The Role of Endothelial $\alpha_v\beta_3$ Integrin in Metastasis

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Abstract

The integrin $\alpha_v\beta_3$ is part of a family of membrane proteins with bi-directional signalling and activity. In this way $\alpha_v\beta_3$ plays an extensive and complex role in cancer biology. This study builds on results that show that knocking out $\alpha_v\beta_3$ in endothelial cells reduces cancer metastasis suggesting that endothelial $\alpha_v\beta_3$ plays a pro-metastatic role. In particular, there has been a focus on the pre-metastatic niche, a pro-metastatic environment set up by the primary tumour before metastatic growth. In this vein, changes to pre-metastatic environments have been studied via immune cell and cytokine profiling. To do this FACS and quantitative analysis of immunohistochemistry has been performed. While this study does not bring about a conclusive explanation of the pro-metastatic role of endothelial $\alpha_v\beta_3$, it opens up other areas of research. These include identifying changes in specific myeloid populations such as polarised neutrophils and identifying differences in the endothelial layer at metastatic sites.

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1. Introduction

1.1 Cancer

1.1.1 Cancer

Cancer is a family of diseases characterised by the uncontrolled and inappropriate proliferation of cells. They may form primary tumours and then, with the development of invasive properties, spread throughout the body and invade organs leading to secondary tumour formation. Cancer is the second most common cause of death in the developed world. Its prevalence in these regions (number of people affected at any one time) is 6 million and the mortality (annual number of deaths) is 2.9 million (Globocan, 2012). Costs to national healthcare systems are extremely high; for example, in the UK the cost to the economy is £18.3 billion annually accounting for the cost of health care and loss of productivity (Department of Health, 2015).

There are a number of risk factors that increase the chance of the development of a cancerous tumour, both environmental and genetic. The environmental factors are wide including obesity, alcohol, age and cancer causing substances (National Cancer Institute, 2015). Understanding these risk factors has been an important factor in reducing incidence of preventable cancers. These increase the chance of developing cancerous mutations as do a number of hereditary conditions (which can pre-dispose a person to developing cancer). These are frequently linked to a family history of cancer.

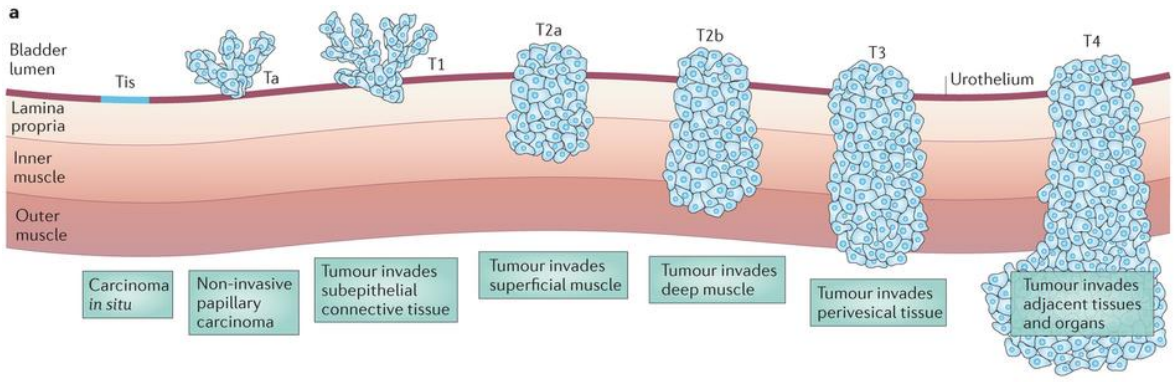

Figure 1: How the tumour develops and invades a tissue as it progresses through the stages of bowel cancer (Knowles and Hurst, 2015)

Cancer is a highly complex disease that has been a focus of research funding for many years. Most (approximately 85%) cancers arise from epithelial cells (Cancer Research UK, 2014) but it can present in a wide range of tissues, organs and cells including the bone marrow. This variety leads to a variable molecular profile and, in clinical practice, several grades of severity. These are scored from grade I to grade IV, the latter being the most invasive and likely to metastasise. In bladder cancer for

example stage I involves the invasion of the epithelial basement layer and in stage II cancer invasion of the muscle is evident. At stage III the cancer invades the perivascular tissue and at the most invasive stage IV the cancer progresses furthest invading new tissues and beginning to metastasise (Knowles and Hurst, 2015). The pathology of these stages is shown in Figure 1.1.

Despite the many complexities and differences in cancers of different stages and different tissue types there are a number of commonalities. These facilitate the switch of cells from normalcy to malignancy and the multi-step process of cancer progression.

1.1.2 Tumour Initiation

It is widely accepted that the origination of a cancerous tumour can be ascribed to mutations in three families of cancer initiating genes. First, the proto-oncogenes (e.g. *c-myc* or ras), where a mutation leads to overexpression of the protein and promotion of cell growth. Second, tumour suppressor genes (e.g. APC), where a mutation of both alleles is necessary to prevent this gene family's negative regulation of cell growth or the genes that control it. Thirdly, in many hereditary cancers, mutations in the DNA repair genes allow an increased probability of mutations in proto-oncogenes and tumour suppressor genes (e.g. the cell cycle repressor Rb) (Tsynes and Bjerkvig, 2007).

