC

CRC is a heterogeneous disease and can manifest via different molecular pathways through the inactivation of genes broadly involved in either proliferation or repair[25,26]. The two pathways that are most commonly observed arise from mutations in different sets of genes that lead to distinct phenotypes with different implications for prognosis, treatment and survival[27].

Unlike other cancers, the developmental pathway and sequential events that occur leading to the formation of an invasive colorectal tumor is very well understood. Inactivation of genes controlling proliferation account for the majority (85%) of CRC cases[28]. This is initiated by loss of heterozygosity of the adenomatous polyposis coli (APC) gene involved in proliferative wingless integrated (Wnt)-β-catenin signaling in colon epithelial stem cells[29,30]. These tumors then develop mutations in *KRAS* and deletions of genes on chromosome 18q that lead to further growth of the precursor adenoma[29,31]. Biallelic loss of the tumor suppressor *TP53* often then mediates the transition of the tumor to a carcinoma **(Fig. 1-1A)**[27,32]. This stepwise pathway was first described by Vogelstein and is referred to as the classical adenoma-carcinoma sequence for CRC[27,29]. Cancers arising along this pathway are characterized as having numerous chromosomal aberrations, aneuploidy, greater metastatic risk and a poor prognosis[27,29]. Although each of these molecular events most commonly occurs sporadically, the hereditary syndrome familial adenomatous polyposis is caused by germline mutation in the *APC* gene that leads to the extensive formation of many polyps throughout the colon many of which follow the same developmental pathway into an invasive carcinoma[12].

It was originally thought that all CRC followed the aforementioned pathway, however upon careful analysis there were a subset of cancers that displayed a unique phenotype[33]. This subset of CRC is initiated by defects in genes involved in DNA repair pathways[33]. Cells from these tumors do not have major deletions of DNA segments indicative of chromosomal instability[31]. Instead, these cells demonstrate deletions and amplifications in repetitive DNA sequences known

as microsatellites, leading to truncations and frameshifts respectively[34–36]. Ultimately it was discovered that these tumors have defects in DNA mismatch repair (MMR) genes that excise mismatched bases and thus accumulate mutations in mono-, di- and trinucleotide repeats of bases[34–36]. In these regions, DNA polymerases are much more prone to slippage leading to a high frequency of incorrect base incorporation and insertions and deletions[36]. Defects in the DNA MMR system can form as a result of an inheritance of a germline mutation in a DNA MMR genes such as *MLH1*, *MSH2*, *MSH3*, *MSH6*, or *PMS2* that initiates disease referred to as hereditary non-polyposis CRC cancer or Lynch syndrome[33,37]. Individuals with one of these mutations are at increased risk for cancer of the endometrium, ovary, stomach, small and large intestine and urinary tract[38–40].

Much more commonly, sporadic cases arise from hypermethylation of the *MLH1* promoter and these tumors display overall perturbations in their genome wide methylation status[27,41]. Aside from defects in methylation patterns, MMR deficient sporadic and hereditary CRC forms follow a similar developmental pattern, each are microsatellite instable (MSI) and accumulate genome wide mutations[12]. Genes that become inactivated typically display nucleotide repeats and include *PI3K*, *TGFβ*, *IGF2R*, *BAX*, *MRE11A*, and *hRAD50* **(Fig. 1-1B)**[27,33]. Less frequently, the tumor can accumulate *TP53* mutations along with inactivation of other tumor suppressor genes[27]. Lynch syndrome and sporadic cases of CRC do differ in mutations observed in *KRAS*, which are more common in the former whereas the V600E mutation in *BRAF* is observed in the later[27,42,43]. MSI CRC that develop through defects in DNA MMR account for 15-20% of cases[27]. Of these cases, only 2-3% are due to a hereditary form while the remaining 12-17% are sporadic cases[12,44,45]. Clinically, MSI CRC are distinct from CRC that develop by the classical adenoma carcinoma sequence, which and are categorized as chromosomally instable (CIN) and microsatellite stable (MSS)[31]. MSI CRC lead to a better prognosis, exhibit poor differentiation, are less invasive, less likely to metastasize and occur more frequently in the ascending colon[27,34,46,47,48,49]. Currently MSI CRC is diagnosed using the Bethesda guidelines which analyzes a panel of five microsatellite markers. Classification of high microsatellite instability requires that greater than 30% of the panel markers be mutated[27,50,51]. Identification of these tumors is important for selection of a chemotherapy regimen as these patients respond differently to standard chemotherapeutic protocols[27,52].

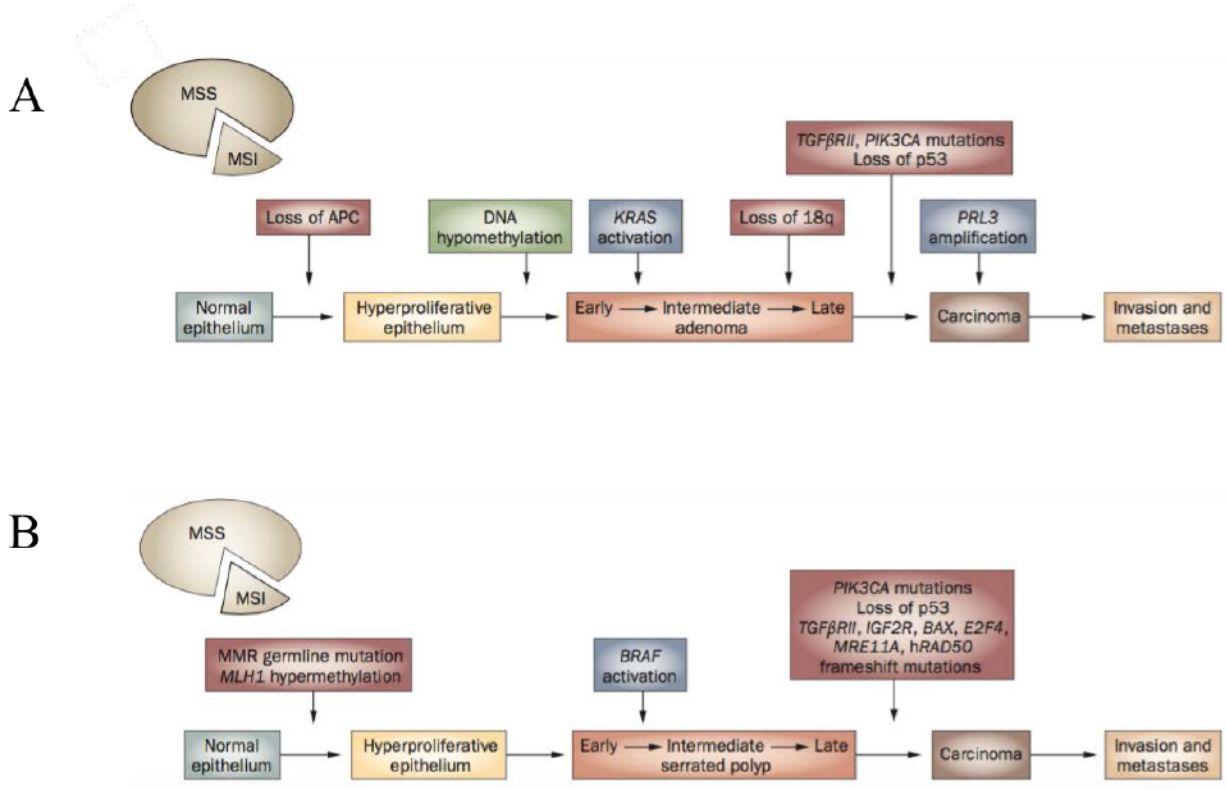


Figure 1-1. Major molecular subtypes of CRC. (A) The developmental pathway of microsatellite stable (MSS) CRC. Loss of the tumor suppressor gene *APC* is a major initiating event in the development of a hyperplastic neoplasia in the colorectum. This is followed by sequential *KRAS* oncogene activation, chromosomal rearrangements and loss of the tumor suppressor *TP53* leading to the carcinoma transition. **(B)** The developmental pathway of microsatellite instable CRC is initiated by the loss of DNA mismatch repair genes through spontaneous hypermethylation or a heritable mutation in a DNA mismatch repair gene *MLH1*. This is commonly followed by oncogenic activation of BRAF and frameshift mutations in genes containing microsatellite repeats leading to the transition to a carcinoma. Figure from: Vilar E, *et al*. 2010. Nat Rev Clin Oncol. 7(3): 153-62. Reprinted with permission.

Immune cells in microsatellite instable CRC

Recently, identification of microsatellite instable tumors has become more important in clinical practice[53]. A unique feature of MSI CRC is the presence of abundant tumor infiltrating lymphocytes (TILs)[37]. Although many immune cells may contribute to anti-tumor immunity, $CD8^+$ T cells have been the most widely studied in CRC. The TILs in MSI CRCs have been found to be predominantly $CD8^+$ T cells and express the cytotoxic markers perforin and granzyme B[54,55]. The presence of these TILs is a unique feature of MSI CRC as MSS CRC have very few TILs[56]. The better prognosis and less invasive nature of MSI tumor is hypothesized to be a result of tumor control by TILs[54,56–58]. Both CRC tumor types contain mutations and chromosomal aberrations, however an excessive number of mutations accumulate in MSI CRC[33,58]. Loss of the MMR system in MSI CRC leads to insertions and deletions causing many frameshift mutations[58,59]. These frameshift mutations lead to the production of a large number of aberrant proteins also known as tumor associated antigens (TAA)[59]. These antigens are also referred to as neoantigens as they are thought to be recognized as non-self by $CD8^+$ T cells which can be detected in peripheral blood of patients[58,60]. The increased TAA load in these tumor cells is thought to drive stimulation of tumor-specific cytotoxic T cells and anti-tumor immunity which has been demonstrated in a number of studies[57,58,61,62]. The presence of these TILs is a positive prognostic marker for overall survival in CRC[37].

Many clinical trials have examined the use of checkpoint inhibitors directed towards cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and PD-1/PD-L1 in a variety of tumors[24,63]. These inhibitors demonstrate the greatest efficacy in tumors that display microsatellite instability including MSI CRC[24]. These findings led to the recent 2017 United States Food and Drug Administration (FDA) approval of the use of pembrolizumab (Keytruda) anti-PD-L1 checkpoint inhibition for all solid pediatric and adult cancers that display microsatellite instability or are MMR deficient. This has significantly increased the importance of detecting this subtype in clinical practice[64–66]. Unfortunately, efficacy of pembrolizumab remains limited to the 15-20% of CRCs and other tumors that display MSI[24]. Combined data from the Keytruda KEYNOTE trials demonstrated a greater than 6-month durable response to pembrolizumab in 78% of patients with MSI or MMR deficient CRC[64–66]. While the response was durable, 7.4% of patients had a

complete response whereas 32.2% of patients a partial response[64–66]. While the number of patients with such a durable response remains limited, these studies were conducted in patients with advanced CRC[24,64–66]. Outcomes of CRC patients with first line Keytruda therapy may be improved with early treatment compared to previously conducted clinical trials. Nevertheless, it is of great interest to find ways to increase the success of immunotherapy in cancers that do not display microsatellite instability, including the majority of CRC cases.

Although it is hypothesized that increased immunogenicity of MSI cancers is solely due to the presence of increased TAA load, other contributing factors have not been thoroughly examined. First, presentation of antigen on major histocompatibility complex I (MHC I) by the cancer cells can be defective because the β-2 microglobulin (β2M) gene required for MHC I formation and TAA display contains microsatellite regions that are often mutated in MSI CRC[67,68]. Decreased expression of MHC I on CRC tumor cells has been demonstrated as a mechanism by which these tumors evade anti-tumor immunity[69]. Second, $CD8^+$ T cells require signals from other immune cells for full activation of cytotoxic capacity[70]. Other immune cells including T cell subsets, B cells, macrophages, NK cells and DC have not been extensively characterized in MSI CRC. Each of these immune cell subsets may have a role in anti-tumor immunity therefore characterization of these cells is important for the development of future therapies. In particular, later stage cancers including MSI CRC, downregulate MHC I, therefore the role of NK cells in detecting these cells will be especially important[71]. Lack of data to support hypotheses other than TAA load suggest more research is needed. Further studies examining the role of antigen presentation, and soluble immune factors released by CRC cells will help our understanding of additional factors contributing to the immunogenicity of MSI CRC. Ultimately these studies will allow us to develop better immunotherapies that extend their efficacy to cancers without microsatellite instability.

Mucosal immune homeostasis in the healthy intestine

The intestine is constantly exposed to a variety of both dietary and microbial antigens. Pathogenic microorganisms pose a threat to the single cell epithelial layer and many immune cells reside in the lamina propria ready to respond[72]. In spite of the many pathogens and dietary

factors that pass through the digestive tract, the majority of bacteria and food antigens from the healthy microbiota do not pose a threat and the immune cells must not recognize them as "foreign antigens[72]." The mucosal immune system has evolved to maintain balance with non-pathogenic antigens than pass through the gut[73]. This system involves tolerization of resident immune cells and a high threshold of activation before responding to an antigen[73]. Many tolerizing factors are present in the gut to prevent overactivation to local antigens[72,73]. DC are key to inducing adaptive immunity, but are also involved in maintaining tissue homeostasis[74]. DC continuously sample the luminal environment and will migrate to the mesenteric lymph node to prime intestine-specific adaptive immunity[72–75]. In addition to DC, T regulatory (Treg) cells are involved in maintaining a tolerant environment and release the suppressive cytokine TGFβ[73]. In addition, retinoic acid (RA) mediates the induction of forkhead box P3 $(FoxP3)^+$ Treg cells and tolerogenic IL-10 releasing DC[75,76]. Furthermore, the normal intestine contains tolerized populations of resident intraepithelial lymphocytes (IEL)[77,78]. This is particularly important in CRC since it remains unclear whether TILs in CRC arise strictly from expansion of the tolerant tissue resident IEL population or whether there is recruitment of additional systemic lymphocytes[58]. Previous experiments have demonstrated the preferential expansion of $CD8^+$ IELs rather than systemic derived $CD8^+$ T cells[79]. These findings are being challenged with the ability to detect numerous populations of immune cells with mass cytometry. More recently, TILs derived from lung and colorectal tumors were found to have specificity for TAA and a wide range of viral epitopes non-specific to the tumor[80]. The specificity of these T cells for non-tumor associated antigens provides evidence for the role in bystander T cell recruitment that contributes to anti-tumor immunity and has been shown to be dependent on $CD4^+$ T cell help[80,81]. The tolerant immune environment of the intestine is hypothesized to contribute to lack of response to abnormal cells that arise in the intestinal epithelium. Especially for the case of MSS tumors, this is thought to contribute to the high prevalence and persistence of this CRC subtype to an advanced stage.

Tumor immunoediting

Immune suppression is also a key hallmark of cancer and is required for the development of clinically detectable tumors[82]. The stages of immune surveillance, recognition and response have

been studied in developing cancers to understand the evolving nature of the interaction between a cancer and the immune system. The tumor immunoediting hypothesis is the paradigm that exists to explain the cancer–immune system relationship[83–85]. This hypothesis describes a stepwise response of the immune system to the tumor starting with the initial tumor detection and ending with the eventual immune failure that enables growth and spread of the tumor. The first stage is elimination of the tumor by the immune system that is mediated by type I interferons[83–85]. This describes the initial recognition of abnormal tumor cells that allows for their killing by cytotoxic T cells. The second phase is equilibrium which describes how some remaining tumor cells that have not been eliminated can remain in a steady state[83–85]. The heterogeneous nature of a tumor allows cells that have developed resistance to immune attack to persist. The last stage of immunoediting is marked by escape of the tumor because of proliferation of immune-resistant clones selected for over time by the tumor microenvironment (**Fig. 1-2**) [83–85]. The selective pressure exerted by the microenvironment in this process is critical. Although MSI status is a positive prognostic marker for cancer, including in the colon, the very existence of these cancers proves they have not escaped the immunoediting cycle[86]. Despite this, it is clear that MSI cancers much more strongly activate anti-tumor immunity than other cancer types[54,56–58]. Studies examining clones from both colorectal and endometrial cancers with defects in *MLH1* show that which specific genes are commonly found to be altered in these cancers is more strongly determined by their anatomic location than their microsatellite instability[87]. These data provide evidence for the essential role of specific cells in the microenvironment in selection of clones over time.

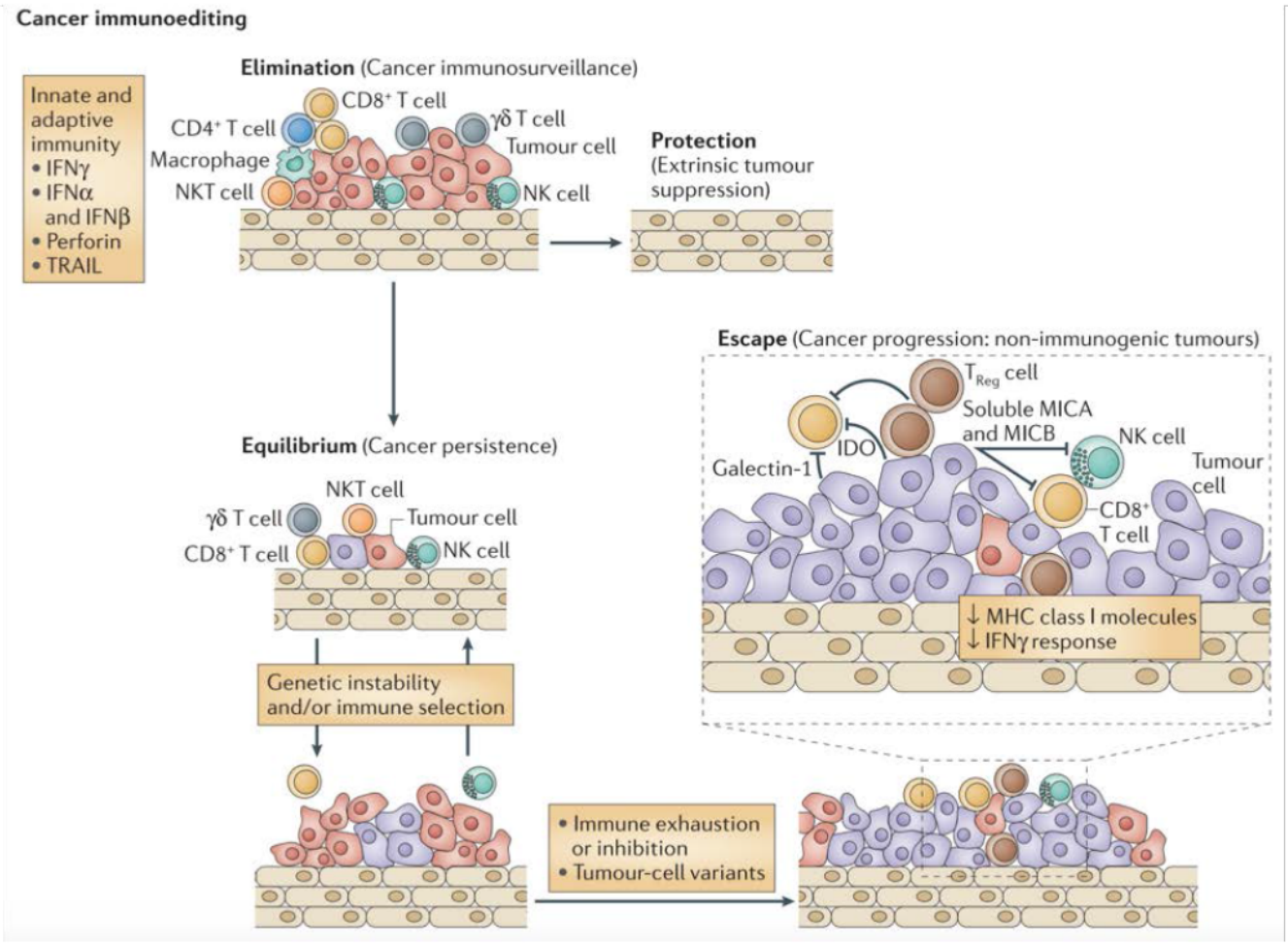


Figure 1-2. The cancer immunoediting cycle. Cancer immunoediting occurs in three phases. The first is recognition of the tumor through immune surveillance by various immune cells including natural killer cells, macrophages, and lymphocytes. Through a type I interferon and Th1 mediated program, these cells drive anti-tumor immunity eliminating cells of the tumor. A balance between tumor reactive and tumor suppressive mechanisms is then reached and the tumor is in an equilibrium state. During this phase, immune resistant clones are not eliminated by the immune system and they persist. Proliferation of these immune resistance clones leads to tumor escape from the immune system. This phase is marked by a myriad of immunosuppressive mechanisms that have been selected for during the course of tumor development and allow for protection of cancer cells from anti-tumor immunity. Figure from: Dunn GP, *et al*. 2006. Nat Rev Immunol. 6: 836-848. Reprinted with permission.

Mechanisms of tumor immune suppression

Mechanisms of tumor suppression from the immune system vary widely across tumor type and location. Many mechanisms of tumor escape from the immune system have been uncovered[88]. The discovery of each mechanisms remains of importance for therapeutic targeting to enhance anti-tumor immunity. However, this approach remains complex as often more than one factor contributes to the prevention of anti-tumor mediated eradication.

To understand various mechanisms of tumor mediated immune suppression, it is important to recognize the existing immunological landscape of the tumor. Three main cancer immune phenotypes have been identified: (1) immune-deserted tumors, (2) immune excluded tumors and (3) inflamed tumors[88]. Each phenotypic group is associated with a group of tumor mediated and microenvironment suppression mechanisms **(Fig. 1-3)** that facilitate tumor escape[88].

The immune desert phenotype **(Fig. 1-3)** is associated with very limited immune infiltration in the tumor over the course of tumor persistence[88]. Although the colorectal mucosa contains many resident IELs, these cells are retained and expanded to a greater degree in MSI compared to MSS CRC. Overall, infiltrating lymphocytes present in MSS CRC are low and this immune deserted phenotype is observed in this subtype of CRC[55,79]. Although specific mechanisms for this phenotype have not be identified for MSS CRC, lack of immune infiltration may be a consequence of a number of factors. This could include the presence of tolerizing factors, lack of signals required for T cell priming and activation, and immunological ignorance[88]. In many tumors, the presence of Treg cells and myeloid derived suppressor cells (MDSCs) are present within the tumor that contribute suppressive cytokines allowing for immunological tolerance to the tumor[88,89]. Although very few immune cells infiltrate MSS CRC, the unique nature of the gut presents a very hostile environment for the infiltration of activated cytotoxic T cells[72,73,76]. Although not directly within the tumor, Treg cells and tolerant DCs that maintain gut homeostasis present in the lamina propria may contribute to the high levels of TGFβ and IL-10 priming the tumor environment for immune exclusion[72,73,76,88]. Immunological ignorance is also likely to play a role in the lack of immune cells observed in MSS tumors. This phenotype is likely to arise from the decreased TAA load observed therefore the immune stimulating antigen

signals do not reach the threshold required for extensive T cell infiltration as observed in MSI CRC[56,58,59].

During the course of tumor development, the stromal environment undergoes many changes to support increased tumor volume that can also function to exclude immune cells from entry into the tumor **(Fig. 1-3)**. The changes in the stromal environment can pose a barrier and prevent the infiltration of immune cells to the tumor[88]. Stromal factors that may play a role include vascular factors, the extracellular matrix and chemokines present in the tumor microenvironment[88]. Immune cells require the vasculature to be permissible to infiltration yet organized allowing for navigation to the tumor. Tumor vasculature is often disorganized and does not allow for proper T cell transendothelial migration[90]. This can occur through the upregulation of VEGF that supports tumor angiogenesis, yet leads to the downregulation of intercellular adhesion molecule-1 (ICAM-1)[91–93]. In addition, elevated levels of VEGF can lead to upregulation of Fas ligand on endothelium thereby inducing apoptosis of infiltrating $CD8^+$ T cells[91,94]. The extracellular matrix of the tumor microenvironment may also create a non-permissible environment for T cell migration[88]. The dense matrix environment and excessive proliferation of cancer associated fibroblasts (CAFs) can inhibit T cell tumor infiltration[95,96]. Necrotic areas commonly found in tumors including MSS CRC have been shown to have a high concentration of potassium ions[27,97]. Although not confirmed in CRC, high extracellular potassium has been demonstrated to lead to ionic reprogramming of T cells reducing effector function by suppressing T cell receptor signaling and IFNγ expression in a melanoma model[97]. The tumor environment may also become altered by stromal cells that contribute to changes in chemokines that may encourage or prevent T cell infiltration[88,91]. In hypoxic regions of the tumor, C-C motif ligand 28 (CCL28) has been shown to become upregulated by ovarian cancer cells allowing for angiogenesis to occur and also leading to the recruitment of Treg cells[98]. Many other chemokines have also been associated with metastatic spread in a number of cancers including CRC cancer through the CXC motif chemokine ligand 12 (CXCL12)- CXC motif chemokine receptor 4 (CXCR4) axis[91,99]. On the other hand, in MSI CRC, production of CXCL9 and CXCL10 by stromal cells are associated with the recruitment of $CXCR3^+$ $CD4^+$ and $CD8^+$ tumor infiltrating lymphocytes in gastric and colorectal tumors[100–102].

While many tumors remain immune deserted over the course of development, some tumors most notably melanoma, lung and gastric cancers can display an inflamed or a “Crohn-like” response as referred to in some CRC[27,88]. This phenotype not only refers to the recruitment of anti-tumor mediated T cells, but also may include the presence of B cells, Treg cells, exhausted T cells or MDSCs[88]. Inflamed tumors often have a cytokine environment rich in type I and II interferon, IL-1β, IL-2, IL-12 and TNFα[88]. The combination of immune stimulating TAA and pro-inflammatory cytokine signals allow for tumor control and elimination by the immune system[57,58,61,62]. This process however can become exhausted preventing the continued activation of $CD8^+$ T cells by a number of processes[103]. Recruitment of suppressor cells that inhibit cytotoxic tumor killing by T cells is often at play in the tumor microenvironment with elevated levels of TGFβ, and IL-10[88]. Many cells in the tumor microenvironment including tumor cells, CAFs, MDSCs, endothelial cells, Treg cells, and B cells express PD-L1[88].

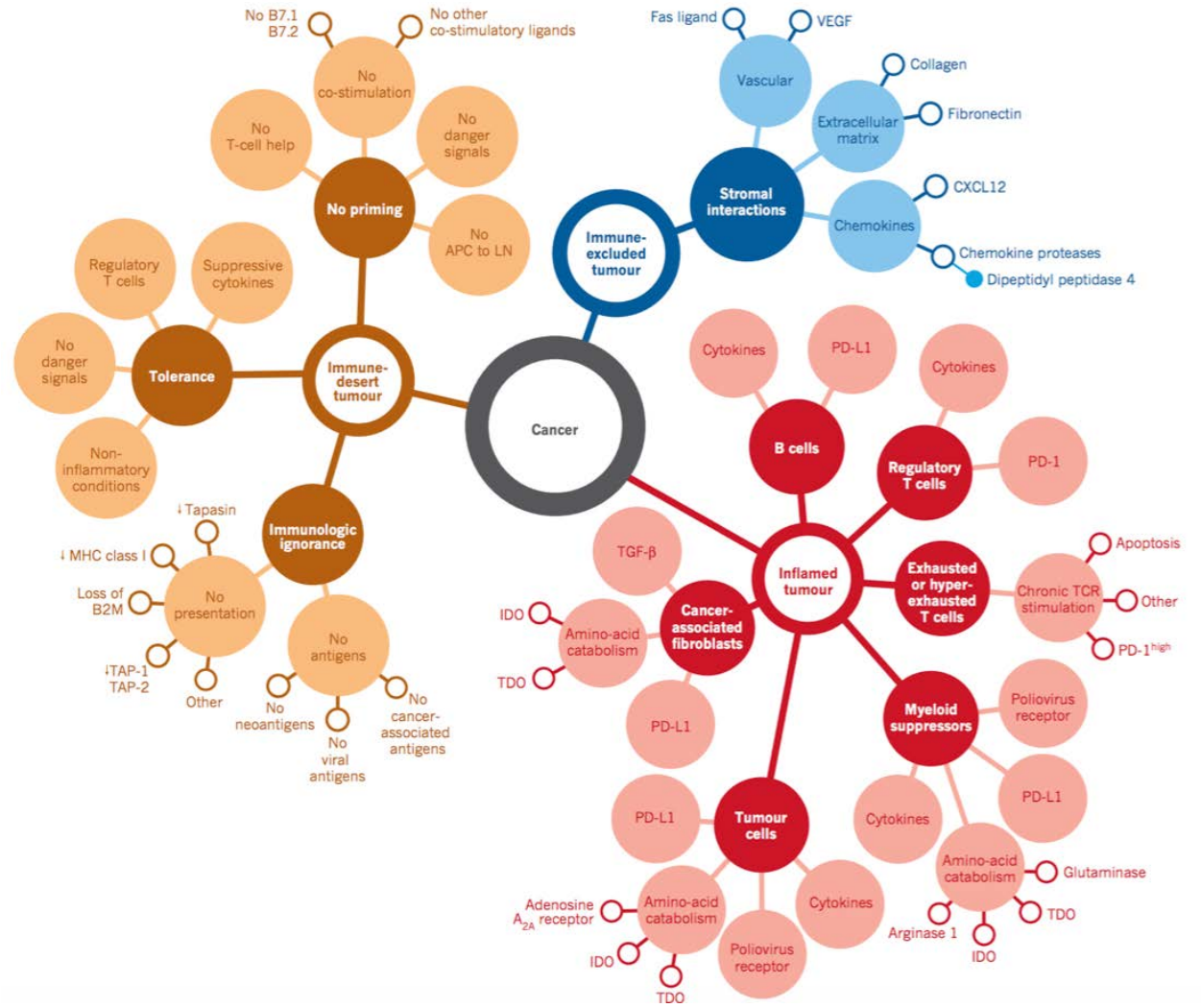


Figure 1-3. The landscape of tumor immunosuppressive and escape mechanisms. Tumors often exhibit one of three immunological landscapes. Each immune phenotype displayed by the cancer is associated with characteristic immunosuppressive and anti-tumor escape mechanisms. The immune desert phenotype (left, in gold) is characteristic of tumors that have little to no infiltrating immune cells present within the tumor. This is a consequence of immune ignorance to the cancer cells, tolerizing factors in play, or lack of contributing factors required for mounting of an anti-tumor immune response. The immune excluded phenotype (top, in blue) is characteristic of tumors that also lack the presence of infiltrating immune cells as a consequence of the tumor microenvironment. The stromal environment can discourage anti-tumor immunity through an unfavorable chemokine environment that fails to attract immune cells. In addition, the

disorganized vasculature and dense extracellular matrix may not allow for the trafficking of immune cells into the tumor. Tumors that exhibit an inflamed phenotype (right, in red) have a greater frequency of infiltrating immune cells present. However, the presence of various immune suppressor cells including B cells, myeloid derived suppressor cells (MDSC) and T regulatory (Treg) cells can dampen anti-tumor immunity by inhibiting activated cytotoxic T lymphocytes (CTL). Expression of inhibitory factors such as PD-L1 on tumor cells, cancer associated fibroblasts (CAFs), MDSC and Treg cells can negatively regulate CTL activity within the tumor leading to lack of control of the tumor by infiltrated immune cells. Figure from: Chen DS, *et al*. 2017. Nat Rev. 541. 321-330. Reprinted with permission.

Escape of the tumor from immune control

Tumors that occur at mucosal sites often display immune cell control despite the tolerant nature of resident immune cell population compared non-mucosal sites[104]. However this immune control is often dysregulated due to the immunosuppressive nature at mucosal sites[105]. The skin, lung and gastrointestinal tract are three sites more heavily exposed to environmental genotoxins. Environmental factors such as the sun, tobacco, microbial and dietary toxins contribute the formation of genetic lesions that lead to melanoma, lung and CRC[106–109]. The large number of genetic aberrations including base substitutions, insertions and deletions lead to a high mutational load in a subset of these cancers[24,110,111]. This high mutational load (especially in MSI CRC) and immune stimulating TAA drive the infiltration of immune cells allowing for anti-tumor control, despite the tolerant nature of the of immune cells that arise at these mucosal sites[104]. However, at some point, an equilibrium is reached, and tumor growth cannot be maintained by immune cells[85,88]. Evidence for this can be observed in profound immune control of melanoma, lung and MSI CRC with anti-CTLA-4 and anti-PD-L1/PD-1 immunotherapy[24,63,64]. However only a small percent of patients display durable complete responses, whereas many patients become refractory to these treatments[24,63,64].

Immune evasion is a hallmark of cancer progression and despite the presence of a high immune stimulating TAA load, it was hypothesized that defects in antigen presentation may be responsible for lack of tumor immune control. Early analysis of this hypothesis proved difficult due to the highly polymorphic nature of the human leukocyte antigen (HLA) locus from human tumor samples[112]. Swanton and collogues along with the TRACERx Consortium present the bioinformatics tool loss of heterozygosity in human leukocyte antigen (LOHHLA) used improve accuracy in determining HLA allele-specific copy number[113]. It was found that 40% of early stage non-small cell lung cancers (NSCLCs) had loss of heterozygosity of the HLA locus and was also associated with significantly increased PD-L1 expression[113]. Another study examined the mechanisms of resistance in melanoma patients that did not respond to anti-CTLA-4, anti-PD-1, or anti-PD-L1[114]. Similar to the previous study a mechanism of immune evasion involving disruption in antigen presentation was found to contribute to lack of response to therapy[114,115]. In contrast, loss of heterozygosity (LOH) of the β2M locus was identified in the 30% of non-

responders[114]. The high frequency of LOH events in the antigen presentation pathway in these tumors suggests immune editing and selection of clones that allows for evolution of tumors with a high mutational burden. The fact that MSI CRC has a high mutational burden but can persist and progress to an advanced stage suggests that immune escape mechanisms by the tumor are in play. Although multiple mechanisms of immune escape are likely to have a role in persistence of MSI CRC, this has not been extensively studied. Recently, analysis of a large number of CRC samples from The Cancer Genome Atlas (TCGA) database were examined to determine possible mechanisms of tumor escape[69]. This study identified the biallelic loss of HLA and β2M locus as a result of both copy number alteration and copy number neutral loss of heterozygosity in MSI but not MSS CRC. In addition, regulators of β2M and HLA loci were examined. *NLRC5* and *RFX5* both containing microsatellite repeats were found to be mutated in 33% and 16% of MSI CRC from the TCGA database[69]. As a direct consequence, downregulation of HLA expression by up to 50% in MSI CRC[69]. In addition, biallelic loss of TAP2 was also observed, however at a much-reduced frequency compared to β2M and HLA LOH[69]. The identification of mechanisms by which tumors undergo immunoediting provides evidence that tumor mutational burden is not the only factor that predicts T cell infiltration. This highlights the importance of continuous presentation of antigen to immune cells to maintain tumor control and present a new area of target for development of new therapies.

Cancer checkpoints and immunotherapy

One of the most promising cancer therapeutics has been the development and implementation of immunotherapy in the clinic. Tumor evasion from the immune system is a major hallmark of cancer allowing for its development and persistence[82]. These mechanisms of immune resistance and suppression were mentioned above and either involve the tumor itself or the microenvironment that mediates suppression of anti-tumor immunity[88]. However, the immune system has a number of pathways and "checkpoints" that allows for the strict maintenance of self-tolerance and modulation of an immune response during active infection[116]. These mechanisms are mediated through receptor-ligand interaction that decrease T cell activation and have implications for cancer therapy since these interactions can be therapeutically blocked to modulate the immune cell response[63,116]. While these receptor ligand interactions prevent the rise

of autoimmunity and tissue destruction by continued activation the immune system under homeostatic conditions these pathways are exploited by tumors to allow for inhibition of anti-tumor immunity and escape of the tumor[63].

The CTLA-4 checkpoint

CTLA-4 is expressed on T cells and is a regulator of early signaling events in the stages of T cell activation[117–119]. Engagement of CTLA-4 on T cells leads to negative regulation of T cell activation which counteracts the activating function of the T cell co-stimulatory receptor CD28[117–119]. T cell activation is initiated by engagement the T cell receptor by cognate antigen in the context of MHC, which becomes amplified by engagement of CD28 binding to its ligands CD80 and CD86 also referred to as B7.1 and B7.2 respectively[63,120–122]. These receptor ligand interactions lead to strong amplification of positive TCR signaling and T cell activation[63,119]. CTLA-4 also shares the same ligands as CD28, however it has been shown that CTLA-4 has a higher affinity for CD80 and CD86[123–127]. CTLA-4 will compete with CD28 for these ligands which leads to negative signaling in the T cell and dampening of T cell activation **(Fig. 1-4)** [63,123–127]. The strict role that CTLA-4 has in regulating the amplitude of T cell activation is demonstrated in whole body *Ctla-4* knockout mice, which have a lethal systemic over activation of T cells[128,129].

Blocking of the interaction of CTLA-4 receptor with its ligand were demonstrated to induce tumor regression in mice which led to the development of humanized CTLA-4 antibodies ilipimumab and tremelimumab[63]. These antibodies entered clinical trials in the early 2000 in late stage cancer patients with advanced melanoma[130–132]. The success of ilipimumab led to the FDA approval of this therapy in 2010 for the treatment of metastatic melanoma which was the first therapy to prolong the survival of these patients[63]. While this therapy did show regression of tumors, immune toxicity is the major side effect[63]. Much work has been done to elucidate the mechanisms the allow for tumor regression. Despite the fact that blockade of the CTLA-4 checkpoint allows for amplification of T cell activation, the main mechanism by which this immunotherapy demonstrates a clinical response is through the depletion of Treg cells within the tumor[133]. CTLA-4 immunotherapy blockade has currently demonstrated the most success in

tumors with a high mutational burden[24,104]. However further work combining this therapy along with other cancer therapy modalities aims to increase the efficacy of CTLA-4 blockade in a wide panel of tumor types.

