The Interface of Metastatic Tumours and the Immune System

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Abstract

Primary tumours are rarely lethal; instead most cancer deaths are due to the spreading of the tumour to other sites in the body (metastasis). One major pathway of metastatic disease is the migration of tumour cells from the primary tumour to the draining lymph node. In this study, we examined the interaction of tumour cells and their apoptotic vesicles (ApoV) with CD169+ macrophages within the subcapsular sinus of the lymph node using CD169+/− mice. As previously shown by our laboratory, lymphoma-derived microvesicles bind to the CD169 receptor expressed by macrophages. In this study, we have successfully confirmed the binding of melanoma-derived ApoV by CD169. We then established lymph node metastatic models of B16 melanoma using forelimb, ear, and skin tumour implantation. In addition, we have attempted a novel flank abrasion method developed by Dr. Jason Waithman (Telethon Institute for Child Health Research, Australia). We next addressed the importance of CD169-macrophage lymph node barriers on tumour spread and the impact of metastatic disease in the lymph node on the immune response against tumours. Strikingly, tumour progression within the draining lymph node was significantly lower in CD169+/− mice compared to wild-type mice. However, this finding was confined only to our murine subcutaneous melanoma model, and not the intravenous lung metastases model. Furthermore, we investigated the effects of melanoma-derived ApoV on the lung tumour progression. Interestingly, pretreatment of mice with tumour ApoV significantly increased the number of metastatic foci on lungs. This suggests that chemotherapy of tumours may cause the release of ApoV as pro-metastatic agents. To our knowledge, this is the first report of apoptotic cell products enhancing tumour metastasis. Therefore our results provide an important starting point to understand the role of CD169 and tumour vesicles in the anti-tumour response and in preventing metastatic disease.
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<tbody>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>ApoV</td>
<td>Apoptotic Vesicle(s)</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell(s)</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin &amp; Eosin</td>
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<tr>
<td>ID</td>
<td>Intradermal/Intradermally</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous/Intravenously</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-Derived Suppressor Cell(s)</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer (cell)</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous/Subcutaneously</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum-Free Media</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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Chapter 1

Introduction
**1.0 Introduction**

Primary tumours rarely kill patients; instead cancer spread (metastasis) is the most common cause of death. For metastasis to occur, it is believed that metastatic traits may be acquired by host selection pressures. A healthy cell may become tumorigenic when it bypasses its own cell-intrinsic tumour suppression mechanisms. This is likely to be founded by the genomic and epigenomic instabilities, caused by spontaneous or artificial mutations in tumour cells. Transformed cells must then circumvent their surrounding microenvironment which provides the chemical (hypoxia and low pH), physical (interstitial and tensional forces), and biological extrinsic barriers (inhibitory cytokines and immune surveillance). It is believed that tumour spread is facilitated by factors intrinsic to tumour cells, including their ability to evade the anti-tumour immune response, which is one of the most important biological barriers. One potential mechanism of immune suppression is the release of soluble materials from tumour cells. For example, lipid-bound micro-vesicles released by tumour cells have the ability to suppress the anti-tumour immune response to achieve tumour metastasis. The McLellan laboratory has found that these microvesicles bind to a molecule (CD169) expressed by a discrete subset of macrophages within the subcapsular region of the lymph nodes and the marginal zone of the spleen; resulting in immune suppression. The progression of metastatic disease in mice genetically deficient in CD169 has not previously been investigated and was researched in the present study.

**SECTION A: The Immune System and Microvesicles**

**The Immune System**

The mammalian immune system possesses both innate and adaptive immune functions. The innate immune system is the first line of defence against foreign materials in a relatively non-specific manner. As opposed to the adaptive immune system, it does not confer a long lasting immunity. Important cells of the innate immune system include the leukocytes: natural killer cells (NK), neutrophils, and macrophages. NK cells are capable of inducing apoptosis to compromised host cells, such as tumorigenic or intracellularly infected cells, whereas neutrophils function as the first responders to inflammation. Phagocytic cells such as macrophages have the
ability to engulf extracellular pathogens and scavenge cellular debris. Macrophages can also present antigens to cells of the adaptive immune system, however, the most specialised antigen presenting cells (APC) of the immune system are the (weakly phagocytic) dendritic cells (DC).\[8\] On the other hand, the adaptive immune system is comprised of highly specialised cells capable of developing immune memory, known as lymphocytes.\[8\] There are two main types of lymphocytes. B lymphocytes (B cells) are antibody secreting cells and thus play a critical role in humoral immunity, through complement activation, neutralisation, and by enhancing phagocytosis.\[8\] The other lymphocytic cell type are the T cells; which form a major component of the cell-mediated (non-antibody) immunity.\[8\] It is important to note that the immune system is actively guided by cytokines (signalling molecules) released by a wide spectrum of cells, including haematopoietic (bone-marrow derived) and non-haematopoietic immune cells.\[9\] Cytokines can be classified as pro-inflammatory or anti-inflammatory.\[8\] The role of important relevant cytokines, as we shall uncover later, can determine the outcome of an immune response, more particularly the fate of a tumour.

**Mechanisms of Immunological Tolerance**

The mammalian immune system undergoes dynamic changes due to its wide network of interactions between itself, other body systems, and the environment. The end result of any of these interactions may lead to immune activation or immune tolerance. The latter is the mechanism by which the immune system tolerates the body's self-antigens which would otherwise result in an autoimmune disorder.\[8\] It has been suggested that the innate immune system has the ability to differentiate self from non-self antigens via three strategies:\[10\] (i) the recognition of conserved antigens unique to microorganisms not found in the body and thus considered as non-self, (ii) the expression of host gene products as well as host metabolic pathway products that aid in blocking the initiation of an immune response, and (iii) by the detection of abnormal self antigens usually due to a viral infection.

On the other hand, the adaptive immune system has developed a sophisticated series of mechanisms to achieve immune tolerance via two major mechanisms. The first mechanism is central tolerance, which involves the elimination or deactivation of potentially autoreactive
immature lymphocytes. This is executed in the lymphocyte maturation regions of the thymus and bone marrow for T cells and B cells, respectively.\cite{8} Occurring in later stages is the second tolerising mechanism, known as peripheral tolerance.\cite{8} This involves the silencing (anergy induction) of autoreactive mature lymphocytes in regions distinct from the sites of early lymphocyte development, known as the periphery. It is important to note that immune cells, such as T cells, recognise processed antigens, namely peptides. Antigen processing in cells can be via the endogenous pathway where the antigen is processed within the cytoplasm by the proteasome.\cite{11} Peptides are then transported into the endoplasmic reticulum where loading onto the major histocompatibility complex class I (MHC-I) molecules takes place.\cite{8} MHC-I / peptide complexes are then transported in vesicles to the cell surface.\cite{8} APC can also cross-present antigens by which exogenous antigens are taken up, processed, and loaded onto MHC-I.\cite{8} Another class of MHC, found exclusively on APC, is MHC-II.\cite{8} Antigens loaded and presented on MHC-II molecules are usually processed from extracellular sources via phagocytosis or pinocytosis, but may also include a large number of self-antigens expressed within cells.\cite{8}

**T cell Subsets and Tolerance**

There are two major subsets of T cells.\cite{8} T helper cells (Th1 and Th2) express cluster of differentiation 4 (CD4). Th2 cells are able to activate B cells whereas Th1 cells can activate CD8+ T cells, which is the second major subset of T cells.\cite{8,12} CD8+ T cells specialise in direct killing of recognised tumorigenic and intracellularly infected cells by releasing pro-apoptotic factors.\cite{13} The activation of CD8+ T cells, however, requires binding of the surface T cell receptor (TCR) and CD8 molecule to the MHC-I and a co-stimulatory molecule of an APC, respectively.\cite{8} For example, CD28 on the T cell binds with the CD80 'B7' molecule of an APC.\cite{8}

Immature DC are known to extensively capture and present self-antigens to autoreactive T cells, which are then deleted or transformed into T regulatory cells (T-reg).\cite{14} T-reg cells are able to circulate the body and act to induce immune tolerance where appropriate. For example, self-antigen-activated T-reg cells usually express high levels of adhesion molecules (such as LFA-1) and be recruited and adhere to APCs (such as DCs).\cite{15} With this, T-reg cells are able to outcompete autoreactive naïve T cells for their interaction with an APC presenting a self-antigen.
The T-reg cells can then downregulate the CD80 of the APC presenting the self-antigen and/or differentiate to kill the autoreactive T cells.\[^{15}\]

Microvesicles (see next section), such as exosomes and apoptotic vesicles (ApoV), have the ability to promote immune tolerance to their tissue of origin. For example, intercellular transfer of self-antigens from medullary thymic epithelial cells (mTECs) to thymic DC has been previously shown. The mechanism of this transfer potentially involves ApoV or directed exosome traffic.\[^{16}\]

Another important population of cells involved in tolerance is the suppressive myeloid-derived suppressor cells population (MDSC).\[^{17}\] The activation of MDSC is influenced by several factors produced by two groups of cells: activated T cells and tumour cells.\[^{17}\] The former directly activates MDSC while the latter promotes the expansion of MDSC, as well as suppresses the differentiation of mature myeloid cells. Factors released from tumour cells include cytokines, such as interleukin-4 (IL-4) or granulocyte macrophage colony-stimulating factor (GM-CSF), that can enhance myelopoiesis in the bone marrow or other haematopoietic organs, such as the spleen in mice.\[^{18}\] T cell activation with excessive production of interferon-γ (IFN-γ) is one major cause of the MDSC stimulation.\[^{19}\] In addition, MDSC express CD11b\(^+\)Gr-1\(^+\) as a marker, and these cells have been found to proliferate particularly in the marginal zone of the spleen where they can present self- or tumour antigens to CD8\(^+\) T cells.\[^{20}\] CD8\(^+\) T cells can then become tolerised; subsequently enhancing tumour progression. Since tumour-derived ApoV have been found in our laboratory to be captured within the marginal zone of the spleen (i.e. the expansion site for MDSC), these MDSC might have an important role in the potential immunosuppressive tumour ApoV (discussed later).