These mutations that will result in the gain or loss of function can modify the gene itself or lead to a change in a separate oncogenic or tumour suppressive gene product that influences gene expression. The alterations will affect the activity of a cancer related protein at one of three levels; genomic transcription, protein translation or post translational modifications. Most fundamentally, there can be changes in the gene itself such as base pair deletions or chromosomal translocations that can lead to mutated proteins. The transcription, and consequently the translation, of a gene can also be increased or decreased in a number of ways including changing the activity of transcription factors, such as p53, or epigenetic modifications such as gene silencing. Finally, post-translational modifications such as phosphorylation and ubiquitination by other proteins can modify the function of a cancer related protein so that its signalling pathways are over or under-active (Fernald and Kurokawa, 2013).

With any of these genetic alterations, tumour initiating cells are formed which will then undergo unrestrained clonal expansion, the first step of carcinogenesis. There are some suggestions that these cells have stem like properties (known as cancer stem cells) with the ability to self-renew and differentiate (Medema, 2013). In this linear model of carcinogenesis as the daughter cells are produced, more mutations occur that facilitate the progression of the tumour to malignancy. In 1976, Nowell suggested that, as these daughter cells produce more aggressive phenotypes, they are selected for in an evolutionary process. The mutations and consequent phenotypes that support cancer progression have been researched in great depth.

1.1.3 Cancer Progression

Carcinogenesis is a multi-step progress where a tumour acquires a number of essential characteristics, or “hallmarks” developing intra tumoural heterogeneity. The aggressive phenotype that the daughter cells gain relies on the accumulation of cancer supporting mutations in the tumour cells.

Part of this evolution of the tumour is thought to be enabled by genomic instability which confers an increased mutative potential on the cells. The cause of this emerging cancer supportive mechanism is still debated but the stage and type of cancer has been shown to reflect the underlying cause. The mutator hypothesis, which seems to mainly be associated with hereditary cancers, suggests that mutations in the DNA repair genes, mentioned previously, grants an increased mutation rate (Negrini *et al*., 2010). In non-hereditary cancer, at least at an early stage, the oncogene-induced DNA replication stress model, a form of chromosomal instability, has been proposed (Negrini *et al*., 2010). This hypothesis suggests that the increased replicative potential that oncogenes have upon the cell leads to replicative stress that is characterised by stalled and collapsed replication forks. As the tumour progresses further factors then contribute to genomic instability (Hills and Diffley, 2014).

After the initial growth and mutations, tumours can remain dormant for years due to a balance between proliferation and growth suppressing mechanisms. Overcoming these growth suppressive mechanisms is an essential step on the path to malignancy. Growth suppressing pathways include negative feedback loops that disrupt proliferative signalling, senescence (where cells enter a non-proliferative yet viable state), and pathways of programmed cell death (Hanahan and Weinberg, 2011).

There are three main forms of programmed cell death; programmed necrosis (a form of cell lysis), autophagy (the breakdown of a cell via the fusion of autophagosomes with lysosomes), and apoptosis (Ouyang *et al*., 2012; Hanahan and Weinberg, 2011). The most studied of these is apoptosis, which presents a barrier that must be overcome as a part of multi-step carcinogenesis. The ability to circumvent apoptosis is a possible mechanism by which certain cancers, such as pancreatic ductal adenocarcinoma, can gain resistance to non-surgical therapies (Arlt *et al*., 2013). The constant stresses that carcinogenesis puts upon a tumour cell can lead to the entrance of programmed cell death pathways. These stresses include genomic instability, proliferative signalling and DNA damage (such as that suggested by the oncogene induced DNA replication stress model) (Fernald and Kurokawa, 2013).

Apoptosis is regulated in a number of ways, via stimuli (such as FasL and TRAIL), inhibitors (like the Bcl-2 family), and promoters (such as Bax) (Arlt *et al*., 2013). All of these can be influenced by upregulating or limiting their expression to overcome

apoptosis in the tumour. One of the most common mutations in a tumour, however, is mutation of the transcription factor p53, a tumour suppressor gene that leads to cell cycle arrest and eventually apoptosis as part of the DNA damage response. This gene has been shown to have a particularly significant role when mutated in carcinogenesis by evidence that its presence increases susceptibility to spontaneous tumour formation in *p53*$^{-/+}$ transgenic mice (Lee and Bernstein, 1995). Once these growth suppressors have been disabled the cancer cell gains "replicative immortality" which is proposed as a hallmark of cancer by Hanahan and Weinberg (2011).

When the tumour overcomes the mechanisms that prevent the over-proliferation of cells and reaches this hallmark it must support its excessive proliferation for the tumour to grow. For this the tumour needs an environment rich with growth factor ligands, such as members of the FGF and HGF families, which stimulate the proliferative signalling pathways of the cells. A number of sources can be used to supply these growth factors. One such source is the surrounding stromal cells such as fibroblast and immune cells which support mitogenic tumour signalling in a paracrine manner (Bhowmick *et al*., 2004; Coussens and Werb, 2002). On the other hand, tumour cells can adapt so that they themselves produce ligands to stimulate their proliferative signalling in an autocrine manner (Hanahan and Weinberg, 2011).

A second mechanism that is essential for sustaining cancer proliferation is the development of a tumour vasculature. As the tumour grows it reaches a size where passive diffusion alone is not enough to oxygenate a tumour. At this point, which occurs at a very early stage of tumour development, the formation of a neovasculature associated with the tumour is essential. Without this new network of blood vessels, the tumour cannot grow past 1 – 2 mm^3 so, beyond this, tumour growth is angiogenesis-dependent (Folkman, 1989). The inhibition of tumour angiogenesis has been studied as a potential therapeutic mechanism, and cellular markers of angiogenesis have been investigated as possible therapeutic targets.