The PD-1 checkpoint

While both CTLA-4 and PD-1 checkpoints exist to promote self-tolerance, PD-1 functions to limit T cell activity in peripheral tissues[134–139]. PD-1 in expressed on T cells, B cells and NK cells[63]. When PD-1 become engaged with its ligand this allows for negative regulation of T cell signaling, thereby limiting T cell activation which also limit the lytic activity of NK cells in the same manner (**Fig. 1-4**)[140]. The ligand for PD-1 and PD-L1 and PD-L2 also known as B7-H1 and B7-DC respectively[141–143]. The PD-1/PD-L1 axis has an important role during the course of a viral infection where both the ligand becomes expressed on epithelial tissue and PD-1 on T cells to limit tissue destruction and maintain self-tolerance preventing autoimmunity[137,138,144]. This pathway is frequently activated and is a major mechanism whereby tumors limit anti-tumor activity of both T cells and NK cells[63,145–147]. PD-1 becomes upregulated on T cells following activation[134–139]. The expression of PD-1 on tumor infiltrating lymphocytes has become a marker of the activity of anti-tumor immunity[148]. Expression of PD-1 on T cells that have engaged with cognate antigen often express PD-1 and are said to be in an anergic state or otherwise "exhausted," and functionally impaired which ultimately prevents destruction of tissue, however limits anti-tumor immunity[63,149].

Tumors use two mechanisms by which this pathway becomes upregulated are referred to as innate and adaptive resistance[63]. Innate resistance is referred to as an inherent signaling pathway that has become dysregulated in a tumor cell that is responsible for the upregulation of PD-L1 on the tumor cell[63]. Pathways that have been identified include active STAT3 and PI3K-AKT signaling in tumor cells **(Fig. 1-5A)**[63,150,151]. In contrast, adaptive immune resistance occurs when, IFNγ released by T cells leads to STAT signaling in tumor and stromal cells in the tumor microenvironment[63,147,152–154]. These cells respond to IFNγ by upregulating PD-L1 **(Fig. 1-5B)** [63,147,152–154]. Blockade of the PD-1/PD-L1 axis in preclinical mouse models demonstrated tumor regression and a less severe immunotoxicity phenotype in *Pdl1* knockout mice[63,137,138]. This led

to the development of the humanized pembrolizumab and blockade of the PD1/PD-L1 axis has demonstrated success in clinical trials with milder side effects than CTLA-4[63,64]. Despite the success of this antibody immunotherapy clinical responses have been limited to tumors that display MSI[24]. While blockade of CTLA-4 leads to the depletion of Treg cells, it is of interest to determine alternative mechanisms by which blockade of PD1/PD-L1 leads to tumor regression other than inhibition of negative T cell regulation[133]. In a mouse model of various CRC cell lines, depletion of MDSC were demonstrated following treatment with anti-PD-L1 of the IgG2 isotype[155]. These studies are important for the future development of the next generation of immunotherapy. It is likely than inhibition of more than one cell type, and regulatory axis will be required for enhanced anti-tumor immunity in patients.

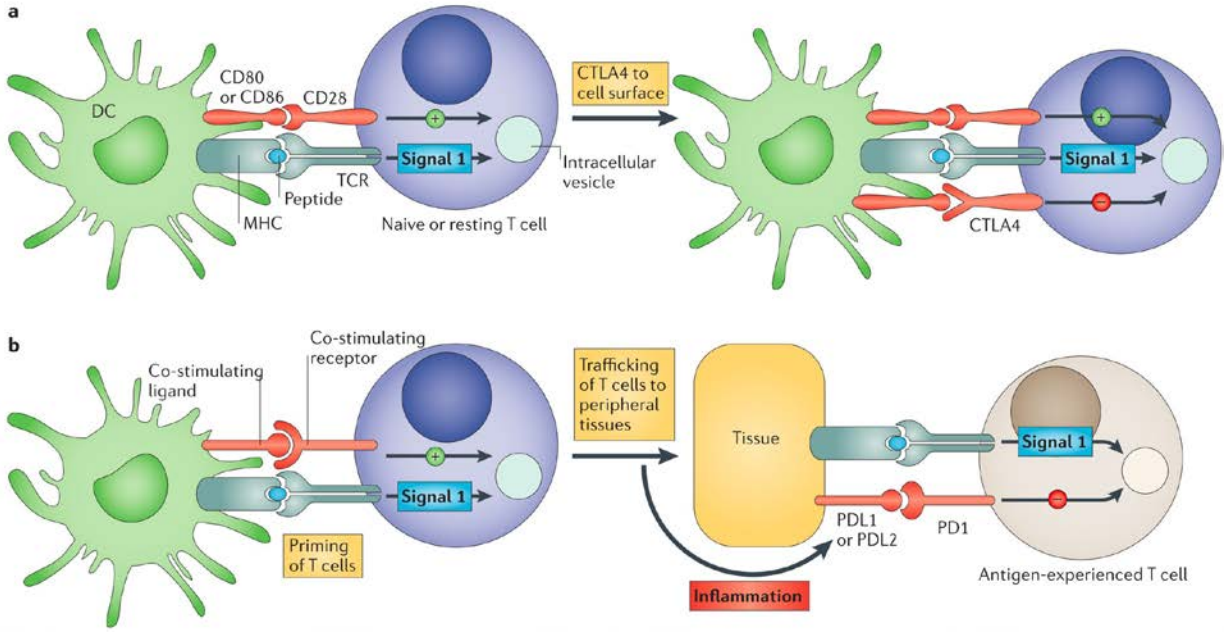


Figure 1-4. The CTLA-4 and PD-1 immune checkpoint. Negative regulators of T cell activation exist to prevent the development of autoimmunity and maintain self-tolerance. Despite the inhibitory nature of engagement of CTLA-4 and PD-1, each has a differential outcome. (A) Ligation of CTLA-4 by either of its ligands CD80 or CD86 leads to dampening of T cell activation mediated in early stages of T cell activation. (B) Following the activation of T cell signaling in response to cognate antigen, T cells traffic to tissue and mediate cytolytic activity directed towards epithelial cells. Upregulation of PD-1 on T cells in peripheral tissues occurs limiting the T cell activity to prevent destruction of tissue under homeostatic conditions. Figure from: Pardoll DM. 2012. Nat Rev Cancer. 12: 252-264. Reprinted with permission.

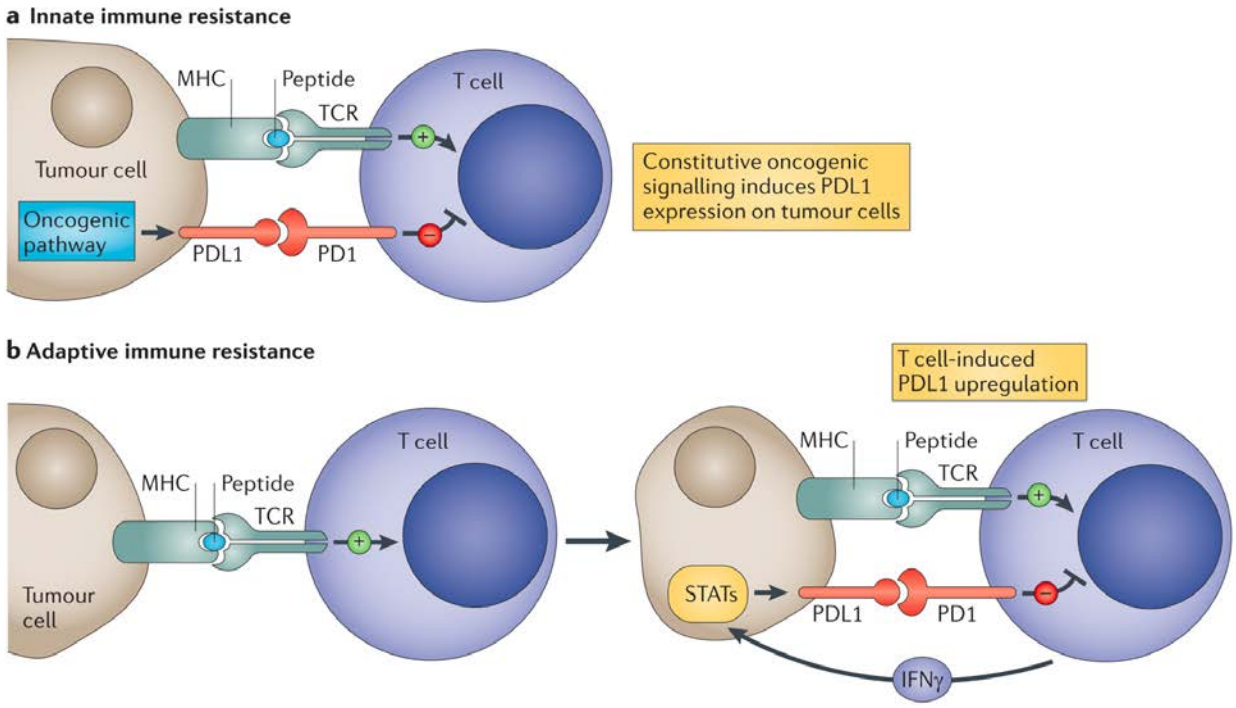


Figure 1-5. Mechanisms of CTL inhibition by the tumor through the PD-1/PD-L1 axis. T cell activity is limited in peripheral tissues following the upregulation of PD-1 on the T cell and interaction with its ligand PD-L1. PD-L1 becomes upregulated on tumor tissue through one of two mechanisms. (A) Innate immune resistance leads to upregulation of PD-L1 through dysregulation of an oncogenic signaling pathway by the cancer cell. (B) Adaptive immune resistance occurs when T cell mediated secreted IFNγ leads to tumor cell STAT signaling that causes upregulation of PD-L1 on the tumor tissue. Figure from: Pardoll DM. 2012. Nat. Rev. Cancer. 12: 252-264. Reprinted with permission.

Dendritic cells

DC possess potent antigen capture, processing and antigen presenting properties. As cells of the innate immune system, DC are often first responders to pathogens. Although DC provide a link to the adaptive immune system that will lead to robust effector T and memory T cell immunity, DC also have major role in maintaining self-tolerance[74]. In the steady state, DC exhibit an immature phenotype[74]. These DC are characterized by the lack of MHC II presented on the cell surface as well as the lack of costimulatory ligands required for T cell activation[74]. Immature DC however, are poised to capture antigen and express numerous endocytic receptors that facilitate phagocytic uptake of pathogens and apoptotic cells[74]. Following the phagocytic uptake, DC are in the activation stage whereby endocytic receptors become downregulated and DC switch to processing of phagocytosed material[74]. Depending on receptors involved in the endocytic uptake, this can dictate which presentation pathway antigen will be shuttled to for presentation to the adaptive immune system. Processed antigen may be presented on MHC to the adaptive immune system, however other signals are required to fully activate these adaptive cells[74]. Not all phagocytosed content will lead to the full activation and maturation of DC. Pathogens that activate DC through toll-like receptors (TLR) can lead to the full maturation and activation of DC[74]. This is characterized by the presence of increased MHC II, expression of costimulatory ligands CD80 and CD86 as well as polarizing cytokines that will induce strong activation of effector and memory T cell activity[74]. Alternatively, DC also have an important role in maintaining tolerance to self, especially in the intestinal mucosa[74]. Intestinal DC that continually sample commensal intestinal bacteria or phagocytose apoptotic cells will remain in an immature state[72,76]. While these DC may process and present antigen on MHC, they lack the high expression levels of CD80 and CD86[74]. The lack of costimulatory ligands leads to T cell tolerance[74]. This may also be facilitated by the expression of the inhibitory ligand PD-L1 and tolerizing TGFβ and IL-10 cytokines[74].

Dendritic cells in cancer

DC possess potent antigen presenting properties, including the unique ability to acquire and

present antigens from cells in their environment on MHC I and MHC II During the course of tumor development, there are many opportunities for DC to acquire tumor derived antigens that can be presented to cross-prime cytotoxic T cells[156,157]. Furthermore, DC can secrete many tumor-inhibiting factors such as cytokines and chemokines that promote and orchestrate anti-tumor immunity[158]. However, recruitment of different subsets of anti-tumor promoting DC to the tumor is required for effective immunity[159,160]. Plasmacytoid DC (pDC) are one subset of dendritic cell found in intestinal mucosal tissue under homeostatic conditions[72]. Although these cells are not effective cross presenters, their stimulation through TLRs leads to the release of high amounts of type I interferon, IL-6, IL-12, TNFα and other proinflammatory cytokines[157,158,161,162,163]. These cells have been shown to provide signals for the development of T helper 1 (Th1) anti-tumor immunity through the production of type 1 interferons, which are central mediators of anti-tumor immunity[85,157,158,161]. In contrast, the conventional DC subset expressing $CD8\alpha^{+}CD11b^{-}CD103^{+}$ effectively captures antigen and favors presentation of antigen on MHC I allowing for cross priming of cytotoxic $CD8^{+}$ T cells[164,165]. These cells have been shown to infiltrate tumors and present antigen to $CD8^{+}$ T cells in both the tumor microenvironment and lymphoid sites[160,162,165]. The ability of DC to present antigen directly at the tumor site suggests these cells can substantially influence anti-tumor immunity mediated by $CD8^{+}$ T cells. Another relevant subset includes circulating inflammatory DC which originate from monocytes[162]. The presence of inflammatory DC in tumors has been shown to enhance $CD8^{+}$ anti-tumor immunity[162].

On the other hand, not all DC promote anti-tumor immunity since one mechanism of tumor escape is the induction of DC dysfunction. Although DC can infiltrate tumors, the hypoxic environment, low pH, and lactic acid have been shown to inhibit the stimulation of $CD8^{+}$ T cell cytotoxicity by DC[166–169]. Other immune cells in the tumor microenvironment such as tissue resident DC and tumor associated macrophages have been shown to produce high amount of IL-10[89,158,166]. This leads to downregulation of MHC I and costimulatory molecules CD80 and CD86 on newly infiltrating DC as well as to decreased production of IL-12 by DC, all of which impair the ability of DC to successfully activate cytotoxic $CD8^{+}$ T cells[166]. As mentioned earlier, continuous presentation of TAA is critical for required for effective anti-tumor immunity. DC dysfunction preventing antigen presentation machinery, upregulation of co-stimulatory ligands

and cytokines can contribute to tumor escape[85,166–168]. As for this reason, DC continue to be of great interest due the role they play in orchestrating anti-tumor immunity.

DCs as a vaccine

Given the ability of DC to orchestrate adaptive anti-tumor immunity by regulating many cell types in their environment, these cells have been used to develop tumor vaccines[161]. Much of the focus in recent years has involved the use of CTLA-4 and PD-1/PD-L1 immunotherapy. However, a search on clinicaltrials.gov with the term "DC vaccine" yields 421 clinical trials, where 253 of these studies occurring in the United States. This highlights the activity and interest in the field to create therapeutic DC vaccines. Priming of cytotoxic T lymphocyte (CTL) responses occur throughout the developmental course of the tumor[85]. Often this process is slowed or inhibited during tumor evolution as immune escape mechanisms are selected for[85]. On the other hand, migration of immune cells may be inhibited by the disorganized tumor microenvironment[91,104]. This could include the trafficking of DC in and out of tumor to the lymph nodes and difficulty of lymph node primed CTL from reaching the tumor site. Spatial challenges are one hurdle that DC based vaccines aim to overcome. Additional challenges are in the ability of DC to prime CTL response that involve acquiring antigen[161]. DC may receive antigen by release of cellular components into the extracellular matrix or release of a bolus of antigen via apoptosis of tumor cells[170]. Although release of antigen is often an active process in the tumor microenvironment, many tumor cells remain in a cellular senescent state whereby this process is slowed and this state can suppress anti-tumor immunity[171,172]. This does not allow for the continuous release of antigens for DC stimulation which provides a challenge for DC to stimulate CTL anti-tumor immunity. Examination of many years of DC and T cell dynamics has allowed for researchers to define the ideal DC vaccine for anti-tumor immunity. A proposed vaccine would allow for DC to present antigen to T cells with high affinity and avidity for tumor antigen[161]. The vaccine would also allow for effective DC maturation leading to the upregulation of MHC I and II, co-stimulatory molecules as well pro-inflammatory cytokines especially IL-12[161]. Activation and selection of high avidity and T cell receptor (TCR) affinity clones that express elevated granzyme B and perforin is optimal for the anti-tumor immunity[161]. In addition, the DC vaccine should allow for the upregulation of trafficking molecules such as CXCR3 on

$CD8^+$ T cell to allow for migration to the tumor site[161]. Not only should the vaccine stimulate $CD8^+$ T cells, but the vaccine should aim to activate $CD4^+$ T cells. Stimulation of $CD4^+$ T cells help by the DC vaccine is ideal for the support for CTL activation and differentiation through various cytokine including IFNγ and IL-21 to allow for CTL expansion[161]. The generation of memory $CD8^+$ T cells is most desirable and dependent on $CD4^+$ T cell help, therefore the vaccine should aim to target this subset of lymphocytes in addition to CTL[161].

Synthetic DC vaccines

Various strategies to develop DC vaccines have been employed and tested over the years. The first method being non-targeted vaccines whereby DC are pulsed with synthetic peptides and injected into patients to induce the generation of anti-tumor specific CTL and induce T cell memory[161]. Many of these vaccines did not produce the desired response in clinical trials. Testing of these synthetic DC vaccines in patients with metastatic melanoma demonstrated that antigen specific $CD8^+$ T cells were present as a result. However, it was identified that the lack of $CD4^+$ related T cell responses did not facilitate robust $CD8^+$ T cell response and induction of long-lived memory[161,173,174]. One factor that determined the activation of various lymphocyte populations in this vaccine strategy was the length of the peptide[161]. Shorter peptides in the 25-50 amino acid length range restricted the T cell response[175]. T cells responses were restricted to $CD8^+$ T cells, due to presentation of peptide on MHC I[161,175]. Engagement of both $CD8^+$ and $CD4^+$ T cells were found to be achieved by attenuation of the amino acid peptide length[161,175]. Longer amino acid lengths led to presentation of peptide on MHC I and MHC II and the activation of $CD8^+$ and $CD4^+$ T cells[161,175]. Despite the use of longer peptides, other factors were found to play a role in the type of T cell response initiated. The tumor suppressor p53 is found to be mutated or exhibit loss of expression in greater than 50% of cancers[176]. The high prevalence of this mutation makes p53 an attractive target for the use in development of new immunotherapies. Since p53 is a self-protein, the $CD8^+$ T cells repertoire is largely restricted by central tolerance[177]. However, circulating $CD4^+$ T cells do not seem to be affected as circulating $CD4^+$ cells with p53 specificity[178]. Given this, a synthetic p53 long peptide was used to vaccinate ovarian and CRC patients[175,179]. While ovarian cancer patients displayed increased IFNγ production by both $CD4^+$ and $CD8^+$ T cells, this was only observed in $CD4^+$ T cells in CRC

patients[175,179]. While the microenvironment of the tumor could have a role in determining the T cell response, the number of vaccinations received by ovarian cancer patients was double compared to CRC patients[175,179]. The length the peptide and the number of vaccinations required to achieve the desired immune response highlights some of the challenges in the development of DC vaccines[161,175]. However, one very important factor to consider is the induction of tolerance by the DC vaccine[161]. Under normal homeostatic conditions DC residing in the lamina propria constantly sample antigen from the luminal compartment[72,76]. DC in the intestine express high levels of RALDH2 and convert dietary vitamin A to retinoic acid that produces tolerant intestinal conventional DC (cDC)[180]. Sampling of antigen by cDC does not lead to DC maturation and immature DCs regulate the induction of $FoxP3^+$ Treg cells in a TGFβ dependent manner[76,161]. Given that DC often induce tolerance in response to antigen this is important to keep in mind during the development of DC vaccines[161]. It is now recognized that full maturation and activation of DCs is important for the induction of CTL response and memory[161]. The addition of adjuvant to stimulate DC along with antigen has been shown to overcome the induction of a tolerant DC phenotype[161]. Many adjuvants that induce TLR stimulation in DCs are being investigated[161]. Potential DC stimulating adjuvant may include the TLR3 ligand polyinosinic : polycytidylic acid (poly I:C) and the TLR9 ligand CpG oligodeoxynucleotide among many others that target surface receptors on DC[161,181,182]. It is currently understood that various adjuvants can alter the type of immune response overserved, however further investigation is still underway.

Ex vivo generated DC vaccines

Another method of DC vaccine involves generation of the vaccine in an ex vivo manner. Many methods exist to prepare the vaccine including the use of synthetic peptides, bulk tumor lysates obtained from patient surgical resection of the tumor and selection of particular patient specific tumor derived antigens[161]. This method has currently been successfully employed in a prostate specific cancer model. The US FDA has approved the use of this Sipuleucel-T DC vaccine for use in metastatic prostate cancer patients[161,183]. This vaccine is prepared through isolation of DC from the patient. The cells are then stimulated with a fusion product of prostatic acid phosphatase and granulocyte macrophage colony stimulating factor (GM-CSF) and injected back into the

patient[161,183]. This therapy has been demonstrated to extend the median survival of castration resistant metastatic prostate cancer patients by 4 months[161,183]. Other studies have specifically targeted the IFNγ producing pDC to stimulate anti-tumor Th1 anti-tumor immunity[184]. These cells were extracted from melanoma patients stimulated with two specific melanoma associated HLA-A2.1 restricted peptides and treated with IL-3 and administered to patients via intranodal injection[161,184]. Migration of pDC to various lymph nodes was overserved and both $CD4^+$ and $CD8^+$ T cell responses were mounted and IFNγ cytokine levels were elevated following injection[161,184]. While many ex vivo DC vaccines have demonstrated some success in mounting anti-tumor immunity, the full potential of this vaccine remains to be determined. A major drawback of these studies is the testing of DC vaccines in late stage and metastatic cancer patients[161]. Often these patients' tumors have acquired extensive escape and immunosuppressive mechanisms that limit the effect of any anti-tumor vaccine[85]. Many clinical studies have also not investigated all possible parameters to determine the extent of anti-tumor immunity. Much of the data collected focuses on extension of median survival, while circulating lymphocyte numbers, and serum cytokine profiles are often not all collected. More work to determine the activation state and trafficking of immune cells to the tumor site is required to determine DC vaccine efficacy.

In vivo targeted DC vaccine

Lastly, another method of DC vaccines focuses on the in vivo targeting of these cells. It is obvious that the prior two methods employed come with financial and feasibility constraints for large cancer patient populations. This method aims to overcome these two factors by targeting these cells using various methods, forgoing ex vivo isolation of these cells. Early studies utilizing this method involved coupling antigen to an antibody specific for a DC surface marker, often DEC205[185]. These early studies identified that target of DEC205 alone lead to the induction of tolerance and the absence of a Th1 cytokine profile[161,185]. However, with the addition of a CD40 agonist, a shift from tolerance to induction of a long-lived T cell response occurred[161,186]. Like ex vivo generated vaccines, the inclusion of an adjuvant such as a TLR ligand has been demonstrated to overcome tolerance and has demonstrated a protective immune response in disease other than cancer including human immunodeficiency virus (HIV) and malaria

infection[161,187]. Careful consideration of the DC surface target is important as this can also dictate the immune response mounted. For example, coupling of antigen to CD40 and the mannose receptor in BDCA1$^+$ monocyte derived DC targets antigen to early endosomes[188]. In comparison target of antigen via DEC205 leads to intracellular trafficking of antigen to late endosomal compartments[161,189]. Even though CD40 demonstrates poor uptake compared to other receptors, target of this receptor led to cross presentation of antigen[161]. Given that anti-tumor immunity is dependent on a CTL response this suggests the choice of target for developing a DC vaccine requires careful consideration.

Vaccines for mucosal tissues

While there are many different methods of creating a DC vaccine, it is imperative to comment on the mucosal nature of the intestine. Preparation of an immunological based vaccine for a mucosal site requires additional consideration. First, the efficacy of vaccines to establish immunity at a mucosal site are highly dependent on the route of delivery[190]. Mucosal vaccines designed for the lower gastrointestinal tract are best established through oral and sublingual administration[190]. Vaccines administered by this route are subject to the same defense mechanisms that pathogens encounter. While this may be more feasible for delivery of an inactivated or encapsulated vaccine, this poses a challenge for a live DC vaccine. While intravenous injection of a DC vaccine is the best route of delivery, there is concern that the T cells that become activated may not effectively traffic to the tumor in the intestinal environment as CD11c$^+$ antigen presenting cells have been shown to imprint CD8$^+$ T cell homing to the colon[191,192]. However, alternative targeting mechanisms may overcome this problem. This has been demonstrated in an orthotopic model of head and neck cancer[191]. The Shiga toxin subunit-B was coupled with tumor specific antigen and delivered intranasally[191]. This delivery system compared to intramuscular injection allowed for the targeting of multiple mucosal lung associated DC[191]. In addition, resident lung but not splenic DC elicited antigen specific T cell activation and led to upregulation of mucosal integrins CD49a and CD103[191]. This subset of T cells demonstrated homing ability to the tumor site where anti-tumor immunity was elicited[191].

Many permutations and combinations exist for developing a DC vaccine, however the identification of the appropriate antigen, adjuvant, and number of immunizations required is still under investigation. Although promising results have been observed using DC vaccines such as this, use of this technique as a main-line cancer therapy is uncommon and has to date only been used and tested in late stage and metastatic cancer patients. However, given the poor success rates of checkpoint immunotherapy in most cancers, it is of interest to identify therapies that can be co-administered in order to optimize the induction of anti-tumor immune responses for most individuals with cancer. DC vaccines are a promising tool for accomplishing this.

An alternative target: Fcγ receptors

The role of Fcγ receptors has often been described as one of the master regulators of an immune response[193]. Fc gamma receptors are involved in binding the Fc region of antibody bound to antigen[193,194]. The outcome of the immune response is governed by a set of receptors which includes three Fcγ receptors with immune activating potential (FcγRI**,** FcγRIII and FcγRIV), whereas the remaining receptor is inhibitory (FcγRIIB) **(Fig. 1-6)**[193–195]. Fcγ receptors are widely expressed on immune cells including DC, monocytes, macrophages, B cells, plasma cells, neutrophils, mast cells and basophils[192]. As the research in this thesis is mainly focused on DC and macrophages, the overview presented here will be limited to these cell types.

As innate cells of the immune system, the central role of these cells is the uptake of pathogens from the cellular environment[74,196]. Following phagocytosis, these cells traffic pathogens to lysosomal compartments and may also present pathogen related antigen to cells of the adaptive immune system[197]. As mentioned earlier, uptake of pathogens most often leads to the induction of tolerance and activation of adaptive immune cells is inhibited[161]. In contrast, pathogens that are opsonized engage Fcγ receptors which leads to increase uptake of these pathogens[193,194]. The pathogens opsonized by antibody is often referred to as an immune complex (IC) [193,194]. Uptake of an IC by Fcγ receptors leads to the initiation of an immune stimulatory program and the shuttling of pathogen related antigens to immune presentation pathways that is facilitated by binding to the intracellular neonatal Fc receptor[193,194]. Crosslinking of Fcγ receptors by ICs leads to early activation events whereby spleen tyrosine kinase is recruited to the tyrosine-based

activation motif (ITAM) [193,194]. Not only can the uptake of pathogen IC lead to shuttling of pathogenic immune stimulating antigens to presentation pathways, it also leads to enhanced recognition of the pathogen through binding to the intracellular tripartite motif-containing protein 21 (TRIM21) receptor[198]. This is facilitated by the presence of phagocytosed intracellular antibody which is potent damage-associated molecular pattern (DAMP) and can lead to pro-inflammatory signaling[198]. Activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) lead to the upregulation of polarizing cytokines, while activation of signal transducer and activator of transcription 1 (STAT1) leads to the upregulation of co-stimulatory molecules[194,199,200]. These early activation events lead to downstream signaling that allow for DC and macrophages to produce polarizing cytokines and co-stimulatory receptors required for effective T cell activation[193,194].

It is currently not well understood whether engagement of Fcγ receptors leads to a specific type of polarizing cytokine. The type of cytokine signature produced by the antigen presenting cells may be dependent on a number of factors including size and type of IC, the extent of antibody opsonization of the target antigen and the Fcγ receptor engaged[201]. The expression of Fcγ receptor varies for antigen presenting cells[194]. In addition, the presence of inhibitory and activating receptors on these cells with also have a major role in response to the IC[194]. The binding of an IC to the inhibitory FcγRIIB bearing an ITIM domain will largely lead to immune cell tolerance and proinflammatory signaling will be avoided[194,202]. On the other hand, IC may bind to an activating Fcγ receptor. Although the activating Fcγ receptors share an ITAM domain, each receptor has a different affinity for the IgG subclass that may bind to it[193–195]. In addition, Fcγ receptors differentially bind IC[203]. Antigen may be complexed to a single antibody or may be opsonized with multiple immunoglobulin which will determine binding to Fcγ receptors[203]. FcγRI is capable of binding to monovalent-Ig IC, whereas FcγRIII and FcγRIV require antigen to be further opsonized by antibody[203]. Given that targeting of antigen to Fcγ receptors shuttles antigen to antigen presentation pathways in DC, this is an attractive avenue for the stimulation of $CD8^+$ anti-tumor immunity.

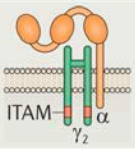
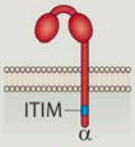
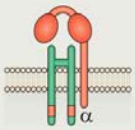
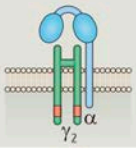
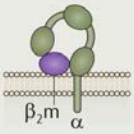
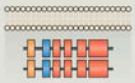
Structure	Name	Gene	IgG1	IgG2a	IgG2b	IgG3	Major function
	FcγRI	*Fcgr1*	NB	$1x10^{8¶}$	$1x10^{5}$	+‡	Activation
	FcγRIIB	*Fcgr2b*	$3x10^{6}$	$4x10^{5}$	$2x10^{6}$	No binding	Inhibition
	FcγRIII	*Fcgr3*	$3x10^{5}$	$7x10^{5}$	$6x10^{5}$	No binding	Activation
	FcγRIV	*Fcgr4*	NB	$3x10^{7¶}$	$2x10^{7¶}$	No binding	Activation
	FcRn§	*Fcgrt*	$8x10^{6}$	+	+	+	IgG recycling and transport
	TRIM21§	*Trim21*	$2x10^{6}$	+	+	+	Activation and proteasome targeting

Figure 1-6. Representation of murine Fcγ IgG receptors on antigen presenting cells. Each of the activating Fcγ receptors (RI, RIII and RIV) shares a common γ chain and the immunoreceptor tyrosine-based activation motif (ITAM) domain. In contrast, the inhibitory Fcγ receptor (RIIB) contains the immunoreceptor tyrosine-based inhibition motif (ITIM) domain. Each Fcγ receptor displays varying affinity for the IgG subclass that becomes engaged upon Fcγ receptor crosslinking. In addition, there are intracellular receptors for IgG including the neonatal Fc receptor (FcRn) and tripartite motif-containing protein 21 (TRIM21). Figure from: Guilliams M. 2014. Nat Rev Immunol. 14. 94-108. Reprinted with permission.

Targeting DC via the Fcγ receptor

Uptake of antigen by Fcγ receptors leads to much more potent immune stimulatory signaling compared to alternative uptake methods[193,194,201]. Induction of an inflammatory anti-tumor immune response at mucosal sites is of great interest since many cancers arise at these sites. One method to facilitate this would be to enhance the inherent cross-presentation capacity of DC to enhance their priming of cytotoxic $CD8^+$ T cell immunity[201]. Increasing antigen uptake by DC is one strategy for increasing their cross-presentation efficiency. DC that are $CD8^-$ are poor cross presenters of antigen that is taken up in soluble form[201,204]. However, previous studies have demonstrated that loading $CD8^-$ DC with IgG complexed antigen allowed the cells to cross present and initiate antigen-specific activation of $CD8^+$ T cells[201,204]. Furthermore, IC have a demonstrated ability to be tumor protective and to successfully initiate anti-tumor immunity[205]. Generation of a DC vaccine with the use of ICs is thus an attractive avenue to promote anti-tumor immunity.

Exosomes

Exosomes are small extracellular vesicles (EVs) released from all cell types upon the fusion of multivesicular endosomal membranes with the plasma membrane[206]. Although there is discrepancy in the literature, these particles are thought to range from 30 – 200 nm in size[207]. Due to current limitations of separation methods, these particles are often also co-isolated with microvesicles. The microvesicles range from 100 – 1000 nm in size and are formed from blebbing of the plasma membrane[208]. Exosomes were once thought to be a disposal system for cells to eliminate extracellular material[206]. Although the EV field remains in its infancy, many studies have demonstrated an important role for exosomes in cell-to-cell communication[206].

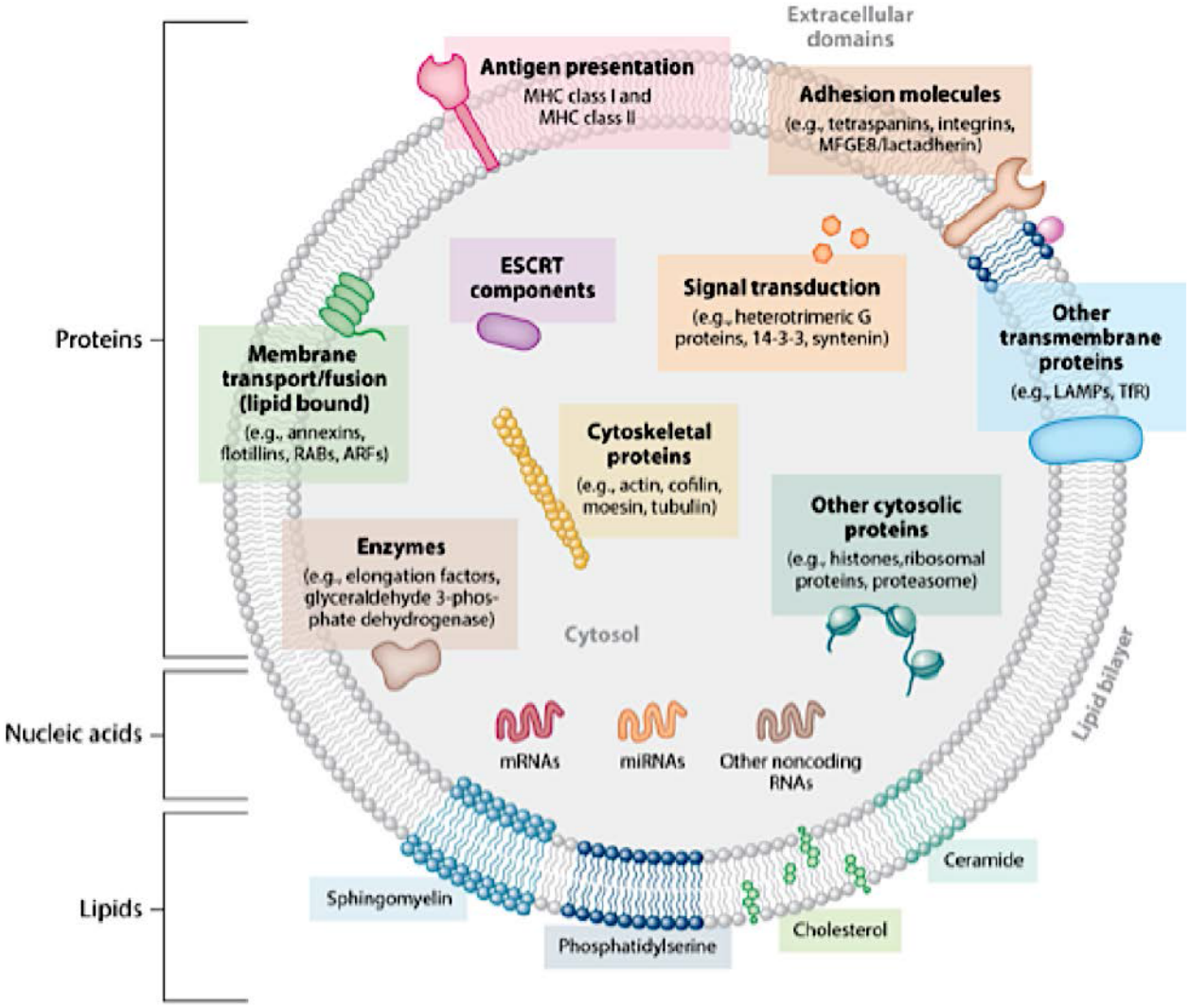


Figure 1-7. Exosomes as carriers of biological molecules. Exosome biogenesis is a highly controlled process. Through this process many cellular components are incorporated within the lipid bilayer and on the exosome surface. Exosomes are carriers of various RNAs including mRNAs, miRNAs and non-coding RNAs. In addition, exosomes are carriers of various cytosolic proteins and transmembrane proteins. Exosomes in particular have a unique lipid membrane structure where the immunogenic lipid phosphatidylserine is exposed on the outer leaflet of the lipid bilayer. Despite the exposure of this lipid and constitutive release of exosomes from all cell types, anti-inflammatory responses do not seem to be directed towards these particles. Figure from: Colombo M, *et al*. 2014. Annu Rev. Cell Dev. Biol. 30: 255-89. Reprinted with permission.

Exosomes in cancer

Many studies have attempted to examine exosomes over the course of tumor progression with the aim of using tumor-derived exosomes as prognostic markers or biomarkers to measure patient response to chemotherapy[209,210,211]. This work has revealed that exosome content and amount may change over the course of tumor development and have conclusively shown that EVs display great heterogeneity at all stages of tumor growth[212]. Exosomes have been shown to be involved in each hallmark of cancer, including inhibition of anti-tumor immunity and in facilitating tumor progression through many mechanisms such as conditioning of new metastatic sites[209].

Exosome biogenesis is a highly controlled process regulating which RNAs, proteins and lipids are incorporated in the vesicles[206]. Much work in the exosome field has focused on exosome RNA cargo. Specifically, the small particles have been shown to carry miRNAs and non-coding RNAs that mediate the inhibition and regulation of gene expression in recipient cells that internalize them[213,214]. Prior to regulation of gene expression by exosome RNA in recipient cells, uptake of exosomes has been shown to follow receptor-ligand interactions mediated by proteins expressed on the exosome surface[206]. Exosome protein cargo has also been shown to contain TAA[215].