**The Lymph Nodes**

Although the lymphatic system has many functions such as the absorption and transport of fatty acids and removal of particulate antigens from interstitial fluids,\[^{21}\] its main function is to initiate the adaptive immune response.\[^{8}\] Organs of the lymphoid tissue can be broadly divided into two categories: central lymphoid organs (where the lymphocytes are generated) and peripheral lymphoid organs (where immature lymphocytes are maintained, activated or...
tolerised). When lymphatic vessels converge, they form a highly organised nodal structure known as the lymph node. As seen on Figure 1, the lymph nodes contain compartments that harbour subsets of cells involved in adaptive immunity. For example, the primary lymphoid follicle, paracortical area, and medullary cords contain mostly B cells, T cells, and macrophages, respectively. The lymph node also contains spherical structures named germinal centres, which mature B cells migrate to, and within, extensively proliferate. Below the marginal sinus exists a population of specialised macrophages that express a surface sialoadhesin-CD169 (discussed later; see page 8).

**Figure 1: Organisation of the lymph node.** (Murphy et al. 2007). The different anatomical compartments contain a majority of specialised immune cells making lymph nodes one of the main storage organs for immune cells.

**Microvesicles**

The extracellular space of multicellular organisms is known to contain ions, metabolites, and nutrients. In addition, it is now clear that this space also contains small lipid-bound particles known as vesicles. There are two important types of extracellular vesicles: ApoV (apoptotic vesicles) and exosomes. ApoV are released from apoptotic cells and can be 500-1000 nm in diameter. ApoV termed 'apoptotic bodies' were first described by Kerr in 1972 while he was...
tracking the cell lineage development in a nematode. Since then, numerous studies have been conducted to unravel their function and their influence on the immune system. Bellone et al., have investigated the uptake of apoptotic lymphoma cells by macrophages in vitro. They found that ApoV-derived T cell epitopes (the immunogenic part of an antigen) can be loaded onto the MHC-I surface molecule and be presented by macrophages. ApoV suppressed the co-stimulatory molecule expression on macrophages and responding T cells became tolerant and non-responsive to antigen. Moreover, when cells undergo apoptosis, they induce an immunosuppressive milieu via the release of transforming growth factor-beta (TGF-β; an immunosuppressive cytokine) that is consistently associated with released ApoV. Further evidence for their extensive immune-interaction is given by the finding that ApoV can also transport auto-antigens, as in the case of the autoimmune disorder systemic lupus erythematosus (SLE).

A wide-spectrum of cells release smaller vesicles (40-100 nm) commonly known as exosomes. After their first discovery by electron microscopy in 1981, studies have revealed that exosomes may function as cell-to-cell messengers. Exosomes express a variety of surface molecules such as adhesion molecules, including ICAM-1 and LFA-1, which enable them to attach to other cells, such as T cells and DC. More importantly, exosomes carrying peptide antigens can fuse with cells and, upon degradation, the processed peptides can be loaded and presented on either MHC-I and/or MHC-II molecules of the recipient cell. This suggests that exosomes may have a role in antigen transfer. Some studies have shown that exosomes derived from B cells or DC are also rich in co-stimulatory molecules; hence there is no surprise that they are currently under investigation for their potential use as a tool to enhance antigen delivery for immunisation. However, due to their size, exhaustive purification protocols, and perhaps the type/maturation stage of their parent cell, scientific reports in this area indicate that the potential of exosomes to modify the immune response is controversial, i.e. can be immunosuppressive/stimulatory. For example, immature DC treated with Th2-derived cytokines, such as IL-4 and IL-10, can secrete anti-inflammatory exosomes that can reduce the severity of established arthritis in mice. Contrary to those findings, Hao et al. purified exosomes from non-treated DC and showed that they are capable of directly stimulating CD8 T cells in vitro. In addition, exosomes have the ability to intracellularly transfer oncoproteins, such as gliomas (brain tumours)-derived exosomes, a feature shared by ApoV. The contradictory findings in
literature clearly indicate that the functions of microvesicles have yet to be determined. Nevertheless, their consistent implication in immune manipulation and in facilitating tumour progression is now well established.

The Sialoadhesin CD169

Macrophages are found in variety of solid tissues, including stromal and lymphatic tissues. A distinct population of macrophages residing in the subcapsular sinus of lymph nodes, marginal zones of spleen, and kupffer cells (macrophages) within the liver express a sialoadhesin molecule known as CD169.\[^{41, 42}\] In addition, a low density of CD169\(^+\) macrophages can be found in the lungs (alveolar macrophages) and colon.\[^{43}\] The presence of CD169 can be to act as a putative phagocytic receptor for cellular vesicles.\[^{44}\] This was shown by Schadee-Eestermans \textit{et al.} when they detected the presence of CD169 within each endocytic vesicle in the CD169\(^+\) macrophages residing in the subcapsular sinus of lymph nodes.\[^{44}\] The CD169 molecule binds to sialic-acid containing glycoproteins, with alpha2,3-linked sialic acids as the preferred ligand. Although the binding of sialic acid has low affinity (3mM), linear increases in sialic acid density results in logarithmic increases in avidity.\[^{45}\]

Interestingly, when T cells, B cells, macrophages, DC, and TK-1 (a myeloid leukaemia cell line) were tested for binding against CD169\(^+\) \textit{in vitro}, TK-1 showed the strongest binding.\[^{46}\] This was predictable as when most cells (particularly lymphoma and melanoma cell lines) undergo tumour transformation, one important feature is the increase in sialylation levels at the cell surface.\[^{46, 47}\] The present study (as well as ongoing research in our laboratory) has attempted to unlock the elusive function of the CD169 molecule. Although it was initially thought that CD169 was not involved in direct uptake of antigens or scavenging materials,\[^{48}\] it is now apparent that CD169 plays a vital role in recognition and uptake of antigens.\[^{45}\]
Section B: Tumour biology and the immune system

Mechanisms of Tumour Progression

A tumour can be characterised by its growth stage; there are five stages of tumour growth escalating from localised stages to the spreading to other organs throughout the body (metastasis). Our knowledge concerning the survival of early stage cancers is poor with the possible exception of virally induced tumours, such as HPV-induced cervical cancer. For example, the hypoxia-inducible factor 1α may be detected in HPV infected patients early on, serving as an early prognostic marker for HPV-induced cervical cancer.\[^{49}\] It may therefore be possible that early stage tumours are eliminated by the immune system. However, once metastasis is established, the failure of the immune system's surveillance mechanisms may be implicated.\[^{50}\] This failure may involve the tumorigenic cells outcompeting their (immune system) elimination, or, as studies have recently shown, the ability of tumours to manipulate the immune system.\[^{50}\] For many of these features, it is still to be determined whether this is an inherent process by the transformed (tumour) cells or the result of selection pressure on transformed cells.

Angiogenesis (the growth of new blood vessels) is the mechanism usually involved in embryo development and wound healing.\[^{51}\] It is also a process extensively induced by tumours to enhance their growth progression.\[^{52}\] Cell cycle checkpoints, the important regulators of cell division, are altered and subsequently defective in tumour cells.\[^{53}\] Another mechanism shared by most tumour cells is the evasion of apoptosis.\[^{54}\] The activation of apoptosis is extensively used by immune cells, such as CD8\(^+\) T cells, NK cells, and activated macrophages, secreting the cytokine tumour necrosis factor (TNF).\[^{8}\] Tumour cells can evade apoptosis via escaping the apoptotic-based anti-tumour immune response.\[^{50}\] Figure 2 summarises some possible mechanisms of tumour immune evasion.

In addition to avoiding immune destruction, other important hallmarks exhibited by tumour cells have also been identified. For example, deregulated cellular energetics (e.g. enhanced anaerobic glycolysis),\[^{55, 56}\] sustaining proliferative signalling, and genome instability and mutation.\[^{57}\] The most dangerous type of skin cancer, melanoma, exhibits properties of immune suppression. For example, they have been observed to reconstruct their supportive
connective tissue into lymphoid-like structures that recruit and maintain regulatory cells to promote immune tolerance.\cite{58} Mouse studies involving either the depletion of NK cells or genetically deficient NK effector molecules coincided with an enhanced tumour growth,\cite{59} which indicates that tumours must also find ways to escape the natural NK anti-tumour attack. How tumours are able to convey immune escape may potentially be related to tumour-released microvesicles.

Figure 2: Mechanisms of tumour-mediated immune evasion. (Mapara & Sykes, 2004) (A) Direct deletion of immune effector cell by expression of death-inducing ligands (apoptosis). (B) Direct tolerisation of tumour-reactive T cells. (C) Suppression of tumour-reactive T cells by regulatory T cells. (D) Ignorance of tumour as a result of spatial separation of T and tumour cells. (E) Tolerisation of host T cells by cross-presentation of tumour-derived antigens. Abbreviations: Fas-L, Fas ligand; TGF-β, transforming growth factor beta; DC, dendritic cell; TCR, T-cell receptors; MHC, major histocompatibility complex.
Mechanisms of Tumour Dissemination

One important prognostic marker of tumour metastasis is the migration of tumour cells from the primary tumour to the draining lymph node, which also indicates the extent of tumour spread (Fig.3).\textsuperscript{60, 61} The first lymph node invaded by tumour cells is referred to as the sentinel or draining lymph node. Excision of the primary tumours is usually accompanied by the removal of the sentinel lymph node because the spread of tumour cells from a tumour-containing lymph node is often a prelude to further tumour spread to vital organs.\textsuperscript{62} Tumour cells invading the sentinel lymph node will eventually spread to other lymphatic nodes in a sequential manner, suggesting that this lymphatic dissemination of tumour cells always starts from the closest (draining) to most distal lymph nodes.\textsuperscript{63, 64} If the draining lymph node is not invaded by the tumour-cells, other distal lymph nodes are unlikely to become invaded.\textsuperscript{65}

One of the lymphatic features that facilitates tumour spread is that lymphatic capillaries lack tight interendothelial (cell-to-cell adhesive protein) junctions, as opposed to blood vessels, rendering the lymphatics relatively leaky to tumour spread.\textsuperscript{66} The detachment from the primary site to the blood stream can occur where it preferentially metastasises in vascular regions such as the liver and lungs.\textsuperscript{67} This complex hematogenous dissemination is also a major means of spread for many cancers. It is thought to occur due to the release of angiogenic stimulating factors increasing vascularity, and thus may contribute to facilitating tumour spread.\textsuperscript{68} The peritoneal cavity can also be invaded by tumour cells, such as in the case of epithelial ovarian cancer, a process known as peritoneal dissemination.\textsuperscript{69}

The diagnostic profile of sentinel lymph nodes from melanoma patients almost always show the initial accumulation of tumour cells within the subcapsular sinus of lymph nodes (Fig.4).\textsuperscript{70} The cause of such accumulation still remains to be identified. However, two explanations could potentially explain the phenomenon: either (i) the accumulation is non-specific and due to initial lymphatic drainage to this area, (ii) the subcapsular region is anatomically the first layer of the lymph nodes as well as a relatively lymphocyte-free area for tumour cells to form a focus, or (iii) the CD169 interacts with tumour cells or their vesicles to induce a local environment permissive to tumour invasion and growth.
Figure 3: Invasion of tumour cells into the draining lymph node. In addition to lymphatic dissemination (white arrows), the diagram shows the overall anatomy of a lymph node and the routes of lymph node flow that supplements percolation through the intercellular spaces of the lymphoid parenchyma. B, B cell area of the lymph node; T, T cell area of the lymph node.