There are a number of ways that this new vasculature can be produced including vasculogenesis, and vascular mimicry. Vasculogenesis is the *de novo* formation of blood vessels using endothelial cell progenitors. There is evidence showing that the inhibition of endothelial precursor activity prevents the initial angiogenic response but whether this effect is directly angiogenic, or whether endothelial precursors support angiogenesis in other ways, is debated (Fox *et al*., 2007). On the other hand, vascular mimicry is where tumour cells mimic an endothelial phenotype forming vascular-like vessels (Hendrix, 2015). While there is some evidence for these processes, much remains unclear and vascular remodelling via angiogenic mechanisms remains the most studied and prevalent response in forming a tumour vasculature.

Angiogenesis is the creation of new blood vessels from a pre-existing vasculature. There are two main forms; sprouting where new blood vessels grow off of old vessels in response to stimuli, and intussusception where a pre-existing vessel splits into two (Carmeliet and Jain, 2000). A number of co-ordinated steps are involved in

angiogenesis. The vessel permeability is increased thereby destabilising the vasculature and supporting the prospective remodelling. The basement membrane, a specialised extracellular matrix, is digested facilitating the migration and invasion of endothelial cells. These cells then proliferate in sprouting angiogenesis and form new capillary lumen. In the final stages of angiogenesis pericytes are attracted forming a new basal lamina and the capillaries stabilise and mature (Wong *et al*., 2009; Nishida *et al*., 2006).

Controlling the expression of a number of oncogenes and tumour suppressor genes can cause a pathological state of angiogenesis in the tumour by affecting the expression of angiogenic factors. Examples include Ras, myc and v-raf, which can upregulate angiogenic factors (Fox *et al*., 2007), and p53 which suppresses VEGF (VEGF-A) and increases TSP-1 expression (Liekens *et al*., 2001). Angiogenesis is controlled by the presence of these pro- and anti- angiogenic factors (Liotta *et al*., 1991), regulating this multi-step process at a number of levels. Pro-angiogenic factors include proteases that facilitate the breakdown of the basement membrane (BM), MMP-9 which digests type IV Collagen and MMP-2 which digests laminin, and integrins such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ which regulate endothelial cell interactions with the ECM (Liekens *et al*., 2001).

One of the most significant factors in angiogenesis is VEGF which binds to VEGFR2 on endothelial cells and supports migration, proliferation and survival, as well as inducing vascular leakage (Yancopoulos *et al*., 2000). This factor is produced by most cells but, under the hypoxic conditions frequently prevailing in the tumour, it can be upregulated by tumour associated macrophages, endothelial cells and tumour cells leading to an angiogenic response (Claesson-Welsh and Welsh 2012; Liao and Johnson, 2007). There are also a number of anti-angiogenic factors that negatively regulate angiogenesis including TSP-1 and endostatin, both of which decrease migration and increase apoptosis of endothelial cells (Liekens *et al*., 2001). The balance of these factors determines whether the angiogenic switch is ‘on’ or ‘off’ and the imbalance in the tumour which causes the constant ‘on’ state of the angiogenic switch leads to an abnormal and disorganised vasculature (Liao and Johnson, 2007). This is characterised by excessive permeability, intermittent and chaotic blood flow, a lack of protective pericytes and smooth muscle cells, and a high turnover of vessels (Carmeliet and Jain, 2000; Claesson-Welsh and Welsh, 2012). Such disorganisation in the vasculature means it is a poor oxygen delivery system causing hypoxic regions in the tumour which in turn, leads to persistent production of VEGF (Fox *et al*., 2007). The disorganised vascular also presents a barrier to effective penetration by potential therapeutic molecules.

Another growing field of research is the investigation of the role that infiltrating immune cells, such as tumour associated macrophages and neutrophils, play in supporting angiogenesis. With the VEGF induced increase in vascular permeability there is an increase in the numbers of tumour associated leukocytes (Claesson-Welsh and Welsh, 2012). These cells produce angiogenic factors such as VEGF, MMP-9 and pro-angiogenic chemokines (e.g. CXCL8 and CXCL1) which perpetuate the pro-angiogenic state of the tumour environment (Tazzyman *et al*., 2009).

1.1.4 The Immune System and Cancer

There has been much debate into the significance of the role played by the immune system in tumour development and whether that role is supportive or antagonistic. That there was a connection between cancer and the immune system was brought to the attention of scientists by a number of early observations made by medical scientists. An increased occurrence of cancer was detected at sites of chronic inflammation, and a link was established between disease regression in cancer patients and increased activity of the immune system.

For years, scientists have been attempting experimentally explore the anti-tumoural role that the immune system can play. One such example has been studies into the incidence of tumours in immunocompromised murine models and humans. One experiment used RAG1$^{-/-}$ mice which have compromised antigen organisation and presentation on T and B lymphocytes. These mice had an increased risk of developing MCA induced sarcomas and spontaneous epithelial malignancies in comparison to WT mice (Dunn *et al*., 2002; Prestwich *et al*., 2008). Studies using a mouse model deficient in another subset of lymphocytes, NK cells, also showed a significant increase in the frequency of spontaneous tumours (Prestwich *et al*., 2008). There is also evidence from immunosuppressed transplant patients and immunodeficient patients that the immune system also plays a role in tumour biology in humans. For example, a Nordic study looking at the data of 5000 immunosuppressed kidney transplant patients between 1964 and 1982 showed a 2-fold to 5-fold increase in a number of cancers including lung, colon and prostate and a 30-fold increase in the prevalence of kidney cancer (Prestwich *et al*., 2008). Despite the complication caused by the prevalence of tumours of viral origin, overall such studies have shown the tumour antagonistic role that the immune system can play.