Although, not as extensively studied as protein and RNA exosome cargo, lipids that compose the exosome have been analyzed[206,216]. Exosomes isolated from various cell lines have been shown to be enriched in sphingomyelin, cholesterol, phosphatidylcholine, phosphatidylserine (PS) and fatty acids (FA) [206,216]. Exosomes in particular have been identified as having a unique lipid membrane structure where the immunogenic PPL PS is found on the outer leaflet of the exosome lipid bilayer **(Fig. 1-7)**[206]. Normally this PPL is contained to the inner leaflet on normal cells through the action of flippases that maintain the asymmetric distribution of PS in the lipid bilayer[206,217]. When cells become apoptotic, PS is exposed on the outer leaflet of cells through the activation of scramblases and apoptotic bodies[221]. Recognition of PS by phagocytic cells facilitate the clearance of these apoptotic cells[221]. The presence of PS has been identified on many exosomes from various cell lines and is maker by which they may be identified.

Exosome regulation of anti-tumor immunity

There is great interest to know how tumor derived exosomes communicate with the immune system. Much work on exosomes indicates that these particles are inhibitory in nature and block anti-tumor immunity through a variety of mechanisms such as inducing immune cell apoptosis and inhibiting immune cell activation and maturation[218]. Exosome miRNA cargo has been shown to regulate expression of innate immune sensing genes in DC following the uptake of exosomes from tumor cells. For example, miRNA 203 carried by exosomes released from pancreatic cancer cells regulated the expression of TLR4 in DC following uptake and subsequent downregulation of pro-inflammatory genes TNFα and IL-12 was observed[219]. Furthermore, uptake of tumor derived exosomes have also demonstrated the capacity to inhibit DC maturation, preventing the presentation of antigen and leading to DC tolerance via TGFβ[220]. Palmitoylated proteins carried by exosomes have also demonstrated the ability to contribute to chronic tumor promoting inflammation in macrophages[221]. Breast cancer derived exosomes demonstrated the ability to increase pro-inflammatory cytokine release of IL-6, TNFα, granulocyte colony stimulating factor (GCSF) and CCL2 in a NF-κB dependent manner following the ligation of palmitoylated proteins with the macrophages[221]. On the other hand, exosomes have also been demonstrated to alter the cytokine profile of DC and macrophages that favors anti-tumor escape mechanisms and facilitates tumor progression and metastasis[222].

Aside from exosomes inhibiting anti-tumor immune responses in DC and macrophages, exosomes have also been demonstrated to directly inhibit CTL critical for anti-tumor immunity[218]. One example is the ability of tumor derived exosomes to inhibit lymphocyte proliferation and mediate apoptosis of lymphocytes that display a Th1 phenotype[223]. Not only have exosomes demonstrated the ability to induce CTL apoptosis through expression of Fas ligand, exosomes have also been demonstrated to impair CTL function by mediating decreased expression of granzyme B and IFNγ[224,225]. In addition, tumors have also shown the ability to release exosomes that promote the generation and expansion of Treg cells mediating escape from anti-tumor immunity[226].

Although there is much existing evidence that demonstrates the ability of exosomes to suppress and inhibit anti-tumor immunity, much of the suppressive nature has currently been attributed to cargo carried by the exosome. Before this cargo is delivered to various immune cells, receptor-ligand interactions between immune cells and exosomes are likely to occur. These interactions responsible for initiating early steps in exosome interaction with immune cells is largely unknown. This is likely the case due to vast heterogeneity in EVs, inconsistent isolation procedures and nomenclature for EVs used throughout literature that account for difficulty in determining these early events whereby exosomes suppress anti-tumor immunity. Various mechanisms by which EVs modulate immune cell activity have been proposed. The major proposed mechanism of uptake of EVs by antigen presenting cells is phagocytosis[206]. Alternatively, studies investigating DC derived EVs suggested that exosomes themselves directly present antigen to T cells[227,228]. In addition, trogocytosis whereby small sections of lipid membrane are exchanged between two immune cells make also be a mechanism by which exosome content is transferred to a recipient cells, however this has not been extensively studied[228]. Many other studies indicate that the presence of tumor derived exosomes are able to inhibit anti-tumor immunity by decreasing proliferation and release of proinflammatory cytokines[227]. Few studies have shown the ability of T cells to take up exosomes, however the majority of studies have not indicated the mechanism by which exosomes alter T cell biology[228]. In other words, it is not entirely clear whether tumor exosomes mediate immunosuppressive effects by the uptake of these particles by T cells. Although a number of different receptor ligand interactions may be responsible for EV mediated immunosuppression, exposed PS on the outer exosome leaflet may have a role.

PS in particular in recognized as a global immunosuppressive factor. This occurs when PS on apoptotic cells including tumor cells interacts with PS receptors on cells of the innate immune system leading to phagocytic engulfment and the release of immunosuppressive TGFβ and IL-10 and decreased proinflammatory cytokines TNFα, IL-1β, and IL-12[229]. In addition to modulating cytokines, phagocytic DC remain in an immature state and expression of costimulatory ligands are suppressed thereby promoting tolerance[229]. PS therefore serves the function of (1) “find me,” (2) “eat me,” and (3) “tolerate me” being thereby inhibiting a proinflammatory immune response against PS positive particles[229]. PS exposed on EVs may also have the same ability to inhibit

anti-tumor immunity through the interaction of exposed PS with receptors on immune cells. One study in particular identified that PS positive EVs derived from ovarian tumor ascites fluid induced arrest in the T cell signaling cascade[230]. This was partially reversed by depletion of PS positive EVs and anti-PS antibody blockade of PS [230]. This study is limited to EVs present in ascites fluid from ovarian tumors. However, PS exposure has been identified on many EVs isolated from various cell lines[206]. Therefore, PS may have a large role in suppression of immune cells by EVs derived from many tumor cell types.

PS exposure on the outer surface is not limited to apoptotic cells and exosomes. PS may also be exposed on activated platelets, tumor cells and viable cells of the immune system[229]. PS is therefore externalized by different mechanisms and can therefore have a different outcome for cells and is not functionally equivalent[229]. PS exposure is highly dysregulated in the tumor microenvironment and is often not recognized as an "eat me" signal, but rather a survival signal and mechanism by which tumor cells circumvent anti-tumor immunity[229]. Viable immune cells that express PS are also not marked for phagocytic clearance[229,231,232]. Many studies of PS exposure indicate that there is a threshold of PS exposure and particular topological organization of PS that is required for the recognition of these cells for phagocytic engulfment[229]. In addition to the amount and organization of PS on various cells, other ligands are likely to have a role in determining whether cells are marked for phagocytic clearance or not[229]. Although PS has been detected on exosomes, conventional flow cytometry have not been able to examine varying levels of PS that may be exposed on exosomes[206]. We currently speculate that the amount of PS exposed on exosomes is sufficient to mark these particles for phagocytic clearance since they have been frequently described to be taken up and metabolized by phagocytic cells[206]. Exosomes are involved in inhibiting anti-tumor immunity and therefore this most likely leads to immune tolerance. EVs carry self-antigen and this is one possible reason that EVs have evolved to be minimally stimulatory[206]. Therefore, it is of great interest to determine how this inherent immunosuppressive tendency mediated by exosomes can be overcome. Alterations to exosomes that, not only overcome the immunosuppressive nature, but other roles these particles have in the Hallmarks of Cancer are a potential area for the development of new cancer therapies.

Not only is PS expressed on exosomes that may inhibit anti-tumor immunity, but PS is highly dysregulated in the tumor microenvironment[229,233]. This has led to strategies to block PS binding to immunoregulatory receptors thereby preventing PS mediated immunosuppression. Pre-clinical studies have shown that binding of annexin V (AV) to apoptotic monocytes, but not PS positive viable monocytes facilitates phagocytic clearance of these cells[229,231]. Blocking of PS mediated immune inhibitory signals with AV has also demonstrated the ability to lead to proinflammatory signaling contributing to anti-tumor immunity[229]. AV coated apoptotic lymphoma tumor cells have also been able to increase the immunogenicity of these cells as well as lead to deceased and increased uptake by macrophages and DC respectively[234]. These studies led to the development of PS targeting antibodies. In preclinical models, the use of anti-PS antibodies have demonstrated the ability to allow for DC maturation, subsequent proinflammatory cytokine release, reduce and increase the number of MDSCs and anti-tumor M1 macrophages respectively[229,235]. In addition, anti-PS antibodies lead to greater anti-tumor effects when in combination with anti-CTLA-4 and anti-PD-L1[229].

While anti-PS antibodies have and are currently being tested in early clinical phase I and II trials, overall response rates remain low[229]. Since PS can be exposed on viable cells, apoptotic cells and exosomes, it is not currently clear which exposed PS is targeted by anti-PS antibody[229]. Much of the anti-PS antibody is likely to be bound to apoptotic tumor cells, however whether these antibodies also bind to PS positive exosome remains unclear. Exosomes display a myriad of proteins including PS on their surface that could be targeted by antibodies[206]. Opsonization of exosomes by IgG has a great deal of therapeutic potential. Uptake of such complexes by antigen presenting cells in the spleen, and in the liver where exosomes are metabolized, could help facilitate the clearance of exosomes so that their tumor promoting effects are inhibited[236–238].

Exosomes are known carriers of TAA and are ideal vesicles for delivering high doses of TAA into antigen presenting DC, that can then prime tumor-targeted immunity[215]. Since exposed PS on cells and exosomes leads to immunosuppressive signals in immune cells, it is of interest to know whether or not anti-PS antibodies also bind to exosomes that could enhance their immunogenicity and allow for DC maturation[229]. Despite evidence that exosomes inhibit anti-tumor immunity, it is unclear how effectively tumor derived antigens are delivered to immune

cells by exosomes. Given that exosomes generally inhibit anti-tumor immunity, it is likely that any tumor derived antigens that are delivered to antigen presenting cells are not presented to $CD8^+$ T cells. It is thus of interest to determine how to enhance the immunostimulatory capacity of tumor exosomes. In addition, complexing exosomes with antibody could facilitate the uptake of IC by DC and allow for cross presentation of TAA to activate $CD8^+$ T cells. However, antibody-targeting of surface proteins on exosomes remains a challenge. Current methods of nanoparticle analysis are not sophisticated enough to characterize surface proteins to choose the appropriate target that is expressed exclusively by exosomes and not the secreting cell[206]. This is particularly difficult for tumor exosomes since the composition of exosome cargo and surface protein expression are known to change over time[206]. Certain tumor microenvironment conditions such as hypoxic conditions and low pH have been shown change exosome protein and lipid composition respectively[239,240]. Specifically, acid conditions were shown to alter exosome lipid content that led to an increase in sphingomyelin and ganglioside GM3 in the lipid membrane of exosomes[240]. While various tumor microenvironment conditions have not been extensively studied in altering the lipid content of exosomes, the unique lipid nature of the exosome membrane could allow for the stable targeting of exosomes by anti-PS antibodies. Since this lipid is contained to the inner leaflet of that majority of non-apoptotic viable cells, antibodies against PS should only target apoptotic cells and exosomes[217]. It is currently understood that exosomes released by almost all cell types are processed and degraded by phagocytes in the liver and spleen[236–238]. Although not currently proven, we hypothesize that the mechanism triggering removal and degradation of circulating exosomes is facilitated by the exposure of PS on the exosome surface. The overall nature of exosomes is immune inhibitory, and this mechanism is likely to exist to prevent over activation of the immune system by exosomes in constant circulation. It is of interest to determine how the targeting of exosome PS by anti-PS antibody changes the overall immune stimulatory capacity of exosome.

Anti-PPL antibodies

Anti-PPL syndrome is an autoimmune condition that is characterized by thrombolytic events and recurrent pregnancy failure[241]. Anti-PPL syndrome is diagnosed upon examination of anti-PPL antibodies[241]. Anti-PPL antibodies are a heterogeneous group of autoantibodies of the IgM and

IgG isotype that include lupus anti-coagulant, anti-cardiolipin or antibodies directed towards anionic phospholipids or phospholipid-protein complexes with β-2-glycoprotein I (β2GPI)[241]. Antibodies directed towards cardiolipin and PS have been the most extensively studied[241]. While these antibodies may be present at low non-pathogenic levels, increased titers of these antibodies have been identified in a number of diseases. Management of anti-PPL is the same across various disease states and consists of anti-coagulants and immunosuppressants. Despite the increased risk of thrombolytic events in anti-PPL syndrome, some patients are asymptomatic and it remains unclear as to whether management of anti-PPL is required in this group[241].

Pathogenesis of anti-PPL antibodies in disease states

The most common autoimmune condition where anti-PPL are elevated are found in patients with systemic lupus erythematosus (SLE). While not all SLE patients have elevated anti-PPL auto-antibodies, 40% present with increased titers[241–243]. While complications of elevated anti-PPL antibodies is usually limited to thrombolytic events, these antibodies have been linked to catastrophic systemic events including organ failure in a number of individual cases[241,244]. The pathogenesis of anti-PPL autoantibodies are not well understood[241]. Dysregulation of complement factors is one potential factor that has been associated with anti-PPL[241]. These antibodies have also been identified in patients with viral infections including HIV[245]. Increased anti-PS in these patients were shown to be a result of increased T cell apoptosis and enhanced antibody dependent cellular cytotoxicity (ADCC) by macrophages[245]. Anti-PPL antibodies have also been shown to be elevated in patients infected various viruses including Epstein-Barr virus, varicella, hepatitis C virus, human T lymphotrophic virus, parvovirus and cytomegalovirus (CMV)[246]. This has led to the "pathogenic hypothesis" of increased anti-PPL. This hypothesis suggests the molecular mimicry of epitopes similar to the epitope of anti-PPL antibodies[246]. Despite the presence of anti-PPL in patients, some studies have indicated that co-factor β2GPI is required for thrombolytic events[241,246]. β2GPI have been shown to share homology with CMV[246]. Injection of peptides with similar structure to β2GPI into mice have been shown to induce generation of anti-PPL[246–248]. Thus, it has been postulated that infection with various viral pathogens may be a step responsible for the generation of anti-PPL in humans.

Anti-PPL antibodies in cancer

Previous work has identified increased titres of anti-PPL antibodies in cancer patients with both haematological malignancies such as lymphoma as well as solid tumors[249,250]. Increased titres of these antibodies have implications for thrombolytic complications in patients and has been suggestive of a marker of poor prognosis in cancer patients[249,250]. However, not all patients who exhibit increased anti-PPL antibodies will experience thrombolytic events and alternative roles for these antibodies in cancer progression have not been examined[249,250]. Continuous apoptotic events during tumor progression due to mechanisms such as hypoxia and immuno-editing could provide a continuous source of the phospholipids PS and cardiolipin that promote production of anti-phospholipid antibodies in cancer patients[85]. This could have profound implications for cancer progression by mechanisms including induction of tumor-promoting inflammation. In addition to their pathogenic functions, anti-PPL antibodies could also promote productive anti-tumor immunity by forming IC with tumor apoptotic bodies and exosomes. The majority of research previously done investigating anti-PPL antibodies and cancer has been limited to correlational studies of serum antibody concentrations in clinical cohorts[241,249,250]. The biological mechanisms by which anti-phospholipid antibodies contribute to either cancer progression or anti-tumor immunity have thus not been explored. Given the rise in the success with immunotherapy and the potential implications phospholipid antibodies may have in cancer immunology, the significance of anti-phospholipid antibodies and the cancer relationship should be revisited.

Role of fatty acids in inflammation

Dietary fatty acids (FA) are in taken up by all cells in the body and incorporated into cell membranes. This includes uptake into cells of the immune system which can influence immune responses through a variety of mechanisms[251–253]. In particular, polyunsaturated FA (PUFA) are preferentially incorporated into the *sn*-2 position of phospholipids including phosphatidylcholine and phosphatidylethanolamine[254,255]. However, PS is enriched in neuronal tissue and incorporated into both *sn*-1 and *sn*-2 positions of PS[254,255]. PUFA in particular are known to modulate inflammatory responses in both innate and adaptive immune cells[254]. PUFA are commonly referred to n-3 and n-6 FA which refers to the location of the last double bond relative

to the terminal methyl ω end of the lipid[254]. FA that are obtained from the diet are fist taken up into cells by FA binding protein and subsequently undergo metabolic conversions in the cell[254]. Not all PUFA acids can be synthesized de novo[254]. Two PUFA in particular that have unique immune modulating properties are linoleic acid (LA) 18:2n-6 and α-linolenic acid (ALA) 18:3n3 which cannot be synthesized de novo are obtained from the diet and metabolized in cells[254]. Following uptake of LA and ALA, these FA undergo saturation, desaturation and elongation to form long chain PUFA AA and DHA respectively **(Fig. 1-8)** [254]. Although AA and DHA can form de novo from dietary LA and ALA, these two pathways compete for the same enzymes and conversion of ALA to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is poor[254]. EPA and DHA is better obtained through dietary sources of these two PUFA from marine fish oil[254].

While n-3 FA are often regarded as having beneficial anti-inflammatory properties many studies are inconclusive and point the importance of some n-6 FA in mediating inflammation[254]. This is observed with dietary supplementation with n-6 PUFA γ-linolenic acid (GLA) 18:3n-6 which forms the elongated FA dihomo-γ-linolenic acid (DGLA) 20:3n-6 which has anti-inflammatory properties[254]. Since both n-3 and n-6 PUFA have been shown to have anti-inflammatory effects, it is now thought that the dietary ratio of n-3 to n-6 PUFA is the important factor in modulating inflammation[254,256]. The change in dietary FA over time has been linked to the number of chronic inflammatory diseases in the Western diet as a result of increased n-6 FA consumption[254,257]. The Western diet has an n-3 to n-6 FA ratio of approximately 1:20-30, whereas populations have higher consumption of fish have a ratio of 1:1-2[254,257]. It is thought that a low n-3 to n-6 FA ratio in cell membranes contributes to chronic inflammation[254]. Although many PUFA have inflammatory modulating properties, n-3 EPA and DHA are by far the most potent anti-inflammatory mediators[254,258]. EPA and DHA are also more potent than their precursor ALA, therefore supplementation with marine fish oils are of particular importance since conversion is poor[254].

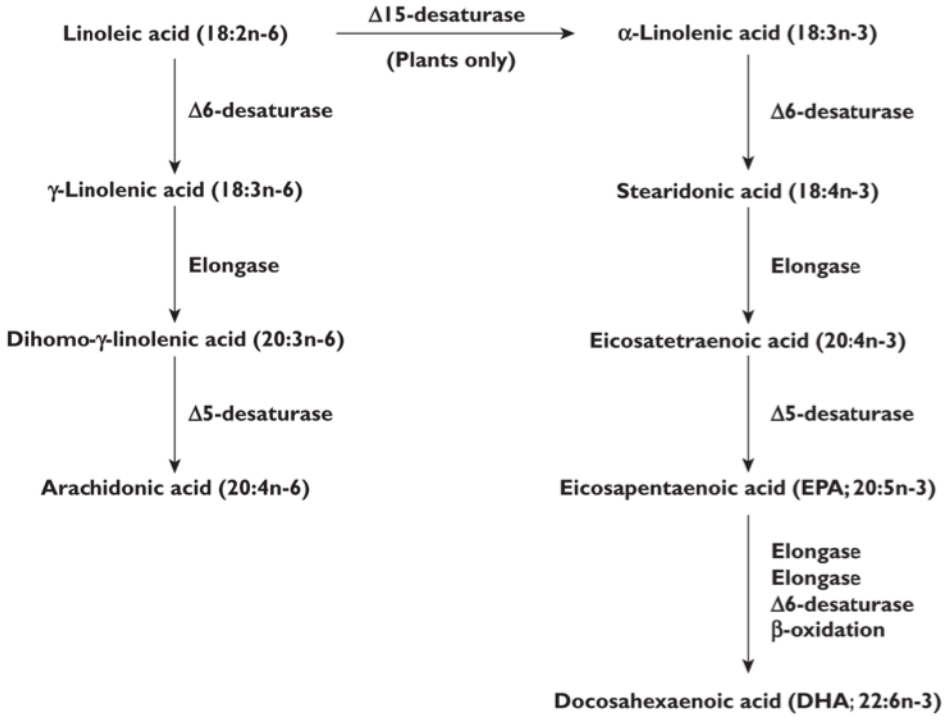


Figure 1-8. Metabolic conversion of dietary n-6 and n-3 FA to AA and DHA. Linoleic acid (LA) 18:2n-6 and α-linolenic acid (ALA) 18:3n3 are converted by a series of enzymatic desaturation and elongation reactions for the conversion of LA to AA and ALA to eicosapentaenoic acid (EPA). Subsequent elongation of, desaturation and β-oxidation are required for the conversion of EPA to docosahexaenoic acid (DHA). Both pathways for conversion of LA and ALA to AA and DHA respectively compete for the same enzymes and conversion of dietary ALA to EPA and DHA is poor. Figure from: Calder P. 2012. Annu Rev. Br J Clin Pharmacol. 75:3 645-662. Reprinted with permission.

Modulation of inflammation by PUFA

PUFA have been suggested to modulate immune cell activity through a number of mechanisms[254]. PUFA are capable of modulating the activity of a number of immune cells including DC, macrophages, NK cells and T cells[254]. The activity of PUFA may affect immune cell activity by modulation of cytokines, surface expression of molecules and receptors, phagocytic function, apoptosis and proliferation of lymphocytes[254]. One mechanism by which PUFA achieve these immune modulating properties is by altering gene transcription[254]. PUFA are most well known to mediate the gene transcription factors peroxisome proliferator activated receptor α and sterol regulatory element binding protein-1c[254]. Alternatively, PUFA may also alter cell signaling of immune cells[254]. This has been studied mainly in T cells where PUFA have been shown to attenuate cell signaling by displacement of early signaling mediators such as Lck, Fyn and LAT from intracellular signaling domains[254,259,260]. PUFA may also exert anti-inflammatory properties through the production of a less potent class of eicosanoid that include thromboxanes, leukotrienes and 3-series prostaglandins and thromboxanes (not pictured), and the 5-series leukotrienes though metabolism by COX and lipoxygenase (LOX) **(Fig. 1-9)**[252,254]. In addition, EPA and DHA can also be metabolized to form resolvins and protectins which are anti-inflammatory and resolve inflammation[252]. PUFA can also alter the lipid membranes that will affect the organization of lipid proteins[254]. PUFA EPA and DHA have been demonstrated to alter the lipid membrane of antigen presenting cells that decreases expression of MHC, which subsequently attenuates T cell activation[254]. While proinflammatory anti-tumor immunity facilitates clearance of tumor cells, excessive inflammation can be tumor promoting[88]. Aside from PUFA modulating immune cell activity, EPA and DHA can also promote tumor progression by decreasing growth receptors on tumor cells and promoting ligands that facilitate the induction of apoptosis[254]. Together, it is of interest to determine how n-3 PUFA can lead to tumor regression by modulating tumor promoting inflammation and decreasing tumor cell growth.

Incorporation of AA and DHA PUFA into lipid rafts of cell membranes can attenuate cell signaling and ultimately have an effect on gene expression[251–253]. However, it is not known

whether exosomes which are rich in lipids can be enriched in AA and DHA and whether these lipids can be transferred to immune cells. AA and DHA can have immunomodulatory properties on DC and macrophages by modulating cell signaling, expression of adhesion molecules and cytokines[251,252]. This raises the possibility that dietary alterations in lipid-membrane composition in exosomes can modulate the anti-tumor immune response following uptake of these exosomes by DC.

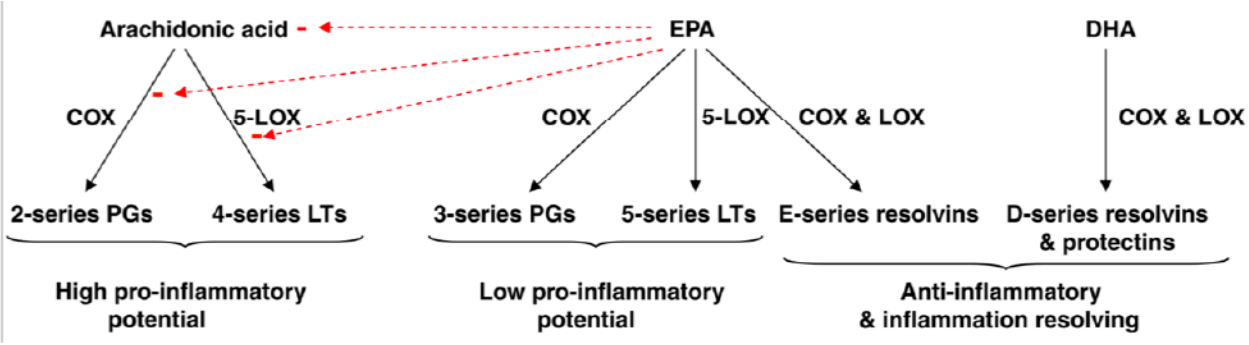


Figure 1-9. Overview of eicosanoid inflammatory mediators and their precursors. Precursors AA, EPA and DHA are metabolized by COX and lipoxygenase (LOX) to form eicosanoids including prostaglandins (PGs) and leukotrienes (LTs) with different inflammatory capacity. Eicosanoids derived from AA and EPA form PGs and LTs with high and low proinflammatory capacity respectively. In addition, EPA and DHA form resolvins and protectins that are anti-inflammatory and inflammation resolving. Figure from: Calder P. 2011. Euro J of Pharmacology. 668: 50-58. Reprinted with permission.

Research Rationale and Objectives

Cancers presenting at mucosal immune sites within the body affect many individuals within the North American population but prove difficult to treat successfully with immunotherapies[24,63,64]. This is thought to be due to the hyporesponsive nature of mucosal immune tissues such as the lung and intestine. This leaves cancers at these sites to be treated only with conventional cytotoxic chemotherapeutic drugs that come with a myriad of side-effect for patients. Since immunotherapy has been successful in the treatment of other cancers, it remains an attractive avenue for treating malignant tumors at mucosal immune sites. However, the overall response rate of immunotherapies remains low and this will require the development of alternative strategies for stimulating immune cells, especially at mucosal sites due to their tolerant state[72,73].

1. Exosomes are involved in all Hallmarks of Cancer and have a role in promoting tumor progression in part by suppressing anti-tumor immunity[209]. PS is a widely recognized immunosuppressive factor that has been identified on the outer leaflet of exosomes[206]. Despite the fact that PS is globally immunosuppressive, only a few studies have suggested that the suppressive nature of exosomes is mediated in part by the exposure of PS[230]. These studies are limited to the role of exosome PS on particular immune cell subsets and have not extended to DC. In order to demonstrate that PS on CRC exosomes has a role in suppression of DC maturation, we will block exposed exosome PS with anti-PS antibody and characterize the immunogenicity of these IC.

2. Many TAA are weakly immunogenic and can lead to a tolerogenic DC phenotype following uptake that results in the subsequent absence of T cell stimulation[74]. More recently, IgG complexed antigen was shown to overcome tolerance and allow for DC to cross present antigen to $CD8^+$ T cells[204]. These studies are limited by the fact that multiple tumor antigens need to be presented by DC to generate polyclonal T cell anti-tumor immunity. Exosomes are carriers of TAA, however it is not well understood whether these TAA may be presented by DC following phagocytic uptake[206]. To study presentation of exosome cargo, we will examine the ability of DC to cross present OVA

to T cells following the uptake of exosomes derived from OVA transfect MC38 cells in vitro and in vivo.

3. Given the ability of lipids incorporated into particular PUFA to modulate inflammation, studies have examined the role of n-3 and n-6 PUFA in modifying lipid rafts, attenuating cell signaling and altering eicosanoid formation[254]. It has yet to be examined whether inflammation can be modulated by delivery of lipids to a recipient cell via exosomes. To study this, we will examine whether AA and DHA can become incorporated into exosomes by modulating the FA content of the parental cell from which the exosomes are derived. Furthermore, we will examine whether these exosomes can modulate proinflammatory cytokine expression in DC.

Specific hypotheses and goals of research

By investigating how adjuvant strategies such as IgG complexing and dietary modification with lipids affect how tumor exosomes modulate the immune microenvironment, we aim to determine how to use exosomes to induce protective anti-tumor immune responses against CRCs.
We hypothesized that complexing exosomes to anti-PS antibody would enhance the immune stimulatory capacity of exosomes that have previously been demonstrated to inhibit immune activation.

Aim 1. Determining if tumor derived exosome-anti-PS IgG IC can prime an immune stimulatory response.

We hypothesized that engagement of Fcγ receptors on DC by tumor exosome IC would lead to presentation of antigen on MHC I and production of Th1 polarizing cytokines, thereby activating IFNγ expressing $CD8^+$ T cells that mediate anti-tumor immunity **(Fig. 1-10)**.

Aim 1A. Determining if lipid alteration can modulate the immunogenicity of CRC derived exosomes.

We hypothesized that two lipids that have immune altering properties, AA and DHA, could become incorporated in exosomes of CRC cells stimulated with them, changing the ability of the tumor exosomes to modulate cytokine expression in DC.

Overall Theses

1. Exosomes form IC following complexing with anti-PS antibody and mediate differential signaling in DC and macrophages. These IC stimulate DC maturation and cytokine production. In addition, following DC stimulation with IC but not exosomes, IFNγ is released by either DC or $CD8^+$ T cells.

2. When murine MC38 CRC cells are modified with PUFA AA and DHA, these lipids become incorporated in exosomes from the parent cell from which they are derived and modulate pro-inflammatory cytokine expression in DC.

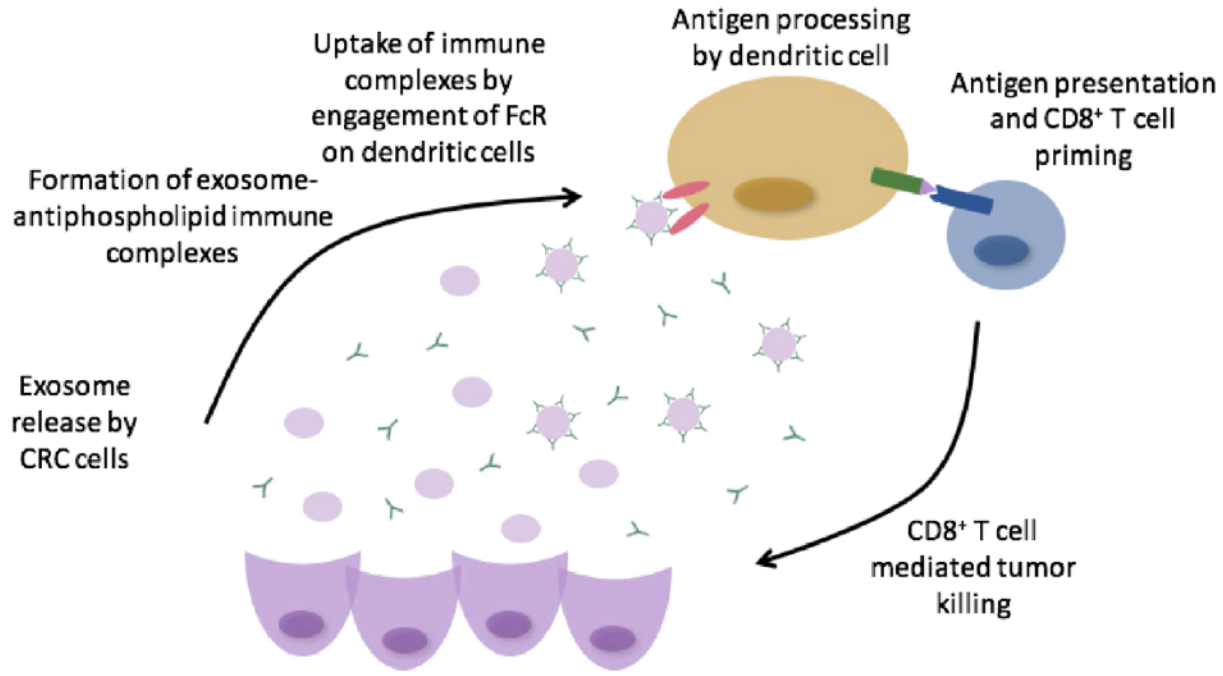


Figure 1-10. The role of exosome IC in stimulating anti-tumor immunity through engagement of Fcγ receptors on DC. Exosomes are released by CRC cells and the display of PS on their surface allows for targeting of the exosome by anti-PS antibodies. We hypothesize that this method will allow for the formation of exosome IC and will initiate pro-inflammatory signaling in DC and the cross priming of CTL. Together, these result in the activation of CTL that may mediate anti-tumor immunity. Figure author: Allison M. McNamara.

CHAPTER 2: Materials and Methods

Mice

Wildtype mice used as recipients in the studies are of the CD45.1 C57BL/6 background. Congenic CD45.2 OT-I mice, which have a TCR that recognizes ovalbumin, were used as a source of antigen-specific $CD8^+$ T cells. Mice used in the study were between the ages of 6-20 weeks old. All strains were originally purchased from Charles River but were bred and maintained in our colony at the Cross Cancer Institute in accordance with protocols approved by the University of Alberta Animal Care and Use Committee.

Cell lines

The MC38 cell line derived from C57BL/6 murine colon adenocarcinoma cells was purchased from Kerafast. The cells were grown in culture with high glucose Dulbecco's modified MEM with 10% fetal bovine serum and 1% penicillin-streptomycin. The MC38 cell line was transfected with the pCI-neo-mOVA ovalbumin-containing plasmid (Addgene) using Lipofectamine 2000 (Life Technologies). The plasmid contains a neomycin resistance cassette and transfected cells were selected and maintained with 200 μg/ml of Geneticin (G-418). Ovalbumin expression was confirmed by Western blot with the ovalbumin antibody.

Knockout of the genes *Mlh, Msh3, Pole, Kras* was CRISPR CAS9 mediated using the guide RNA for each gene with the pSpCas9-2A-Puro PX459 plasmid (Addgene). Cells were selected by using 2 μg/ml of puromycin and knock out was confirmed by sequencing and Western blot. The following gRNAs were used to create the cells:

Mlh1: caccgGGTAGTGAACCGCATAGCGGCGG
aaacCCGCCGCTATGCGGTTCACTACCc
Msh3: caccgCTCTGTTGGCACAGACAGGTCGGAGGGAAG
aaacCTTCCCTCCGACCTGTCTGTGCCAACAGAGc
Pole: caccgGGCTTGGGCCTATCCGAGAGGGG
aaacCCACGTGGCCCCCGACGTAGGTCc
Kras: caccgGCCGCCTGCCGAATCGAGCCCGG
aaacCCGGGCTCGATTCGGCAGGCGGCc

Isolation of Exosomes

MC38 cells were seeded in T175 flasks in high glucose DMEM. At 24 hrs, the cells were washed and the medium was replaced with exosome-production medium. This medium was high glucose DMEM containing 10% FBS that was depleted of contaminating FBS exosomes by ultracentrifugation at 100,000 x g for 15 hrs at 4°C as described in Théry et al (2006)[261]. Following ultracentrifugation, the supernatant of the FBS was removed and filter-sterilized with a 0.22 μm filter. The cells were grown for another 48 hrs and the cell culture medium was collected for exosome isolation by differential ultracentrifugation as described in Théry et al (2006)[261]. Specifically, the medium was subjected to a series of centrifugations each at 4°C and the supernatant was collected after each step and then spun at the next speed: 300 x g for 10 min, 2000 x g for 20 min, 10,000 x g for 30 min and 100,000 x g for 70 min. The pellet was then collected, resuspended in PBS to wash and spun at 100,000 x g for 70 min (**Fig. 2-1**). After the wash, the exosome pellet was resuspended in PBS and stored at -80°C for use in future experiments.

For purification of serum exosomes, blood was collected by post-mortem cardiac puncture on non-tumor bearing wildtype C57BL/6 mice. Whole blood was incubated at room temperature for a minimum of 15 min, centrifuged at 14,000 rpm for 15 min and the serum was collected and stored at -80°C. For exosome isolation, serum was diluted in an equal volume of PBS and was subjected to a series of centrifugations each at 4°C. At each step, the supernatant was collected and used in the next spin: 2000 x g for 30 min, 12,000 x g for 45 min, 110,000 x g for 2 hrs. The pellet was resuspended in PBS and filtered through a 0.22 μm filter. The filtrate was centrifuged for 110,000 x g for 70 min twice with one wash step in between, and the remaining pellet was resuspended in PBS and stored at -80°C for future use.

Exosome yield was determined using the NanoSight LM10, the Bradford assay or the bicinchoninic acid (BCA) assay.

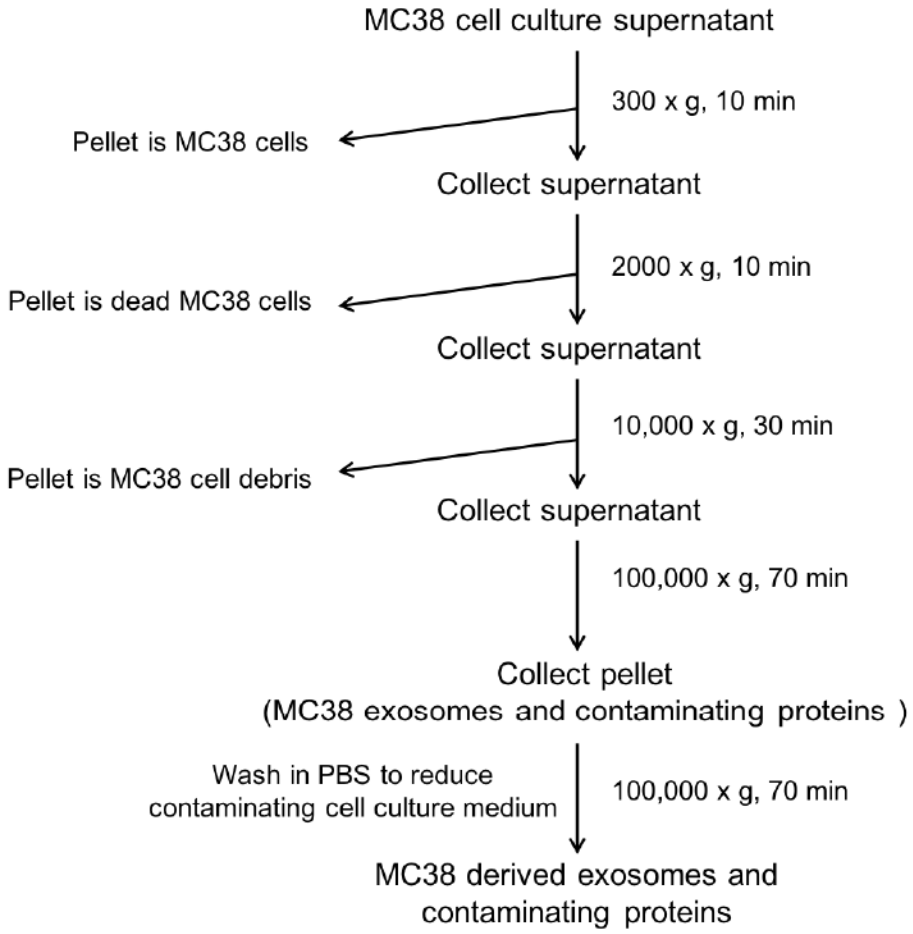


Figure 2-1. Exosome purification work flow. A series of centrifugations are done prior to exosome isolation to remove cells, dead cells and cellular debris. Figure author: Allison McNamara.