Figure 4: Sentinel lymph node biopsy with extensive metastatic foci. (Dadras et al. 2011). The two immunohistochemical stains HMB-45 and Melan-A (A and B respectively) show an accumulation of melanoma cells within the sub-capsular sinus of the right calvicular node. The sections are derived from a metastatic melanoma patient.
Tumour Vesicles and CD169+ Macrophages

Previous studies have shown that a potential mechanism that may induce immune tolerance involves the release of tumour antigen-bearing microvesicles.[31] The McLellan laboratory has recently discovered that exosomes and apoptotic bodies bind to macrophages in the spleen and lymph nodes in a sialic acid-CD169-dependent manner, resulting in peripheral immune tolerance (unpublished data).[6] Figure 5 represents the model by which tumour vesicles are captured by CD169. Previous data from our laboratory of tumour-(lymphoma-) derived vesicles captured by CD169 are represented in Figure 6.

Furthermore, our laboratory also found that the alpha-2,3 linkage of sialic acid to galactose are the predominant carbohydrate ligands present on vesicles that bind to the CD169 on macrophages (unpublished data). However, the identity of these sialylated molecules on the vesicles is yet to be identified and is currently being investigated in our laboratory. This novel finding suggests the potential role of tumour-derived microvesicles to aid tumours in immune escape and thus progression.

Aims of this Project

In order to investigate tumour progression, a reliable mouse model for metastatic melanoma was needed to be established to allow the efficient invasion of (melanoma) tumour cells into the sentinel lymph node. As mentioned before, the effects of CD169 on tumour progression remain to be identified. Thus the second aim was to test the metastasis of melanoma cells in CD169 deficient mice, compared to wild-type mice. The third aim was to test the effects of melanoma tumour vesicles on tumour dissemination, in attempt to reveal any potential therapeutic or prophylactic interventions for metastatic disease.
Figure 5: Schematic diagram of CD169 capture of tumour vesicles. Lymph fluid draining into its lymph node must pass through the subcapsular region. The diagram illustrates a model by which the flow of lymphatic fluid can carry tumour vesicles, generated from the primary tumour site, that are captured by macrophages expressing the sialoadhesin CD169.

Figure 6: Microvesicle capture by CD169+ macrophages within the subcapsular sinus of the lymph node. B6 mice were injected subcutaneously under the forearm with biotinylated (Bio)- lymphoma-derived ApoV. Five minutes after, mice were sacrificed, the draining lymph node (axillary) was removed, sectioned and stained with DAPI (nuclear stain), anti-CD169 (MOMA-1) detected with rat anti-mouse IgG (Alexa-488, green), and streptavidin (Alexa-594, red) to detect ApoV. (A) represents the CD169+ macrophage subpopulation residing within the subcapsular region whereas (B) indicates that ApoV are captured within the subcapsular region; (C) shows a merge of images A and B. Yellow areas indicate co-localisation of CD169 and ApoV. Photo source: McLellan Laboratory.
Chapter 2
Materials and Methods
2.0 Materials and Methods

2.01 Mice

C57BL/6 mice, originally obtained from Jackson Laboratories, were bred at the Hercus Taieri Resource Unit (HTRU), University of Otago, under specified pathogen free conditions. CD169 deficient mice (C57BL/6), originally given by Prof. Paul Crocker - University of Dundee - Scotland, were also bred at the HTRU, University of Otago. Depending on the protocol, mice were euthanised either with CO₂ asphyxiation or a lethal dose of pentobarbital (100 mg/kg) injected in the peritoneal cavity using a 31G needle. Animal studies were approved by the University of Otago Animal Ethics Committee.

2.02 Media and Solutions

Dulbecco's Phosphate Buffered Saline (PBS)

For 1 L: 1 sachet GIBCO PBS (Gibco cat. # 21600-010)

Total volume of 1 L was made up using milli-Q water

pH 7.3

Filter sterilised

10× Tris Buffered Saline (TBS)

For 1 L: 90 g NaCl

12.1 Tris

Total volume of 1 L was made up using milli-Q water

pH 7.6

Filter sterilised
**1× TBS**

For 1 L: 100 ml of 10× TBS

Total volume made up to 1 L with milli-Q water

pH 7.6

Filter sterilised

**RPMI Medium 1640**

For 1 L: 1 sachet RPMI Medium 1640 (Gibco cat. # 31800-022)

2 g NaHCO₃

1/100 penicillin (100 µg/ml) / streptomycin (50 µg/ml) (Gibco cat. # 15140)

55 mM β-mercaptoethanol (Gibco cat. # 21985)

Total volume of 1 L was made up using milli-Q water

pH 7.3

Filter sterilised

**R5 / R10**

For R5/R10: 95% / 90% RPMI Medium 1640

5% / 10% Foetal Calf Serum (FCS) (PAA cat. # A15-101)

**10 % Bovine Serum Albumin (BSA) in PBS**

For 100 ml: 10 g of GIBCO BSA (Gibco cat. # 30063-572)

Total volume made up to 100 ml with milli-Q water

Filter sterilised
Serum-Free Medium (SFM)

For 1 L:
- 489 ml DMEM/F12 (Gibco cat. # 11320)
- 489 ml RPMI-1640 (antibiotic free)
- 10 ml penicillin (100 µg/ml) / streptomycin (50 µg/ml) (Gibco cat. # 15140)
- 10 ml of 10% BSA / PBS (5 µg/ml)
- 1 ml bovine insulin (5 µg/ml) (Sigma cat. # I-5500)
- 1 ml bovine holo transferrin (5 µg/ml) (Sigma cat. # T1283-50MG)
- 100 µl sodium selenite (10 nM) (Sigma cat. # S5261)
- 100 µl Hydrocortisone (50 nM) (Sigma cat. # H0888)
- 16.2 µl of Triiodothyrinine (5 pM) (Sigma cat. # IRMM469-IEA)

Coomassie Blue G-250

0.25% Brilliant Blue G-250
- 40% methanol
- 10% acetic acid
- 49.75% milli-Q water

Coomassie destain

For 1 L:
- 100 ml acetic acid
- 250 ml methanol

Total volume of 1 L was made up using milli-Q water
3-Amino-9-Ethylcarbazole (AEC) substrate:
For 8 ml: 500 µl acetate buffer (1 M, pH 5)
  9 ml milli-Q water
  500 µl AEC stock (10 mg/ml)
  5 µl of 30% H₂O₂

Fekete's fixative solution
For 460 ml: 400 ml 70% ethanol
  20 ml glacial acetic acid
  40 ml 37-40% formalin

Sorenson's buffer
For 1 L: 49.2 ml Na₂HPO₄ (9.5 g/L)
  50.8 ml KH₂PO₄ (9.1 g/L)
  Na₂HPO₄/ KH₂PO₄ solution pH adjusted to 6.8
  900 ml milli-Q water
  Used at a 1/20 dilution
10× Hanks Buffered Salt Solution (HBSS)
For 1 L: 0.48 g Na$_2$HPO$_4$
4 g KCL
0.6 g KH$_2$PO$_4$
80 g NaCl
10 g glucose
Total volume made up to 1 L with milli-Q water
Filter sterilised

1× HBSS
For 1 L: 100 ml 10× HBSS
4.7 ml sodium bicarbonate (7.5%)
Total volume made up to 1 L with milli-Q H$_2$O
pH 7.3-7.4
Filter sterilised

Medium for Fluorescence Activated Cell sorting (FACS)
Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco cat. # 12440)
1/100 ml penicillin (100 µg/ml) / streptomycin (50 µg/ml) (Gibco cat. # 15140)
1/1000 β-mercaptoethanol (55 mM) (Gibco cat. # 21985)
10% FCS
1/1000 DNase I (Roche cat. # 1 284 932)
Cytotoxic drugs

Staurosporine (2 mg/ml) (Sigma cat. # S4400)
Doxorubicin (2 mg/ml) (Dunedin hospital, New Zealand)
Mitoxantrone (2 mg/ml) (EBEWE Pharma, Australia)
Dacarbazine (2.54 mg/ml) (Dunedin hospital, New Zealand)

Ultraculture medium

For 100 ml: 10 ml penicillin (100 µg/ml) / streptomycin (50 µg/ml) (Gibco cat. # 15140)
0.1 ml β-mercaptoethanol (55 mM) (Gibco cat. # 21985)
1 ml 10% BSA / PBS
0.1 ml bovine holo transferrin (5 mg/ml) (Sigma cat. # T1283-50MG)
0.1 ml bovine insulin (5 µg/ml) (Sigma cat. # I-5500) in 12 mM HCl
97.7 ml IMDM (Gibco cat. # 12440)

2.03 Melanoma B16 Cell Lines

The B16.F1 melanoma cell line transfected with full length green fluorescence protein gene (B16.GFP) using a pMIG vector was obtained from Dr. Jason Waithman, Ludwig Institute for Cancer Research, Australia. The B16.F10 melanoma cell line transfected with full length ovalbumin gene (B16.OVA) using a pAc-neo vector was obtained from Drs. Edith Lord and John Frelinger (University of Rochester Cancer Center, USA). The B16.F10 melanoma cell line was obtained from the Malaghan Institute of Medical Research, New Zealand. The cell lines B16.GFP and B16.OVA were selected for transfected cells using fluorescence-activated cell sorting (FACS) and by the addition of 50 µg/ml Geneticin (Gibco Invitrogen), respectively. The cell count was determined via standard microscopic techniques using a haemocytometer. As a starting culture, cells were incubated at a concentration of $1 \times 10^5$ cells/ml in either R5, R10, or SFM at 37˚C with 5% CO₂ within BD 75 cm² cell culture flasks.
2.04 FACS of B16.GFP Cells

Melanoma B16.GFP cells were first filtered through a 70 µm filter then centrifuged at 453 ×g for five minutes. The cells were then resuspended in FACS medium on ice prior to being sorted for GFP expression using the FACSaria (Becton-Dickinson). Melanoma B16.F10 was used as a control. The software FlowJo was used to analysed the data.