It has been proposed that, as part of these antagonistic activities, the immune system detects and destroys cells undergoing neoplastic transformation in an effort to maintain homeostasis (Mellman *et al*., 2011). Known as the immunosurveillance theory, it was suggested that tumour initiation happens more regularly than the occurrence of detectable tumours would suggest. There appears to be a number of triggers that initiate these mechanisms in the immune system; ‘danger signals’ and tumour antigens (Mellman *et al*., 2011). Endogenous signals, such as RNA/DNA, from stressed or dying cells in states such as hypoxia or necrosis, or cells targeted by NK cells which initiate cell lysis, will alert the immune system (Prestwich *et al*., 2008). This allows the presentation of tumour antigens by antigen presenting cells that will lead to an immune response. Antigens are presented on the cell by membrane proteins such as HLA, in humans, and MHC, in mice (Rosenberg, 2001). They can be tumour specific antigens produced by mutations in genes, for example chromosomal translocations, or by post-translational modifications (Prestwich *et al*., 2008). They can also be normal proteins with an abnormal expression profile, both increased and decreased (Tinn, 2012). Examples of tumour antigens include

MAGE-1 an antigen in testicular cancer, and p53, the expression of which is frequently reduced in tumours (Rosenberg, 2001).

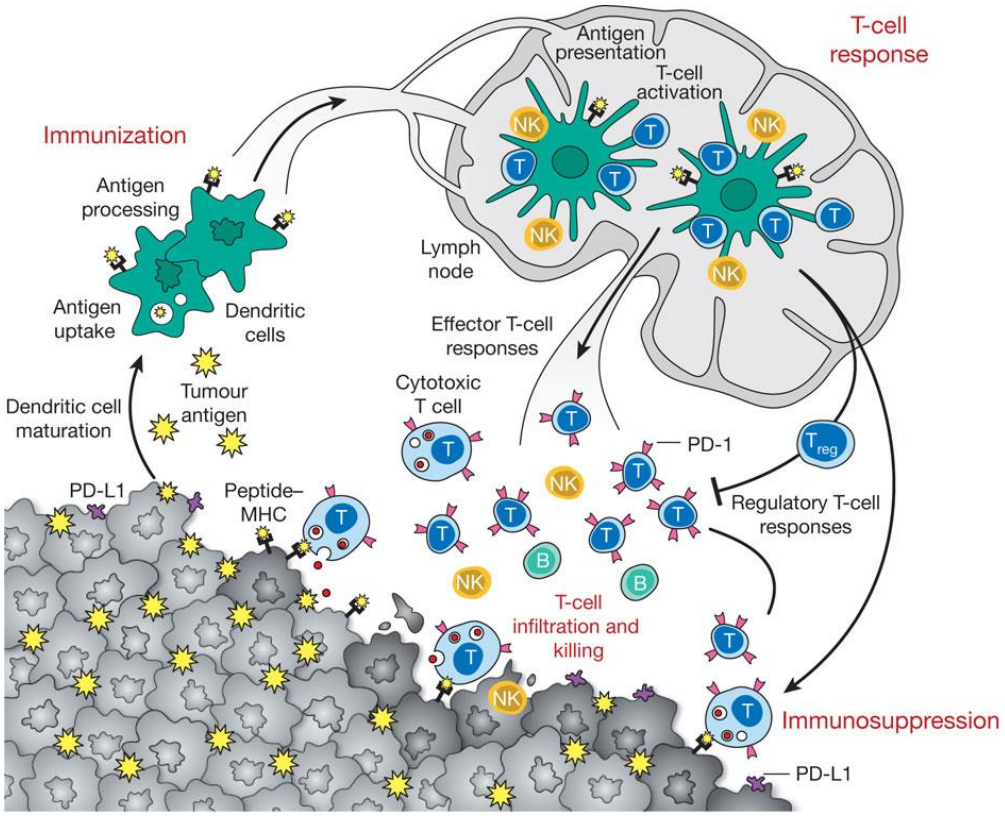

Figure 2: The regulation of the immune system during tumour development. This image shows how anti-tumour immunity can be developed by tumour antigens and the regulation of this response by immunosuppressive cells such as T_{reg} cells (Mellman *et al*., 2011)

The predominant mechanism behind the anti-tumour immune response initiated by these factors is adaptive immunity, much of which is shown in Figure 1.2. This is based on the production of tumour infiltrating lymphocytes with anti-tumour immunity, the presence of which correlates to a better prognosis (Mantovani *et al*., 2008). To generate these, antigen presenting cells (APCs) such as dendritic cells, must be exposed to tumour antigens which they then collect (Krieg and Boyman, 2009). The APCs then present the tumour derived fragments to immature lymphocytes in the lymph nodes such as CD4 T helper cells and CD8 cytotoxic T cells. With the stimulus these cells are activated and undergo clonal expansion before homing to and infiltrating the tumour (Finn, 2012; Mellman *et al*., 2012; Pardoll, 2012). The relevance of such cells in humans was shown in an 11-year study in Japan that followed 3625 individuals predominantly older than 40. They found that higher cytotoxic activity of peripheral blood lymphocytes, assessed by a chromium release assay and immunological markers, was associated with decreased risk of cancer (Imai *et al*., 2000).