Analysis of Exosomes by Flow Cytometry

Exosomes fall into the nanometer size range making their analysis by conventional flow cytometry unreliable. To overcome this problem, we complexed exosomes to beads in the detectable size range of a conventional flow cytometer which is described in Théry et al (2006)[261]. Latex beads of 1μm and 4μm were tested to determine the appropriate size to use. 10μl of surfactant-free aldehyde-sulfate, 4% (w/v) 4μm latex beads (ThermoFisher Scientific) were incubated with 5 μg of exosomes isolated from mouse serum or derived from MC38 cells. PBS was added and the beads were incubated for 2 hr on a rotator at room temperature. Free binding sites on the latex beads were then saturated by the addition of 110 μl of 1 M glycine, gently mixed and incubated at room temperature for 30 min. The beads were centrifuged at 4000 rpm for 3 min at room temperature, followed by 3 washes in 1 ml PBS/0.5% BSA and resuspended in 500 μl of PBS/0.5% BSA. 10 μl of beads were then stained for flow cytometry in PBS/0.5% BSA for 30 min at 4°C, washed twice and acquired on the LSRFortessa.

Exosomes were also later analyzed on the Apogee Flow Systems micro flow cytometer suited for the analysis of individual particles in the nanometre size range. Exosomes were not complexed to beads and were suspended in 0.1 μm filtered PBS to eliminate contaminating particles when acquiring samples.

Analysis of phospholipid antibody specificity

A semi-quantitative bead-based flow cytometric assay was used to identify the specificity of anti-PS antibodies and detect endogenous anti-PS antibodies in tumor bearing mice. This method is described in Stewart MW, et al 1993[262]. It is comparable to a standardized ELISA and allows for the detection of various isotypes of PPL antibodies. 6 μm polystyrene beads were washed 4 times in absolute ethanol and centrifuged at 2000 x g for 2 min. The beads were then coated with phosphatidylserine, phosphatidylcholine or cholesterol in ethanol and sonicated to allow for dispersion of the beads and even coating and were then incubated on a rotator at 4°C for 24 hr. The lipid coated beads were then washed 3 times with 10 mM tris/140 mM NaCl pH 7.2 with sonication in between washes. The beads were then blocked in 10 volumes of 10% (v/v) FBS in

10 mM tris/140 mM NaCl pH 7.2 at 37°C for 30 min. The beads were then rapidly cooled in an ice bath. The beads were washed and 3.0 x 10^6 beads per sample were stained with serum from tumor bearing mice at a 1:100 dilution or with 1μg/ml of polyclonal or monoclonal anti-PS antibody as a control for 30 min on ice. The beads were centrifuged at 4500 x g for 5 min at room temperature and then stained with anti-mouse IgG for 30 min on ice in the dark. The beads were washed, resuspended in fluorescent activated cell sorting buffer (FACS) buffer (2% FBS and 1 mM EDTA) and acquired on the LSRFortessa.

Immune complex (IC) formation

Exosomes from the MC38 CRC cells that had a confirmed size range of 50-150 nm by NanoSight LM10 analysis were used to form IC. For in vitro experiments, either 1.0 x 10^6 DC or macrophages were stimulated with 1.0 x 10^8 exosomes whereas 5.0 x 10^5 DC were stimulated with 5.0 x 10^7 exosomes to assess in vivo T cell priming ability for a final ratio of 1:100 (cell:exosomes) in all experiments. In vivo tumor experiments used 10-50 μg of exosomes per dose. In both cases, exosomes were complexed with 100 μg/ml of monoclonal or polyclonal anti-mPS or anti-pPS antibody respectively in PBS for 1 hr at 37°C. For highly complexed exosomes, an additional 25 μg/ml of both anti-CD9 and anti-CD63 Abs for 1 hr at 37°C.

In vivo tumor model

Mice were subcutaneously injected with 1 x 10^6 MC38 parent or OVA expressing cells into either the left or right flank, respectively. Tumor growth was monitored until tumor volumes reached between 500 and 1500 mm^3. At this point, mice were intravenously or intratumorally injected with either PBS, anti-PS antibody (anti-PS Ab), 10 – 50 μg of exosomes, or 10 – 50 μg of exosomes complexed with either (anti-PS Ab) alone or along with anti-CD9 and anti-CD63 (3-6 mice per group). Exosomes injected into mice were derived from the MC38 OVA expressing cell line. The following day, tumor-bearing mice were intravenously injected with 2 x 10^6 CFSE-labeled OT-I $CD8^+$ T cells. T cells were prepared by harvesting the spleens and lymph nodes from C57BL/6 OT-I mice. The tissues were homogenized, passed through a 40 μm filter, washed and treated with ammonium-chloride-potassium (ACK) lysis buffer to lyse red blood

cells (RBC) . CD8 T cells were suspended in EasySep media (PBS, 2% FBS, 2 mM EDTA) and were isolated using the EasySep Mouse $CD8^+$ T cell Isolation Kit (StemCell Technologies). Isolated cells were washed and labeled with 1.25 μM CFSE in PBS at 1 x 10^7 cells/ml for 20 min at 37°C with regular mixing. After CFSE labelling the reaction was quenched with RPMI (10% FBS, 10 mM HEPES, 1% penicillin/streptomycin), the cells were washed and resuspended in PBS for injection of 2 x 10^6 cells per mouse intravenously by tail vein. Tumors and spleens were harvested from the mice 1-6 days post injection of OT-I CFSE labeled $CD8^+$ T cells.

Mice were euthanized by CO_2 asphyxiation. Blood, spleen and tumor were harvested in PBS. Spleens and tumors were chopped as finely as possible and fragments were transferred to 10ml of pre-warmed enzyme digestion cocktail (0.5 mg/ml collagenase IV and 10 μg/ml DNase I in complete RPMI 10% FBS, 1% penicillin-streptomycin and 1% HEPES) and were incubated shaking for 30 min at 37°C. Tissues fragments were vigorously dissociated by pipetting before filtering through a 100 μm strainer. The cells were centrifuged, resuspended and washed 2% FBS / in PBS. Splenocytes were treated with ACK lysis buffer to lyse RBC. Lymphocytes were further isolated from tumor tissue using density gradient centrifugation. Specifically, tumor or spleen cells were resuspended in 3ml of 40% Percoll in HBSS and overlaid on 80% Percoll. The cells were centrifuged with the break off at 2000 rpm for 30 min at room temperature. Epithelial cells were aspirated from the top layer and lymphocytes were isolated from the interface of the 40% and 80% percoll. Cells were washed in 2% FBS in PBS at 1500 rpm for 10 min at 4°C. Cells were filtered through a 40 μM strainer, centrifuged and resuspended in RPMI (10% FBS, 10 mM HEPES and 1% Penicillin/Streptomycin) for ex vivo phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation prior to staining for flow cytometry.

Ex vivo stimulation

Lymphocytes isolated from spleens and tumor tissue were stimulated with PMA (5 ng/ml), ionomycin (500 ng/ml) and monensin (2 μM) in RPMI (10% FBS, 10mM HEPES and 1% penicillin/streptomycin) for 4 hr at 37°C in a 24 well flat bottom plate. After stimulation, the cells were centrifuged and subsequently stained for flow cytometry.

Antibodies and Flow Cytometry

*S*taining was performed in round-bottom 96-well plates. Prior to antibody (Ab) labeling, cells were incubated with zombie aqua viability dye in PBS for 30 min in the dark. Cells were washed, resuspended in FACS buffer (PBS, 1% FCS, 0.02% sodium azide, 1 mM EDTA [pH 7.2]) and incubated with anti-Fc receptor block for 10 min at 4˚C. Cells were then stained with surface Abs in FACS buffer for 15 min at 4°C in the dark. Intracellular staining for IFNγ was performed using the eBioscience Foxp3 Staining Buffer Set. After staining cells were acquired on a BD LSRFortessa and analyzed with FlowJo software (TreeStar). Antibodies used for flow staining can be found in Table 2.

Generation of macrophages and DCs

Bone marrow was harvested from wildtype mice, passed through a 40 μm filter and cultured with supernatants from B16 that secrete GM-CSF (BMDC) or L929 cells that secrete G-CSF (BMDM) in RPMI (5% FBS, 10 mM HEPES and 1% Penicillin/Streptomycin, 50 μM 2-mercaptoethanol) for up to 7 days changing the medium every 2 days. The cells were used in experiments immediately or stored in liquid nitrogen.

Generation of the F(ab')$_2$ Ab

The CD16/CD32 F(ab')$_2$ Fc receptor antibodies were generated from the CD16/CD32 Ab (BDBiosciences) with the Pierce F(ab')$_2$ Preparation Kit (ThermoFisher Scientific). Enzymatic digestion of the Fc region was confirmed by flow cytometry with anti-mouse Fc IgG-FITC (BDBiosciences) showing lack of staining.

Stripping of endogenous IgG from exosomes

Exosomes isolated from the serum of wild type non-tumor bearing mice were quantified by the BCA assay. In order to remove endogenously bound antibodies, 10 μg of exosomes were added to protein G sepharose (Sigma). The tubes were incubated on a rotator for 2 hr at room temperature to allow coating of the sepharose by exosomes. The sepharose resin and bound

exosomes were centrifuged at 4500 x g, resuspended in acidic stripping buffer (0.1 M citric acid pH 2.75), incubated for 15 min and centrifuged. The supernatant was removed and the acid strip of the sepharose-bound exosomes was repeated. pH of the supernatant fractions containing stripped exosomes were neutralized. IgG stripping efficiency was evaluated by the Apogee Flow Systems micro flow cytometer before using them to stimulate DCs.

Activation of DC, macrophage, and T cells by IC

Fc receptors on DC and macrophages were blocked with 0.25 μg/ml anti-CD16/CD32 IgG or 6.0 μg/ml CD16/CD32 $F(ab')_2$ for 30 min at 37°C in RPMI. The cells were washed and subsequently stimulated with MC38-OVA exosomes or exosome ICs for 15, 24 and 48 hrs after which the cells were lysed for isolation of RNA and the supernatant was collected for analysis of cytokines IL-1β, IL-6, IL-12, and TNFα with the ELISA Ready-SET-Go kit (eBiosciences and BD Biosciences IC or exosomes for 5, 10, 30 and 60 min at 37°C after blocking Fc receptors and the cells were subsequently lysed for Western blotting.

In some experiments, DC were first loaded with 1μg/ml of soluble OVA for 30 min at 37°C, before being stimulated with IC or exosomes derived from the MC38 OVA cell line. The cells were washed and co-cultured with CFSE-labeled OT-I $CD8^+$ isolated T cells and 1 μg/ml of IL-2. At 24 and 48 hr, the supernatant was collected for analysis of IFNγ with the ELISA Ready-SET-Go kit (eBiosciences).

In additional experiments, DC were stimulated with serum derived exosomes complexed with anti-PS antibody or not. Polyclonal CFSE-labeled $CD8^+$ T cells isolated from the spleen were added and cocultured for 48 hr. Proliferation and surface expression of activation markers were examined by flow cytometry.

RNA isolation and reverse transcription

Cells were lysed with Trizol, incubated first for 5 min at room temperature and then at -80°C for 1 hr. The samples were thawed and chloroform was added. Samples were mixed and incubated

for 3 min followed by centrifugation at 12,000 x g for 15 min at 4°C. The aqueous phase was removed and Glycoblue followed by isopropanol were added and the extract was incubated at -80°C for 1 hr. The extract was thawed, centrifuged at 12,000 x g for 20 min at 4°C. The supernatant was removed and the RNA was washed with 75% cold ethanol, centrifuged at 7,400 x g for 15 min at 4°C and the supernatant was removed and the RNA was allowed to dry for 1 hr. The RNA was resuspended in nuclease free water for 3 min, quantified using the NanoDrop spectrophotometer and stored at -80°C. RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit without RNase inhibitor (Life Technologies). qPCR was performed with the EvaGreen 2X qPCR MasterMixROX (ABM) and samples were run on the Quant Studio real time qPCR machine. Primers for the genes examined in this study are found in Table 1.

Western Blotting

Protein extracts were prepared from stimulated macrophage and DC with the addition of lysis buffer consisting of (50 mM Tris-HCl, 150 mM NaCl, 50 mM sodium pyrophosphate, 1 mM EDTA, 0.5% NP40, 1% Triton X-100, 1:100 protease inhibitor and 1 mM sodium orthovanadate). Lysates were rotated for 30 min at 4°C, then centrifuged at 14,000 rpm for 15 min and supernatants were aliquoted and stored at -80°C. The protein concentration of the cell lysates was determined using a BCA assay. Equal amounts of protein from each treatment were separated by SDS-PAGE on either 10 or 15% polyacrylamide gels. Protein separation was monitored with the PINK prestained protein ladder and Blu Elf prestained protein ladder. Proteins were electrophoretically transferred to 0.22 μm nitrocellulose membranes for 70 min. Membranes were washed in TBST (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% v/v Tween-20) and subsequently blocked for 30 min at room temperate in 5% w/v powdered milk (Carnation) in TBST. Membranes were washed in TBST after blocking and incubated overnight with primary antibody diluted 1:1000. Membranes were washed and incubated with HRP conjugated secondary antibody at 1:2000 in 5% milk in TBST. Membranes were developed using chemiluminescence (ECL Prime) detection kit (Amersham Biosciences) and exposed to film (Kodak). The antibodies used for Western blotting can be found in Table 2.

Assessing in vivo priming

DC were loaded with exosomes derived from the MC38 OVA cell line in the form of an IC with monoclonal anti-PS Ab or not and incubated for 1 hr at 37°C. The 5 x 10^5 DC were washed, suspended in PBS and injected 4 mice in two separate experiments into opposite hind footpads of wildtype mice adoptively transferred with CFSE-labeled OT-I $CD8^+$ T cells that had been isolated with the EasySep Mouse $CD8^+$ T cell Isolation Kit (StemCell Technologies) as described for the tumor model above. Draining left and right popliteal lymph nodes were harvested at 72 hrs, homogenized and stimulated ex vivo with PMA/ionomycin prior to staining for flow cytometry.

Fatty acid conjugation

Arachidonic acid (AA) and docosahexaenoic acid (DHA) (Matreya) were conjugated to delipidated bovine serum albumin (BSA) (Sigma) at a ratio of 2:1 DHA:BSA prior to addition to the cell culture medium. Lipids were dissolved in 100% ethanol and added to BSA/PBS in 50 μl increments dropwise every 30 min under the flow of nitrogen gas to allow for proper conjugation so that the final concentration of BSA conjugated DHA and AA was 4 mM. The lipid-BSA solution was passed through a 0.22 μm filter and stored in glass vials under nitrogen gas at -80°C.

Growth analysis of lipid stimulated cells

MC38 cells were seeded and grown in high glucose DMEM. After 24 hr the culture medium was replaced with medium containing 25, 50, 75, 100 and 150 μM of either AA or DHA and incubated for 72 hr, during which the medium was not changed. After 72 hr, the cells were stimulated with 50 μg/L of EGF (Sigma-Aldrich) at 37°C for 15 min, then lysed for analysis by Western blot. To ensure fatty acid treatment was not toxic to MC38 cells, we seeded cells and at 24 hrs, replaced the medium with 25, 50, 75, 100, 150, 250 and 300 μM concentrations of AA or DHA in DMEM, allowed the cells to growth for 12 hr and conducted proliferation experiments using the thiazolyl blue tetrazolium bromide (MTT) assay.

Fatty acid Composition Analysis

MC38 cells were treated with 30, 60 and 100 μM of AA or DHA and lipid composition of the membrane was examined to determine the concentration where maximal incorporation of AA or DHA was observed for use in future experiments. Cells were seeded in flasks and at 24 hrs, the medium containing AA or DHA was added. At 72 hours, cell culture supernatant was collected for the isolation of exosomes. Lipids from whole cells and isolated exosomes were extracted using a modified Folch procedure as follows: 0.8 ml of methanol, 2.0 ml of chloroform/methanol (1:1, v/v), 2.7 ml of chloroform and 2.5 ml of chloroform/methanol (2:1, v/v) were added in succession, followed by the addition of 1.6 ml of 0.1 mM KCl. The samples were vortexed vigorously and allowed to stand overnight at 4°C. The phospholipid layer was collected, and the tubes were dried under nitrogen on the heat block. Fatty acid methyl esters were prepared by methylation with the addition of 1.5 ml of BF_3 and 2 ml of hexane to the samples which were vortexed vigorously and heated to 110°C for 1 hr. Samples were allowed to cool at room temperature before 1 ml of ddH_2O was added and the samples were allowed to stand overnight at 4°C. The hexane layer was then removed and transferred to a GC vial. The vials were then dried under nitrogen, rinsed with hexane and subsequently dried under nitrogen. The vials were stored at -35°C until they were acquired on the GC machine.

Statistical Analysis

Mean, SD and p values were determined using Prism software (GraphPad). The p values were calculated using a two-tailed unpaired t test with a 95% confidence interval.

CHAPTER 3: RESULTS

Anti-PS antibodies are present in tumor bearing mice

Anti-phospholipid antibodies could play a role in IC formation and lead to inflammation. We thus decided to investigate whether these antibodies were elevated in mice bearing subcutaneous MC38 colorectal adenocarcinoma tumors with a knockout in one of the following genes *Mlh1*, *Pole*, *Msh3,* or *Kras.* These genes were chosen because loss of either *Mlh1*, *Pole* and *Msh3* in CRC is associated with a particularly immunogenic phenotype that we reasoned would manifest in particularly high anti-PS levels[27,33,69]. For detection of elevated serum anti-PPL antibodies, we used a bead-based assay in which we coated beads with PS and then incubated these with the serum sample and then a fluorescently-conjugated secondary antibody to detect antibodies of the IgG isotype[262]. We validated the specificity of the test using known anti-PS antibody as our positive control. This antibody demonstrated specificity to PS and not PC or cholesterol (**Fig. 3-1A**). While, anti-PS antibodies were detected in all tumor bearing mice, levels were particularly high in two mice with either *Mlh1* or *Pole* deletion. However, there was variability in the other mice also bearing the same gene knockout CRC that did not display elevated antibodies to the same extent (**Fig. 3-1B**). Detection of anti-PS antibodies in mice bearing MC38 tumors provided preliminary evidence that the presence of growing CRC could lead to increases in serum anti-PS antibody levels.

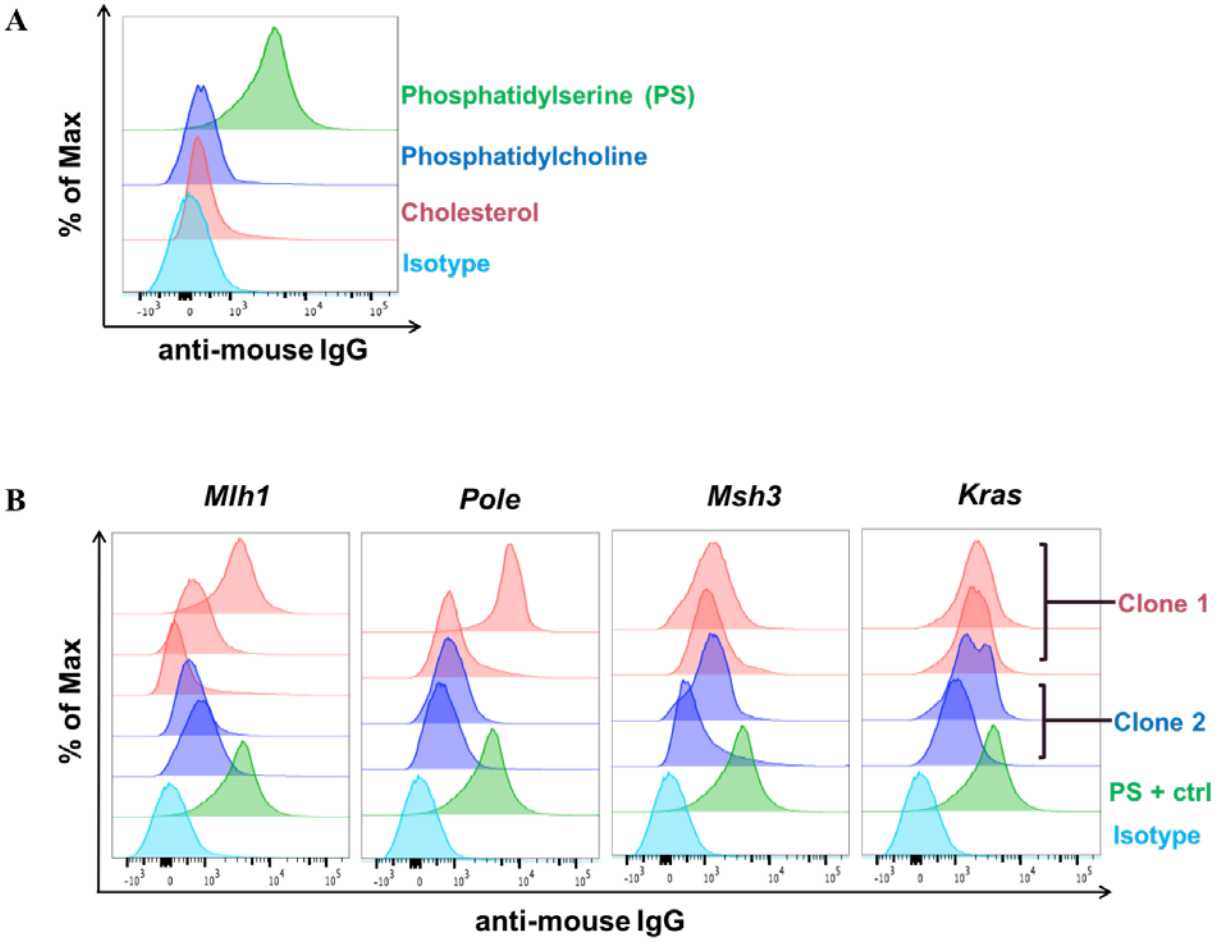


Figure 3-1. Mice bearing hypermutable tumors show evidence of increased anti-PS antibodies. (A) Polystyrene beads were coated with PS, phosphatidylcholine, or cholesterol. The lipid coated beads were subsequently stained with anti-mPS followed by fluorescently labelled anti-mouse IgG and analyzed by flow cytometry to examine the specificity of anti-mPS for the phospholipid PS. **(B)** PS coated polystyrene beads were stained with serum from mice bearing mutations in *Mlh1, Pole, Msh3* or *Kras* – two clones for each tumor cell line each grown in duplicate or triplicate (*Mlh1* clone 1) in separate mice. The beads were subsequently stained with anti-mouse IgG and analyzed by flow cytometry in order to examine the level of anti-PS antibody present in the serum of these tumor bearing mice. The experiment in panel **(A)** and **(B)** were repeated twice and once respectively.

Isolated exosomes are identified by common exosome markers

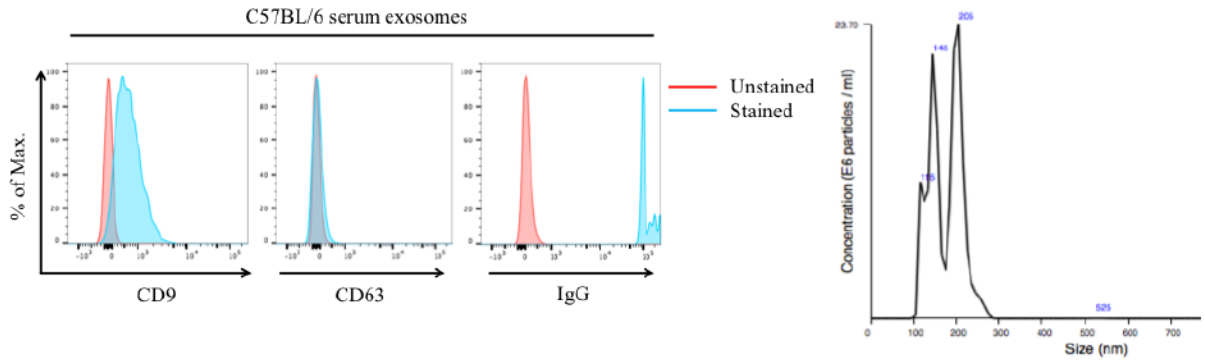
Given the observable, yet limited evidence of elevated anti-PS antibodies in mice bearing the hypermutable *Mlh1* and *Pole* tumors, we decided to investigate the possibility that such endogenous anti-PS antibodies could form IC with exosomes released from colorectal cells. This required developing and validating stringent protocols for isolating exosomes since these can easily be mistaken for other forms of EVs. We selected ultracentrifugation as our method of choice for purifying exosomes since this is currently the gold standard method used by leaders in the field[261,263]. In order to validate our methods, we relied on the well characterized size properties of exosomes as well as on their expression of known exosome markers[242]. Following purification, we evaluated the size of the isolated exosomes using a NanoSight instrument, which uses Nanoparticle Tracking Analysis based on the principles of Brownian motion and light scattering to measure size and quantity of particles[264]. Exosomes isolated from MC38 cell culture medium fell into the expected size range for exosomes and had a mean of 145.2 nm (**Fig. 3-2B**). This is consistent with the accepted size range for exosomes cited in many other studies and is in line with current analysis methods in the field[265].

In order to evaluate whether the isolated exosomes expressed known exosome markers on their surface, we used flow cytometry based assays on either a standard flow cytometer or microparticle specific instrument. The exosomes we isolated from serum expressed the common exosome marker CD9 but not another common marker, CD63, and were highly bound by endogenous IgG (**Fig. 3-2A**). MC38 cell-derived exosomes, in contrast, were positive for both CD9 and CD63. In addition, exosomes from these cells contained HSP70, which is commonly incorporated into exosomes during their biogenesis (**Fig. 3-2B and 3-2C**)[206,261]. We also confirmed that the purified exosomes expressed PS on their surface using an AV binding assay. Since exosomes are produced by most cells in the body, identifying which serum exosomes arise from the colon requires a marker for this type of cell. We determined that cytokeratin-20, a colon epithelium specific cytokeratin, was enriched in MC38 exosomes as well as exosomes from the human colorectal HCT116 p53 wildtype cell line (**Fig. 3-2D**) and could thus be used as a marker for exosomes originating from these cells in future experiments[266]. In addition, the presence of

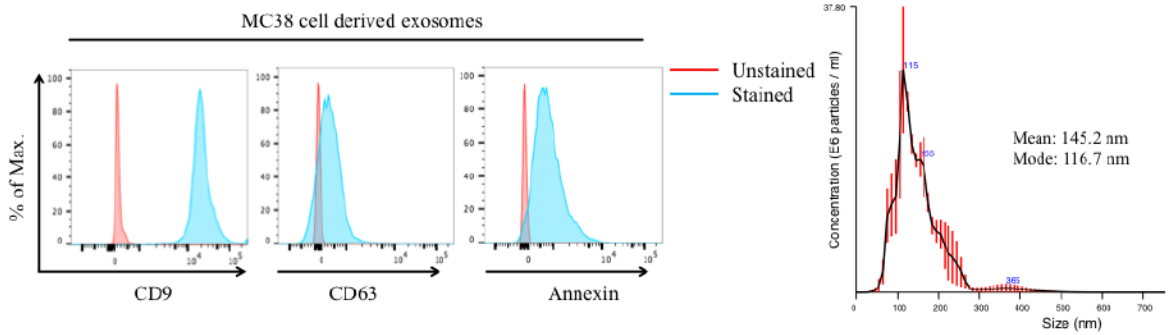
cytokeratin-20 in these exosomes may suggest that it could be feasible to isolate human serum exosomes and identify exosomes that are derived from the colon epithelium for analysis that could facilitate future use of exosomes as biomarkers.

One of our main interests in studying exosomes is the possibility that they serve as TAA delivery vehicles that could contribute to priming anti-tumor immunity. As a model for this, we are using an MC38 CRC cell line that has been stably transfected with the ovalbumin (OVA) gene, which acts as a model tumor antigen in our system. It was thus necessary to confirm that this tumor antigen could be incorporated into exosomes. We used Western blotting to confirm that OVA was incorporated in exosomes derived from the MC38 OVA cell line that was used in the majority of subsequent studies (**Fig. 3-2E**). We also tested whether or not cellular contaminants that can accumulate during the isolation of exosomes were present but found this to be minimal, as demonstrated by the absence of calnexin in the exosome lysate[261]. These data suggest that the majority of microparticles isolated from serum or cell culture medium by our methods were truly exosomes and could be identified by common exosome markers.

A


C57BL/6 serum exosomes
% of Max.
CD9
CD63
IgG
Unstained
Stained
Concentration (E6 particles / ml)
Size (nm)

B


MC38 cell derived exosomes
% of Max.
CD9
CD63
Annexin
Unstained
Stained
Concentration (E6 particles / ml)
Size (nm)
Mean: 145.2 nm
Mode: 116.7 nm

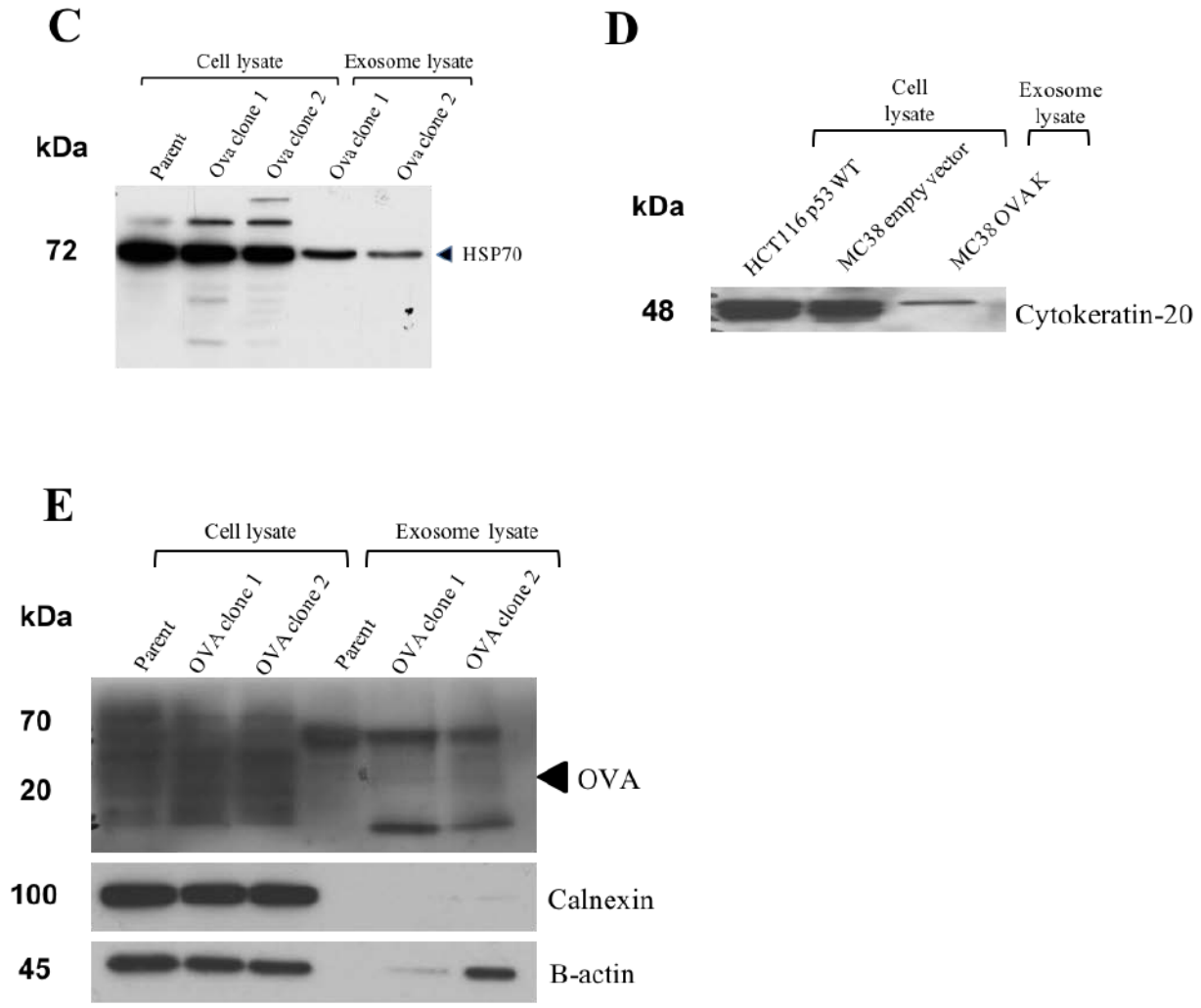


Figure 3-2. Validation of the exosome purification protocol. (A) C57BL/6 mouse serum and **(B)** MC38 cell culture supernatant was collected and exosomes were isolated, complexed to beads, stained and analyzed by conventional flow cytometry for common exosome markers CD9, CD63 and the presence of bound endogenous IgG or binding of AV. Nanoparticle Tracking Analysis was used to identify the size range of exosomes isolated (right panel). **(C)** Exosomes were isolated from OVA transfected MC38 cells or **(D)** HCT116 p53 wildtype human colorectal cancer cells. Exosomes were lysed and analyzed by Western blot for the presence of the common exosome marker HSP70 and colon specific cytokeratin (cytokeratin-20). **(E)** The presence of OVA in MC38 OVA isolated exosomes was confirmed by Western blot. Contamination of the isolated exosomes by cellular material was assessed by presence of calnexin by Western blot.

Experiments in panels **(A)** and **(B)** were repeated three times and experiments in panels **(C)** and **(D and E)** were repeated twice and once respectively.

Endogenous IgG bound serum exosomes are not sufficient to elicit inflammatory gene expression in DC

In humans, anti-PS antibodies may be present at low, non-pathogenic levels that do not result in clinical autoimmune manifestation or thrombolytic events[241]. It is not known whether these antibodies function in binding to apoptotic bodies, thereby facilitating phagocytic clearance, or if they also have an additional role in clearance of released exosomes from various cell types. Our finding that endogenous exosomes isolated from the serum of mice are already highly bound by IgG suggest that this could be a possibility **(Fig. 3-2A)**. We aimed to remove exosome bound IgG using an acid strip followed by protein G pulldown of the stripped antibodies to characterize them for PS specificity. Although we were able to remove some bound IgG from the serum-isolated exosomes, stripping efficiency was poor **(Fig. 3-3A)** and the acidity of the acid stripping buffer may have led to denaturation of the immunoglobulin, interfering with epitope characterization. As an alternative, we decided to see if all PS binding sites on the exosomes were saturated by attempting to complex the serum exosomes with additional polyclonal anti-PS antibody. This yielded highly complexed exosomes that were more highly bound than serum exosomes alone and indicated that if endogenous anti-PS antibodies were binding to exosomes, it was not in sufficient quantity to saturate the available binding sites **(Fig. 3-3A)**.

We next aimed to investigate the immunogenic potential of anti-PS-complexed exosomes. In early studies, it was thought that only $CD8a^+$ DCs could mediate cross-presentation of antigen[201,204]. However, it has since been demonstrated in numerous studies that the opsonisation of soluble antigen forming an IC permits poor cross-presenter $CD8a^-$ DCs to become potent cross-presenting cells[204]. This means that anti-PS-bound exosomes could allow for targeting of multiple DC subsets to induce priming of anti-tumor $CD8^+$ T cells. This also allowed us to use bone marrow derived GM-CSF matured $CD8a^-$ DC throughout our experiments to study the potency of the exosome IC in initiating an inflammatory response. Since additional anti-PS complexing led to more highly IgG-bound exosomes, we investigated how this changed the immune stimulatory capacity of exosomes in DC by stimulating DC with serum exosomes for 15 hr and then examining proinflammatory gene expression by qRT-PCR. Serum exosomes alone led to little to no upregulation of COX-2 or the proinflammatory genes IL-1β, IL-6, IL-12 and

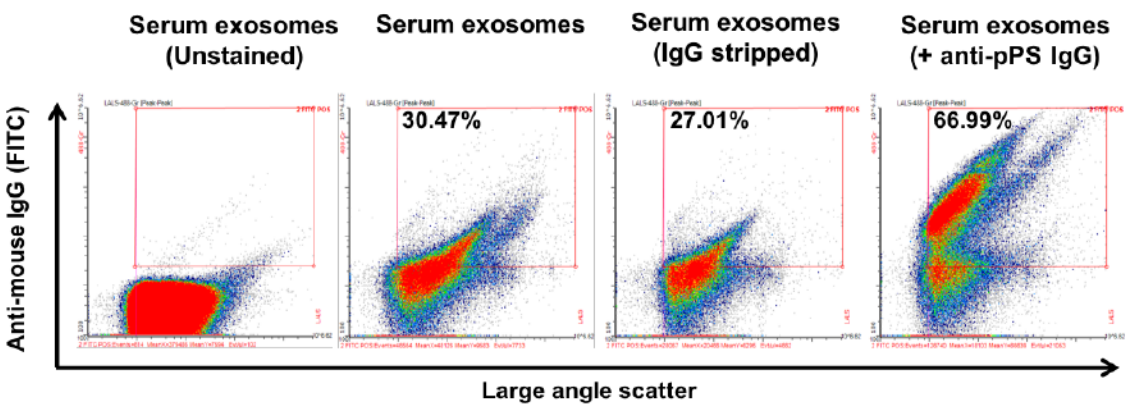
TNFα **(Fig. 3-3B)**. In contrast, the complexing of anti-PS to serum exosomes greatly increased expression of COX-2 and these proinflammatory cytokines, and significantly increased IL-6 expression ($p < 0.05$).