2.05 Bone Marrow Derived Dendritic Cells (DC)

Femurs and tibias from naïve C57BL/6 mice were excised and bones freed from flesh using scissors and then placed in a Petri dish. Bones were sterilised in 70% ethanol for two minutes and then washed in PBS in the same Petri dish. A 27 gauge needle was used to flush out bone marrow plugs. Cells were then filtered through a 70 µm filter using warm R10 and were pelleted at 453 ×g for five minutes. Cells were then plated at 5 × 10^6 in 10 ml R10 plus 5% exosome depleted culture supernatant from murine GM-CSF secreting Ag8653 myeloma line in Petri dishes and incubated at 37°C with 5% CO₂. On day three, the cells were fed by the addition of 10 ml of R10 + 5% exosome depleted GM-CSF; whereas on day six, 10 ml of the media was replaced with 10 ml of fresh R10 + 5% vesicle depleted GM-CSF. The semi adherent cells (DC) were harvested between day seven and nine and centrifuged at 453 ×g for five minutes. The pellet was resuspended in sterile PBS.

2.06 DC Stimulation by OVA or Lipopolysaccharide (LPS)

For OVA stimulation, the DC were treated with 0.2 mg/ml of OVA (Worthington cat.# 3054) the day before the cells were harvested. For LPS stimulation, the cells were treated with 200 ng/ml LPS derived from Salmonella Typhimurium (Sigma cat. # L6511-10MG) for six hours prior to their harvest.
2.07 Apoptotic Vesicle Release

The melanoma B16.OVA cells were allowed to grow in SFM or R10 until a 175 cm$^2$ BD cell culture flask was fully confluent (~ 13.6 × 10$^6$ cells). The media was removed to deplete any exosomes that may be present. The cells were then resuspended (by cell scraping) in fresh SFM and were either incubated with 0.25 µM staurosporine or 50 µM doxorubicin. The cells were incubated at room temperature for 30 minutes then left for two nights at 37˚C with 5% CO$_2$.

2.08 Apoptotic Vesicles Purification

The cells were first pelleted at 453 ×g for five minutes. The supernatant was then transferred into a fresh tube and spun at 2000 ×g for 20 minutes to remove any debris. After that, the supernatant was transferred into Nalgene Oakridge centrifuge tubes and spun at 25,000 ×g for 60 minutes at 4˚C. The pellet was then washed with PBS and 5 mM EDTA (to prevent vesicle aggregation) and pooled into one 1.5 ml Eppendorf tube before it is passed through a second spin at 25,000 ×g for 60 minutes at 4˚C. The final pellet was resuspended in 200 µl PBS.

2.09 Determining the Best Mouse Model for Metastatic Melanoma

Mice were divided into three groups receiving 1 × 10$^6$ B16.GFP cells in 25 µl PBS: (i) subcutaneously (SC) under the forearm, (ii) SC within the ear, or (iii) intradermally (ID) in the flank. Mice from group (ii) and (iii) were first anaesthetised using Ketamine (75 mg/kg) and Domitor (1 mg/kg). While under anaesthesia, mice were placed on a heating pad set to 37˚C and mineral oil was applied to protect the eyes. Antisedan (1 mg / kg) was used to reverse Domitor post-tumour implantation. Mice were monitored daily for pain symptoms, severe dehydration and weight loss, then euthanised at day 14 or when they reached primary humane end points, i.e. and maximum tumour diameter of 12 mm, 5 mm, and 14 mm for the SC forearm, SC ear, and ID flank tumour group, respectively. Maximum tumour diameter for SC forelimb and ear tumour groups was reached on day 12, whereas the ID flank tumour group, at day 14. Both appropriate draining and non-draining LN from each group were dissected out from each mouse and sectioned (method 2.12).
2.10 Prophylactic Immunisation and Tumour Challenge

Mice were intravenously (IV) immunised either with $1 \times 10^5$ LPS activated DC-OVA, $1 \times 10^5$ DC-OVA with 50 µg ApoV, 50 µg ApoV, $1 \times 10^5$ DC-OVA, $5 \times 10^4$ DC-OVA, $1 \times 10^4$ DC-OVA, or PBS as a control. Mice were then challenged seven days later with an IV injection of $1 \times 10^5$ B16.OVA cells in 1× HBSS. Mice were monitored daily. as described in method 2.09, then euthanised on day 19 using a lethal dose of pentobarbital (100 mg/kg) injection in the peritoneal cavity. Lungs were excised and metastasis counted (method 2.14).

2.11 Flank Abrasion and Tumour Implantation

This method has been developed by Dr. Jason Waithman (Telethon Institute for Child Health Research, Australia). Mice were anesthetised using Ketamine (75 mg/kg) and Domitor (1 mg/kg). While under anaesthesia, mice were placed on a warm plate set to 37°C and mineral oil was applied to protect the eyes. Flank hair was partially removed and then depilated using Veet® depilation cream. A small (2 mm²) patch of skin is then lightly abraded for 15 seconds using an abrasive patch (15 mm diameter) attached to hand held rotary motor tool ('Dremel' M9400) removing only the keratin and epithelial layers. A drop (10 µl) of $1 \times 10^5$ B16.GFP cells in Matrigel (BD cat. # 356234) was then applied to the abraded area. To contain the inoculum at the abraded site, it was covered with a piece of Op-site™ Flexigrid (second skin). The torsi of the mice were then wrapped with first a soft latex-free tape (hypoallergenic) followed by a strong porous polyethene tape to keep the first tape in place. Mice were then administered with Domitor (1 mg / kg) and allowed to recover. Mice were monitored daily (as described in method 2.09), bandages removed on day six, and sacrificed using CO₂ asphyxiation when maximum tumour size of 200 mm² is reached. Both draining and non-draining LN were then excised and sectioned (method 2.12).

2.12 Tissue Excision and Cryostat Sectioning

The excised tissue was first blotted on filter paper to remove excess fluid. The tissue was then transferred into a plastic mould containing Optimal Cutting Temperature (OCT) freezing media
(SAKURA Finetek, USA). The mould was then filled with OCT after which it was frozen using dry ice. The tissue was then stored for at least 24 hours at -80°C before sectioning. A cryostat (LEICA CM 1850 UV) was used to cut frozen tissue into 6 µm thin sections that were then mounted onto Histobond® adhesion slides (Marienfeld, Germany). Slides were dried for two hours in moving air or overnight at room temperature.

2.13 Modified Stamper-Woodruff Assay

The original Stamper-Woodruff assay[72] was modified and optimised previously in our laboratory (Sauderson, S., PhD). Lymph nodes from either C57BL/6 (wild-type) or CD169− mice were dissected and sectioned as described above (method 2.12). Slides were rinsed in PBS and then blocked with 1% BSA/PBS at room temperature for ten minutes. The following steps were performed in a humid box at room temperature. The lymph node sections are then treated with 50 µg/ml of B16-derived biotinylated apoptotic vesicles diluted in 100 µl of ultraculture for two hours at 37°C. After that, the sections were rinsed with PBS then fixed with 1% paraformaldehyde (PFA) for ten minutes followed by another wash with PBS. Slides were quenched with 100 mM glycine/PBS for ten minutes before being blocked with 1% goat serum/PBS for another ten minutes. The slides were then incubated with 4 µg/ml anti-CD169 (MOMA-1) for one hour. After that, the slides were treated with 1/200 streptavidin (Alexa-594, red), rat-anti-mouse IgG (Alexa-488, green), and 1/50 DAPI (all in 1% goat serum/PBS) in the dark for one hour. After a final rinse with PBS, cover slips were fixed onto the slides with ProLong® Gold antifade reagent (Invitrogen cat. # P36930).

2.14 Metastatic Lung Excision and Fixation

Mice were euthanised with pentobarbital (200 mg/kg) then lungs immediately excised. The lungs were then washed thoroughly with PBS, submerged in Fekete's solution, and left to whiten for at least three days. The Fekete's solution was replaced at least twice during the incubation period. Lung metastases was counted using a 3× magnifying lens. Each black dot was considered as an individual tumour focus.
2.15 Immunofluorescence

Mice were injected with biotinylated apoptotic vesicles (ApoV) (1 mg/ml biotin: 50 µg ApoV) into left forelimb. Five minutes later, axillary and brachial LN were excised, prepared and sectioned as described above (method 2.12). Sections were washed with PBS before being fixed with 1% PFA/PBS for ten minutes followed by a 10 minute incubation in 100 mM glycine/PBS. The following steps were performed in a humid box at room temperature. The sections were blocked with 1% goat serum/PBS for ten minutes prior to being incubated with 4 µg/ml of the primary antibody anti-CD169 (MOMA-1) for one hour. After that, the sections were rinsed with PBS then incubated with secondary antibody; 1/200 streptavidin (Alexa-594, red), rat-anti-mouse IgG (Alexa-488, green), and 1/50 DAPI (all in 1% goat serum/PBS) for one hour in the dark. A final rinse with PBS was given to the slides, then cover slips were fixed onto the slides with ProLong® Gold antifade reagent (Invitrogen cat. # P36930).

2.16 Haematoxylin and Eosin (H&E) Staining

All H&E staining was performed under standard conditions by the Department of Pathology, University of Otago.