This anti-tumour immune response that seems to be in a large part mediated by lymphocytes, is reliant on chemokines and cytokines produced by immune cells.

These membrane bound and secreted proteins play a number of roles in the tumour, including the homing of immune cells, and the stimulation of intracellular signalling pathways that manipulate the activity of both immune and stromal cells. One cytokine that has been of particular scientific interest has been IFN-γ, which is released by T effector cells. This protein exerts an anti-proliferative and pro-apoptotic influence on tumour cells as demonstrated in IFN-γ KO mice which have been shown to have an increased risk of both spontaneous and chemically induce tumours in comparison to WT mice (Prestwich *et al*., 2008; Dunn *et al*., 2002). IFN-γ also stimulates the expression of CXCL-9, -10, and -11 by T-lymphocytes which also support anti-tumour immunity by facilitating the homing of lymphocytes and exerting angiostatic effects on endothelial cells (Liu *et al*., 2011). Chemokines play an important part in immunity by directing immune cells that express specific receptor to sites of inflammation. One example is CCL21 which attracts leukocytes via its receptor CCR7 and plays a part in inducing T effector cell infiltration (Krieg and Boyman, 2009). Understanding how this process of anti-tumour immunity works has been important in the development of potential therapies.

Overall, it is the immunogenicity of a tumour that will determine the success of the immune response in combating the malignancy. Based on this concept there have been a number of attempts to harness these elements of the immune system to develop cancer immunotherapies. The idea that the immune system could be harnessed for clinical benefit was proposed years before the theory of immunosurveillance in the 1890s by William Coley, a surgeon. He carried out a procedure, known as Coley's toxin treatment, in which he injected patients with attenuated strains of *Streptococcus pyogenes* (Mellman *et al*., 2011). Though inconsistent results have been obtained using this treatment, it marked the beginning of a long struggle to use the immune system against cancers. Cancer immunotherapy has been approached from a number of angles; passive, where a pre-existing and active immune response is enhanced, and active, where components of the immune system are activated.

One example of passive immunotherapy is the antibody rituximab which increases B cell lymphoma identification by components of immune system (Finn, 2012). Another is the use of vaccines such as peptide vaccine based on tumour antigens. These can be combined with cytokines such as IL-2 to increase their effectiveness (Rosenberg, 2001). Cytokines can also be taken advantage of by active immunotherapies such as the intra-tumoural injections of gene modified dendritic cells that overexpress active CCL21. This therapy has gone through Phase 1 pre-clinical trials for non-small lung cell carcinoma and has been shown to induce $CD8^+$ T cell infiltration (Lee *et al*., 2014). Another cell-based method of immunotherapy is the exposure of immune cells (such as dendritic cells) to tumour antigens *ex vivo* and the re-injection of these tumour targeting cells into patient (Krieg and Boyman, 2009). A Phase I/IIa clinical study exposed autologous dendritic cells to multiple tumour associated antigens, including MAGE-1, left 9 of 12 patients who had undergone previous clinical treatment tumour free up to 24 weeks and increased the tumour-specific immune response (Lee *et al*., 2015). One example of success in immunotherapy has been the preventative human papilloma virus vaccines based

on the E6/E7 antigens (Rosenberg, 2001), which are available on the NHS. These target the oncogenic virus rather than harnessing the immune system against the cancer itself. Overall there has been limited success but with increased understanding of the relationship between cancer and the immune system the number of clinical trials of immunotherapies is now rapidly increasing. Indeed, several novel drugs with immune targets have recently been approved for clinical use (Kwek *et al*., 2012; Schmidt, 2015).

It has now been shown that the interaction between tumours cells and the immune system is not simple, and that tumours can in fact respond to challenge and evolve to evade destruction. According to immunoediting hypothesis, there are three stages that a cancer will go through in its relationship with the surveillance components of the immune system; elimination, equilibrium and escape. Elimination is the anti-tumorigenic behaviours of the immune system shown above. In equilibrium the tumour goes through cycles of growth and destruction by the immune system. Finally, escape is where the tumour enters a state where it avoids destruction by the immune system (Dunn *et al*., 2002).

The destruction of tumour cells that is associated with elimination and equilibrium exerts a selective pressure on cancer cells that enables them to enter into a new relationship with the immune system known as escape. There are a number of genetic changes to tumour cells themselves that will enable them to avoid immune surveillance. These include the downregulation of antigen presenting receptors, and suppressing the activity of signalling pathways that enable immune destruction such as impaired perforin binding or downregulating the Fas receptor. Tumours may reduce stimulatory signals such as danger signals and co-stimulatory molecules so that the immune response is attenuated (Prestwich *et al*., 2008; Rosenberg, 2001). A number of immunosuppressive cytokines, such as TGFB and IL10, can directly decrease the activity of immune cells by inhibiting the function and activation of anti-tumour cells (Krieg and Boyman, 2009; Coussens and Werb, 2002). These factors can be produced by tumour cells or by cellular components of the immune cell that regulate the inflammatory response. Examples of such cells include regulatory T cells, which interfere with effector T cell function and produce immunosuppressive factors, myeloid derived suppressor cells which suppress the cytotoxic activity of $CD8^+$ T and NK cells, and tumour associated macrophages (Mantovani *et al*., 2008; Krieg and Boyman, 2009; Prestwich *et al*., 2008).