Prior to stimulating DC with serum exosomes, we also decided to block CD16 (FcγRIII) and CD32 (FcγRII) receptors with anti-CD16/CD32 IgG prior to exosome addition. Fc receptors can mediate IC internalization, and by blocking these receptors we aimed for the IC to be taken up by an alternative mechanism other than Fc receptor mediated uptake. Blockade of these receptors would allow us to examine the stimulatory capacity of IC internalized by the Fc receptor mediated route. We expected uptake of IgG bound exosomes by alternative uptake routes to be less efficient and to decrease proinflammatory gene expression. However, we observed increased proinflammatory gene expression compared to serum exosomes alone or IgG stripped exosomes when the DC were first blocked with anti-CD16/CD32. Even though IgG stripping efficacy was poor, we expected relatively similar proinflammatory gene expression in comparison to serum exosomes alone. Since IgG binding to Fc receptors can be stimulatory in and of itself by crosslinking the receptors, we tested whether the blocking of CD16 and CD32 Fc receptors led to increased proinflammatory gene expression by DCs in the absence of serum exosomes. We found that anti-CD16/CD32 IgG antibodies downregulated IL-1β, IL-6, TNFα and COX-2 expression compared to the absence of blocking antibodies **(Fig. 3-3C)**. This suggests that the mechanism by which the blocking antibodies increase proinflammatory gene expression in our original assay is dependent on the presence of exosomes. We attempted to resolve this by generating anti-CD16 and CD32 $F(ab')_2$ fragments of our blocking antibodies since these would lack the Fc region that binds to Fc receptors but still have specificity to the Fc receptors to block their ligand binding. We compared the immunostimulatory capacity of the anti-CD16 and CD32 IgG antibody and $F(ab')_2$ fragment. IC along with the anti-CD16 and anti-CD32 IgG antibody had a synergistic effect and led to sustained pAKT and pERK signalling in DC 24 hr post stimulation **(Fig. 3-3D)**. In contrast, the expression of these signalling mediators was decreased in the presence of IC and anti-CD16/CD32 $F(ab')_2$ fragments. Despite proteolytic cleavage of the Fc region of the anti-CD16/CD32 IgG, the $F(ab')_2$ fragments remained immunostimulatory in the presence of IC and we have yet to find the exact explanation for this observation.

In addition to cytokine production, the activation of DC can be ascertained by expression of certain activation markers that are upregulated upon DC stimulation. We thus also examined surface marker expression of DC stimulated with serum exosomes and found upregulated expression of H-2Kb in DCs stimulated with anti-PS complexed serum exosomes **(Fig. 3-3E)**. We saw no change in I-Ab or CD86 expression. However, upregulated expression of H-2Kb, which is an MHC I molecule that contributes to antigen presentation, suggested that anti-PS complexed exosomes enhanced the cross-presentation ability of these DC. We tested whether this could increase presentation of self-antigens, which would facilitate anti-tumor immunity, by first stimulating DC with serum exosomes complexed or not with anti-PS IgG and then co-culturing these DC with polyclonal $CD8^+$ T cells. This is a situation similar to that which would be found early in tumor development in the absence of large amounts of TAA. The anti-PS-complexed exosomes, however, did not result in greater T cell proliferation and instead seemed to decrease T cell proliferation compared to the uncomplexed exosomes **(Fig. 3-3F)**. This suggests that large amounts of a specific TAA in exosomes may be a prerequisite to their ability to stimulate productive anti-tumor immunity when bound by anti-PS IgG. This finding also indicates that endogenously occurring highly IgG-bound exosomes may contribute to general immune homeostasis in the absence of a specific pathogenic antigen.

Collectively, this set of experiments suggests that not all, if any, PS sites on exosomes are saturated with immunoglobulin under homeostatic conditions and that additional IgG complexing of exosomes is required to induce upregulation of proinflammatory genes and H-2Kb on DC. Despite enhanced H-2Kb expression on DC stimulated with exosomes carrying self-antigen, the lack of T cell proliferation observed suggests mechanisms of tolerance may play a role in preventing an immune response to exosomes that become highly bound by Ig early in tumor development.

A


Serum exosomes (Unstained)
Serum exosomes
Serum exosomes (IgG stripped)
Serum exosomes (+ anti-pPS IgG)
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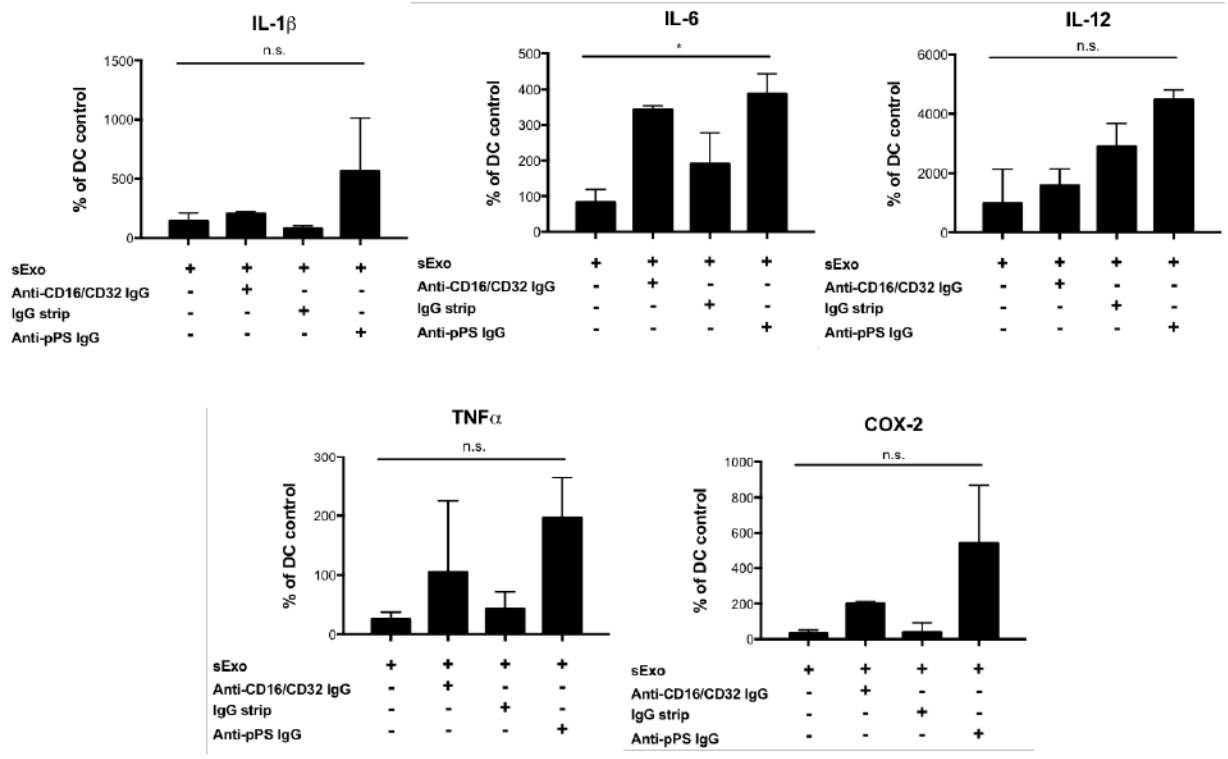
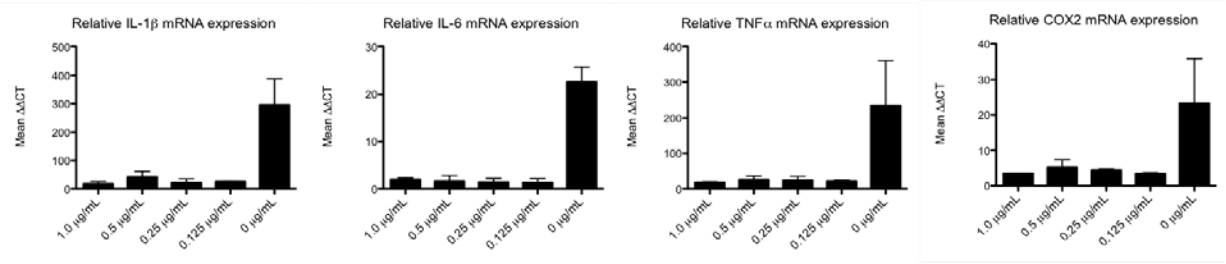
27.01%
66.99%
Anti-mouse IgG (FITC)
Large angle scatter

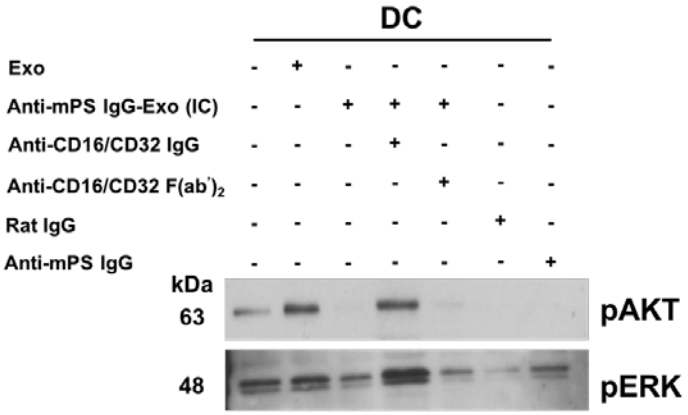
B


IL-1β
IL-6
IL-12
TNFα
COX-2
n.s.
*
% of DC control
sExo
Anti-CD16/CD32 IgG
IgG strip
Anti-pPS IgG

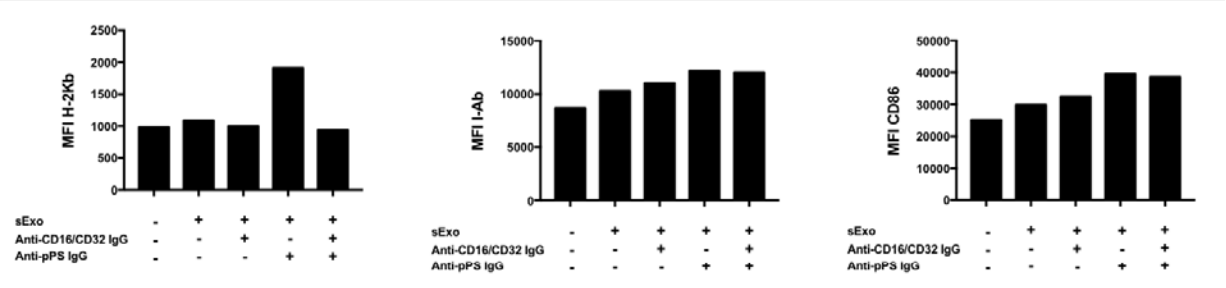
C


Relative IL-1β mRNA expression
Relative IL-6 mRNA expression
Relative TNFα mRNA expression
Relative COX2 mRNA expression
Mean ΔΔCT
1.0 μg/mL
0.5 μg/mL
0.25 μg/mL
0.125 μg/mL
0 μg/mL

D


DC
Exo
Anti-mPS IgG-Exo (IC)
Anti-CD16/CD32 IgG
Anti-CD16/CD32 F(ab')2
Rat IgG
Anti-mPS IgG
kDa
63
48
pAKT
pERK

E


MFI H-2Kb
MFI I-Ab
MFI CD86
sExo
Anti-CD16/CD32 IgG
Anti-pPS IgG

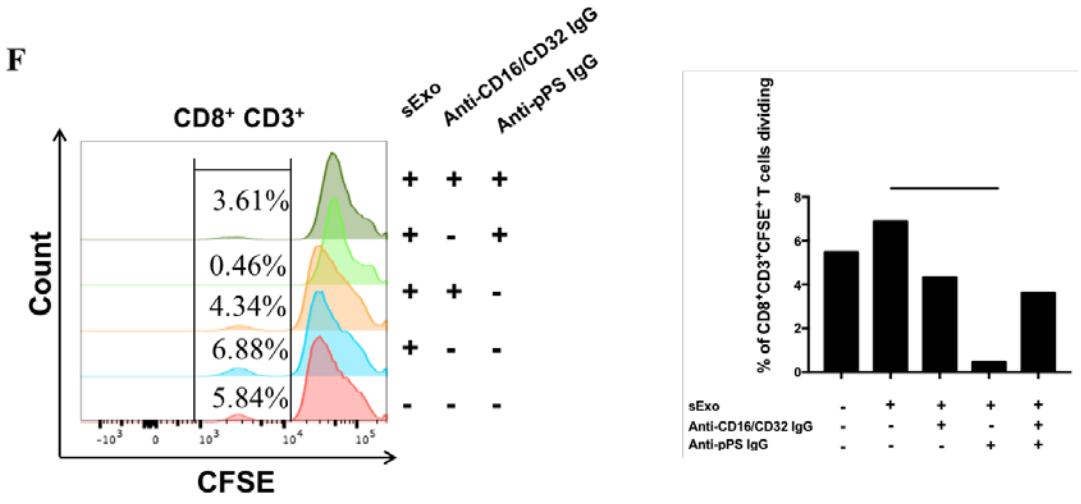


Figure 3-3. IgG complexed serum exosomes elicit inflammatory gene expression in DC. (A) Non-tumor bearing C57BL/6 mouse serum derived exosomes (sExo) were stripped of endogenous bound IgG or complexed with additional anti-pPS IgG. Bound IgG was detected with a secondary fluorescently labelled anti-mouse IgG antibody. PS sites on serum derived exosomes were not saturated with IgG, allowing for the formation of highly IC exosomes upon addition of anti-pPS. **(B)** Fc receptors on DC were blocked with anti-CD16/CD32 antibodies. The DC were then stimulated with serum derived exosomes, IgG stripped sExo or sExo complexed with additional anti-pPS antibody for 15 hr. mRNA expression of inflammatory genes was evaluated by qRT-PCR and analyzed using the ΔΔCT method and compared to unstimulated DC. **(C)** DC were stimulated with various concentrations of anti-CD16/CD32 IgG in the absence of exosome for 15 hr and proinflammatory gene expression was evaluated by qRT-PCR using the ΔΔCT method. **(D)** DC were blocked with either anti-CD16/CD32 IgG, anti-CD16/CD32 $F(ab')_2$ or rat IgG where indicated for 30 min prior to stimulation with exosomes or anti-mPS IgG exosome IC. Samples were stimulated for 24 hrs, lysed and analyzed by Western blot for downstream signaling mediators. **(E)** DC were stimulated with sExo complexed with additional anti-PS antibody or not. At day 3 post-stimulation, DC and **(F)** co-cultured polyclonal T cells were stained and analyzed by flow cytometry for surface marker expression or dilution of CFSE respectively. DC surface marker expression is presented as the mean fluorescent intensity (MFI). Each experiment was performed once, except for panel **(B)** which was repeated three

times. Data is depicted as mean ± SD; * $p < 0.05$, n.s. not significant. Asterisks over data represent statistical significance, as determined by the *t* test.

CRC exosomes complexed with anti-PS IgG mediate proinflammatory gene expression in DC

Since serum derived exosomes complexed with additional anti-PS IgG were capable of eliciting proinflammatory gene expression, we next examined the ability of MC38 CRC cell derived exosomes complexed with anti-PS antibodies to mediate proinflammatory signalling in DC. We previously observed that anti-CD16/CD32 IgG blocking antibodies alone increased proinflammatory gene expression in the presence of exosomes. We therefore decided to use the anti-CD16/CD32 F(ab')2 generated above for blocking studies to minimize the chance of non-specific results from receptor crosslinking by the blocking antibody.

The outcome of Fc receptor mediated uptake of IC is guided by the subclass of IgG bound to the antigen[193–195]. IgG subclasses have varying affinity for Fcγ receptors and can influence downstream signalling events[193–195]. In general, polyclonal compared to monoclonal antibody responses are more stimulatory since multiple different Fcγ receptor types can be engaged. We tested the inflammatory response of DC stimulated with MC38 derived exosomes complexed with either monoclonal (unknown IgG subclass) or polyclonal anti-PS antibodies (anti-mPS or anti-pPS IgG) for 15 hr and analyzed inflammatory gene expression by qRT-PCR. Overall, exosomes complexed with anti-mPS antibody greatly increased COX-2 and significantly increased IL-12 mRNA gene expression ($p < 0.05$) compared to exosomes alone. In contrast, exosomes complexed with anti-pPS antibody only resulted in a minimal increase in COX-2 and IL-12 mRNA gene expression **(Fig. 3-4A)**. When exosomes were complexed with either anti-CD9 or anti-CD63 antibodies of the IgG2a subclass which has the affinity for FcγRI-IV, there was a minimal increase in COX-2 and IL-12 mRNA gene expression in both cases. This is the case despite the fact that MC38 exosomes display greater surface expression of CD9 that could lead to more highly complexed exosomes with anti-CD9 IgG compared to either CD63 and PS, which were expressed at more moderate levels **(Fig. 3-2B and Fig. 3-4A)**. However, when exosomes were complexed with anti-mPS IgG in addition to anti-CD9 or -CD63, we observed an additional increase in COX-2, but not IL-12 expression in comparison to exosomes complexed with only anti-mPS. Interestingly, this additive effect was not observed with the polyclonal antibody. These experiments provide preliminary evidence to suggest that exosomes alone may

not be sufficient to initiate gene expression required for DC maturation, activation and skewing of the immune response towards a Th1 cytokine profile. This may explain the inability of CRC exosomes to efficiently stimulate anti-tumor immunity in patients but suggests that IgG complexing of these exosomes could significantly enhance immune-mediated tumor detection.

Efficient cross presentation of TAA to $CD8^+$ T cells is highly dependent on the activation and maturation of the DC[201,204,205,267]. Cross-presentation of antigen in the form of an IC is highly regulated by the ratio of activating and inhibitory Fcγ receptors on DC[201]. This, in turn, is highly influenced by cytokines present in the tumor microenvironment[193,194]. IFNγ is a cytokine that can be present in an inflammatory tumor microenvironment and has been shown to have opposing functions on Fcγ receptor expression and can either up- or downregulate activating Fc receptors on DC[89,193,194]. We investigated the effect of IFNγ on Fcγ receptor expression in DC and macrophages. While expression of Fcγ receptors on DC was lower compared to macrophages, IFNγ decreased mRNA expression of the inhibitory receptor FcγRIIB on DC and significantly in macrophages ($p < 0.01$). IFNγ also increased the expression of activating receptors FcγRIII and FcγRIV ($p < 0.001$) on DC, however the expression of these receptors was still lower compared to macrophages **(Fig. 3-4B)**.

IFNγ decreased the expression of FcγRIIB in DC and we investigated whether this would polarize DC to a type-1 phenotype. Thereby enhancing DC to upregulate proinflammatory Th1 related genes in response to anti-pPS-complexed MC38 exosomes, which had limited stimulatory capacity in non-polarized DCs. Interestingly, exosome IC significantly downregulated COX-2 mRNA gene expression ($p < 0.05$) and the proinflammatory genes IL-1β, IL-6, IL-12 and TNFα in IFNγ-primed DC compared to non-polarized DC **(Fig. 3-4C)**. Since the polarization of these DC with IFNγ did not enhance inflammatory gene expression induced by exosomes complexed with the polyclonal anti-pPS antibody, we continued our experiments with the monoclonal anti-mPS antibody that had greater stimulatory capacity in DC despite being less representative of an endogenous immune response.

Exosome IC lead to differential signalling in DC and macrophages

DC are not the only antigen presenting cell type that can be present in the tumor microenvironment[85]. Fcγ receptor-expressing macrophages may also be present and can contribute either tumor promoting or anti-tumor inflammation, depending on the cytokine milieu[85,89,193,194]. Crosslinking of Fcγ receptors on macrophages has previously been shown to induce inflammatory gene expression[191,192]. We therefore compared the proinflammatory gene expression in DC and macrophages in response to stimulation with exosomes complexed with anti-mPS IgG. Proinflammatory gene expression was modulated by exosomes and exosome IC to a greater extent in DC than macrophages **(Fig. 3-4D)**. Inconsistent with our previous observation, we observed upregulation of IL-12 by exosomes alone which was significantly downregulated by exosome IC in DC ($p < 0.01$).

Although the isolation method of cell culture derived exosomes remained consistent, quantification of final exosome yields was an estimation and therefore there was some variability in the number of exosomes used to stimulate DC and macrophages in different experiments. This may explain the change in gene expression pattern that we observed, as might our switch to monoclonal rather than polyclonal anti-PS antibodies, which could lead to differential downstream Fc receptor signaling. However, we continued to observe increased IFN-β1, IL-6 and TNFα and IL-1β ($p < 0.05$) expression in DC stimulated with exosome IC, similar to our previous observation **(Fig. 3-3B)**. In contrast to DC, gene expression following stimulation by exosome IC was largely unchanged in macrophages except for a similar downregulation of IL-12 expression. Unlike DC, the proinflammatory genes IFN-β was significantly downregulated ($p < 0.5$) and a decrease in IL-1β was also observed response to IC in macrophages **(Fig. 3-4D)**. Despite observing upregulated proinflammatory gene expression in DCs and macrophages stimulated with MC38 exosome IC, no difference in secretion of these cytokines at 24 or 48 hr post stimulation was observed except for a significant release ($p < 0.01$) of IL-6 by DC at both time points **(Fig. 3-4E)**. The intrinsic role DC and macrophages being antigen presentation and pathogen clearance respectively likely owes to the differential expression of proinflammatory cytokine genes observed here.

Cross-linking of Fcγ receptors modulates macrophage phagocytosis and clearance of pathogens[191,192]. Many opsonized pathogens lead to the downregulation of IL-12 expression and upregulation of IL-10 in macrophages as a mechanism of pathogen survival and limitation of macrophage over activation[268–270]. This mechanism to prevent over activation may be responsible for the lack of proinflammatory gene expression observed in macrophages in response to exosome IC. On the contrary, Fcγ expression in DC largely leads to modulation of antigen presentation. Cytokines released by DC are one signal required for mounting of adaptive immunity along with presentation of antigens. Greater proinflammatory gene expression observed in DC compared to macrophages may facilitate this process.

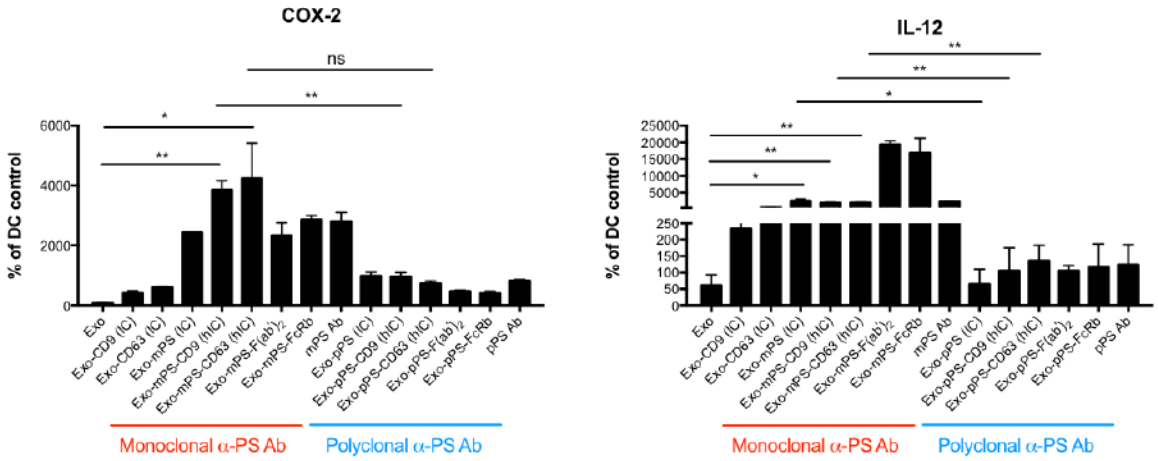
Effective anti-tumor immunity is largely mediated by cytotoxic $CD8^+$ T cells that have been primed towards a specific TAA. We thus next examined the capacity of DCs to cross present a model tumor antigen, OVA, delivered in the form of anti-mPS IgG-complexed exosomes from OVA-expressing MC38 CRC cells, to OVA-specific OT-I $CD8^+$ T cells. We did this by measuring IFNγ production by OT-I T cells when cultured in vitro with DC stimulated as in the previous experiments. OT-I T cells co-cultured with DC treated with anti-mPS IgG complexed exosomes released more IFNγ ($p < 0.01$) than did those co-cultured with DC treated with exosomes alone or DC treated with soluble OVA (sOVA) **(Fig. 3-4F)**. Additionally, when DC were stimulated with exosomes alone, OT-I T cells released less IFNγ than unstimulated DC ($p < 0.01$) providing additional evidence that exosomes in the absence of IgG complexing antibodies inhibit an inflammatory response in DC. Interestingly, it is important to note that Fc receptor blocking on DC with anti-CD16/CD32 IgG, anti-CD16/CD32 $F(ab')_2$ or rat serum did not lead to greater IFNγ release by co-cultured OT-I T cells **(Fig. 3-4E)**. This suggests that blocking of CD16 and CD32 Fcγ receptors does effectively decrease the ability of anti-mPS-IgG complexed MC38 exosomes to induce the type of antigen-specific immune priming that is known to effectively drive anti-tumor immunity.

In spite of creating the anti-CD16/CD32 Ig F(ab')$_2$ fragment, the absence of the Fc region of the antibody failed to prevent upregulation of proinflammatory cytokines by DC **(Figs. 3-4C, D and E)**. The presence of the either the anti-CD16/CD32 IgG and F(ab')$_2$ along with exosomes often led to greater proinflammatory gene expression by DC than did exosomes in the absence of Fc receptor blocking. Since F(ab')$_2$ fragments can still crosslink Fc receptors, our data suggest that Fc receptor crosslinking in general is more proinflammatory than exosomes or exosome ICs. Although CD16/CD32 IgG and CD16/CD32 F(ab')$_2$ may crosslink Fc receptors and lead to upregulation of proinflammatory gene expression, this could be explained by an alternative mechanism. Anti-CD16 and anti-CD32 antibodies bind FcγRIII and FcγRIIB respectively, however FcγRIV and the high affinity FcγRI receptors remain available to binding of IgG. This could allow for the IC which may normally bind to a lower affinity (FcγRIII) or inhibitory receptor (FcγRIIB) to be directed towards the activating FcγRI or FcγRIV receptors. This could explain why we observed higher than expected expression of proinflammatory cytokine genes, release of inflammatory cytokines and increased expression of signalling mediators in the presence of Fcγ receptor anti-CD16/CD32 blocking antibodies in both DC and macrophages stimulated with IC.

Ultimately, we are interested in uptake of exosome IC by Fcγ receptors and the model used to study this mechanism should be modified for future experiments. To determine whether exosome IC are in fact taken up by Fcγ receptors, the use of DC and macrophages derived from FcRγ$^{-/-}$ mice would provide a useful model. Each activating FcγR elicits intracellular signalling following crosslinking via the ITAM domain which is associated with the γ-chain[202,271]. One limitation of this model is the requirement of the FcRγ chain for surface expression of the activating Fcγ receptors. This poses a challenge for determining the role of exosome IC in ITAM dependent and Fcγ receptor functions. An alternative model that could circumvent this would be mice containing mutant FcRγ-chain[202,271]. These mice exhibit normal expression of activating γ-chain associated Fcγ receptors, however show abrogated γ-chain ITAM-dependent in vivo and ex vivo effector functions[202,271]. Unlike anti-CD16/CD32 Fcγ receptor blocking antibodies this model would be more appropriate for determining the role of exosome IC interaction with activating Fcγ receptors.

Together, our data provides evidence that exosomes in the absence of IgG complexing antibodies are not sufficient to alter or upregulate proinflammatory cytokine expression in DC and macrophages, where they may play a more regulatory role. In contrast, immune complexing the exosomes with anti-mPS IgG led to enhanced production of numerous proinflammatory cytokines and significant secretion of IL-6 by DC. More significantly in the context of anti-tumor immunity, only when in the form of an IC are exosomes able to efficiently prime tumor-specific $CD8^+$ T cells. Our data do not point to the existence of a polarizing cytokine signature. In particular IL-6, is known to be a negative regulator of IFNγ expression in lymphocytes[272,273]. Although we do not know the role of IL-6 in this model system, it is possible that this cytokine could have a role in attracting other immune cells to the tumor site to facilitate in anti-tumor immunity. The increased amount of IFNγ released by these T cells suggests that exosomes are capable of initiating Th1 mediated immunity. One role of IFNγ is the upregulation of MHC I on infected cells and tumor cells[274]. MSI CRC have a greater number of CTL present in the tumor which is dependent on the presentation of TAA on MHC I[27,69]. Our data could provide a mechanism by which MHC I becomes upregulated on MSI CRC tumor cells though the release of exosomes that are phagocytosed by DC and prime CTL responses leading to greater CTL tumor infiltration.

A


COX-2
% of DC control
6000
4000
2000
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ns
**
*
Exo
Exo-CD9 (IC)
Exo-CD63 (IC)
Exo-mPS (IC)
Exo-mPS-CD9 (hIC)
Exo-mPS-CD63 (hIC)
Exo-mPS-F(ab')2
Exo-mPS-FcRb
mPS Ab
Exo-pPS (IC)
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pPS Ab
Monoclonal α-PS Ab
Polyclonal α-PS Ab
IL-12
% of DC control
25000
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15000
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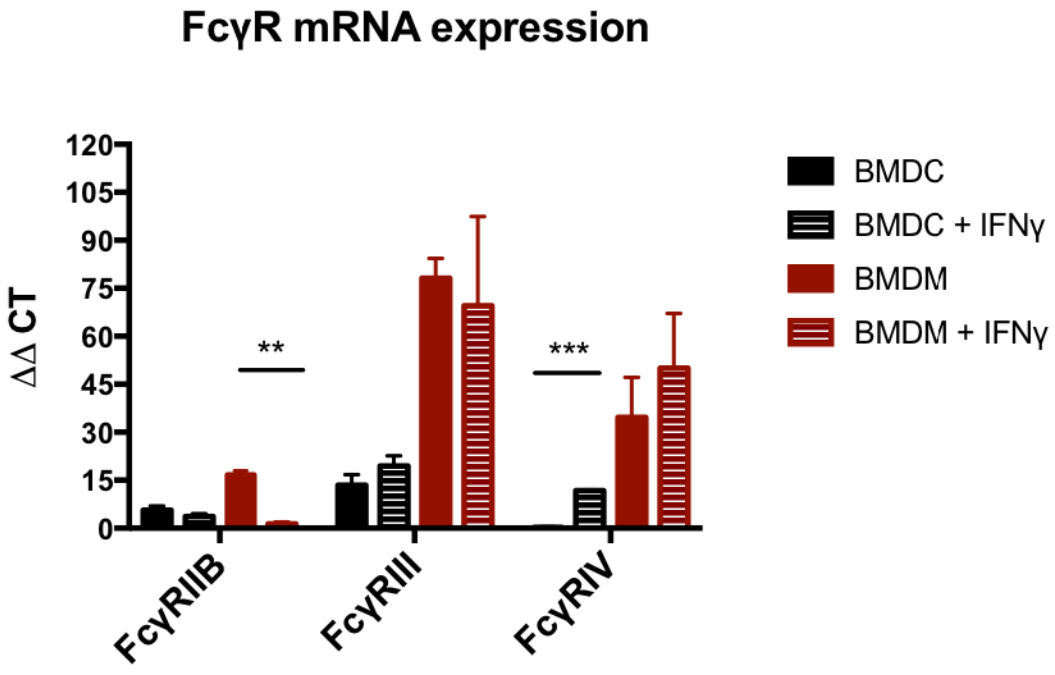
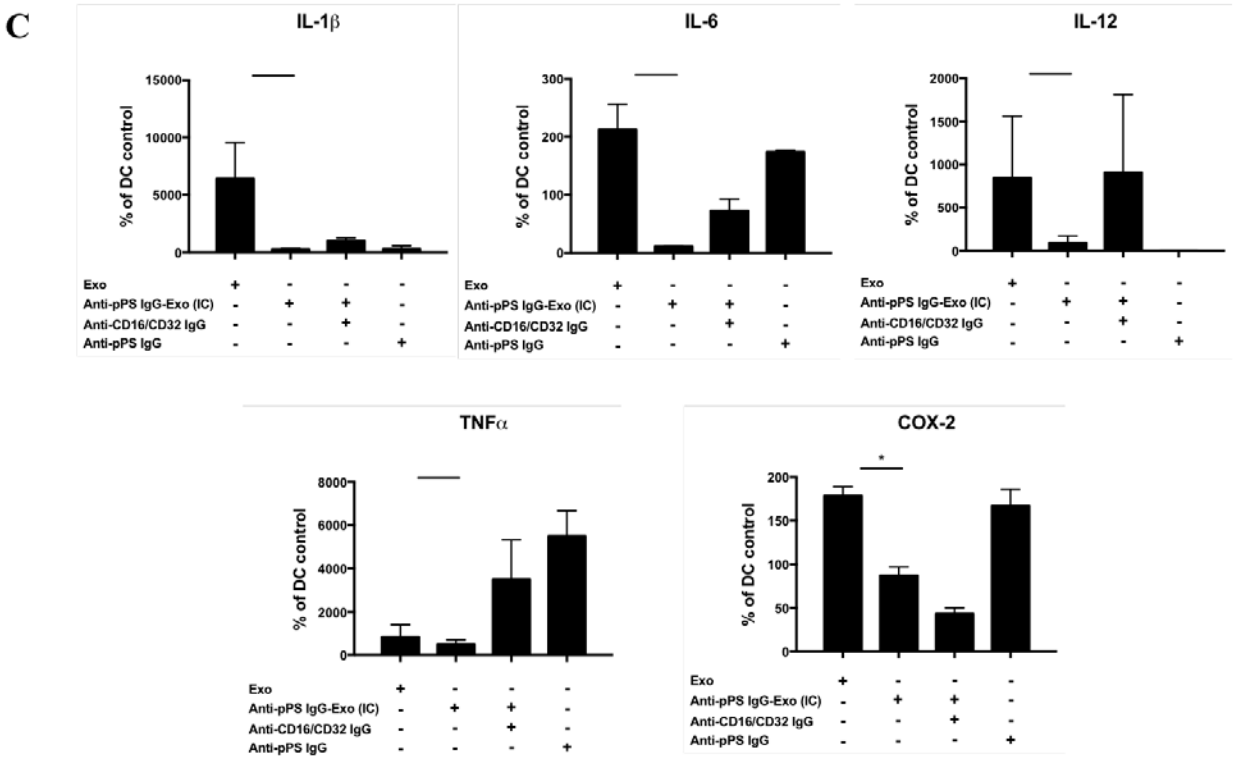
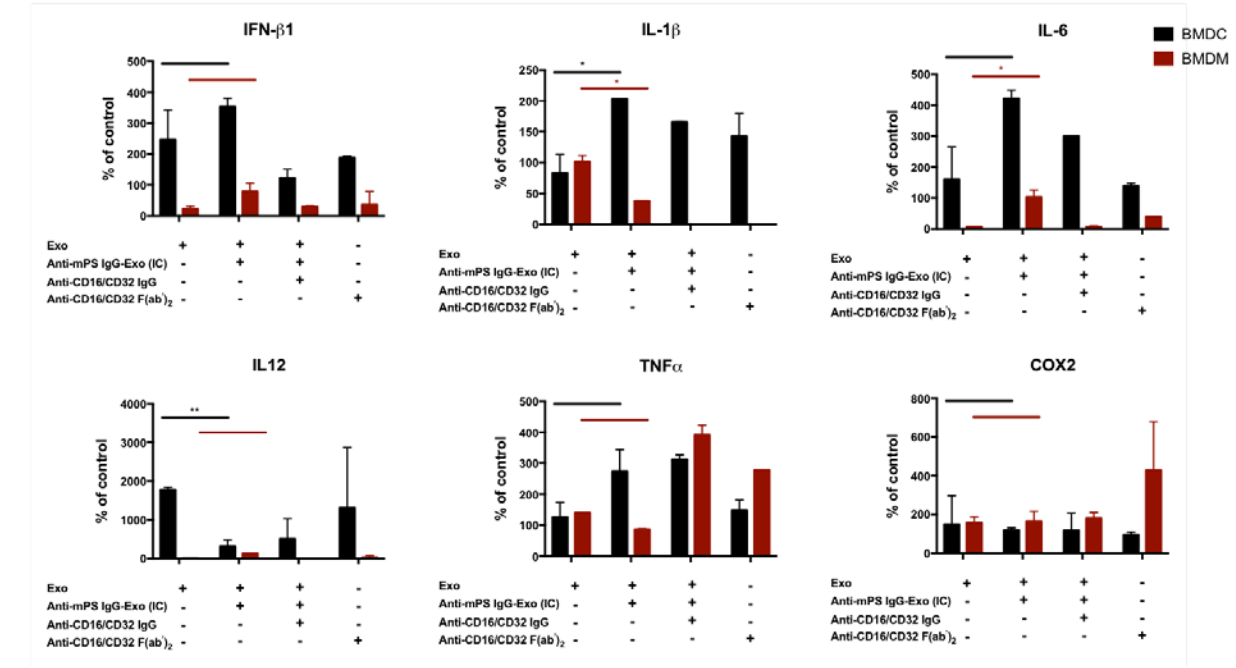
*
Monoclonal α-PS Ab
Polyclonal α-PS Ab