2.17 Immunohistochemistry

Frozen lymph nodes were cut into sections using a cryostat, as previously described in method 2.12. Sections were fixed in 25% ethanol / 75% acetone for ten minutes at room temperature then allowed to air dry for ten minutes at room temperature. After that, the slides were rinsed in PBS before being blocked with 1% goat serum / PBS for ten minutes. The following steps were performed in a humid box at room temperature. After the blocking solution was tapped off, primary antibody (mouse αCD45-biotin) at 1 µg/ml in 1% goat serum/PBS was applied and left to incubate for 30 minutes. The antibody IgG2a, κ isotype was used as a control (1 µg/ml in 1% goat serum/PBS). Following that, the slides were washed with 0.05% Tween20/PBS before they were incubated with streptavidin-HRP diluted 1/1000 in 1% goat serum / PBS for 30 minutes. The slides were then washed once with 0.05% Tween20/PBS and once with PBS only before
being treated with AEC substrate for 10-20 minutes. The slides were washed with PBS, counterstained with Meyer's haematoxylin for ten minutes and rinsed with TBS. Glycerol-gelatine was used to mount the slides.

2.18 Cytotoxicity Assay

Each well of a 12-well plate was supplemented with $1 \times 10^5$ B16.F10 in 2 ml R5. After an overnight incubation at 37°C with 5% CO$_2$, the wells were supplemented with either doxorubicin, dacarbazine, or mitoxantrone. The cytotoxic drug treatment was at either 500 µM, 50 µM, or 5 µM. Control wells were incubated with 100 µl PBS. After a 48 hour incubation at 37°C with 5% CO$_2$, the wells were stained with May-Grunwald Giemsa (Section 2.19).

2.19 May-Grunwald Giemsa Staining

The media was gently pipetted out from each well of a 12-well plate. Each well was then flooded with freshly prepared May-Grunwald stain (0.25 g/100 ml methanol; Sigma) and left to incubate for ten minutes. The wells were then washed with distilled H$_2$O for one minute followed by a 10 minute staining with Giemsa stain diluted 1:10 in Sorenson's buffer. The wells were finally washed in distilled water for one minute. The wells were left to air-dry before visualisation using an inverted microscope. Live cell count / well was estimated using the software ImageJ.

2.20 Coomassie Blue Protein Concentration Spot Test

A standard was prepared from 1 mg/ml BSA and diluted in doubling dilutions in PBS. Apoptotic vesicle protein was diluted 1/3, 1/9, and 1/27 in PBS. The standard and apoptotic protein were pipetted onto filter paper in 1 µl spots and allowed to air dry at room temperature. Samples were then flooded with Coomassie Blue and then incubated with Coomassie Destain for 20 minutes on a rocker at room temperature. Protein concentration was estimated by eye comparison to the standard.
2.21 Spectrophotometry

Doxorubicin-derived ApoV (0.45 mg/ml) or doxorubicin standard (0.5 mg/ml in PBS) were added (150 µl) to a well of a standard 96-well black opaque Optiplate (Nunc, Thermo Fischer, Auckland, New Zealand). Both samples were 1/3 serially diluted in 0.5% Triton-X/PBS within the same black plate. Empty wells (containing Triton-X/PBS only) were used as a negative control. Wells were then analysed for doxorubicin fluorescence (excitation/emission 485/600 nm) using a spectrophotometer (TECAN infinite M200).

2.22 Descriptive and Analytical Statistics

Data was analysed using GraphPad Prism®. Results were expressed as means ± SEM and analysed for normality using the Kolmogorov-Smirnov tests and significant differences calculated using unpaired parametric t-tests.
Chapter 3

Results
3.0 Results

3.01 Capture of melanoma-derived ApoV by CD169⁺ macrophages.

The *in vivo* capture of melanoma-derived ApoV by CD169⁺ macrophages in the subcapsular sinus of lymph nodes has been shown before in our laboratory.[⁶] In addition, as mentioned before, our laboratory has found that the CD169 mediated binding was linked to the enrichment of surface sialic acids found on the vesicles (unpublished data). However, when CD169⁻/⁻ mice were SC injected with melanoma-derived ApoV, the vesicles were also captured by the subcapsular sinus of the draining lymph node in a CD169 independent manner (Fig. 7).

![Figure 7: Co-localisation of melanoma B16/F10 ApoV by CD169⁺ macrophages within the subcapsular sinus of the lymph node (Laboratory data). C57BL/6 (WT) (A-C) or CD169⁻ (KO) (D-F) mice were injected SC under the forearm with biotinylated (Bio)- melanoma-derived ApoV. Five minutes after, mice were sacrificed, the draining lymph node (axillary) was removed, sectioned and stained with DAPI (nuclear stain), anti-CD169 (MOMA-1) detected with rat anti-mouse IgG (Alexa-488, green), and streptavidin (Alexa-594, red) to detect ApoV. (A & D) captured ApoV within the subcapsular region; (B & E) CD169⁺ macrophage subpopulation residing within the subcapsular region; (C) and (F) show a merge of images (A & B) and (C & D) respectively. Areas of yellow indicate co-localisation of CD169 and ApoV. Photos were viewed under ×20 objective lens. Results are representative of three repeats.](image)
The CD169-independent capture of ApoV within the subcapsular sinus of the lymph node of a CD169"−/−" mouse may have been due to the fact that when lymph fluid drains into a lymph node it must pass through the subcapsular region first.[73] Thus the collection of ApoV within the subcapsular sinus may just be a result of anatomical constraints within the subcapsular sinus. To show that this is the likely scenario, a modified in vitro (Stamper-Woodruff) binding assay was performed (Fig. 8).

![Figure 8: Direct interaction of B16/F10-derived ApoV with CD169"+" macrophages within the subcapsular sinus of the lymph node.](image)

The fact that the in vitro binding of melanoma-derived ApoV was exclusive to the wild-type strain (Fig. 8) suggests that CD169 mediates the specific binding of ApoV in the subcapsular sinus of lymph nodes.
### 3.02 Establishing a mouse model for metastatic melanoma (pilot study)

In order to determine a possible role for CD169 in tumour metastatic spread, we next tested several mouse models for the generation of reliable metastatic melanoma spread to the lymph nodes. To achieve this, C57BL/6 mice were injected either SC in the forelimb, SC in the ear, or ID in the flank with melanoma B16.GFP cells. To eliminate the possibility of having melanoma cells with low or no expression of GFP, the melanoma B16.GFP cells were first sorted for the highest GFP fluorescence (Fig. 9). Prior to sorting, the B16.GFP cell culture contained a high population of cells that had no GFP expression. This was displayed by the high peak beside the control peak (Fig. 9A). The sorted cells, for very high fluorescence (Fig. 9B) were then injected into each mouse via the appropriate injection route. All mice showed a primary tumour growth within the site injected (Fig. 10). Mice were culled when primary humane end points were reached i.e. when the diameter of the primary tumour reached 12 mm, 5 mm, or 14 mm for the SC forelimb, SC ear, or ID flank tumour group, respectively.

![Figure 9: Melanoma B16.GFP cell sorting.](image)

**Figure 9: Melanoma B16.GFP cell sorting.** The B16.GFP cell line was sorted for GFP fluorescence against B16/F10 as a control (dashed line). Two populations of B16.GFP were sorted; high (A) and very high (B) GFP fluorescence. The sorted ‘high’ and ‘very high’ fluorescent populations are indicated by the arrows. Blue stars indicates the population of cells expressing very low (or no) GFP.
Figure 10: Implantation strategies of melanoma cells in C57BL/6 mice. Mice were injected with $10^6$ B16.GFP either (A) subcutaneously (SC) in the forelimb, (B) SC in the ear, or (C) intradermally (ID) in the flank. Mice were monitored daily and euthanised using CO$_2$ asphyxiation when tumour diameter reached 12 mm (SC forelimb), 5 mm (SC ear), and 14 mm (ID flank). Arrows indicate tumour growth within the site of tumour implantations on day of culling. Pilot results are representatives of six mice/group.

A novel (unpublished) method was recently developed by Dr. Jason Waithman (Telethon Institute for Child Health Research, Australia). This method produces metastasis (i.e. lymph node invasion) at a reported success rate of 100%. This was the flank abrasion method (Fig.11) and involves the inoculation of melanoma cells mixed in Matrigel within an abraded area in the flank (see method 2.11). On day one, mice had caught their feet in the surgical tape and showed symptoms of distress. For ethical reasons, this experiment was terminated immediately.
Figure 11: Melanoma tumour implantation using the abrasion of the flank. This method was developed by Dr. Jason Waithman (Telecom Child Health Institute, Perth, Australia). Mice were anaesthetised with Ketamine/Domitor prior to the surgery. Arrow indicates the abraded site for tumour implantation.

Following euthanasia of the mice, the draining and non-draining lymph nodes from each group were removed (Fig.12). Due to the black appearance of melanin in melanoma cells, lymph nodes that were invaded with melanoma were easily detected. The only exception was the ID flank tumour group which showed low melanin (black) presence. There was also an expansion of the draining lymph nodes’ size compared to the non-draining lymph nodes within the SC tumour groups. This was expected, as in cancer patients, the incidence of lymph node metastases was found to be accompanied by an increase in the size of the invaded lymph nodes.\cite{74}
The draining lymph nodes from all three groups were then sectioned and analysed for GFP fluorescence as an indicator of melanoma B16.GFP invasion. It was found, however, that GFP was downregulated in vivo. Based on current literature, different GFP transgenic melanoma
cell lines (e.g. B16.F1, B16.F10, or B16.F0) exhibit a stable GFP expression in vivo.\textsuperscript{[75-77]} One possible explanation to the GFP downregulation may be due to the instability of the plasmid vector used (pMIG). Another more feasible explanation is that B16.GFP cells have been sorted for very high GFP fluorescence (Fig. 9). High GFP have been found to be toxic to cells,\textsuperscript{[78]} and hence there might have been a selection pressure against GFP expressing cells which may drive a shift for cells to downregulate GFP.