Part of this anti-inflammatory response, is the hijacking of mechanisms that are essential in preventing auto-immune diseases. Other examples of homeostatic regulatory proteins involved in cancer immune escape are the immune checkpoint proteins. In tumour biology PD1, found on T cells, binds to the PDL-1 ligand, expressed by untransformed epithelial cells and by tumour cells, forming a complex. It is known that this inhibits lymphocytic cell function and hides tumour cells from the immune system, though the intricacies of this mechanism are still unclear (Schmidt, 2015; Pardoll, 2012). While PD1 regulates effector T cell activity, other immune checkpoint proteins regulate their activation. On such protein is CTLA4, which plays a part in co-stimulation of T cells by antigen presenting cells and dampens the

tumour specific T cell response. T lymphocytes are activated by APCs by a co-stimulatory mechanism; TCR recognises its cognate antigen and CD28 binds to CD80/86 amplifying TCR signalling. Upon T cell activation by APCs CTLA4 is transcribed and expressed on the surface of the cell at sites of APC/T cell engagement. This protein also binds to CD80/86 and with a greater affinity thereby dampening the activation response (Kwek *et al*., 2012; Pardoll, 2012). The discovery of these has brought about a number of cancer immunotherapies that enhance the immune response by overcoming immune checkpoints. Examples include ipilimumab (CTLA4) and nivolumab (PD1), both of which have been approved by the FDA for clinical use (Schmidt, 2015).

As with the anti-tumour components of the immune system, tumour supportive inflammation does not only help to control anti-cancer inflammation but can directly influence the steps in the progression of a tumour described earlier. The increased incidence of colon cancer in chronic inflammatory colon diseases such as Crohn's Disease and Chronic Ulcerative Colitis is one example of evidence that supports this (Coussens and Werb, 2002). A particularly well studied constituent of pro-tumorigenic inflammation, mentioned earlier, is tumour associated macrophages (TAMs). The polarisation of macrophages to TAMs is induced by a number of cytokines including IL-10, produced by tumour cells, tumour associated stromal cells and T regulatory cells (Mantovani *et al*., 2008). Apart from suppressing the adaptive immune response these cells can promote tumour development by the production of pro-tumorigenic cytokines. These include MMPs which support tissue remodelling, VEGF which is plays an important role in angiogenesis, and growth factors that stimulate cancer growth. Finally, TAMs have been shown to increase the metastatic potential of a cancer (Noy and Pollard, 2014).

1.1.5 Metastasis

It is not primary tumours that cause the majority of cancer deaths; rather it is the metastatic burden which results in 90% of cancer mortalities (van Zijl *et al*., 2011). As the tumour proceeds down the road of carcinogenesis it develops the hallmarks and mechanisms that support its proliferation. The hallmarks described previously are frequently shared with benign tumours, but the ability of a cancer to invade new organs and metastasise is not, making this the most definitive characteristic of malignancy. The complexity of the metastatic process, which involves many steps, means that only a small proportion of cells metastasise successfully, yet metastases are established as the numbers of malignant cells involved is so large.

Metastasis, leading to secondary tumour formation, occurs through three main processes; invasion, intravasation and extravasation and the steps involved in these processes are broken down in Figure .3. The first mechanism involved in metastasis is the dissemination of cells from the primary tumour by changes to the adhesive properties of tumour cells. This increases their motility and allows their migration to a transport system. The basement membrane and extracellular matrix surrounding the system is invaded so that the cells enter a circulatory system, either the lymphatic or vascular system. Upon entering the circulatory system cancer cells evade the immune system until they reach a site where they can bind to the endothelium. At this point a secondary tissue is invaded where the metastatic cells will proliferate and set up a secondary tumour. This metastatic process has been dubbed the metastatic cascade.

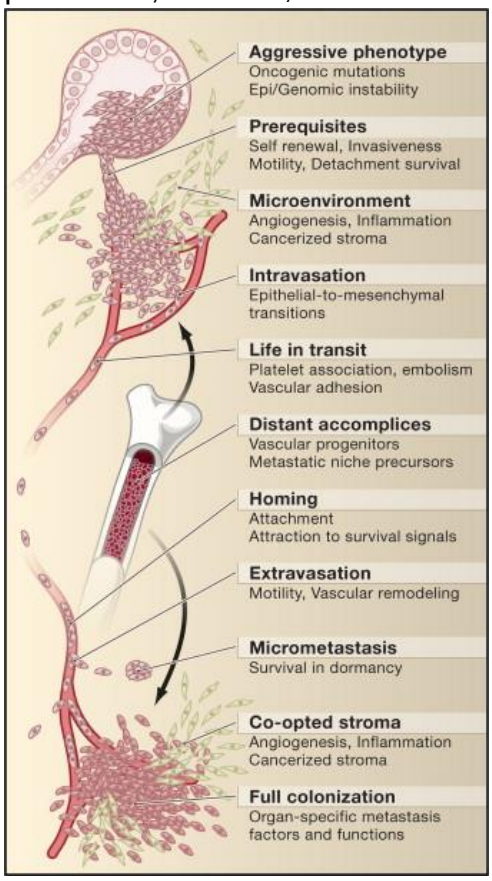

Figure 3: The steps of the metastatic cascade and the processes involved in each of these steps (Gupta and Massagué, 2006)

There are a number of factors that will cause a transformation to a metastatic phenotype in tumour cells and the subsequent mechanisms that comprise the metastatic cascade. Examples of pressures that select for a metastatic phenotype are hypoxia, reactive oxygen species and the immune system. When oxygen is low and cells become hypoxic the HIF-1 protein is stabilised and promotes angiogenesis and invasion by promoting the expression of proteins such as CXCR4 and LOX. With these factors the cells metastasise in a number of complex and not necessarily successful steps that are described below (Gupta and Massagué, 2006).