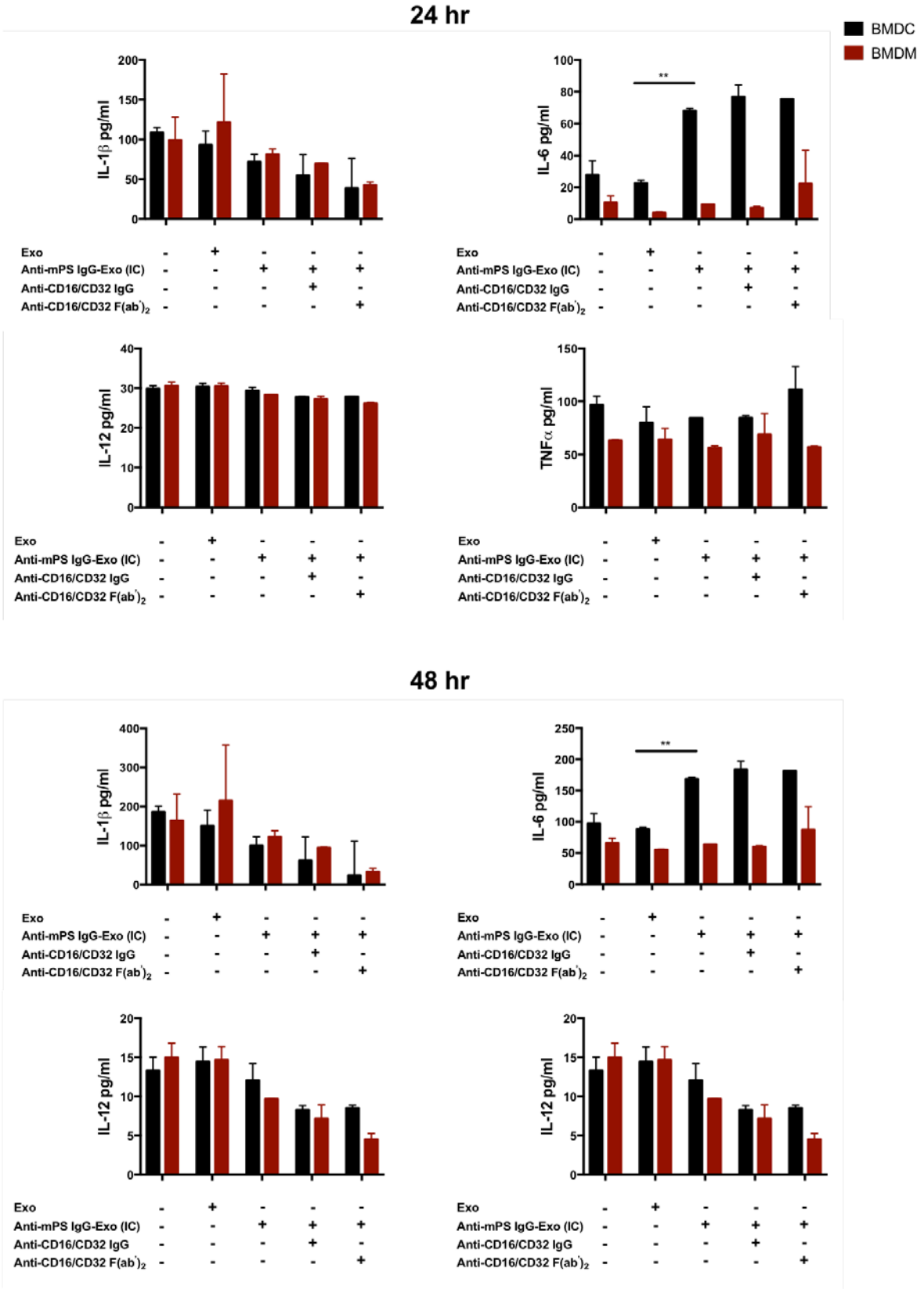
B


FcγR mRNA expression
ΔΔ CT
120
105
90
75
60
45
30
15
0
**
***
FcγRIIB
FcγRIII
FcγRIV
BMDC
BMDC + IFNγ
BMDM
BMDM + IFNγ


C
IL-1β
IL-6
IL-12
TNFα
COX-2
% of DC control
Exo
Anti-pPS IgG-Exo (IC)
Anti-CD16/CD32 IgG
Anti-pPS IgG


D
IFN-β1
IL-1β
IL-6
IL12
TNFα
COX2
BMDC
BMDM
% of control
Exo
Anti-mPS IgG-Exo (IC)
Anti-CD16/CD32 IgG
Anti-CD16/CD32 F(ab')2

E


24 hr
BMDC
BMDM
IL-1β pg/ml
IL-6 pg/ml
IL-12 pg/ml
TNFα pg/ml
**
Exo
Anti-mPS IgG-Exo (IC)
Anti-CD16/CD32 IgG
Anti-CD16/CD32 F(ab')2
48 hr

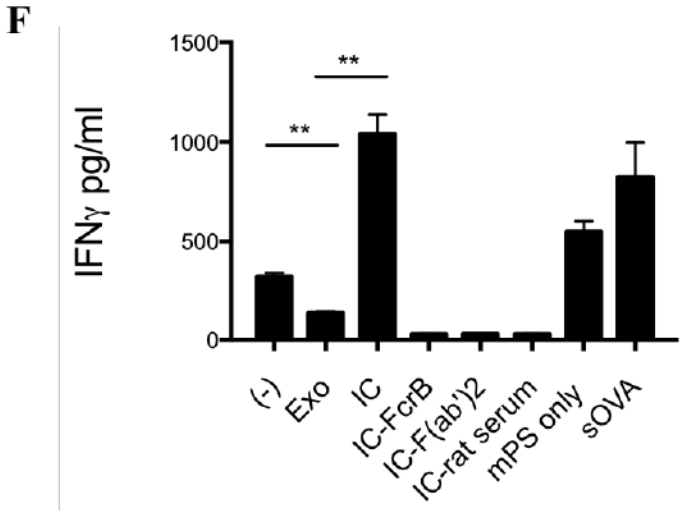


Figure 3-4. IgG-complexed serum exosomes elicit proinflammatory gene expression in immature DC. (A) DC were simulated with MC38 exosomes derived from MC38 cells transfected with OVA and complexed with either monoclonal (anti-mPS) or polyclonal (anti-pPS) IgG along with anti-CD9 or CD63 for 15 hr. mRNA expression of inflammatory genes COX-2 and IL-12 was evaluated by qRT-PCR. **(B)** DC and macrophages were stimulated for 24 hrs with IFNγ or not and mRNA expression of FcγRIIB, FcγRIII and FcγRIV inflammatory genes was evaluated by qRT-PCR. **(C)** DC were polarized with IFNγ overnight and DC were subsequently stimulated with MC38 OVA derived exosomes complexed with anti-pPS IgG or not for 15 hrs. Where indicated, Fc receptors were pre-blocked for 30 min with anti-CD16/CD32 antibodies. mRNA expression of inflammatory genes was evaluated by qRT-PCR. **(D)** DC and macrophages were stimulated with MC38 OVA exosomes complexed with anti-mPS antibodies or not for 15 hrs. Where indicated, Fc receptors were pre-blocked for 30 min with anti-CD16/CD32 antibodies. mRNA expression of inflammatory genes was evaluated by qRT-PCR and **(E)** cytokine release was examined at 24 and 48 hrs. **(F)** Fcγ receptors on DC and macrophages were pre-blocked for 30 min with anti-CD16/CD32 IgG (FcrB), anti-CD16/CD32 ($F(ab')_2$), or rat serum. DC and macrophages were stimulated with soluble OVA (sOVA) or MC38 OVA exosomes complexed with anti-mPS antibodies or not for 14 hrs prior to the addition of OT-I $CD8^+$ T cells. IFNγ release was examined at 24 and 48 hrs by ELISA. Where

proinflammatory gene expression was evaluated by qRT-PCR, data were analyzed using the ΔΔCT method and expressed as a percent of unstimulated DC or macrophages. Experiments in panels (**A), (B) and (F)** performed once and experiments in panels (**C)** – (**E)** were repeated three times. Data is depicted as mean ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant. Asterisks over data represent statistical significance, as determined by the *t* test.

CRC exosomes complexed with anti-mPS IgG activate STAT1 signalling in DCs and macrophages

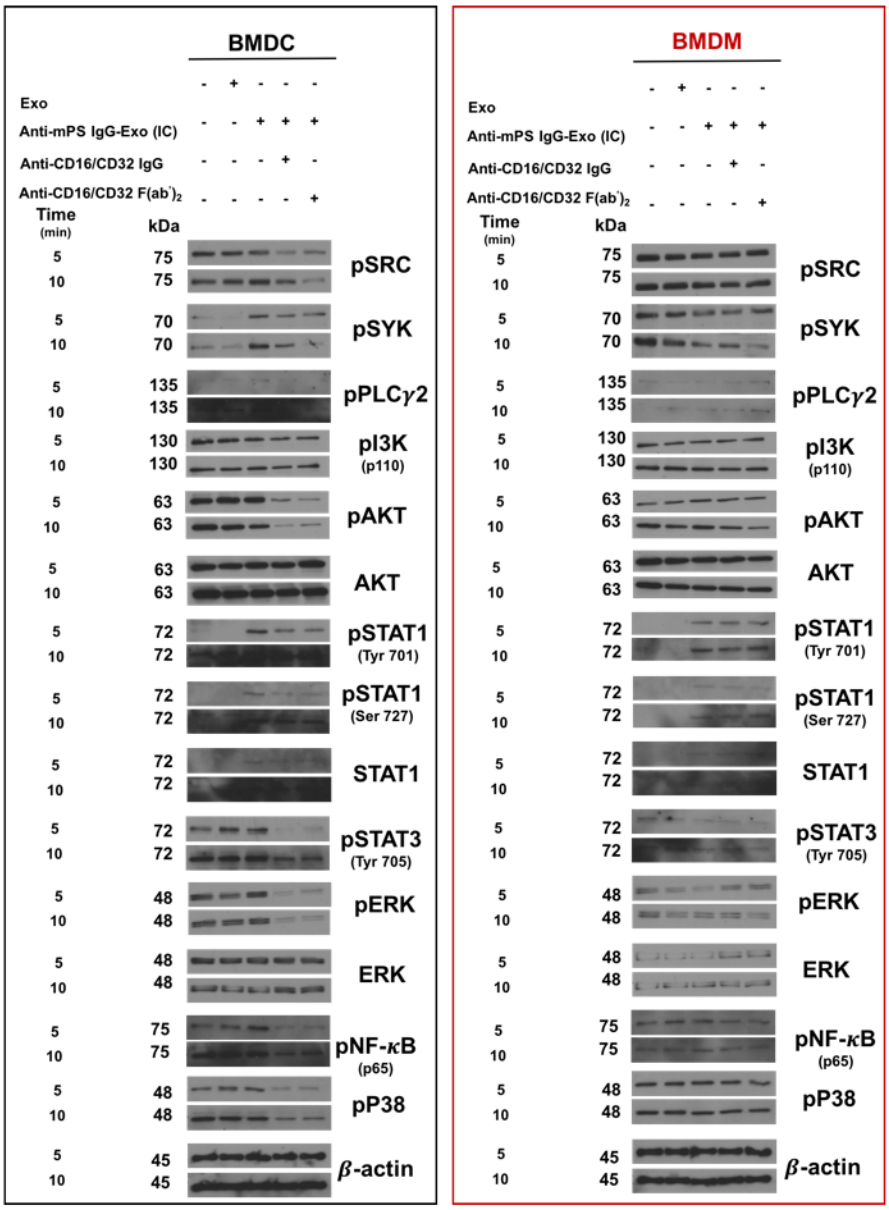
Cross linking of Fcγ receptors bearing an ITAM domain by IC has previously been demonstrated to lead to phosphorylation of the ITAMs by kinases in the SRC family[193,194]. This leads to the recruitment of SYK-family kinases and activation of downstream signalling mediators PI3K and PLCγ[193,194]. In addition, the RAS-RAF-MAPK and the STAT1 pathways have both been shown to become activated in response to Fcγ receptor crosslinking in macrophages and DC, respectively[193,194,275,276]. We observed maturation of DC stimulated with exosome IC that led to upregulation of H-2Kb, proinflammatory cytokines and the release of IL-6. These DC may have also had the ability to efficiently activate tumor-antigen specific OT-I T cells. Given this, we next investigated what downstream signalling events occur in the DC following stimulation with exosome IC for 5 and 10 min.

We observed phospho-STAT1 (Tyr 701 and Ser 727 sites) as early as 5 min in DC and macrophages stimulated with exosome IC, but not exosome alone, and this signal was sustained at 60 min **(Fig. 3-5A and B)**. Upstream of STAT1, we observed no increase in phospho-SRC, PI3K, PLCγ or phospho-AKT, however there was an increase in phospho-SYK in DC but not macrophages stimulated with exosome IC suggesting that the activation of STAT1 in macrophages may be independent of the SYK-family kinases. Proinflammatory cytokines including IL-6 are often transcribed in a STAT3 dependent manner and despite observing increased IL-6 gene expression and cytokine release in DC **(Fig. 3-4D and E),** we did not detect elevated phospho-STAT3 in response to stimulation with exosome IC[277]. On the other hand, increased phospho-ERK and NF-κB were seen as early as after 5 min in DC but not macrophages stimulated with exosome IC. This signal became extinguished by 60 min. These data suggest that anti-mPS-IgG-complexed exosomes led to rapid activation of the STAT1 pathway in both antigen presenting cell types but did so via different mechanisms of activation. In both cell types, this mechanism appears independent of early activation of the classical Fcγ receptor signalling mediators SRC, PI3K and PLCγ. However, signalling events following crosslinking of Fcγ receptors is rapid and likely not captured by 5 and 10 min timepoints. Moreover, the additional activation we observed of the ERK and NF-κB signaling mediators

upon stimulation with exosome IC in DC compared to macrophages may explain the greater extent of proinflammatory gene expression, especially IL-6, observed in DC.

The nature of exosomes, which are composed of proteins, nucleic acids and lipids, adds complexity to identifying one common signaling pathway induced by anti-PS-IgG complexed exosomes as each of these components is able to trigger multiple signaling pathways. The presence of Fcγ receptor expression on DC and macrophages will also direct downstream signalling[194]. We observed differential expression of Fcγ receptors on both antigen presenting cells types which may explain our observation of activation of different signalling mediators in DC compared to macrophages. Nonetheless, our data shows that IgG-complexed CRC exosomes consistently activate early STAT1 signaling, IL-6 cytokine production in DC. In turn, we have also shown that these DC have the potential to activate $CD8^+$ T cells.

A


BMDC
BMDM
Exo
Anti-mPS IgG-Exo (IC)
Anti-CD16/CD32 IgG
Anti-CD16/CD32 F(ab')2
Time (min)
kDa
pSRC
pSYK
pPLCγ2
pI3K (p110)
pAKT
AKT
pSTAT1 (Tyr 701)
pSTAT1 (Ser 727)
STAT1
pSTAT3 (Tyr 705)
pERK
ERK
pNF-κB (p65)
pP38
β-actin

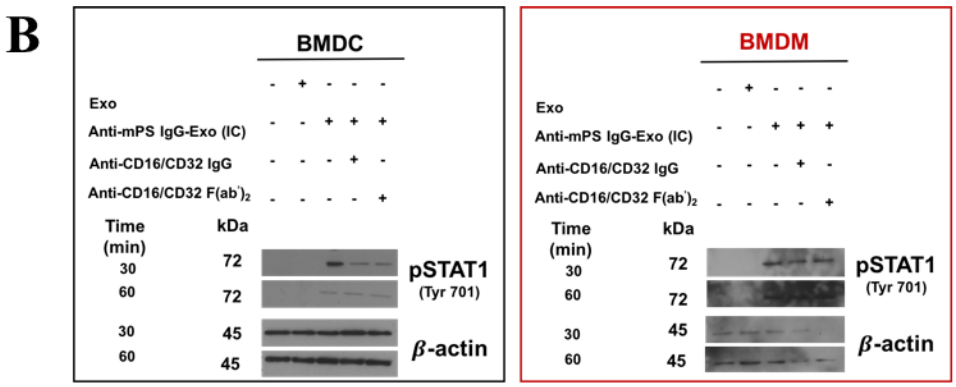


Figure 3-5. Engagement of Fcγ receptors by exosome IC initiates the proinflammatory STAT1 signalling pathway in DC and macrophages. DC and macrophages were stimulated with exosomes isolated from the MC38 cell line transfected with OVA and complexed to anti-mPS IgG (anti-mPS IgG-Exo IC) for **(A)** 5 and 10 min or **(B)** 30 and 60 min. The cells were lysed for analysis of downstream signaling mediators by Western blot. Where indicated, Fc receptors were pre-blocked for 30 min with CD16/CD32 IgG or CD16/CD32 $F(ab')_2$. This experiment was independently repeated three times.

Exosome IC are not sufficient to induce cross presentation by DC and generate a $CD8^+$ T cell response

Fcγ receptor cross linking has previously been demonstrated to lead to DC activation, inducing maturation following IC uptake and cross presentation of antigen mediating Th1 polarization through activation of STAT1 signalling. Since we confirmed activation of STAT1 signalling in DC following stimulation with MC38 OVA exosome IC and greater IFNγ which may have been released by OT-I T cells in vitro, we next assessed the ability of these DC to cross present and induce a $CD8^+$ T cell response in vivo. DCs were pulsed with MC38 OVA exosomes in the form of an IC or not and adoptively transferred to the opposite hind footpads of recipient mice that had previously been injected with CFSE labeled OT-I T cells the previous day **(Fig. 3-6A)**. Ipsilateral popliteal lymph nodes (pLN) were harvested after 72 hr and antigen specific OT-I T cell proliferation was assessed. DC loaded with exosome IC did not induce greater proliferation of adoptively transferred OT-I T cells in comparison to DC loaded with exosomes **(Fig. 3-6B)**. Although we did not observe robust proliferation, we did notice the OT-I T cells in both conditions have downregulated CD8 and the first cell division was initiated. This data suggests that exosome IC loaded DC were not sufficient to cross prime antigen specific T cells that would lead to robust proliferation of these cells in vivo.

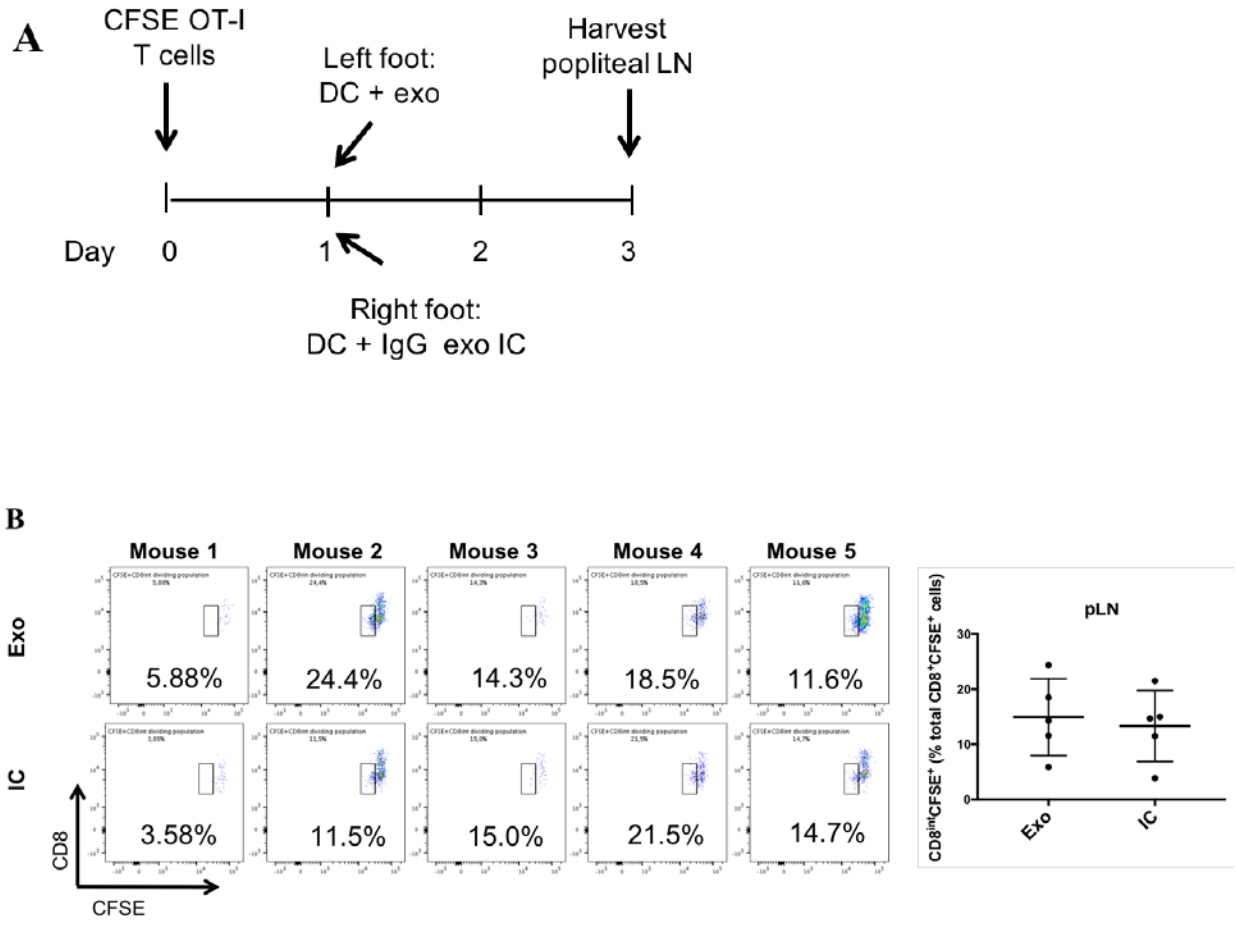


Figure 3-6. In vivo cross-presentation of IgG-complexed exosome IC by adoptively transferred DCs does not lead to greater T cell proliferation compared to exosomes alone. **(A)** Experimental scheme for the footpad assay. CFSE labeled OT-I T cells were adoptively transferred into wildtype C57BL/6 mice. The following day, DC were pulsed with exosomes isolated from the MC38 cell line transfected with OVA and complexed with anti-mPS IgG or not. Stimulated DC were then injected into the hind left (DC:exo) and right (DC:IC) footpad of C57BL/6 mice. Draining ipsilateral popliteal lymph nodes (pLN) were collected at day 3 and staining for analysis by flow cytometry. **(B)** T cells isolated from the draining pLN as described in **(A)** were not effectively cross primed by DC loaded with IC or exosomes which did not allow

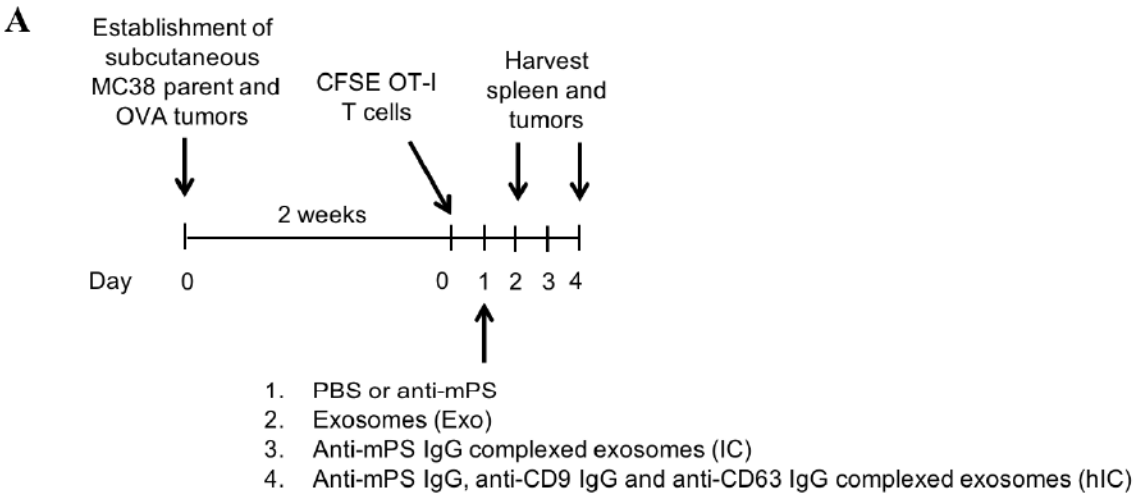
for proliferation of T cells in a 3-day time course. This experiment was repeated twice. Data is depicted as mean ± SD from a minimum of five mice per group.

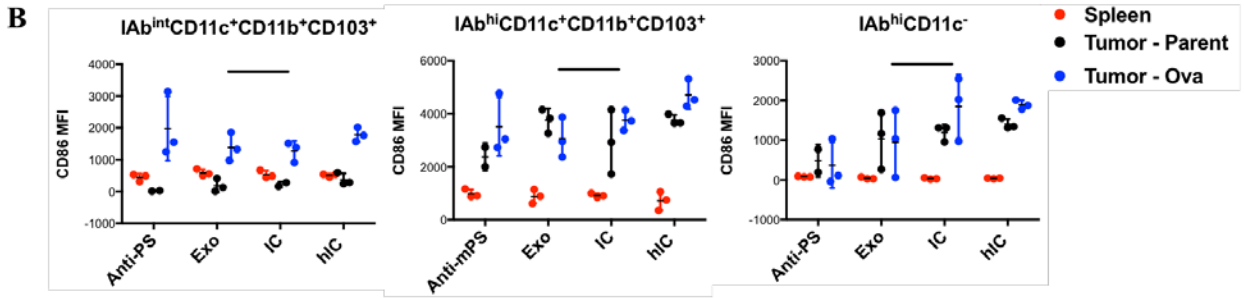
Anti-PS-IgG complexed exosomes increase DC and $CD8^+$ T cell activation in mice bearing MC38 CRC tumors

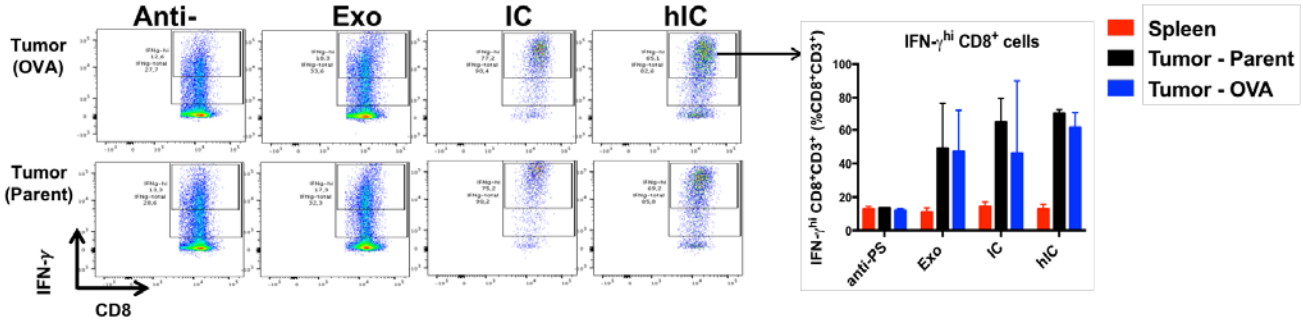
Earlier we observed exosomes complexed with both anti-mPS IgG and additional IgG against CD9 and CD63 stimulated enhanced expression of COX-2 in DC, which induces DC maturation. We aimed to evaluate whether additional exosome complexing, which would increase the IgG to exosome ratio of the IC and thus the strength of the activating signal it delivered to APCs, could enhance activation of DC and macrophages enough to provoke subsequent anti-tumor T cell activation and IFNγ production in our in vivo tumor model. To examine the immune-stimulating capacity of exosomes, we used the model outlined in **(Fig. 3-7A)**. Our model involved subcutaneously injecting parental MC38 CRC cells into one hind flank of a recipient mouse and OVA-expressing MC38 CRC cells into the opposite hind flank. Once the tumors were established, we adoptively transferred CFSE labeled OT-I T cells into the recipient mice. The following day, MC38 exosomes complexed with anti-mPS IgG (IC) or highly immune complexed (hIC) exosomes with anti-PS IgG in addition to anti-CD9 and anti-CD63 IgG and delivered by IV injection. We then harvested the spleen and tumors from the mice at day 2 and 4 for analysis. At day 2 in the DC ($IAb^{hi}CD11c^+CD11b^+CD103^+$) and the macrophage "like" population ($IAb^{hi}CD11c^-$), we observed an increasing trend for CD86 expression when mice were injected with MC38 OVA exosome IC or hIC compared to exosomes alone **(Fig. 3-7B)**. This indicated a greater level of APC activation by IgG complexed exosomes but did not identify a differential effect for a greater IgG to exosome ratio in the IC.

We were not able to detect the CFSE OT-I T cells in either the spleen or tumor using flow cytometry and thus we analyzed the bulk lymphocyte population to determine if complexing exosomes with anti-mPS IgG could induce greater cytokine-mediated $CD8^+$ T cell activation. Despite an increasing trend in CD86 expression in the APC populations, we did not observe greater frequencies of $IFN\gamma^{hi}CD8^+CD3^+$ T cells in the tumor or spleen when the mice were injected with exosome IC or hIC compared to exosomes alone (**Fig. 3-7B**). In a separate

experiment, we analyzed the spleen and tumors at day 4 post exosome injection and did not detect a greater frequency of $IAb^{hi}CD11c^{+}CD11b+CD103+$ DCs or CD11b+CD11c-$Ly6c^{hi}F4/80^{+}$ macrophages **(Fig. 3-7C)**. This suggests the initial DC activation we observed 2-days post injection was very transient and insufficient to produce durable immune consequences. In this experiment, adoptively transferred OT-I T cells were detected, however this was only in the spleen and not the tumors. Nonetheless, we were able to ascertain that mice that received exosome IC had a greater frequency of $IFN\gamma^{+}CD8^{+}CD3^{+}CFSE^{+}$ T cells in the spleen in comparison to mice that received exosomes alone but that this had not induced their proliferation. These data suggest that anti-mPS IgG exosome IC, but not exosomes alone, can lead to early upregulation of the co-stimulatory molecule CD86 on DC and a later increase in the systemic frequency of activated $IFN\gamma^{+}CD8^{+}CD3^{+}CFSE^{+}$ T cells in an in vivo tumor model.


A
Establishment of subcutaneous MC38 parent and OVA tumors
CFSE OT-I T cells
Harvest spleen and tumors
2 weeks
Day 0 0 1 2 3 4
1. PBS or anti-mPS
2. Exosomes (Exo)
3. Anti-mPS IgG complexed exosomes (IC)
4. Anti-mPS IgG, anti-CD9 IgG and anti-CD63 IgG complexed exosomes (hIC)


B
IAb^int CD11c+CD11b+CD103+
IAb^hi CD11c+CD11b+CD103+
IAb^hi CD11c-
CD86 MFI
Anti-PS
Anti-mPS
Exo
IC
hIC
Spleen
Tumor - Parent
Tumor - Ova


Anti-
Exo
IC
hIC
Tumor (OVA)
Tumor (Parent)
IFN-γ
CD8
IFN-γ^hi CD8+ cells
IFN-γ^hi CD8+CD3+ (%CD8+CD3+)
anti-PS
Exo
IC
hIC
Spleen
Tumor - Parent
Tumor - OVA

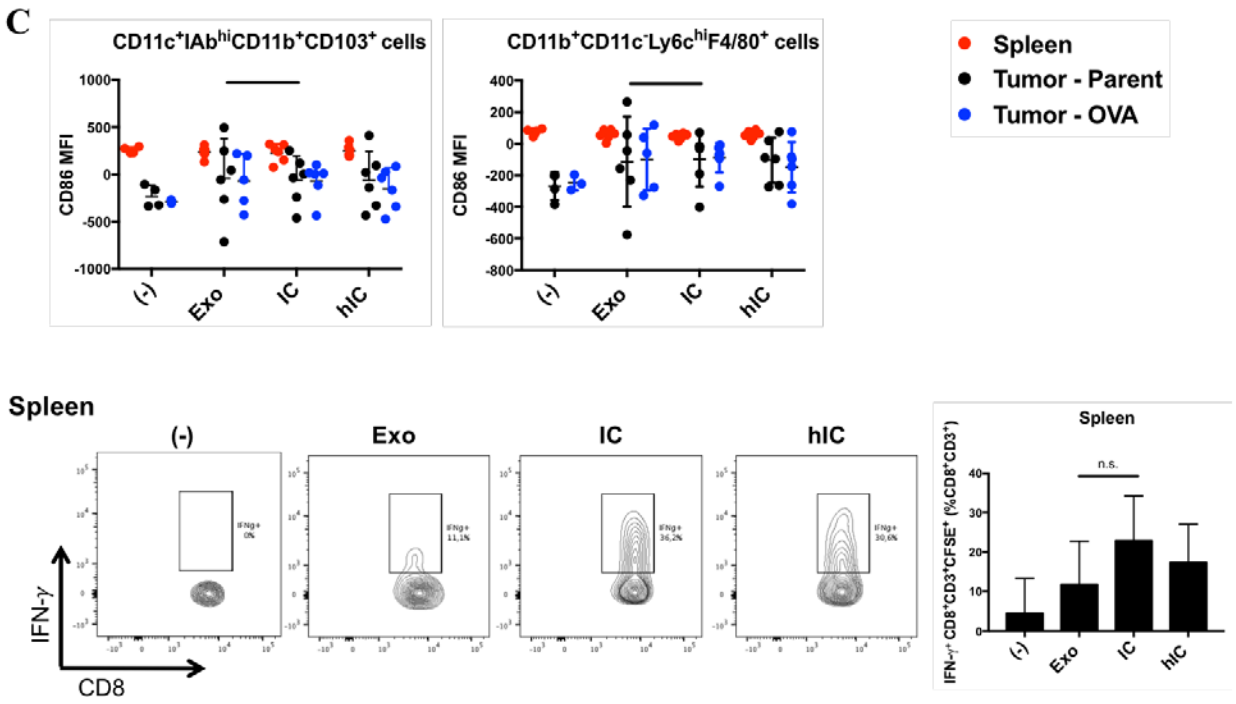


Figure 3-7. IgG-complexed exosomes lead to early activation of DC and late activation of CD8$^+$ T cells. (A) Experimental scheme for the tumor model. MC38 cells that were transfected with OVA or not (MC38 parent) were subcutaneously injected into C57BL/6 mice. Once tumors were established, MC38 exosomes complexed with anti-mPS IgG (IC), anti-mPS IgG and anti-CD9/anti-CD63 IgG (hIC), or not and intravenously injected into tumor bearing mice. CFSE labeled OT-I CD8$^+$ T cells were then adoptively transferred the following day. Immune cells from tumors and spleens were harvested at days 2 and 4 post injection of CFSE T cells. Prior to staining for flow cytometry, immune cells were stimulated ex vivo with PMA/ionomycin for analysis of intracellular IFNγ expression. **(B)** Mean fluorescent intensity (MFI) of CD86 in the DC (IAb^{int}CD11c$^+$CD11b$^+$CD103$^+$ and IAb^{hi}CD11c$^+$CD11b$^+$CD103$^+$) and macrophage "like" (IAb^{hi}CD11c$^-$) population and frequencies of polyclonal bulk IFNγ^{hi} CD8$^+$ T cells isolated from the spleen and tumors on day 2. **(C)** CD86 MFI on tumor infiltrating DC (CD11c$^+$IAb^{hi}CD11b$^+$CD103$^+$) and macrophage (CD11b$^+$ CD11c$^-$Ly6c^{hi}F4/80$^+$) populations and frequency of CFSE-labeled CD8$^+$ IFNγ$^+$ OT-I T cells in the spleen isolated on day 4.

Experiments in panel **(B)** and **(C)** were repeated once and twice respectively. Data is depicted as mean ± SD from a minimum of three mice per group. n.s. not significant as determined by the *t* test.

Intratumoral delivery of anti-PS IgG complexed exosomes does not enhance tumor infiltration or activation of immune cells

We next investigated whether delivery route of exosome IC would enhance immune cell activation and infiltration in an in vivo tumor model. In choosing a route of delivery for the exosomes, we considered previous work evaluating the distribution and pharmacokinetics of intravenously injected exosomes. This revealed rapid clearance from circulation with exosomes having a serum half-life of only 2 min with initial sites of uptake being the liver and lungs[236]. 4 hr post-injection, the major sites of exosome retention have previously been reported as the lung and spleen[236]. The degradation of exosomes in tissue where they become concentrated has been understudied and it is not clear if exosomes can recirculate following entry into a tissue. Thus, given their rapid clearance from circulation, we decided to compare both intravenous (IV) and intratumoral (IT) delivery of exosomes to determine if direct delivery of exosome IC was better able to induce immune cell activation and infiltration into the tumor. As illustrated in (**Fig. 3-7A)**, we thus repeated the experiment described above but used both IV and IT delivery of MC38 exosomes complexed with anti-mPS IgG the following day after adoptive transfer of CFSE labeled OT-I T cells into the recipient mice. The tumors and spleens were evaluated at days 1 and 4 post injection for immune cell infiltration and activation.

We aimed to isolate and analyze the adoptively transferred CFSE OT-I cells that had infiltrated into the tumors and spleen, however the OT-I T cells were not detectable in either the dissociated spleens or tumors. Therefore, our analysis was restricted to polyclonal non-antigen specific immune cells, including $CD4^+$ and $CD8^+$ T cells, as a readout of immune activation. We reasoned that differences in non-specific cytokine-induced activation should be obvious if intravenously delivered exosomes were truly cleared so fast that they never entered the tumors in substantial amounts. Interestingly, the route of exosome IC delivery did not differentially impact frequency of $CD8^+$ T cell ($IFN\gamma^+CD8^+CD3^+$) or DC ($I\text{-}Ab^{hi}CD11c^+CD11b^+CD103^+$) populations in the spleen or tumor, although the frequency of $CD8^+$ T and $CD11c+IAb^{hi}$ cells were slightly greater in the MC38 OVA tumor at day 4 following IV injection compared to the MC38 OVA tumor following IT injection **(Fig. 3-8A)**. We repeated this experiment to analyze the NK cells these cells express Fcγ receptors and are involved in antibody-dependent cell-mediated

cytotoxicity, we evaluated the frequency of this population at day 4 post injection of IC by IV or IT delivery and found a low frequency of NK cells in the spleen and MC38 tumor that did not differ according to the route of exosome IC delivery **(Fig. 3-8B)**. While these data did not allow us to address our initial question about tumor antigen-specific T cell activation, we were nonetheless able to ascertain that direct delivery of exosome IC by IT injection did not enhance the frequency or activation of immune cell populations that infiltrated the tumor. As discussed below, this is possibly because IgG has a very long half-life which may have prolonged the circulating time of anti-PS-IgG-complexed exosomes.