When the lymph node sections were viewed under a light microscope without the use of any cell staining, dark spots were detected. Although these were likely to be melanin, their presence was inconsistent with the melanoma cells within each lymph node (data not shown). Thus alternatively, draining lymph nodes from each mouse group were sectioned and stained using H&E (Fig. 13A-13D). As predicted, both SC forelimb and ear tumour groups exhibited a better melanoma invasion compared to the ID flank tumour group. ID tumour implantations are known to show low invasion.\textsuperscript{[79]} Since 6/6 of mice from the SC forelimb tumour group showed good metastatic models for melanoma, it was concluded to be the best method (Fig. 13E).
Figure 13: Haematoxylin and eosin (H&E) section of metastatic sentinel lymph nodes. C57BL/6 mice were injected with $10^6$ B16.GFP via three various routes (see Fig. 10). Draining and non-draining lymph nodes were dissected, sectioned, and stained with H&E. (A) SC forelimb; axillary, (B) ID flank; inguinal, and (C) SC ear; auricular. Negative control (D) represents an auricular lymph node from a C57BL/6 mouse treated with PBS. Typical melanoma cells are pointed by the black arrows. Sections were visualised under ×20 objective lens. (E) Table showing the number of mice with metastatic draining lymph node(s)/group. Pilot results are representatives of six mice/group.
3.03 Metastasis of cutaneous melanoma in C57BL/6 wild-type and CD169⁻/⁻ mice

The pilot study (see result 3.02) has led to the conclusion that injecting mice (SC) in the forelimb with melanoma B16 cells is the most efficient method to generate a mouse model for metastatic melanoma. Injecting tumour cells SC in ear was unpredictable and some primary tumours exhibited necrosis and vascular invasion. If this was used as a routine method, it could have resulted in additional animal welfare complications. Therefore, the method was not adopted. Instead, the SC forelimb method was applied to test the melanoma metastatic progression between wild-type and CD169⁻/⁻ mice. Due to the fragility of some axillary lymph nodes during cryo-sectioning, caused by high levels of melanoma invasion, draining brachial lymph nodes were examined (Fig. 14A and 14B).
Figure 14A: Metastatic draining brachial lymph nodes in wild-type mice. C57BL/6 wild-type mice were injected SC in the forelimb with $10^6$ B16.GFP and euthanised at day 12 post-injection. Draining brachial lymph nodes were removed, sectioned, and stained with H&E. Photos taken at ×4 objective lens. Each section represents one mouse.
Figure 14B: Metastatic draining brachial lymph nodes in CD169<sup>−/−</sup> mice. CD169<sup>−/−</sup> mice were injected SC in the forelimb with 10<sup>6</sup> B16.GFP and euthanised at day 12 post-injection. Draining brachial lymph nodes were removed, sectioned, and stained with H&E. Photos taken at ×4 objective lens. Each section represents one mouse.
Each section (from Fig. 14A and 14B) was then analysed under ×10 objective lens of a light microscope. The percentage area of melanoma tumour invasion within each lymph node was calculated in proportion to the entire area of the appropriate lymph node using the software ImageJ. Melanoma cells were distinguishable from lymphoid cells in terms of morphology. For example, melanoma tumour cells have a larger cytoplasm compared to lymphoid cells. Another feature of melanoma tumour cells is their low absorption of the haematoxylin stain. In addition, control samples and previous literature showing H&E staining of invaded lymph nodes sections by tumour cells were used as a guide.

Figures 15A and 15B represent the areas invaded by melanoma cells (shaded) within each lymph node (from Fig. 14A and 14B). It is important to note that melanoma invasion within lymph nodes caused a loss of integrity of some sections resulting in suboptimal sectioning.
Figure 15A: Draining brachial lymph nodes in wild-type mice (tumour invasion shaded). C57BL/6 wild-type mice were injected SC in the forelimb with $10^6$ B16.GFP and euthanised at day 12 post-injection. Draining brachial lymph nodes were removed, sectioned, and stained with H&E (see Fig. 14A). Areas of melanoma invasion within lymph nodes sections were shaded. Photos taken at ×4 objective lens. Each section represents one mouse.
Figure 15B: Draining brachial lymph nodes in CD169−/− mice (tumour invasion shaded). CD169−/− mice were injected SC in the forelimb with 10^6 B16.GFP and euthanised at day 12 post-injection. Draining brachial lymph nodes were removed, sectioned, and stained with H&E (see Fig. 14B). Areas of melanoma invasion within lymph nodes sections were shaded. Photos taken at ×4 objective lens. Each section represents one mouse.
To confirm that the tumour margins (Fig. 15A and 15B) were accurately placed, the draining brachial lymph nodes from both strains were stained for the Leukocyte Common Antigen (CD45) using immunohistochemistry (Fig. 16). Despite the fact that some infiltrated CD45+ leukocytes were detected within the shaded area, CD45-negative tumour cells made up the majority of cells found within the previously designated tumour areas. Following an ImageJ analysis using area calculations, it was found that tumour progression to draining lymph nodes was slower in CD169+ mice compared to wild-type mice (Fig. 17).

Figure 16: Immunohistochemical analysis of melanoma invaded draining lymph nodes. C57BL/6 mice were injected SC in the forelimb with 10⁶ B16.GFP and euthanised at day 12 post-injection. Draining brachial lymph nodes were removed, sectioned, and stained with haematoxylin (blue) and anti-CD45 (red). M (melanoma) indicates areas containing the majority of cells that are CD45 negative, whereas I represents areas that are CD45 rich. L represents areas containing lymphoid cells as a majority. The white arrows point to melanoma (CD45 negative) cells. Sections are visualised under ×20 objective lens. Confirmatory results are representative of four mice.
Figure 17: Quantification of melanoma tumour progression in C57BL/6 wild-type and CD169⁻/⁻ mice. Draining brachial lymph nodes from mice immunised with B16.GFP SC in the forelimb were sectioned and stained with H&E. Areas with invaded melanoma tumour cells that were shaded (see Fig. 15 A and 15B) were calculated with respect to the entire associated lymph node area. CD169⁻/⁻ mice show a lower melanoma tumour progression as opposed to wild-type mice. Results are representative of six mice/group.

3.04 Metastatic melanoma progression in the lungs in C57BL/6 wild-type and CD169⁻/⁻ mice

As there was a significant difference in terms of lymph node tumour invasion between the two strains in a cutaneous model, we next determined if similar findings would arise using a different metastatic model. Based on clinical findings, the late stages of melanoma involves the dissemination of melanoma cells into other organs of the body, including the lungs.¹² However, due to the lethality of rapidly growing primary melanomas, it was not possible (or ethical) to prolong experiments until the lungs were invaded by the melanoma cells disseminated from the primary site. However, it is well established that following direct IV injection of melanoma B16 tumour cells in mice, tumour cells preferentially metastasise to the lungs.¹³
The model was first optimised for effective lung dissection and fixation techniques. Fekete's and formalin solutions were tested for their relative efficiency (Fig. 18). In addition to being a preservative, Fekete's solution has 'bleaching' properties mediated by acetic acid, which also lyses erythrocytes in tissues.\cite{84} Efficient whitening of the lung stroma would enable clear identification of melanoma foci on lungs. In contrast to buffered formalin, Fekete's solution maintained good integrity of the lungs with excellent whitening properties (Fig. 18).

When CD169\textsuperscript{+} mice and C57BL/6 mice were compared, only a slight increase in the number of metastases was observed in the CD169\textsuperscript{+} strain. This difference was not statistically significant (Fig. 19).

![Figure 18: Optimisation of the lung fixative.](image.png)

**Figure 18: Optimisation of the lung fixative.** Freshly removed lungs from naïve C57BL/6 mice were treated with either 10\% formalin or Fekete’s solution for two days. Note whitening and superior preservation of lung morphology observed with Fekete’s solution. Anterior (top) and posterior (bottom) views of lungs are shown from a single pilot study.
Figure 19: Metastatic melanoma progression between wild-type and CD169<sup>−/−</sup> mice in lungs. C57BL/6 wild-type (4 mice) or CD169<sup>−/−</sup> mice (4 mice) were injected (IV) with B16.OVA and monitored until euthanasia on day 19 post-tumour challenge. (A) Lungs were excised and fixed in Fekete’s solution for at least two days. (B) Metastases were then counted on each lung using a 3× magnifying lens and the mean number of metastases per set of lungs was plotted. Standard error of the mean (SEM) is represented by the error bars (n=4 mice group). An unpaired parametric t-test was used to analyse the data collected. NS = not significant.

3.05 The effects of ApoV on the anti-tumour response

In order to investigate the effects of ApoV on the anti-tumour response, staurosporine, an inhibitor of several protein kinase C isoforms, was used to induce apoptosis in the B16.OVA melanoma cell line. The OVA-transfected B16 line was used to provide a tumour associated antigen. The experimental plan initially involved immunising mice (IV) with DC pulsed with the tumour antigen ovalbumin (DC-OVA), with or without the presence of ApoV derived from melanoma B16.OVA. Seven days later, the mice were challenged (IV) with melanoma B16.OVA and euthanised on day 19 post-tumour challenge. Initially, there were four groups of mice each receiving a different treatment of either: (i) PBS, (ii) DC-OVA, (iii) DC-OVA/ApoV, and (iv)
ApoV. The aim was to reveal the effects of ApoV (if any) on the anti-tumour immune response directed by the DC-OVA prophylactic treatment.

There were, however, some issues encountered during this protocol. The first issue presented was that the yield of ApoV generated by staurosporine was low. As such, only three mice were able to receive the DC-OVA/ApoV treatment instead of the originally planned four mice per group. Furthermore, due to this severely low ApoV yield, the mouse group receiving a prophylactic treatment of ApoV alone was not able to be tested. The second complication arose when the availability of CD169+/− mice was limited. Thus, comparing the immunising treatments between the wild-type and CD169+/− strain was not possible. The third problem encountered was that mice that received the DC-OVA with ApoV exhibited immediate adverse effects, including paralysis and spasms. The injection of ApoV derived from lymphoma cell lines into mice is regularly performed in our laboratory and there has been no record of such reaction from mice.

Nevertheless, the first prophylactic experiment (Fig. 20) has revealed that the DC therapy was very efficient. Both mouse groups immunised with DC-OVA and DC-OVA/ApoV showed similar results i.e. no tumour load on lungs. It was possible that the potential immunosuppressive effects of the ApoV could not overcome the highly immunostimulatory DC vaccine. Alternatively, ApoV might simply have no effect on the murine anti-tumour immune response.
Figure 20: Effects of ApoV on DC-OVA immunisation against melanoma B16.OVA. C57BL/6 mice were immunised (IV) either with PBS (4 mice), $10^5$ DC-OVA (4 mice), or $10^5$ DC-OVA + 50 µg ApoV derived from B16.OVA (3 mice). On day seven, all mice were challenged (IV) with B16.OVA and monitored until euthanasia on day 19 post-tumour challenge. (A) Lungs were excised and fixed in Fekete’s solution for at least two days. (B) Metastases were then counted on each lung using a 3x magnifying lens and the mean number of metastases per set of lungs was plotted. Standard error of the mean (SEM) is represented by the error bars. An unpaired parametric t-test was used to analyse the data collected. NS = not significant.