Epithelial cells have a number of junctions that facilitate their attachment to other cells. When cells undergo carcinogenesis the up/down-regulation of specific proteins within these adhesion complexes controls cell-cell interactions and the ability of a tumour cell to successfully metastasise. Tumour cells are thought to be less adhesive than normal, homeostatic epithelial cells but there are a number of mechanisms that will encourage development of this phenotype (Oppenheimer, 2006). One such mechanism of particular interest that regulates this detachment is the epithelial to mesenchymal transition (EMT), specifically Type III EMT (Martin *et al*., 2000). In this process cells switch from a highly connected epithelial phenotype to a detached mesenchymal phenotype. EMT can be stimulated in cells by a number of proteins including TGFβ and FGF. Recently a role for microRNAs has also been discovered in regulation of this process. These proteins have been shown to have

a number of effect such as inducing the expression of a number of transcription factors including Snail, Slug, Twist and ZEB1 which activate the EMT programme (Kalluri and Weinberg, 2009). This can lead to the dissociation of B-catenin from adheren junctions, cell-cell junctions which contain the critical adhesion proteins cadherins, and link the actin cytoskeleton to the cell membrane. With B-catenin no longer sequestered in the cytoplasm it can be influenced to translocate to the nucleus where it leads to the expression of metastasis supporting genes (Xu *et al*., 2009). One of the most definitive changes to the cell during EMT is the change of these adheren junctions with loss of E-cadherin expression, which has adhesive functions, and the increased expression of N-cadherin, which encourages a migratory phenotype. There are a number of experiments that show the link between decreased expression of this E-cadherin and the invasiveness of cancer cells (Cavallaro and Christofori, 2001; Bremnes *et al*., 2002).

During EMT there is a rearrangement of the cytoskeleton whereby cells lose their epithelial arrangement and gain a migratory phenotype that supports their movement from the primary tumour to a transport system (Farahani *et al*., 2014). When carcinoma cells move they undergo a complex process of extension and retraction based on the dynamism of the cytoskeleton. Actin polymerisation leads to the extension of the leading edge of the cell via Rac-1 dependent protrusions that are functionally equivalent to lamellopodia. These form integrin based focal adhesion with components of the ECM. This leads to actomyosin contraction, and the disassembly of focal contacts and retraction at the trailing edge (Yamaguchi *et al*., 2005; Gupta and Massagué, 2006; van Zijl *et al*., 2011). Cancer cell migration is directed towards a transport system that is incorporated into the tumour by lymphangiogenesis by VEGF-C and VEGF-D (van Zijl *et al*., 2011), and the angiogenic switch. One way that the direction of this migration is mediated has been shown in breast carcinoma *in vivo* by the interaction of tumour-associated macrophages with carcinoma cells. To attract TAMs to the tumour the cells secrete CSF-1 which acts a chemoattractant for macrophages expressing the receptor that exists in the vascular system. Once the macrophage receptor binds CSF-1 it secretes EGF which is a chemoattractant for the breast cancer cells which express EGFR (Condeelis and Pollard, 2006). It has been shown that EGF can then stimulate migration by upregulation of cofilin and components of the Arp2/3 complex that facilitate actin polymerisation (Yamaguchi *et al*., 2005). In this way TAMs can act as a guide for cancer cell migration towards the vascular system. It has also been proposed that VEGF in its role as a vascular permeability factor can increase the number of cancer cells in transport systems by negatively regulating tight junctions in the vascular endothelium. Another mechanism of guiding tumour cells in the lymphatic system has been suggested to be based on the CCL21-CCR7 axis that mediates homing of lymphocytes to lymph nodes during an immune response.

Upon reaching a transport system cancer cells penetrate it in a process known as intravasation. As part of the EMT the expression of a number of proteases is upregulated by transcription factors such as Snail which increases the transcription of MMP-2 and MMP-9. These proteases not only digest components of adhesion protein complexes but, with a number of others including serine proteinases and

cysteine proteinases, they can cleave components of the extracellular matrix (Arya *et al*., 2006; Martin *et al*., 2010). When cells reach the transport system they adhere to the endothelial layer and form actin based protrusions known as invadopods that are rich in these proteins and which allows them to penetrate the basement membrane (Condeelis and Pollard, 2006). This enables them to reach the endothelial layer which they migrate through thus gaining entry to the vessels.

Once cancer cells have successfully entered a circulatory system they experience such harsh conditions that only an estimated 0.1% of circulating tumour cells survive (Farahani *et al*., 2014). These adverse conditions include shear forces which can cause physical damage, and destruction by circulating anti-tumour immune cells such as Natural Killer cells (Arya *et* al., 2006; Oppenheimer, 2006). One mechanisms that tumour cells use to survive is the co-opting of platelets via integrin binding to form 'clots' that protect the cancer cells from both physical and immune stresses in circulation (van Zijl *et al*., 2011; Arya *et al*., 2006).