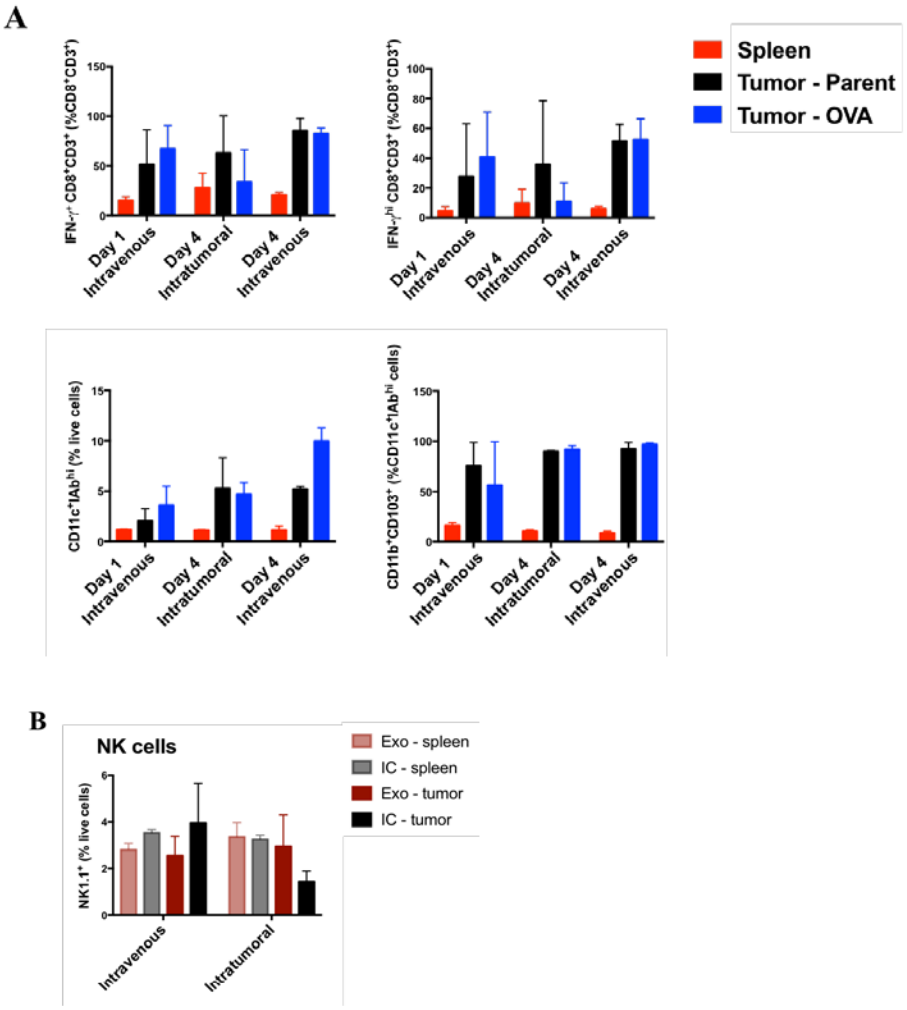


Figure 3-8. Intratumoral delivery of IgG-complexed exosomes does not lead to greater frequency of IFNγ$^+$ T cells or tumor infiltrating immune cells. (A) MC38 cells that were transfected with OVA or not (MC38 parent) were subcutaneously injected into C57BL/6 mice. Once tumors were established, MC38 exosomes complexed with anti-mPS and either injected into the tumor bearing mice via intratumoral (both the MC38 OVA and parent tumor) or intravenous delivery. CFSE labeled OT-I CD8$^+$ T cells were then adoptively transferred the following day. Immune cells were isolated and evaluated from tumors and spleens at days 1 and 4 post injection of CFSE T cells. Prior to staining for flow cytometry for analysis of DC and T cells, immune cells were stimulated ex vivo with PMA/ionomycin for analysis of intracellular IFNγ expression. **(B)** MC38 parent cells were subcutaneously injected into the mice. Once

tumors were established, the mice were intratumorally or intravenously injected with MC38 parent exosomes complexed with anti-mPS IgG (IC) or not. Immune cells were isolated from tumors and spleens at day 4 post injection of IC, stained and analyzed by flow cytometry and the frequency of NK cells were analyzed. Experiments in panel **(A)** and **(B)** were repeated twice and once respectively. Data is depicted as mean ± SD from a minimum of two mice per group.

Incorporation of long chain PUFAs AA and DHA into exosome membranes alters their ability to stimulate DCs

Dietary FA can influence inflammatory immune responses through a variety of mechanisms[251–253]. One of these is the incorporation of these FA into membrane lipid rafts in a way that can attenuate cell signaling and ultimately affect gene expression[251–253]. Immune modulatory lipids include AA and DHA which exhibit inflammatory and anti-inflammatory properties, respectively[251–253]. While it is known that cell membranes can be modified with AA and DHA, it is not known whether this will alter the lipid membrane composition of exosomes. Since AA and DHA can exert immunomodulatory properties on DC and macrophages, by modulating cell signaling or expression of adhesion molecules and cytokines, we aimed to determine whether altered lipid-membrane composition in exosomes could modulate the anti-tumor immune response seen following uptake of these exosomes by DC[251–253].

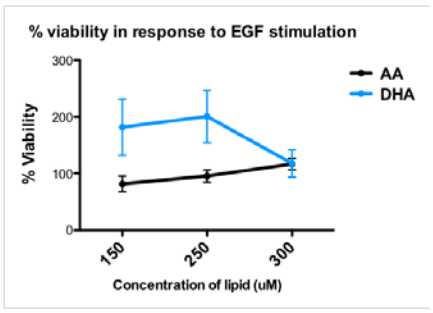
First, we tested whether or not we could influence the membrane FA composition of MC38 CRC cells by supplementing their growth medium with PUFA to determine what concentration of AA and DHA resulted in maximal incorporation into the cell membrane **(Fig. 3-9A)**. We determined that DHA was more efficiently incorporated into the cell membranes at all concentrations examined. Since MC38 cells express the epidermal growth factor receptor (EGFR) which can be altered by AA and DHA, we examined signaling mediators downstream of MC38 cells stimulated with these PUFA and epidermal growth factor (EGF). No differences in phospho-AKT or phospho-ERK were detected in MC38 cells modified with varying concentrations of AA or DHA **(Fig. 3-9B)**. Whether the lipids altered phosphorylation of the EGFR was difficult to discern due to non-specific banding patterns in our Western blots, however concentrations of AA between 50-150 μM may have increased phosphorylation of EGFR. We also tested whether AA or DHA influence MC38 CRC cell viability. Even stimulating MC38 cells with concentrations of AA or DHA up to 300 μM did not affect the viability of the cells **(Fig. 3-9C)**. This gave the us the option of stimulating our cells with very high concentrations of PUFAs to examine their effect on modulating exosome membrane composition although we chose to work with a

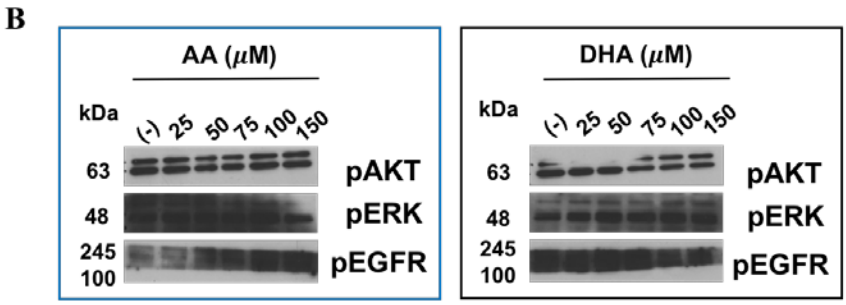
concentration frequently used in other publications and likely to be physiologically compatible with other cell types we may examine in the future.

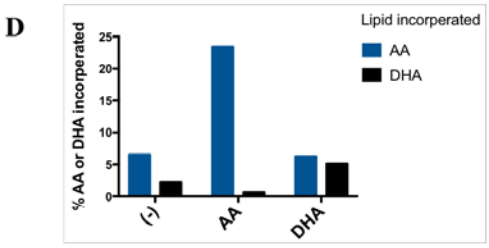
We next stimulated MC38 CRC cells with AA or DHA in order to determine if exosome membrane composition was also modulated by exogenous PUFAs. Following stimulation, exosomes were isolated from the cell culture medium and we compared the lipid content of exosomes and isolated lipid rafts from MC38 cells supplemented with AA or DHA. After stimulating MC38 CRC cells with 30 μM of AA or 60 μM of DHA, which were that those concentrations led to maximal incorporation of the respective PUFA in the MC38 cell membrane **(Fig. 3-9A)** we purified exosomes from the cell culture supernatants. Lipid membrane composition of the exosomes was analyzed and while both PUFA were found to be incorporated into the exosome membrane, this occur to a much greater extent with AA than DHA **(Fig. 3-9D)**. The immunogenicity of the lipid-altered exosomes in the absence of complexing anti-PS IgG was next tested by using them to stimulate DC. Exosome isolated from MC38 AA modified cells significantly increased COX-2 expression in DC ($p < 0.05$). Both AA ($p < 0.05$) and DHA ($p < 0.01$) exosomes significantly decreased IL-6 expression. Both the change of COX-2 and IL-6 gene expression occurred in a dose dependent manner but did not become significant until 10ug of exosomes were used to stimulate DC **(Fig. 3-9E)**. These data suggest that dietary lipids not only alter epithelial cell membrane composition but also influence the membrane composition of exosomes released from the CRC cells. This, in turn, alters their capacity to alter inflammatory gene expression in DCs in vitro and raises the possibility of dietary lipid modification of CRC exosomes and then complexing these with antibodies of different lipid specificities to generate a more tunable anti-tumor immune response.

A

DHA (μM)	% incorporation
30	10
60	20
100	20
AA (μM)	% incorporation
30	10
60	13
100	11







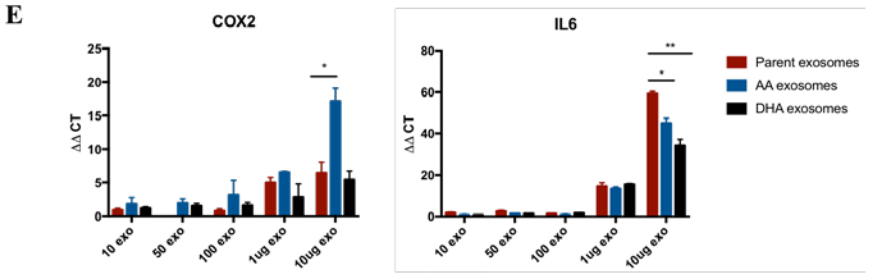


Figure 3-9. Long chain PUFA AA and DHA are incorporated into exosomes and alter inflammatory gene expression in DC. (A) MC38 cells were cultured with various concentrations of AA and DHA PUFA for 3 days and lipid membrane composition was analyzed by gas chromatography (GC) to determine the concentration that would result in maximal incorporation of AA or DHA into the lipid membrane. **(B)** MC38 cells were cultured with varying concentrations of AA and DHA PUFA for 3 days then stimulated with EGF, lysed and

analyzed by Western blot to determine optimal concentration for signaling activation. **(C)** Viability was assessed by MTT assay in cells treated as in **(B)** to determine lipid toxicity. **(D)** MC38 cells were treated with AA or DHA and exosomes were isolated from the cell culture supernatant at 48 hrs after the addition of the lipid containing medium**. T**he lipid composition was then analyzed by GC. **(E)** Varying amounts of exosomes (10 exo/DC, 50 exo/DC, 100 exo/DC, 1 μg exo and 10 μg exo) isolated from AA and DHA treated MC38 cells were used to stimulate DC. RNA was isolated from stimulated DC after 24 hrs and inflammatory mRNA gene expression was analyzed by qRT-PCR using the ΔΔCT method. Experiments in panels (**A)**, (**B)** and (**E)** were performed once and (**C)** and **(D)** were performed twice. Data is depicted as mean ± SD; * $p < 0.05$, ** $p < 0.01$, n.s. not significant. Asterisks over data represent statistical significance, as determined by the *t* test.

CHAPTER 4: DISCUSSION

Exosomes have been described in the literature to play a role in cell-cell communication through various exosome-ligand and recipient cell-receptor interactions[206]. It is currently unknown whether expression of various ligands on the exosome surface may become bound by antibody to form an IC and subsequently taken by DCs through an Fcγ receptor mechanism. We hypothesized that exosome IC form via binding of PS-specific IgG to PS on the exosome surface and that the ICs interact with DC through Fcγ receptors to facilitate cross presentation and enhance $CD8^+$ T cell activation. In this present study, we demonstrate that complexing MC38 CRC cell-derived exosomes to anti-mPS IgG induced STAT-1 signalling in DC following IC uptake and this in turn led to the production of proinflammatory IFNγ by antigen specific $CD8^+$ T cells. Additionally, we show that exosome IC enhance co-stimulatory molecule and IFNγ expression in DC and $CD8^+$ antigen specific T cells, respectively, in the spleen of mice bearing MC38 CRC tumors.

Rationale for choosing anti-PS to form exosome IC

Exosomes are perfectly suited to initiating an anti-tumor immune response because of several unique characteristics. These characteristics include the fact that exosomes contain a bolus of TAA and display a wide array of transmembrane proteins and lipids, including the immunogenic PS[206,215]. Many groups have attempted to characterize TAA carried by exosomes from a wide array of cancer cells to elucidate the role these nanoparticles may play in initiating an anti-tumor immune response. The characterization of exosome TAA remains a challenge in part due to tumor evolution over the course of disease progression[212,239,240]. One explanation for the difficulty in TAA characterization is the heterogeneity observed in tumor cells[85,206]. One mechanism by which tumor cell heterogeneity may arise in CRC is through the loss of the DNA MMR system[27]. Although, mutations caused by defective MMR are not completely random, the microsatellite repeat regions that acquire mutations will vary from cell to cell[27]. Through analysis of mutations in MSI CRC, it appears that mutations are more likely to be acquired in certain genes compared to others[27,33,69]. The selection of these tumor clones containing these mutations is a direct effect of the microenvironment including tumor immunoediting[85]. While many clones

will be eliminated by the immune system, others harbour mutations that allow the tumor cell to escape immune control and persist further increasing TAA heterogeneity[85]. Not only does the diversity of TAA vary from one tumor to the next, it is difficult to predict which TAAs will be presented to the immune system[113]. Computational software exists for TAA prediction, however further work is needed to increase accuracy of peptide-MHC predication that accounts for high degree of polymorphisms in HLA genes[113]. Overall, the constant evolution and heterogeneity from the cancer-immunity cycle provides great challenge in the characterization of TAA[85,113]. Despite the fact the exosomes carry immune stimulating antigens, substantial evidence indicates that tumor derived exosomes can suppress both non-specific and antigen specific anti-tumor immunity[218].

In this present study, we aimed to determine whether the immunostimulatory capacity of exosomes could be enhanced by coupling the particles with antibody to form an IC. Despite the diverse array of transmembrane proteins that could be selected for this purpose, careful consideration of the antibody target was required. First, evolution of tumors over time is known to change both exosome cargo and surface expression of proteins[212,239,240]. The potential for these to change requires careful selection when determining which protein is the best candidate for therapeutic target. Due to the evolution and selection of clones over time, it is in one's best interest to select a target that remains consistently expressed throughout tumor development. Second, many of the surface targets on exosomes are also found on the parent cells from which the exosomes are derived and are shared by both malignant and normal cells[206]. In anticipation of targeting exosomes in vivo, we selected PS as a target. By selecting PS, we aimed to minimize the possibility of targeting normal cells, which maintain PS on their surface to a greater extent, and to thus limit IC formation to exosomes and apoptotic cells. While PS is most widely recognized as the apoptotic phagocytic "eat me" signal, not all PS is functionally equivalent, and PS may also be expressed on viable immune cells and tumor cells[229]. PS in the tumor microenvironment is highly dysregulated and PS positive tumor are often not susceptible to phagocytic clearance as the cells have developed other immune evasion mechanisms [229]. On the other hand, immune cells that express PS are not susceptible to phagocytic uptake[229]. This suggests that there is a threshold of surface PS exposure required for phagocytic uptake and a role for other receptors indicating cell death [229]. The dynamics of PS exposure on exosomes

during tumor progression are not known. However, PS may be consistently present on exosomes due to loss of flippase activity compared to transmembrane proteins that are selectively incorporated into exosomes[206]. PS exposure on apoptotic cells is a known immunosuppressive ligand which inhibits DC maturation and induces anti-inflammatory signalling[229]. Therefore, the immunosuppressive nature of PS along with its likely consistent presence on exosomes makes PS an attractive target for IgG complexing antibody.

Due to the fact that anti-PPL antibodies, including anti-PS, are elevated in some cancer patients, we conducted a preliminary study to investigate if anti-PS was upregulated in an immunogenic CRC type[249,250]. We also tested whether the PS sites on their exosomes were already saturated with antibody, which would have suggested that anti-PS-exosome IC are unlikely to be therapeutically useful. Published studies detecting increased anti-PPL antibodies remain limited and do not extend to many tumor types, including CRC[249,250]. Although anti-PPL antibodies are known to increase the risk for thrombolytic events, it is unknown whether these antibodies may be predictive of other factors such as an anti-tumor immune response[241]. Hypermutable *MLH1*- and *POLE*-deficient CRC have a better prognosis and we hypothesized that higher levels of anti-PS antibodies in these patients could be partly responsible for enhanced anti-tumor immunity[27,278]. To address this, we investigated whether anti-PS antibodies were elevated in mice bearing MC38 colorectal adenocarcinoma tumors engineered to be deficient in *MLH1*, *POLE*, *MSH3,* or *KRAS.* The greatest levels of anti-PS IgG antibodies were observed in mice bearing *Mlh1* or *Pole* tumors, however, there was great variability between mice within each experimental tumor group (**Fig. 3-1B**). Although our data suggest that hypermutable tumors may lead to elevated anti-PS antibodies, the small sample size requires further studies to confirm if there are really differential amounts of these antibodies in specific subtypes of CRC. Given that we did not detect high levels of anti-PS IgG antibodies in the serum of tumor bearing mice, this suggested that PS sites on exosomes may not be bound by endogenous anti-PS IgG antibody.

It was next important for us to characterize the PS target on the exosomes that we isolated from the serum of non-tumor bearing mice and from the MC38 CRC cell line directly. The particles isolated from MC38 CRC cells had a mean size of 145 nm **(Fig. 3-2A)**. Exosomes are currently defined by a size range of 30 – 100 nm or 50 – 200 nm, whereas microvesicles are cited as 50 –

2000 nm or 100 – 1000 nm[206,263]. Despite the discrepancy in size ranges found throughout the literature, the particles we isolated throughout the study are highly likely to contain a heterogeneous population of EV. There is currently no consensus or "gold standard" methodology for the isolation specific subsets of EVs. We chose to isolate exosomes by differential ultracentrifugation, which is by far the most commonly utilized method[263]. Other methods, including the use of commercially available kits, have not demonstrated their ability to isolate a pure population of a specific EV types[206,263,279]. In our experiments, we have chosen to employ an empirical definition to characterize our isolated particles as exosomes. By this definition, exosomes are vesicles that sediment after centrifugation at 100,000 x g (utilized in our isolation protocol), whereas microvesicles sediment following 10,000 x g centrifugation[279]. We thus refer to our EV isolates as exosomes, however it is important to highlight that the population is not exclusive to this type of EV.

The use of markers to define EV populations remains limited since a specific set of markers has not been well defined for each cell population and could vary between cells type[206,279]. Regardless of the fact that the field of EV research remains in early stages of development, best common practices for particle research have been defined. Minimal requirements to claim the presence of EV are: (1) the use of transmembrane extracellular proteins, (2) cytosolic proteins, (3) intracellular proteins[208]. We chose to examine the most commonly defined markers CD9, CD63 and AV binding on exosomes. We found that both CD9 and CD63 were highly expressed on MC38 cell-derived exosomes, however only CD9 was identified on serum exosomes (**Fig. 3-2A, B**). In addition, we detected AV binding to MC38 derived exosomes. Notably, we also found that serum exosomes were highly bound by IgG, but, for reasons discussed below, we did not investigate the specificity of the antibodies involved. To exclude the possibility of contaminating cellular components in our exosome preparations, we evaluated the endoplasmic reticulum associated protein calnexin and found little to none present in MC38 CRC cell-derived exosomes. Given the presence of common exosome surface transmembrane proteins, an endosome-membrane binding protein and the absence of contamination from cellular compartments other than the plasma membrane, our data suggest that our purified EVs are exosomes of both endosomal and plasma membrane origin.

In our initial characterization studies, we examined our exosome isolates with conventional cytometry by complexing exosomes to 4 μm beads. Conventional flow cytometers have a minimum detection size of 200 – 500 nm and are not able to discriminate nanoparticles with a difference in size of 100 – 200 nm or less. While it is possible to circumvent this by complexing the nanoparticles to larger beads, as we did above, conventional cytometry is ineffective at analyzing complex EV preparations. Specifically, it cannot identify subpopulations of nanoparticles that may express different levels of a particular marker. For these reasons, we conducted our subsequent exosome analyses on the Apogee micro flow cytometer, which is designed for resolving particles down to 70 nm in size. Our data showed that exosomes from mouse serum were highly bound by IgG. Since we aimed to target PS for IC formation, we investigated whether this IgG could be bound to exposed PS on exosomes using the micro flow cytometry. We first characterized the antibody that we used to complex exosomes. This antibody was produced by the immunization of mice with liposomes containing 70% PS and 30% phosphatidylglycerol, therefore we examined the specificity of the antibody for PS[280]. We determined that the anti-mPS antibody was specific for PS and not for cholesterol or phosphatidylcholine **(Fig. 3-1A)**. Specificity of this anti-mPS antibody has also been previously shown to not cross react with other phospholipids as well as cardiolipin[281]. Upon complexing the serum derived exosomes to anti-pPS followed by detection of bound IgG, we observed a two-fold increase in IgG bound to the exosomes (**Fig. 3-3A**). This suggests that not all exposed PS sites were previously bound by endogenous IgG and indicates that exogenously-administered anti-PS IgG could efficiently bind to CRC exosomes. Previous studies have investigated exosome PS content either by mass spectrometry or by conventional flow cytometry. Neither of these two methods we able to identify the amount of exposed PS sites on the exosome surface. Our data is thus to first to show this. Since there seems to be a threshold of PS exposure that is required for phagocytic uptake, characterization of the amount of surface PS is of importance[229]. High exposure of PS also suggests the high IgG complexing can be achieved which may have greater potential to lead to proinflammatory signalling when phagocytosed by DC[229]. Furthermore, this data suggests that a suitable number of PS sites are present on CRC exosomes to facilitate the formation of an exosome IC that could be therapeutically active.

Anti-PS-exosome IC stimulate DC

Exosome IC could be potent stimulators of DC, which express abundant Fcγ receptors on their surface[194]. We next sought to investigate if we could use our purified exosomes to stimulate DCs. The majority of vesicles in our preparations were within the size range of exosomes, however we did note some larger particles in our purified samples. The presence of a heterogeneous population of both exosomes and microvesicles should not negatively impact our experiments for several reasons. First, both exosomes and microvesicles display PS on the surface, therefore both are capable of forming IC with anti-PS antibodies[206]. In addition, despite the discrete origin of the two populations, exosomes and microvesicles have both been shown to carry immune stimulating TAA[206,215]. Lastly, the release of EV is a constitutive process and these cells are likely to be continuously releasing both EV types[206]. These shared characteristics made us confident that we could use our purified population of EVs to examine how exosomes interact with antigen presenting cells upon formation of an IC.

We began by investigating the immunostimulatory capacity of serum derived exosomes complexed to anti-PS antibodies in DC. When stimulated with the anti-pPS complexed exosomes, COX-2 and the proinflammatory genes IL-1β, IL-6, IL-12, TNFα were upregulated in DC (**Fig. 3-3B**). These data provided us with preliminary evidence that exosome IC were able to modulate proinflammatory gene expression and provided support for our antibody target choice being PS.

Ultimately, it is of great interest to determine whether TAA in CRC exosomes are cross presented to $CD8^+$ T cells. A number of signals from DC provide T cells with key signals that result in cross priming[74]. We observed an increase in IL-12 production by the DCs stimulated with serum exosomes complexed to anti-pPS antibody, suggesting that DCs may become polarized to initiate a Th1 response. These are not the only signals that are required for efficient cross priming. Presentation of antigen on MHC I and expression of co-stimulatory molecules are required[74]. Although not statistically significant, our data show that DC stimulated with serum exosomes complexed with anti-pPS IgG upregulated the maturation markers H-2Kb and CD86, but not I-Ab (**Fig. 3-3E**). Not only are immune polarizing cytokines important for the activation

of T cells, but the upregulation of MHC I and costimulatory molecules are required for DC activation and presentation of antigen to T cells[74].

Presentation of TAA on MHC is a key step for the initiation and ongoing activity if tumor infiltrating CTL[69]. This is demonstrated by the ability of high mutational burden MSI CRC to escape anti-tumor immunity by the down regulation of MHC I antigen presentation machinery[69]. In addition, although not specifically demonstrated in CRC, tumors may also inhibit DC function and therefore antigen presentation. These data demonstrate the potential exosome IC have to modulate DC activation and maturation. On the contrary, these DC did not have the capacity to stimulate $CD8^+$ T cells to robustly proliferate (**Fig. 3-3F**). Exosomes used in these experiments were derived from non-tumor bearing mice, therefore the majority of exosome cargo is self-antigen. While not of exosome origin, IC consisting of self-antigen have been identified to play a role in autoimmune disease pathogenesis. Deposits of these IC have been identified in the kidneys of patients with SLE and rheumatoid arthritis[282,283]. Failure to clear these IC leads to progression of disease, however the detailed mechanistic roles IC have in activation of adaptive immune cells in poorly understood[282,283]. The immature DC phenotype and lack of robust proliferation of T cells that we observed in the presence of anti-PS-complexed exosomes suggest tolerance mechanisms are in play to avoid activation and maturation of DC in the absence of non-self-antigen. This mechanism seems to arise from the fact the DC did not present all of the stimulation signals required for the induction of a T cell response.

Given the potential exosome IC may have in stimulating an anti-tumor immune response, we turned our attention to exosomes derived from MC38 adenocarcinoma cells that, as described below, we expect to carry TAAs rather than just self-antigens. We complexed MC38 exosomes to either a monoclonal or polyclonal anti-PS antibody and assessed proinflammatory gene expression in DC. We also included a condition we refer to as “highly IC exosomes” where we complexed exosomes to anti-PS IgG in addition to antibodies against the common exosome markers CD9 or CD63. This was done to determine if the formation of a greater IgG to exosome IC forming ratio was more immunostimulatory than IC formed with anti-PS alone. Our data show that both types of IC, but not exosomes alone, were able to upregulate COX-2 and IL-12 expression in DC (**Fig. 3-4A**). Previous groups have identified high levels of the common

exosome markers CD9 and CD63 on exosomes[206]. In our analysis, we also identified high levels of CD9 present on MC38 exosomes. Interestingly, complexing these exosomes with only CD9 or CD63 antibody did not upregulate COX-2 expression to the nearly the same extent as exosomes complexed with only anti-mPS. Of note, additional complexing with CD9 or CD63 antibody to form "highly IC exosomes" did not have and additive effect on increasing COX-2 or IL-12 expression. One central question in IC-Fcγ receptor biology is how the antigen to antibody ratio (size of the IC) modulates uptake of the IC by Fcγ receptors, nevertheless cross presentation of the antigen[201]. Although this has not been studied in the context of cross presentation, one study demonstrated that alteration of IC size did not abrogate Fcγ receptor binding to the IC[284]. This was a result of altering the Fc region of IgG by mutagenesis, thereby partially altering glycosylation of the Fc region and subsequent Fcγ receptor binding[284]. In contrast to our study, we did not attenuate Fc glycosylation, however we investigated whether increased IgG presence on the IC modulated proinflammatory signalling in DC. Given that "highly complexed" exosomes did not enhance proinflammatory signalling our data suggests that increased IC size may not enhance proinflammatory signalling. We experimented with a higher degree of IgG complexing, that did not lead to greater proinflammatory signaling. The amount of anti-PS antibody used for complexing was high (100 μg/ml). Future experiments should investigate a lower amount of anti-PS antibody to investigate whether this could allow for the formation IC that are aggregates of exosomes captured by a single anti-PS antibody to see if this heightens proinflammatory signaling.

We hypothesized that complexing exosomes to one of anti-PS, anti-CD9 or anti-CD63 would have led to the same proinflammatory gene expression signature in DC. This was not in line with our findings given that anti-mPS leads to much greater COX-2 and IL-12 gene expression. Exposure of PS on apoptotic cells plays a regulatory role allowing for phagocytosis of cellular material[217]. The exposure of PS on the exosome surface is also likely to play a role in continued clearance of these particles preventing an inflammatory response mediated towards them. One possible explanation of enhanced immunogenicity of anti-mPS complexed exosomes is the blocking of this regulatory response by DC to exosome. The presence of antibody bound to PS may allow for steric hindrance between PS and a PS receptor allowing for exosome IC to be taken up by an alternative mechanism allowing for proinflammatory signalling compared to

recognition of PS as the "eat me" signal by phagocytes. Overall this observation provides support for targeting PS, a component of cell membranes and not a transmembrane protein on the exosome surface, for IC formation.

Effective anti-tumor immunity is dependent on the activation of a CTL effector program[85,285]. In order to facilitate this effector program, NK cells or $CD4^+$ T cells may also provide help for the development of a strong Th1 response[85,285]. This Th1 response is mediated by the release of IL-12 from antigen presenting cells including DC which is facilitated in response to IFNγ in the DC environment[74,186,285,286]. In order to facilitate the release of IL-12, we polarized GM-CSF matured bone marrow-derived DC with IFNγ prior to stimulating them with MC38 derived exosomes complexed with anti-pPS antibody. In some conditions, we also included an FcγR blocking antibody, the CD16/CD32 IgG, in order to confirm the response was Fcγ receptor-dependent. Contrary to our expectations, exosome IC significantly downregulated expression of COX-2 and the proinflammatory cytokines IL-1β, IL-6 and, notably, IL-12 (**Fig. 3-4C**). Even though the $CD8^-$ DC produced by GM-CSF maturation of bone marrow cells are capable of presenting antigen on both MHC I and II, our result could suggest that uptake of exogenous exosomes may not facilitate the development of type-1 polarized effector DC[201]. Alternatively, cytokines are known to modulate expression of Fcγ receptors on antigen presenting cells, which could affect our results. IFNγ has been shown to upregulate activating Fcγ receptors on antigen presenting cells which would enhance uptake of IC[193,194]. On the contrary, IFNγ polarization of DCs can also decrease antigen uptake by downregulation of activating Fc receptors[193,194]. This has been demonstrated after antigen uptake and is attributed to the ability of IFNγ to stimulate DC maturation and transition into antigen presentation mode[74,193,194]. Our data suggest the Fcγ receptors became downregulated by our IFNγ maturation of DC, leading to their ignorance to IC stimulation. However, mRNA analysis of Fcγ receptor expression in DC polarized with IFNγ demonstrated that activating FcγRIII and FcγRIV become upregulated. This highlights the inhibitory nature of exosomes that, in this case, was not overcome by complexing with anti-pPS antibody. Many cytokines may be present in the tumor microenvironment that can influence Fcγ receptor expression besides IFNγ[193,194]. We therefore decided to analyze how IC modulate DC

signalling in the absence of cytokines. Therefore, we changed our experimental protocol to eliminate the IFNγ maturation step we had initially used to achieve Th1 polarization of our DC.

Many immune cell types are present in the tumor environment and can influence the development of anti-tumor immunity in CRC. We thus investigated exosome IC responses in both macrophages and DC in parallel. We found that proinflammatory gene expression was modulated by exosomes and exosome IC to a greater extent in DC than macrophages (**Fig. 3-4D**). Consistent with earlier data using serum derived exosomes, we observed upregulation and significant release of IL-6 from DC and macrophages stimulated with exosome ICs but not exosomes alone. In addition, we observed upregulation of IL-1β mRNA, but not secreted protein, by DC and macrophages stimulated with IC. Exosomes derived from B16 melanoma cells have previously been demonstrated to upregulate IL-6 and TNFα secretion in bone marrow precursor GM-CSF stimulated cells in vitro and in B16 mouse melanoma tumor model[287]. These exosomes were found to inhibit differentiation of these myeloid precursors into DC. The exosomes instead expanded the precursor cells into $CD11b^{+}Gr\text{-}1^{+}$ MDSC via a MyD88-STAT3 dependent mechanism[288]. The MDSC population is well known to suppress $CD8^{+}$ T cell expansion[88,89]. Alternatively, simultaneous upregulation and downregulation of IL-6 and IL-12 in DC is associated with promotion of Th2 immunity and limitation of a Th1 response[74,85]. In addition, IL-6 release by DC has been shown to overcome $CD4^{+}CD25^{+}$ Treg mediated suppression of $CD4^{+}$ effector T cells[289]. While the role of IL-6 in response to IC in our system is unknown, further investigation will be required as IL-6 is often a tumor promoting cytokine and may indicate the induction of Th2 immunity[89]. While Th2 immunity is often regarded are being tumor promoting, there is evidence indicating the anti-tumor activity of $CD4^{+}$ Th2 cells[290]. Exosomes are exogenous antigen and uptake of exosomes by DC may thus result in their entry into the MHC II presentation pathway. Although this is a possibility, engagement of Fcγ receptors on DC by an IC has been shown to preferentially shuttle antigen into the MHC I pathway even in poorly cross-presenting $CD8^{-}$ DC[201,204]. As an extension of this, our analysis focused on induction of Th1 promoting cytokines, which are the main players in cross-presentation, however exploration of Th2 cytokine induction cannot be ignored in future studies given the highly expressed cytokine signature we observed.

In contrast with our previous data, we observed low levels of COX-2 gene expression and a significant decrease of IL-12 expression in DC treated with exosome ICs (**Fig. 3-4D**). Given that these DC had not been matured with IFNγ and were thus more immature, our finding suggests that the effect of exosome IC on DC varies with DC maturation state and possibly the DC subset. Nonetheless, there are also experimental reasons we have observed this differential response. The isolation method for cell culture derived exosomes remained consistent throughout our studies however the specific state of the producing cells during each preparation could have introduced batch-to-batch variability. In addition, variability in the number of exosomes used in each experiment could have been introduced during the quantification of our exosomes. The majority of studies in the literature currently fail to report the number of exosomes used in experiments. Groups that do attempt to quantify and report these numbers often have quantified exosomes using a BCA assay which estimates exosome amount based on protein quantification. This is currently an acceptable method of quantification of exosomes in the nanoparticle field, however it is certainly not the most accurate[261]. Exosome protein content can change over time during tumor evolution or due to the variability in exosome size and capacity to carry varying amounts of protein[212,239,240]. In addition to our exclusion of an IFNγ DC maturation step, this variability in exosome number throughout experiments may provide an explanation for the lack of increased COX-2 and IL-12 expression observed in this experiment in contrast to the previous stimulation.

Exosome IC activate STAT1 signaling in DC and macrophages

Our earlier analysis demonstrated the capacity exosomes IC could have in stimulating a proinflammatory immune response. However, the type of immune response that may be induced still remains elusive when looking at the cytokine output of stimulated DC. Therefore, we investigated the more immediate signalling events in DC following IC stimulation to gain insight into which signalling programs are initiated.

JAK-STAT signalling is often induced by various stimuli that trigger the upregulation of cytokines[291]. Which STAT family member becomes activated in each case varies depending on the stimuli and can induce a distinct cytokine signature[291]. IL-6 cytokine expression is often

mediated by STAT3 rather than STAT1 signalling[291]. However, our data did not demonstrate active STAT3 signalling in response to IC. This suggests the upregulation of IL-6 observed in DC is not mediated in a STAT 3 dependent manner. Crosslinking of Fcγ receptors bearing an ITAM domain leads to rapid recruitment of SRC followed by SYK family kinases, leading to activation of downstream signalling pathways[193,194]. It is thought that the RAS-RAF-MAPK pathway is essential for activation of these downstream signalling targets[193,194]. We observed increased phospho-SYK in both DC and macrophages in response to exosome IC, however this was more pronounced in DC. Despite not detecting increased activation of immediate downstream signalling mediators of SYK (PI3K and PLCγ2), we observed increased phospho-ERK and phospho-NF-κB in DC, but not macrophages. Activation of this pathway in DC but not macrophages points to the existence of differential signalling pathways that are activated in these cell two cell types in response to exosome IC. Earlier, we demonstrated that IC have greater immunomodulatory capacity in DC than macrophages and can now conclude that this can be partially explained by enhanced activation of the signalling pathways in DC.

Signalling through ERK and NF-κB has been shown to be responsible for providing signals promoting adaptive immunity, including polarizing cytokines and co-stimulatory molecules[193,194]. Fcγ receptor crosslinking by IC has also been shown to active JNK and p38 MAPK signalling mediators[193,194]. Specifically, activation of p38 has been shown to lead to upregulation of TNFα[193,194]. Since we did not observe ERK activation in macrophages we examined p38, however this was not found to be activated in response to IC. The STAT1 pathway has also been identified to contribute to DC maturation leading to Th1 polarization[193,194]. We observed increased phospho-STAT1 activation in both DC and macrophages in response to exosomes IC. In addition, we observed activation of both the tyrosine and serine residues of STAT1, required for maximal activation of downstream transcription factors[292]. The activation we observed of the STAT-1 pathway is suggestive of induction of signals required for Th1 polarization, however the cytokines signals seen in our earlier experiments are suggestive if Th2 induction. Our data thus suggest that exosome IC can deliver signals for Th1 and Th2 T cell immunity.