To determine if the DC therapy (Fig. 20) was too efficient to block the potential immunosuppressive effects of ApoV, the number of IV injected DC-OVA was titrated using $1 \times 10^5$, $5 \times 10^4$ and $1 \times 10^4$ DC-OVA per mouse. The mice were challenged (IV) with melanoma B16.OVA at day seven and euthanised 19 days post-tumour challenge as previously described in Figure 20. Remarkably, the DC-OVA therapy using only $1 \times 10^4$ DC was still highly efficient, eliciting a strong anti-tumour response against melanoma B16.OVA cells (Fig. 21). Although I did not have time to repeat the ApoV experiments using a lower DC dose, the results strongly suggest that future ApoV inhibition should be carried out using a lower DC-OVA dose than that used in the current experiments.
Figure 21: Effects of DC-OVA titration against melanoma B16.OVA in mice. C57BL/6 mice were immunised (IV) with either PBS (4 mice), $1 \times 10^5$, $5 \times 10^4$, or $1 \times 10^4$ DC-OVA (2 mice per group). On day seven, all mice were challenged (IV) with B16.OVA and monitored until euthanised on day 19 post-tumour challenge. (A) Lungs were excised and fixed in Fekete’s solution for at least two days. (B) Metastases were then counted on each lung using a 3× magnifying lens and the total mean of metastases per set of lungs was plotted. Standard error of the mean (SEM) is represented by the error bars. Due to the limited group size (n=2) in the DC-vaccinated mice groups, statistical analysis was not performed.

3.06 Optimisation of ApoV administration

Due to the low yield of ApoV encountered in the previous experiment (Fig.20), the generation of melanoma-derived ApoV has been optimised. Other clinically relevant cytotoxic drugs were tested for their efficacy to generate the tumour apoptotic vesicles (Fig. 22).
Figure 22: Optimisation of cytotoxicity on melanoma B16.F10 cells. (A) Each well was incubated overnight with $10^5$ melanoma B16/F10 cells before being treated with either dacarbazine, mitoxantrone, or doxorubicin. Each well was treated with a dose of either 500, 50, or 5 µg/ml of the appropriate cytotoxic drug for 48 hours. Control wells were supplemented with PBS. Wells were then stained using May-Grunwald Giemsa staining. (B) Live cell number was estimated using the software ImageJ. Pilot experiment.

Following the cytotoxicity assay (Fig. 22A), both mitoxantrone and doxorubicin exhibited strong cytotoxic effects on melanoma B16.F10 cells. Despite its use as a frontline drug for human melanoma, dacarbazine had almost no cytotoxic effect on melanoma B16.F10 cell. Finally, when the cytotoxic drugs' effects were quantified (Fig. 22B) doxorubicin possessed the greatest cytotoxic effects, inducing complete cell death at 50 µg/ml.

Doxorubicin provided a five-fold higher yield of ApoV, compared to staurosporine (data not shown). However, following administration of ApoV generated from doxorubicin, one mouse showed the similar symptoms observed earlier (see results 3.05). To ensure that contaminating cell culture products (including doxorubicin) were removed, the ApoV were then washed twice with high speed centrifugation (see method 2.08). Washed ApoV showed greatly decreased adverse effects post-administration. To further investigate the cause of the adverse effects, the level of doxorubicin in the ApoV preparations was determined. Since doxorubicin is
fluorescent\textsuperscript{[87]} it was possible to calculate the amount of doxorubicin within ApoV before and after the extra washing. A standard curve was plotted against fluorescence values (ex480/em600) of doxorubicin using a fluorescence spectrophotometer (Fig. 23). Using the GraphPad Prism\textsuperscript{®} software, a cubic spline interpolation was plotted to determine the level of doxorubicin present in ApoV. It was found that the amount of doxorubicin in TritonX-100-lysed ApoV was 1.317 \( \mu \text{g} \pm 0.007 \) SD (pre-wash) and 0.536 \( \mu \text{g} \pm 0.004 \) SD (post-wash) per 50 \( \mu \text{g} \) ApoV injection (n=2). The (IV) LD50 of doxorubicin in mice is reported to be 1.2 mg/kg\textsuperscript{[88]} (approximately 30 \( \mu \text{g} / 25\text{g} \) mouse). Since the weight of mice used in the present study ranged between 20-25 g, the amount of doxorubicin administered was well below 30 \( \mu \text{g} \) and is unlikely to be the sole cause of the adverse effects.

To further investigate the effects of ApoV on the immune system, mice were then administered with extensively washed ApoV. On day seven, mice were challenged with \( 10^5 \) B16.OVA melanoma cells and then euthanised on day 19 post-tumour challenge. ApoV significantly increased number of tumour metastases in C57BL/6 mice. The effect of ApoV on CD169\textsuperscript{+} mice awaits further study.
Figure 24: Effects of melanoma-derived ApoV on metastatic progression in wild-type. C57BL/6 wild-type mice were immunised (IV) either with PBS or 50 µg ApoV derived from B16.OVA (4 mice per group). On day seven, all mice were challenged (IV) with B16.OVA and monitored until euthanasia on day 19 post-tumour challenge. (A) Lungs were excised and fixed in Fekete’s solution. (B) Metastases were then counted and the mean number of lung metastases per mouse plotted. Standard error of the mean (SEM) is represented by the error bars (n=4 mice group). An unpaired parametric Student t-test was used to analyse the data collected.
Chapter 4

Discussion & Conclusion
4.0 Discussion and Conclusion

Although the mechanisms of cellular transformation for tumourogenesis are well understood and much progress has been made in this field,[3, 57] the interface of host factors, including the immune system, with tumours is still poorly understood. In spite of 30 years of research into the immune response to tumours, there has been limited progress in the area of immunotherapy for cancer.[89, 90] In particular, knowledge is lacking in areas such as the identity of clinically relevant tumour-associated antigens, optimal methods for stimulating effective anti-tumour responses, and how to form long lived anti-tumour memory cells.[91-93] In addition, a lack of understanding of the complex interplay between tumour and host factors has delayed progress in developing vaccines for tumours, such as melanoma.[94, 95]

Previous work in this laboratory has identified a potential role of microvesicles and the CD169-vesicle receptor in immune suppression.[6] In this project, I investigated the role of CD169 and microvesicles in lymph nodes and lung metastatic disease, as well as their effect on an anti-tumour vaccination strategy.

In order to achieve my aim in unlocking the effect of CD169 on metastatic progression, I first investigated mouse models for metastatic melanoma. Tumours, for example melanoma, usually form metastases via lymphatic dissemination.[96] Since the subcapsular sinus of lymph nodes contains a high density of the CD169 receptor (Fig. 5 and 6),[46] the aim of the pilot study was to generate a metastatic melanoma model with good lymphatic dissemination to the draining lymph nodes. In classical metastatic melanoma mouse models, resection of the primary tumour and long term housing of the mice (around 6 months), is required for the development of metastatic lymph node disease - similar to that observed in melanoma patients.[97] However, this was not possible to conduct in this study due to time constraints. Moreover, the endpoint of the resection method is usually death.[97, 98] My pilot study showed that the SC forelimb method was the optimal mouse model for metastatic melanoma (Fig 10-13). Our laboratory has previously shown that SC injections of microvesicles in the forelimb results in a good lymphatic drainage.[6] This was also demonstrated by my results with 6/6 mice showing successful invasion of melanoma cells into the brachial and axillary draining lymph nodes. Similarly, the SC tumour ear method also resulted in a good metastatic model and invasion of the auricular draining lymph node. Bobek et al. reported that the SC ear method showed melanoma invasions within various
lymph nodes and, interestingly, the liver and spleen in some mice.[99] Unfortunately for my experimental mouse ear models, primary tumours exhibited necrosis and vascular invasion. Therefore, the method requires careful animal monitoring. However, this method could be optimised perhaps by the incorporation of a gelatinous protein mixture, such as the one used in the flank abrasion method (Matrigel; see method 2.11) to better contain tumour cells at the injection site.

On the other hand, the ID route resulted in poor lymph node infiltration with B16. One possible reason for this might be because ID injections are known to induce a higher degree of local inflammation than that of SC injections.[100] A local inflammation provides a TNF-α-rich local microenvironment which is an important cytokine for DC maturation.[79, 101] Thus, perhaps tumour antigens were presented more effectively to T cells which caused lymphatic dissemination of melanoma cells to be hampered by the primed T cell response.[79] Another potential scenario could be due to the high availability of APC in the cutaneous region, such as Langerhans cells.[79]

Interestingly, when the SC forelimb tumour method was applied to CD169−/− mice, tumour progression was significantly lower than that observed in wild-type mice (Fig. 17). This novel finding could be explained by the 'soil and seed' hypothesis of specific metastatic tumours such as melanoma.[102-104] This hypothesis states that tumour cells/microvesicles act as 'seeds', while the niche they invade acts as the 'soil'. Since there was a similar growth of the primary tumour in both mouse strains (data not shown), we can assume that the difference relates to the metastatic processes, rather than the growth of the primary tumour. One possibility is that exosomes or ApoV released from the tumour are captured and processed by distinct mechanisms in wild-type and CD169−/− mice. Consistent with this hypothesis, it has been previously found that melanoma cells release exosomes to facilitate their invasion into organs, including the sentinel lymph nodes.[5, 105] In addition, melanoma-derived exosomes have the ability to promote endothelial angiogenic responses.[106] Exosomes may carry RNA and other proteins that can potentially elevate tumour growth.[107] Hood et al. showed that exosomes derived from a glioblastoma (aggressive brain tumour) cell line can be endocytosed into recipient cells, carrying their functional RNA which can then be translated using the host cell machinery. In addition to RNA materials, the exosomes also carried proteins. These proteins consisted of angiogenin, IL-6,
IL-8, and vascular endothelial growth factor (VEGF); all of which are known to promote angiogenesis. Furthermore, melanoma-derived exosomes can express the transmembrane Fas ligand (FasL).\textsuperscript{[108]} FasL (member of the TNF family) is a ligand for its receptor FasR (or CD95).\textsuperscript{[8]} A receptor expressed on many mammalian cells including B and T lymphocytes and, when bound with its ligand FasL, initiates cell apoptosis.\textsuperscript{[109]} Thus with the fact that tumour-derived vesicles possess certain tumour growth enhancing characteristics, the difference between the degree of tumour progression between wild-type and CD169\textsuperscript{−} mice (Fig. 17) may be explained in the following potential mechanism:

Tumour-derived microvesicles have been described to express molecular markers characteristic of their parent tumour cells.\textsuperscript{[110]} Among these markers were glycoproteins (possibly sialylated proteins) as identified by Taylor \textit{et al.} It is now clear, however, that tumour cells and their microvesicles contain surface glycoproteins rich in surface sialic acids.\textsuperscript{[111-113]} Melanoma secreted exosomes may travel from the primary tumour site via the afferent lymphatic to the subcapsular sinus of the draining lymph node where they bind (in wild-type mice) avidly to CD169\textsuperscript{+} macrophages in a CD169-sialic acid manner.\textsuperscript{[6]} Some exosomes may be carrying tumour antigens and thus, when endocytosed by CD169\textsuperscript{+} macrophages, will be expressed on MHC-I or -II and their tumour antigens presented to T lymphocytes. It is not clear at this point if antigen presentation by lymph node CD169\textsuperscript{+} macrophages results in immune tolerance or immune stimulation. It is possible that in CD169\textsuperscript{−} mice, microvesicles are accessed by more potent APC deeper within the lymph node cortex, resulting in a more potent anti-tumour immune response.\textsuperscript{[114]}

In contrast to the SC model, no difference was noted in the IV model in terms of metastatic load between wild-type and CD169\textsuperscript{−} mice (Fig. 19). When a mouse is IV injected, the injectate traffics to the right ventricle, then to the pulmonary artery and lungs.\textsuperscript{[115-117]} After that, the content will return to the left ventricle and access the aorta and thus all major non-pulmonary organs, including the liver, spleen and brain.\textsuperscript{[115-117]} It is therefore evident that the IV-administered melanoma cells, or their products, primarily pass through, and will be trapped within, the lung tissue. Nevertheless, it is possible that a subset of melanoma cells could reach the spleen, particularly when immunosurveillance in the lung is compromised.\textsuperscript{[118]} Since the spleen receives IV-administered tumour cells only after they have been filtered through the
vascular beds of the lung, the spleen receives only a limited amount of tumour material after the formation of lung metastases. As opposed to the SC model (Fig. 19), both CD169+/− and wild type mice showed metastases regardless of presence of CD169. This might indicate that in the IV model, interactions of the immune system, in particular CD169+/− macrophages, with tumour or tumour products might be a case of 'too little, too late' and unlikely to influence tumour development.\[115-117\] In contrast, in the SC model, tumour metastasis is delayed and tumour growth and dissemination could be influenced by an immune response generated in the regional lymph node. Furthermore, the intravenous route differs from the subcutaneous route in terms of the role of CD169 in the immune response.\[119, 120\] For example, the Tanaka laboratory injected dead tumour cells either via the IV or SC route. They found that the CD169+ macrophages functioned as immunosuppressors and immunostimulators following the IV and SC immunisation, respectively. In addition, the spleen is involved in the generation of immune tolerance to IV-administered antigens.\[120-123\] Thus there is evidence that the two organs (i.e. lymph nodes and spleen) function in quite different ways.

The late stages of my project focused on the effects of ApoV on the tumour metastases using an IV model. Strikingly, when wild-type mice were administered with ApoV seven days prior to tumour challenge, they exhibited a higher metastatic load (Fig. 24). One possibility is that administration of ApoV alters the susceptibility of the lung for metastatic invasion, e.g. via formation of micro-thrombi. For example, Parish and colleagues showed an essential role for platelets in the formation of melanoma lung metastases.\[118\] Platelet activation plays a major role in the enhancement of thrombi formation.\[124\] When Parish laboratory depleted platelets in mice, melanoma lung metastases were undetectable compared to the control mice.\[118\] Moreover, when they intravenously challenged a platelet-deficient mouse (c-mpl-/-) with melanoma cells, their findings were similar to the platelet-depleted model. Thrombi assist tumour metastasis in various ways; for example, a thrombus can shield tumour cells and protect them from shear stress.\[125\] Alternatively, platelets can release angiogenic factors that promote tumour cells' metastases as well as migration.\[126\] Interestingly, Kopp et al. revealed that platelet-derived TGF-β can directly suppress the NK cell-mediated anti-tumour response.\[127\] Enhanced platelet activation could also explain the immediate adverse effects observed following the IV injection of ApoV (see results 3.04 and 3.06). These adverse effects may be the result of acute respiratory distress syndrome (ARDS) caused by thrombi formation in the vascular beds of the lungs.\[128\] Although platelets
may have contributed to the pro-metastatic response founded by the administered ApoV, the vesicles may have potentially induced immune tolerance via mechanisms discussed below.

Our laboratory has previously found that when administering mice IV with ApoV, the ApoV are captured by the CD169+ macrophages in the marginal zone of the spleen.\[6\] It is known that the marginal zone of spleen is a primary site for the induction of immune tolerance.\[120, 123, 129\] Tolerance may be due to direct death or anergy of tumour antigen reactive lymphocytes that have been stimulated in the absence of co-stimulation by non-professional APCs.\[8, 93, 130\] For example, melanoma tumour cells can lack the expression of co-stimulatory molecules (such as B7) which may lead to T cell anergy.\[131\] Chen et al. transduced the B16 melanoma cell line with the B7 gene (B16-B7).\[93\] When they challenged mice either with B16 or B16-B7 melanoma, the CD8+ T cell immune response only prevented the growth of the B16-B7. Tumour antigen-specific CD8+ T cells can also be deleted or become hyporesponsive when exposed to T-reg cells.\[132\] The augmented number of T-reg cells in melanoma patients\[133\] suggests that tumour cells may secrete factors (e.g. TGF-β) that upregulate their induction \textit{de novo} during an immune response to tumour antigens.\[134\]

ApoV may also act on MDSC to produce the observed immunosuppression / tumour progression. The marginal zone of the spleen is also the expansion site of MDSC.\[17, 18\] MDSC can be activated by numerous factors including IL-10, VEGF, and TGF-β.\[17, 135\] Previous evidence has shown that ApoV derived from tumour cells such as lymphoma can carry TGF-β.\[136\] A study performed by Xie et al. investigated the anti-tumour T cell response with regards to ApoV and exosomes.\[136\] Using EG7 (an ovalbumin-expressing lymphoma cell line) as the source of microvesicles, their findings indicated that ApoV express membrane-bound TGF-β1 and thus suppress CD8+ T cells. In contrast, their study revealed that exosomes enhanced the anti-tumour T cell response; survival in tumour mice studies confirmed these divergent effects of ApoV and exosomes. These results, however, were not convincing; one issue was that the differential ultracentrifugation purification protocols\[36\] for both microvesicle types were identical. Despite the fact that they used irradiation to induce apoptosis, exosomes are naturally secreted into the extracellular environment, i.e. there is a high possibility that the vesicle preparations were not pure. This fact was confirmed by their electron microscopy illustration of exosomes and ApoV by which both appeared identical. In a human study, it was revealed that
serum isolated from melanoma patients contained tumour-exosomes associated with TGF-β.\textsuperscript{[137]} They found that downregulation of endogenous anti-tumour immune response of the patients correlated with high levels of circulating MDSC. Nevertheless, there is still no evidence that melanoma-derived ApoV contain TGF-β or any other MDSC-stimulating factors. It is however likely that such melanoma vesicles possess immunosuppressive factors that increase tumour metastases in the lungs (Fig. 24).

A further potential mechanism might involve NK cell suppression. It is well known that inhibition of lung metastases is greatly dependent on NK cells.\textsuperscript{[138]} Two functions of NK cells have been well characterised: (i) the direct cytotoxic activity and, (ii) IFN-γ production.\textsuperscript{[139]} IFN-γ recruits Th1 cells that, in turn, direct a CD8\textsuperscript{+} T cell response (CTL). Mattes et al. found that CTL responses are only effective against early (non-solid) tumours, as opposed to the Th2 (humoral/antibody producing) response.\textsuperscript{[140]} Thus, NK cells are thought to be important in controlling tumours in their early development. It is now clear that MDSC can induce NK cell anergy through TGF-β.\textsuperscript{[141]} Therefore, the prophylactic administration of ApoV is likely to have impacted on the endogenous anti-tumour response via the upregulation of MDSC division which, in turn, anergised circulating NK cells.

Finally, the dependence of these potential immunosuppressive mechanisms on CD169 remains unknown. A vital future experiment would be to replicate the ApoV / IV metastases experiment (Fig. 24) in a SC setting, both in CD169\textsuperscript{-/-} and wild-type mice.

Our prophylactic DC therapy was shown to be highly efficient; this may explain why there was no difference observed between the DC-OVA alone compared to the ApoV / DC-OVA (Fig. 20 and 21). Even when IV DC-OVA dose was titrated down to 10\textsuperscript{4} cells, highly efficient immune responses were observed, as evidenced by very low metastatic load. As stated in method 2.06, DC were pulsed with the OVA protein, which may result in presentation of OVA antigens on MHC-II and (with cross-presentation) MHC-I.\textsuperscript{[8]} Post-immunisation, DC-OVA would have migrated primarily to the spleen where they present the OVA antigen to naïve CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells.\textsuperscript{[142]} Due to the low expression of MHC molecules on B16,\textsuperscript{[143]} it is unlikely that the primed CD8\textsuperscript{+} T cells were directly responsible for the elimination of the melanoma cells. Instead, it has been shown that elimination of closely associated non-malignant stromal cells by cytokine
secreting lymphocytes can efficiently ablate tumours via NK cell and T cell dependent mechanisms.\textsuperscript{144, 145}

In conclusion, this thesis provides evidence for a role of CD169 in tumour progression. Although chemo- and radiation therapy kills tumour cells, these agents may also cause the release potentially immunosuppressive tumour ApoV. This may explain in part why such standard cancer therapies can fail. Unlocking the mechanisms of the myriad of interactions between tumours and the immune system may allow us to block immunosuppression during chemical and radiation therapy to enhance patient outcomes. This thesis provides an important starting point to understand the role of CD169 and tumour vesicles in the anti-tumour response and in preventing metastatic disease.
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