When investigating the uptake of IgG complexed antigen by Fcγ receptors, it is important to consider factors that will initiate signalling pathways in response to the uptake of exosome IC. While both activating and inhibitory Fcγ receptors will phagocytose an IC following ligation, the type of Fcγ receptor is thought to influence the intracellular trafficking and fate of the antigen[193,194]. This has been demonstrated with antigen phagocytosed following by the inhibitory Fcγ receptor. FcγRIIB ligation has been shown to traffic antigen to a non-degradative pathway allowing for antigen recycling to the cell surface[293]. Presentation of antigen via this pathway activates B cells and leads to induction of humoral immunity[293]. However, this may be unlikely since FcγRIIB mRNA expression is low in both DC and macrophages **(Fig. 3-4B)**. In contrast, the presence of the ITAM domain in activating Fcγ receptors leads the recruitment of SYK and allows for trafficking of antigen to lysosomes[294]. Activating Fcγ receptors are thought to allow for the trafficking of antigen to a degradative route that facilitates processing and presentation of antigen to T cells[201,204]. Following uptake of IC by activating Fcγ receptors, the neonatal Fc receptor (FcRn) for IgG is responsible for binding of antigen in endocytic compartments that is favoured at a pH $\leq$ 6.5[201,204]. This has been demonstrated to facilitate the loading of antigen on MHC I and induction of a $CD8^+$ T cell response[201,204]. In contrast to FcRn, another intracellular receptor (TRIM21) for IgG has been described[295]. The role of this receptor has been studied in the context of IgG opsonized viruses and has been demonstrated to limit viral infection through "antibody dependent intracellular neutralization[198]." This occurs following uptake of viral IC where binding of TRIM21 to the invariant domain targets virions to the RING domain with E3 ubiquitin ligase activity[198]. From these studies, intracellular antibodies are recognized as a potent DAMP as this pathway leads to the upregulation of inflammatory cytokines IL-6 and TNFα[295,198]. While we did not observe significant TNFα in response to exosome IC uptake in our studies, we did observe significant IL-6 upregulation and secretion of protein. Whether or not TRIM may initiate a signalling in response to exosome IC following Fcγ receptor ligation is currently unknown. Not all IgG opsonized viruses are susceptible to TRIM21 mediated degradation. Some virus have evolved to avoid this type of virion degradation, while others including rhinovirus utilize TRIM21 degradation to facilitate intracellular uncoating prior to replication[198]. It is not entirely clear which viruses are the most susceptible to TRIM21 degradation, however enveloped compared to non-enveloped viruses are thought to be more

resistant[198]. Although exosome content differs from viruses, the lipid nature of the exosomes like enveloped viruses might suggest resistance to TRIM21. This raises the point that the unloading of exosome cargo in DC is well understudied. While the initial crosslinking of Fcγ receptors owes to the initial signalling events, unloading of cargo by different pathways in DC will be responsible for secondary signalling events. Given that exosomes contain a bolus of nucleic acids, lipids and proteins (including TAA), a number of different pathways may become activated. The content of these exosomes has the potential to allow for unloading of antigens that may stimulate TLR or pattern recognition receptor signalling. In addition, the nucleic acid content may stimulate cytoplasmic nucleic acid receptors RIG-I or MDA5[193,194]. While many of these pathways are most likely inhibited as a regulatory mechanism to allow for tolerance of these EVs, these pathways may become activated when exosomes are taken up by DC or macrophages in the form of an IC. In addition, of extent of TAA incorporate in exosomes and how these are unloaded in DC degradative pathways will also play a role in activation of T cells. It is already difficult to predict which TAA will be presented by tumors to the immune system. The complexing of exosomes to IgG and the uptake of these IC adds additional level of complexity to predicting TAA presented to the adaptive immune system. Further investigation of innate signalling in DC and macrophages is needed to fully elucidate how the type of immune response initially triggered by exosome IC in DCs becomes translated into $CD8^+$ T cell activation.

DC response to anti-PS IgG exosome ICs varies with antibody subclass and Fcγ receptor expression

The substantial difference in COX-2 and IL-12 gene expression observed between exosome IC formed with monoclonal or polyclonal anti-PS antibodies provides important information about the influence of IgG subclass on DC binding of the IC. Surface expression by flow cytometry along with RNA analysis have previously described Fcγ receptor expression on murine antigen presenting DC and macrophages derived in vitro by culture of bone marrow with GM-CSF and G-CSF respectively[194]. From these studies, high expression of FcγRIII, intermediate expression of FcγRI and RIV and low expression of FcγRII were identified on both monocyte derived DC and macrophages[194]. We confirmed this by examining mRNA expression of Fcγ receptors and

found greater expression of FcγRIII, FcγRIV and low FcγRIIB on both DC and macrophages. This is important because the different Fcγ receptors have different affinities for each IgG subclass. The subclass identity of the monoclonal and polyclonal anti-PS antibodies used in our experiments has not been defined. However, given the substantial difference in expression of COX-2 and IL-12 between the monoclonal and polyclonal anti-PS exosome ICs, we may predict the subclass identity of these IgG antibodies. Generally, IgG antibodies of the 2a and 2b subtype bind Fcγ receptors with a higher affinity and they are capable of binding all Fcγ receptors[193–195]. IgG1 only binds to FcγRIIB and FcγRIII while IgG3 only binds FcγRI although this has been under debate[193–195]. The expression of Fcγ receptors on the cell surface as well as the ratio of activating to inhibitory receptors are not the only factors that will affect the immunostimulatory capacity of IC uptake[193–195,201]. FcγRI and FcγRIV have high affinity binding capacity[193–195,201]. Well opsonized antigen is not required for immunostimulatory signalling as FcγRI and FcγRIV and the intracellular FcRn will bind monomeric IgG[203]. Therefore, the monoclonal antibody in our experiment is most likely of the IgG2 subtype. On the other hand, for the polyclonal anti-pPS antibody, the IgG1 and IgG2 subclasses are most likely both present as they are the most common in serum but IgG1 is expected to be dominant since it is 3-4-fold more abundant than IgG2[296].

Antibodies have also been identified in patients with anti-PPL syndrome[241]. These PPL-specific antibodies of the IgG class are predominantly of the IgG1 and IgG2 subclasses[297]. Like the monoclonal antibody, it is difficult to predict which Fc receptors are preferentially engaged by exosomes complexed with the polyclonal antibody. Given that there is increased expression of immune-stimulatory COX-2 and IL-12 in DC exposed to the polyclonal exosome IC, we predict that the ICs are most predominantly binding to the activating receptors FcγRI, FcγRIII or FcγRIV, rather than FcγRIIB.

Despite the inherent binding affinity of various IgG subtypes to Fcγ receptors, modifications can be made to the Fc region to enhance binding. IgG engineering has already been exploited in monoclonal antibody therapy. It was discovered that the Fc region of IgG contains a conserved amino acid sequence, that allow for glycosylation at specific residue including asparagine 297

(N297)[203]. While antibody based therapies have demonstrated clinical success for a number of cancers, the efficacy of this type of therapy remains limited in subgroups of patients[24]. One explanation for the reduced efficacy of antibody therapy in some patients can be explained by polymorphisms in genes coding for Fcγ receptors[203]. Some polymorphisms have been linked to certain diseases namely SLE[193,194]. Disease states that have been linked to specific polymorphisms have been shown to have decreased inhibitory FcγRIIB function as well and increased binding affinity for IC by activating Fcγ receptors increasing immune stimulatory signalling[195,203]. The outcome of these polymorphisms in the Fcγ receptor genes modulate IgG binding affinity for Fc receptors[203].

The significance of Fcγ receptor polymorphisms has been demonstrated in clinical trials. Two polymorphisms in the FcγRIIIa gene V158 and F158 have been identified to have higher and lower affinity for binding of IgG1 respectively[203]. In a number of clinical trials investigating monoclonal therapeutic antibodies including cetuximab, trastuzumab and rituximab, patients with V158 had better outcome than patients with the F158 polymorphism[298–300]. Many monoclonal antibody therapies were originally designed to inhibit ligation of growth factors with their respective receptor including cetuximab (anti-EGFR) and trastuzumab (anti-human epidermal growth factor receptor 2 – HER2) [298–300]. By the blocking of this receptor-ligand interaction this would inhibit the growth of cells that were overexpressing or had a mutation in these receptors[298–300]. It is now better understood that this is not the only mechanism by which these types of therapies work. Recruitment of NK cells to tumor cells over expressing EGFR or HER2 marked by cetuximab or trastuzumab antibodies respectively enhances anti-tumor immunity through ADCC, cytokine release and further recruitment of neutrophils[193–195]. In addition, IC formation may occur allowing for uptake of opsonized tumor cells by antigen presenting cells. The later can lead to presentation of TAA on DC and stimulation of $CD8^+$ T cells. Immune cells in the tumor microenvironment may also become of target of ADCC following antibody therapy. This is demonstrated with monoclonal antibodies directed towards CTLA-4 and PD-L1[132,155,301,302]. CTLA-4 is expressed on Tregs and has a role in maintaining self-tolerance[140]. CTLA-4 monoclonal antibody therapy has been demonstrated to lead to selective depletion of Treg cells in the tumor microenvironment of mice with antibodies that

contain the appropriate Fc domain to facilitate Fcγ receptor binding[301–304]. Depletion of Treg cells by ADCC has been shown to be largely mediated by the low affinity FcγRIII and FcγRIV receptors restricted to $CD11b^+$ cells[301]. Studies in cancer patients have also shown the reduction in $CD4^+CD25^+CD62L^+$ which may consist of a regulatory subset of T cells[305]. On the contrary, other studies have shown that anti-CTLA-4 antibodies including ipilimumab (IgG1) and tremelimumab (IgG2) do not lead to depletion of Tregs but may impair the suppressive function of Tregs[306,307]. In the case of PD-L1, one study has suggested the ability of an IgG2 antibody subtype to lead to the depletion of myeloid cells expressing high levels of PD-L1 in certain tumor mouse models[155]. Much recognition of the role of Fcγ receptors in contributing to tumor regression has remained largely unrecognized. These studies suggest that the Fc region of antibodies can be further modified to enhance Fcγ mediated depletion of immune inhibitory cell subsets and highlight an area for future research.

Given the multiple roles of the immune system in antibody-based therapy, it is of interest to enhance the immunostimulatory capacity of these therapies in patients. Various IgG Fc engineering methods aim to do just this. There have been IgG mutants that have demonstrated the ability of antibody to enhance complement activation, Fcγ effector function and also the half-life of IgG[203]. The conserved N297 in IgG is glycosylated with N-acetylglucosamine (GlcNAc) and contains additional sugar moieties including fucose, mannose and sialic acid[203]. Glycoengineering of this region by removal of fucose has demonstrated success in enhancing carbohydrate interaction between IgG and Fcγ receptors[203]. Another method involved alteration of amino acid sequence which can be achieved by point mutagenesis[203]. This leads to a change in glycosylation to enhance or decrease IgG binding with Fcγ receptors[203]. Although these methods for modulating IgG affinity for Fcγ receptors is attainable, screening of mutants with the most desirable effects remains a challenge. For example, some triple IgG mutants have demonstrated ability to enhance IgG binding to Fcγ and increase ADCC, while other double mutants have only demonstrated ability of enhancing Fcγ receptor binding of IgG[203]. Much of the work presented in this thesis is suggestive that exosome IC may have the potential to induce immune stimulatory signalling. One area for future direction to consider is mutagenesis of the anti-PS IgG to allow for enhanced binding to Fcγ receptors. Overall, screening of these mutants to achieve efficacy of

antibody based therapy in a greater patient population taking into consideration various polymorphisms is an area with active interest to make the next generation of antibody based therapies.

Anti-PS IgG exosome complexes can prime tumor-specific CD8$^+$ T cells in vitro

The ultimate aim of delivering IgG-complexed exosomes to DCs is to stimulate tumor-targeted CTL. Despite the lack of IL-12 released from DC upon exosome IC stimulation, the cells successfully induced significantly more IFNγ release by tumor-antigen specific CD8$^+$ OT-I T cells than did exosomes alone (**Fig. 3-4F**). This effect was directly attributable to the additional stimulation delivered by Fcγ receptors since T cell activation was abrogated in the presence of Fc receptor blocking conditions. Interestingly, when DC were loaded with exosomes alone, we observed a decrease in IFNγ release by the T cells in comparison to DC that did not receive exosomes at all. This finding is consistent with the literature as exosomes have been demonstrated to supress both innate and adaptive inflammatory immune responses. By co-culturing exosome IC stimulated DC with OT-I T cells we aimed to determine OT-I T cell activation by analysis of IFNγ release. Although the IFNγ detected in this experiment may have been released by the OT-I T cells, our data also suggest that DC are also responsible for secreted IFNγ. The ability of the anti-mPS antibody alone had led to an increase in IFNγ release that occurred without the presence of soluble OVA. This suggests the IFNγ release is not antigen specific and therefore not mediated by OT-I T cells, but rather by the DC themselves. Release of IFNγ by DC is important for the maturation of DC in an autocrine fashion[308]. This could suggest that exosome IC can facilitate the maturation of DC following uptake. This could subsequently enhance presentation of antigen to OT-I T cells, leading to IFNγ release by these cells. Future investigation should focus on determining the source if IFNγ by a means such as intracellular cytokine staining to determine the activation of these immune cells following IC stimulation.

Many studies have reported that tumor derived exosomes inhibit proliferation, induce Treg generation or induce apoptosis in CTL[218]. The inhibition of cytotoxic T cell function by exosomes has been attributed to miRNAs, immunosuppressive cytokines and Fas ligand carried by exosomes[218]. Many groups have attempted to overcome the immunosuppressive nature of

exosomes by changing exosome composition such as miRNAs and transmembrane proteins. Some attempts to alter exosomes have included in vitro modification of the parental cell from which the exosomes are derived using heat shock or transfection with plasmids expressing cytokines IL-2, IL-12 or antigen presenting cell chemoattractants GM-CSF[309]. Other groups have modified surface expression of ligands on the exosome surface[309]. Our data suggests that complexing exosomes to antibodies to generate an IC increases the proinflammatory capacity of exosomes. This technique provides an alternative method of enhancing immunostimulatory capacity that does not require extensive manipulation of exosome in vitro compared to alternative methods. Other methods to increase the immunostimulatory capacity of exosomes require the isolation of patient derived exosome for modification. While this may be necessary, this process remains complicated since all cell types release exosomes. By targeting PS on exosomes and apoptotic bodies with IgG, we aim to overcome this isolation step. This would allow for targeting of exosome to form IC allowing for therapeutic binding to Fcγ receptors and immune stimulatory signalling in patients.

Anti-PS IgG exosomes variably induce tumor-reactive CD8$^+$ T cells in vivo.

Exosomes are particularly attractive from a therapeutic standpoint because they offer the promise of being able to stimulate a cancer patient's own immune system to fight the tumor. Given the ability T cells to release IFNγ following in vitro stimulation with IC pulsed DC we decided to investigate if similar responses could be induced in vivo. We used antigen specific OT-I T cells to focus on examining whether OVA expressing MC38 CRC-derived exosome IC become cross-presented in vivo. Since IC are rapidly cleared following intravenous injection, we first tried a strategy that did not rely on injecting the pure exosome IC. We instead pulsed DC with the exosome IC in vitro prior to injection into the footpad of the mice. CFSE OT-I T cells that had been injected the previous day were harvested from the draining pLN 72 after adoptive transfer of the DC. No difference in T cell proliferation or IFNγ expression was observed when the DC were stimulated with IC, compared to exosomes alone (**Fig. 3-6B**). While some other groups have demonstrated activation of exosome induced anti-tumor immunity, many studies report otherwise inhibitory functions of exosomes. Our in vivo data support other studies that have also demonstrated tumor exosomes inhibiting the activation and maturation of DC showing decreased

expression of MHC II. Our work differentiates from these studies as we complexed exosomes to IgG prior to injection into mice. It has been demonstrated this group that exosomes induced IL-4 and TGFβ expression by DC and reduced T cell IFNγ expression[218]. Other groups have used similar isolation protocols and have created DC vaccines by stimulating the cells with particle isolates that range from 50 – 400 nm, with a similar mean particle size (153 nm) that we have observed in our experiments.

Our experiments did not yield a cytotoxic phenotype, other groups have observed anti-tumor immune responses. Exosomes from melanoma and lymphoma, but not MC38 CRC, cell lines have commonly been investigated in these studies suggesting immune stimulatory capacity of exosomes could be partially dependent on the tumor type from which the EV was derived. One striking difference in our experimental setup is the number of particles used to stimulate DC. Much of the studies done using EV have not reported particle numbers used to stimulate cells. Reporting of this information is becoming more common however, to date, this has posed challenges in the field. Groups that have pulsed DC with exosome commonly utilize 10 μg of exosomes determined by the BCA assay[218]. Although the physiological concentrations of exosome in tissues is poorly understood, we have chosen a much more realistic number of exosomes to stimulate DC with. Based on NanoSight quantification, we estimate stimulating DC with approximately 100 exosomes per cell. Although this a high load of antigen, especially when complexed to antibody, we have taken into account that not all exosomes will come into contact with the DC during the short stimulation time of 5 – 10 min we chose to capture rapid Fcγ receptor signalling. It is also far lower than what would be delivered by 10 μg of exosome protein. The 100 exosomes per DC value we used to stimulate DC can only be determined by NanoSight Nanoparticle Tracking Analysis, as this number of exosomes is not sensitive enough to be detected by the BCA assay as no current standard curve exists to estimate a direct correlation between exosome number and BCA values at such a low protein concentration. We conclude that the exosome:DC ratio we have selected likely recapitulates the interaction of exosome and DC in an in vivo setting, as DC are not he only cell type that will take up exosomes. Even though our experiments may be a more realistic indication of what may happen in vivo, we cannot conclude that cross presentation does not occur. Future studies may require using a greater number of exosomes to determine whether or not exosome IC are capable of

stimulating antigen specific T cell activation in vivo. In addition, future experiments with exosomes derived from cells lines other than the MC38 lines may be useful in determining whether these exosomes may carry immunosuppressive factors compared to other cell derived exosomes.

MC38 CRC cells are widely used by a variety of labs, however public data regarding the genome and transcriptome of these cells is not available. These cells have nonetheless been identified as immunogenic and are widely used a model for studying tumor immunoediting and immunotherapy. Recently, one study evaluated the genetic profile of MC38 cells and identified mutations in the mismatch repair gene *MSH3* and the exonuclease proofreading domain of *DNA polymerase delta* 1 (*POLD1*)[310]. The presence of a mutation in these two DNA repair genes in the MC38 cells may confer a hypermutable phenotype to these cells. This, in turn, could lead to high TAA production that causes these cells to be more responsive to immunotherapy than other murine colorectal carcinoma cell lines. Of the 2743 TAA that were identified in the MC38 cells, 1399 TAA were predicted to bind strongly to MHC H-2Kb[310]. Although genetic divergence will occur during passage of MC38 cells in culture, it is likely that the MC38 cells used in our experiments express similarly abundant TAA. Prediction of TAA incorporation in exosomes has not been examined, however it is likely a fraction of these antigens will be present in exosomes.

Given the inherently high mutational burden of these cells, we examined the ability of MC38 CRC exosome IC to induce DC maturation and activation of T cells in vivo. We investigated priming and activation of tumor antigen specific OT-I T cell in vivo following IV injection of IC or exosome in mice bearing subcutaneous MC38 parent and OVA expressing tumors. In mice that received anti-PS IC exosomes or highly complexed exosomes containing anti-PS, anti-CD9 and anti-CD63, we observed greater CD86 expression in the $IAb^{hi}CD11c^{+}CD11b^{+}CD103^{+}$ DCs isolated from the MC38 OVA tumor (**Fig. 3-7B**). This suggests that IC initiate signalling in DC that can provide the upregulation of the co-stimulatory molecule CD86 on the DC surface to facilitate T cell activation. This was also observed in the $IAb^{hi}CD11c^{-}$ population isolated from the OVA tumor, which should be macrophages but additional markers to confirm macrophage identity were not included in this experiment. Given that we observed differential signalling in DC and macrophages in response to exosome IC (**Fig. 3-5A**), both of these populations

demonstrated the ability to upregulate CD86 costimulatory expression in vivo. While STAT1 signalling has been shown to mediate upregulation of costimulatory signals, NF-κB also contributes to the upregulation of these signals which we only observed to be active in DC[193,194]. This may suggest that NF-κB signalling may not be required for presentation of costimulatory molecules on macrophages. However, it is worth mentioning that given the lack of macrophage markers to identify this cell population, our interpretation of the data could be inaccurate since the $IAb^{hi}CD11c^{-}$ compartment may have DC present. Given the greater expression of CD86 on the DC population, we analyzed activation of the $CD8^{+}$ T cell populations in the tumors. Although we aimed to examine antigen specific $CD8^{+}$ T cells, we were not able to identify the CFSE OT-I adoptively transferred T cells in the tumor or spleen and therefore analyzed the bulk population of $CD8^{+}$ lymphocytes that we reasoned could have been activated by the other non-OVA antigens in the MC38 exosomes. However, we did not observe greater expression of IFNγ by $CD8^{+}$ T cells in mice that had received IC compared to exosomes alone (**Fig. 3-7B**). Since we analyzed these immune cell populations on day 2 post injection of IC, we repeated a similar experiment examining the DC, macrophages and T cells at day 4 post IC injection. At day 4 post IC injection, we did not observe a greater frequency of antigen presenting cells present in the tumor and there was no difference in CD86 expression in DC or macrophages in the mice that received exosome IC as observed from the former experiment analyzed at day 2. This could be due to the number of exosomes used in the experiment. In the earlier experiment, we injected 25 μg of exosome, whereas mice in the latter received 10 μg of exosomes. We decided to change the dose of exosome used in the in vivo experiments for two reasons. The first being that exosomes are inhibitory in nature and fewer exosome may allow us to examine their immune stimulatory capacity as this results in a greater IgG to exosome ratio. Second, since there is variability in expression of markers of activation in the DC and T cells, we increased the number of mice in each group from n = 3 to n = 6. This led to the need for greater exosome production and it is much more feasible to produce enough exosomes for a smaller injection dose with larger groups of mice. When using this smaller dose of exosomes, we did observe greater IFNγ expression in antigen specific T cells isolated from the spleen of mice at day 4 post IC injection that received exosome IC complexes (**Fig. 3-7C**). Given that we examined a later time point following IC injection, this could suggest that IC lead to early DC maturation, following by priming of antigen specific T cells. The lack of increased CD86 expression on DC at day 4 could

suggest that CD86 becomes downregulated. This seems to allow for maximal IFNγ expression at day 4, but also limits T cell over activation.

Here in these in vivo experiments, we were able to isolate antigen specific T cells from the spleen, but not the tumors **(Fig. 3-7C)**. This may suggest that despite activation of these cells in the spleen, the T cells may not have had the capacity to migrate and infiltrate the MC38 OVA tumor. This could be a result of various factors including the tumor microenvironment and the T cells themselves. One major factor that contributes to the lack of antigen specific $CD8^+$ T infiltration could be due to the overall poor vascularization of these MC38 tumors. Earlier it was discussed that disorganization of the tumor vasculature or the presence of inhibitory factors on endothelial cells such as PD-L1 could prevent T cell in filtration[88,91]. While this may be the case for many tumors, our specific model does not seem to allow for much vascularization to occur in the first place. Despite the MSI nature of MC38 cells this does not slow down the growth of these cells in vivo, as the cells form large tumor within 2 weeks following subcutaneous injection. In this model, it does not appear to be adequate time for vascularization as the tumors already have extensive necrosis by this point. While subcutaneous tumor models have the accepted gold standard for studying various cancers, it is recognized that these models are not optimal for examining the tumor microenvironment, especially the immune cells. Previous models of CRC included injection of CRC cells or surgical transplantation of a subcutaneous CRC tumor into the cecum of mice[311]. While this model may better recapitulate the tumor microenvironment of CRC, there are obvious limitations with this model. One major limitation with this model when studying the immune cells is would be inflammation that occurs as a result of injection or surgical transplantation of the CRC cells into the cecum. This would make it difficult to study tumor driven inflammation. More recently a new orthotopic model has tested the use of lower endoscopy in mice to seed cells into the colon. This model has allowed for the successful growth of tumors in vivo which recapitulates the CRC environment. This model may allow for better infiltration of antigen specific $CD8^+$ T cells into a tumor in the colon and we aim to repeat our experiments with exosome IC in this model. The lack of antigen specific $CD8^+$ T infiltration in our subcutaneous model could also be due to the lack of T cell activation in the spleen. This lack of T cell activation may be a result of too many OT-I T cells in competition for priming. While mice that received exosome IC have the greatest IFNγ expression, these cells

may not express the chemokine receptor CXCR3 for trafficking into tumor tissue. Alternatively, the tumor may not express the ligands CXCL9 and CXCL10 to facilitate T cell infiltration into the tumor[88,91]. Our data may suggest that even though DC and T cells may become activated following IC injection, the complex nature the tumor microenvironment may not allow for activation T cells to mediate anti-tumor immune due to complex mechanisms of immune suppression.

Given that exosomes are rapidly cleared from circulation following intravenous injection, we tested an intratumoral injection route for the exosome IC. We further investigated priming and activation of tumor antigen specific OT-I T cell in vivo following IV and IT injection of IC or exosome in mice bearing subcutaneous MC38 parent and OVA expressing tumors. Although we observed a slight increase in the frequency of $CD11c^{+}IAb^{-}$hi DCs in the MC38 OVA tumor of mice that received exosome IC by IV delivery (**Fig. 3-8A**). However, we did not observe a difference in the frequency of $IFN\gamma^{+}CD8^{+}CD3^{+}$ or NK cells in the tumors of mice that received exosome IC by IV or IT route of delivery. Overall, exosomes IC injected at the tumor site did not enhance activation or frequency of immune cells in the tumor beyond that of intravenously injected exosomes. The pharmacokinetics of exosomes injected intratumorally is not well understood however it is likely that the IC will eventually reach circulation and be rapidly cleared by phagocytes, however uptake of IC by tissue resident phagocytes may also occur. Our data suggest that route of IC delivery did not enhance the immuostimulatory capacity of exosome IC, despite the fact the exosomes are cleared rapidly from circulation following delivery intravenously.

Exogenous factors can modify tumor exosome composition

Many ideas have been proposed to alter the immunosuppressive nature of exosomes[309]. Our own data showing initiation of Th1 signaling in DC by exosome ICs that instead yields Th2 cytokine production suggests that the composition of exosomes strongly influences the outcome of their uptake by DC. Ex vivo manipulation of exosomes is possible, however is likely not feasible in a clinical setting. Dietary lipids have a known role in modulating immune cell signalling. Given the lipid nature of exosome membranes, we investigated whether exogenous PUFA could

become incorporated into exosomes and subsequently alter cytokine gene expression in DC that take these particles up. We thus examined the membranes of exosomes from MC38 cells influenced with AA and DHA. Both of these PUFA have been chosen for analysis due to their immune modulating properties. A greater ratio of n-6 to n-3 PUFA can lead to chronic inflammation and increased cancer risk[256]. While metabolism of AA can produce eicosanoid mediators of inflammation, DHA can counteract this effect. The presence of DHA was detected in exosomes, however incorporation of additional DHA into exosomes was minimal in cells grown with DHA **(Fig. 3-9D)**. In comparison, AA was significantly increased in exosomes of cells grown with AA. We used these exosomes to stimulate DC, then analyzed cytokine gene expression. Both AA and DHA are metabolized by COX-2 to form inflammatory eicosanoids[251]. We observed a relative increase in COX2 expression when DC were stimulated with exosomes derived from MC38 cells influenced with AA that occurred in a dose dependent manner. Exosomes from MC38 cells influenced with DHA- and AA-influenced cells induced less IL-6 expression, however no difference in IL-12 expression was observed **(Fig. 3-9E)**. Despite minimal incorporation of DHA into exosomes, these particles were still able to modify IL-6 gene expression, indicating that small changes in exosome composition can have large immune effects. Our studies did not investigate the mechanisms by which lipid modified exosomes alter cytokine gene expression in DC. Future investigation should examine the incorporation of exosome AA and DHA incorporation in the DC cell membrane which may alter cell signalling, metabolism and binding of these lipids to intracellular receptors when may influence gene transcription.

In earlier experiments, we overserved an increase in IL-6 expression in DC that were stimulated with exosome IC. Cytokine signalling networks in CRC have previously focused on immunosuppressive cytokines found in cells in the intestinal lamina propria, however IL-6 has gained new attention[89]. IL-6 is a known inducer of both inflammation and adaptive immunity[89]. The chronic elevated levels of IL-6 in the tumor microenvironment may not lead to tumor-promoting inflammation but instead contributed to anti-tumor immunity. IL-6 is produced by various immune cells in a tumor microenvironment is a major regulator of STAT3 and NF-κb signalling[89]. Although cytokines signalling networks have been most extensively studied in the more common MMR proficient cancers, a unique role of IL-6 has been described for MMR

proteins in CRC cell lines[89]. In MMR, proficient CRC elevated IL-6 has been shown to contribution to tumor promoting inflammation as well as angiogenesis and tumor metastasis[89,312]. On the contrary, treatment of non-transformed colon epithelial cells and MMR proficient CRC cell lines led to the relocalization of the MMR protein *MSH3* from the nucleus to the cytoplasm, leading to more abundant frameshift mutations in these cells[89,313]. Abundant frameshift mutations can generate more immune stimulating TAA, which would be expected to promote anti-tumor $CD8^+$ T cell responses. However, it has not been confirmed whether IL-6 can actually contribute to better antitumor immunity. Currently IL-6 has been identified as an inflammatory cytokine for therapeutic target, as chronic elevation of IL-6 have more tumor promoting properties than inhibiting properties. Early trials of the IL-6 targeting antibody siltuximab have not demonstrated clinical responses[314]. This may be due to the role of IL-6 in stimulating anti-tumor immunity of other cells in the microenvironment. Our data demonstrating that exogenous DHA alters exosomes in a way decreases IL-6 signalling in DC points to a new avenue of therapeutic intervention of this inflammatory cytokine through dietary modification by incorporation of n-3 PUFA. Dietary modification through addition of n-3 PUFA provides the opportunity for modulation of inflammatory cytokines that does not require isolation of exosome and manipulation of these particles in a laboratory setting. Future studies we conduct aim to identify whether the anti-tumor activity initiated by Fcγ receptor uptake of exosome IC can be enhanced though addition of n-3 PUFA to decrease anti-tumor supressing or promoting immune activity.

Conclusion

Exosomes are a newly recognized form of distant cell to cell communication. Despite the small size of these particles, they are carriers of biological messages that have been demonstrated to have a role in tumor progression though the transfer of tumor exosome cargo to recipient cells. Exosomes have a likely role in each hallmark of cancer, therefore using them in a therapeutic capacity requires special attention as the literature points to their capacity to mediate tumor progression through a variety of mechanisms[205]. One mechanism is the ability of exosomes to modulate immune cell signalling in the tumor microenvironment. Exosomes have been shown in many studies to inhibit anti-tumor immunity[214]. This restraint that exosomes place on the immune system adds to the complex number of resistance and suppression mechanisms used by

the tumor to escape immune evasion. Thus, it is of great interest to find ways of altering the immune stimulating capacity of exosomes to shift the tumor microenvironment from an inhibitory one to one where a productive proinflammatory immune response can gain control of tumor growth. This rationale provided the basis for the research in this thesis, which investigated whether the immune stimulating capacity of exosomes could be altered by the addition of exosome targeting IgG.

To do this, we investigated the immunogenic anionic phospholipid PS as a potential antibody target due to its conserved structure in exosomes released by CRC cells. Exosomes incubated with anti-PS antibody formed IC containing both TAAs and Fcγ receptor-stimulating IgG. While exosome IC did modulate proinflammatory signalling in DC and led to the upregulation of inflammatory cytokine genes, we did not observe a consistent pattern of immune modulation by these exosome IC. The complex nature of Th1 and Th2 polarizing cytokines that were found to be upregulated highlight why the complex nature of exosomes needs to be considered. Even though the literature indicates polarization towards Th1 mediated immunity following Fcγ receptor crosslinking, intracellular exosome unloading, and processing of cargo may stimulate pathways that contribute to Th2 signalling. While these complexes may not stimulate sufficient proinflammatory signalling to mediate a durable CTL-mediated anti-tumor response, we did not investigate whether targeting of exosomes with IgG inhibited the ability of these particles to mediate other tumor promoting cell-cell communications within and beyond the immune system. This remains an area of future investigation.

The current therapeutic use of exosome remains limited. The isolation of exosomes from the tissue of interest remains a challenge as all cells release extracellular EVs. In addition, strategies to overcome rapid clearance of potential therapeutic exosomes have not been overcome, which remains a major hurdle in the use of therapeutic exosomes. One mechanism to overcome this would be the development of a DC vaccine with the use of exosomes. Our initial investigations of an exosome IC-DC vaccine did not suggest the ability of these DC to mediate efficient cross priming of antigen specific $CD8^+$ T cells in vivo in the tumor microenvironment despite the fact that we observed T cell activation systemically and in vitro. However, our work in this area remains preliminary. One major avenue of enhancing the potential immune stimulatory capacity

of these exosome ICs is to alter the Fc region of the targeting antibody. This is most easily achieved through point mutagenesis. The ultimate goal would be to form an exosome IC with this mutated anti-PS that would sufficiently enhance binding of the IC to activating Fcγ receptors to induce effective T cell priming. Further work is also needed to investigate whether the nature of specific exosome cargo components is a major factor responsible for the inhibitory nature of exosomes. Finding specific inhibitory factors could shed light on why we did not observe robust T cell activation in CRC despite uptake of IC by activating Fcγ receptors and indicate new ways that this could be achieved.

It is not currently understood whether the lipid content of exosomes can be altered to modify their immune modulating capacity. Here, we provided preliminary evidence that PUFA AA and DHA can become incorporated in exosomes to modulate proinflammatory cytokine gene expression. The ability of these dietary lipids to enable this allows for another avenue of therapeutic exosome modification. We demonstrated that both AA and DHA incorporated into exosomes were able to downregulate IL-6 in DC, while only AA exosomes led to upregulation of COX-2. This strategy would provide a potentially "tunable system" to achieve the desired level of immune modulation by exosomes to initiate anti-tumor immune stimulation without tumor promoting inflammation. Future investigations will allow for combinatorial approaches including mutagenesis of the anti-PS Fc region of the antibody, development of optimal IC and DC vaccine conditions, the incorporation of immunomodulatory lipids or combination with other immunotherapies.

CLOSING REMARKS

The work in this thesis aimed to challenge the traditional notion that exosomes exert immune modulating properties on immune cells that are inhibitory in nature. Though we do not have substantial evidence to indicate that the inhibitory nature of exosomes can be modulated with the addition of immune complexing IgG, our work provides evidence that IgG targeting of exosomes has the potential to stimulate proinflammatory signalling and initiation of anti-tumor immunity through further modifications to the exosome IC. The lack of a clear immune polarizing cytokine signature highlights the complex nature of exosomes and their interaction with cells of the immune system. If anything, these data provide evidence for the fact that exosomes alone are indeed involved in mechanisms of tumor immunosuppression. Further work on modulating the immune stimulatory capacity of exosomes is needed to break this mechanism of tumor immune suppression.

TABLES

Table 1. List of primers used in experiments.

Primers	**Sequence (5'→ 3')**	**Purpose**
IFNβ1 forward	CGTGGGAGATGTCCTCAACT	qRT-PCR
IFNβ1 reverse	AGATCTCTGCTCGGACCACC3	qRT-PCR
IL1β forward	TTCAGGCAGGCAGTATCACTC3	qRT-PCR
IL1β reverse	GAAGGTCCACGGGAAAGACAC3	qRT-PCR
IL6 forward	TAGTCCTTCCTACCCCAATTTCC3	qRT-PCR
IL6 reverse	TTGGTCCTTAGCCACTCCTTC3	qRT-PCR
IL10 forward	GCTCTTACTGACTGGCATGAG3	qRT-PCR
IL10 reverse	CGCAGCTCTAGGAGCATGTG3	qRT-PCR
IL12 forward	ACGAGAGTTGCCTGGCTACTAG	qRT-PCR
IL12 reverse	CCTCATAGATGCTACCAAGGCAC3	qRT-PCR
TNFα forward	CCCTCACACTCAGATCATCTTCT3	qRT-PCR
TNFα reverse	GCTACGACGTGGGCTACAG3	qRT-PCR
TGFβ forward	CTCCCGTGGCTTCTAGTGC	qRT-PCR
TGFβ reverse	GCCTTAGTTTGGACAGGATCTG	qRT-PCR
COX2 forward	TGAGCAACTATTCCAAACCAGC3	qRT-PCR
COX2 reverse	GCACGTAGTCTTCGATCACTATC3	qRT-PCR

Table 2. List of antibodies used in experiments.

Antibody	Fluorophore or secondary	Purpose	Source
CD3	APCcy7 or APC fire	FACS	Biolegend
CD4	PerCpCy5.5	FACS	Biolegend
CD8	APC	FACS	Biolegend
CD9	PE	FACS	Biolegend
CD11b	PeCy7	FACS	Biolegend
CD11c	APCR 700	FACS	Biolegend
CD16/CD32	PE	FACS	Biolegend
CD24	BUV 737	FACS	BDBiosciences
CD44	BUV395	FACS	BDBiosciences
CD63	PerCpCy5.5	FACS	BDBiosciences
CD64	BV786	FACS	BDBiosciences
CD69	PECy7	FACS	Biolegend
CD86	APC or BUV395	FACS	Biolegend or BDBiosciences
CD103	PE	FACS	eBiosciences
F4/80	Alexa 647	FACS	BDBiosciences
H-2Kb	PE		BioLegend
I-Ab	PerCpCy5.5	FACS	Biolegend
IFNγ	PE	FACS	Biolegend
NK1.1	FITC	FACS	BDBiosciences
Ly6c	V450	FACS	BDBiosciences
IgG1 Isotype (rat)	PE	FACS	Biolegend
Phospho-AKT (ser 743)	Anti-rabbit IgG HRP	Western	CST
Phospho-ERK	Anti-rabbit IgG HRP	Western	CST
Phospho-SRC (Tyr 416)	Anti-rabbit IgG HRP	Western	CST

Phospho-ZAP-70 (Tyr 319)/SYK (Tyr 352)	Anti-rabbit IgG HRP	Western	CST
Phospho-STAT1 (Tyr 701)	Anti-mouse IgG HRP	Western	Santa Cruz
Phospho-STAT1 (Ser 727)	Anti-mouse IgG HRP	Western	Santa Cruz
Phospho-STAT3 (Tyr 705)	Anti-rabbit IgG HRP	Western	CST
Phospho-NF-κB p65	Anti-mouse IgG HRP	Western	Santa Cruz
Phospho-PLCγ1 (Tyr 783)	Anti-rabbit IgG HRP	Western	CST
Phospho-PLCγ2 (Tyr759)	Anti-rabbit IgG HRP	Western	CST
Cytokeratin-20	Anti-mouse IgG HRP	Western	
HSP70	Anti-rabbit IgG HRP	Western	CST
Calnexin	Anti-rabbit IgG HRP	Western	Fisher/EMD Millipore
β-actin	Anti-rabbit IgG HRP	Western	CST
Ovalbumin	Anti-rabbit IgG HRP	Western	
Phosphatidylserine Monoclonal (mPS)	-	Immune complex	Millipore Sigma clone 1H6
Phosphatidylserine Polyclonal (pPS)	-	Immune complex	

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