Selectins expressed on endothelial cells plays an important role in the trans-endothelial migration of leukocytes. Tumours can use a similar mechanism by upregulating the selectin ligands such as sLe^x and sLe^a (Farahani *et al*., 2014; Arya *et al*., 2006). This allows interactions between endothelial cells and tumour cells to be developed at this point as the first step of the second invasion process of the metastatic cascade, extravasation. Upon entering the secondary tissue, the mesenchymal phenotype is reversed back to an epithelial phenotype in a MET regaining an apical-basal cell polarity and cell-cell adhesion junctions (Martin *et al*., 2010).

This cascade of events is a highly complex process with a low success rate and when metastatic cells reach the secondary tissue there are a number of fates they can undergo. The metastatic cells may be successful and form interactions with their new environment enabling proliferation. They may also remain dormant for years or be destroyed due to environmental factors such as the adaptive immune system. The success of the cancer cells depends on the environment of the secondary tissue that they enter (Gupta and Massagué, 2006; Oppenheimer, 2006; Farahani *et al*., 2006). As such, the formation of a pre-metastatic niche can improve the chances of proliferation and the setup of a successful metastatic tumour.

1.2 Pre metastatic niche

1.2.1 Pre- metastatic Niche Formation

Metastasis is an inherently inefficient process with very few circulating tumour cells reaching the metastatic site. Even then these metastatic tumour cells may never lead to a secondary tumour (experiments show that only 0.02% of injected tumour cells form macrometastases (Massagué and Obenauf, 2016)) and in some cases tumour cells will remain dormant for many years. There is now a growing body evidence that the primary tumour may influence the secondary tissue so that it will

be favourable to the seeding and growth of metastatic cells. This environment has been called the dubbed the pre-metastatic niche.

The idea that certain cancers more disposed/have greater success when metastasising to specific organs was noted many years ago. This was first described in 1889 by Stephen Paget who presented the 'seed and soil' hypothesis showing that the time, pattern and site of cancer spread is influenced by the cancer type. The frequency of metastasis could not be explained simply by blood supply suggesting a favourable soil. He argued that while the primary tumour is of great importance to the understanding of cancer, there should also be a concerted effort to understand the metastatic environment (Fidler, 2003). This was challenged by Ewing who hypothesised that the predilection of certain cancers for particular organs is explained by the anatomy lymphatic and vascular systems associated with the primary tumour (Psaila and Lyden, 2009). While this has been the accepted theory for a number of years there is now a range of data that backs up Paget's theory of a favourable soil. Fidler in particular was important in presenting some of the first evidence that the soil plays an important role. He showed that metastatic cells can be found in the capillary beds of multiple organs, but only at a select number of sites do metastases consistently develop (Peinado *et al*., 2011). This has been supported by Weiss (1992) who showed that while regional metastasis might be put down to anatomy, many metastasis (particularly distant metastasis) cannot be explained by blood flow demonstrating that distant metastasis is site specific. This recent focus on understanding the soil has led to the proposal of a pre-metastatic niche.

Part of what determines where circulating tumour cells will arrest is their expression of different receptors and at this stage the seed is significant. Cancer cells can be directed to specific sites by homing mechanisms such as the CXCR4-CXCL12 axis (Mendoza and Khanna, 2009). The interactions of cells with the endothelium will then determine which organs the metastatic cells will enter in the docking and locking process. First the docking though selectins and then the firm adhesion though integrins such as $\alpha_v\beta_3$ which will bind with greater success to bone endothelium when expressed by prostate cancer cell lines (Cooper and Pienta, 2000).

After the identification of a permissible environment, it has been suggested that the pre-existing niche will assist in a number of processes that metastatic cells must go through before the establishment of a metastatic tumour. The mechanisms that facilitate this are summarised below and are described in detail in 1.2.2 and 1.2.3. First, invasion can be assisted by supporting invadopodia formation, and locomotion (Rafii and Lyden, 2006; Feller *et al*., 2012). The transition from a mesenchymal to epithelial phenotype can then be backed by factors that influence tumour signalling pathways (Quail and Joyce, 2013). With cells established in the secondary organ they then grow using stem cell growth pathways that are frequently overactive. This growth can be supported by the presence of pre-existing ligands of this pathway (Massagué and Obenauf, 2016). The pre-metastatic niche can also be influenced so that the angiogenesis switch is activated. As with the primary tumour, this will facilitate the formation of macro-metastases (Psaila and Lyden, 2009). In metastasis

tumour cells also remove themselves from the immunoprotective environment of the primary tumour when they metastasis. As such the pre-metastatic niche can support metastasis by mechanisms that protect from metastatic cells from the immune system (Sceneay *et al*., 2013). There are many links between the primary tumour niche and the pre-metastatic niche because of the similar process that the primary and secondary tumour must go through to progress. These all rely upon bone-marrow derived cells and tumour derived secreted factors which determine the state of a pre-metastatic organ (Kruger, 2015).

1.2.2 The Role of Cytokines in the Pre-Metastatic Niche

The pre-metastatic niche is dependent on the existence of the primary tumour and the signals that it conveys. Most of these signals are cytokines and chemokines that will then activate tissue components to niche similar to the primary tumour with a pro-metastatic phenotype. This will decrease the chance of cells entering dormancy and enabling the metastatic progression though the mechanisms mentioned above.

One mechanism mentioned above that supports some of the very first steps of metastasis is the CXCL12/CXCR4 and CCL21-CCR7 chemokine axis (Strieter, 2001). CXCL12 is a chemotactic factor that plays roles in the trafficking during inflammation of leukocytes that express the CXCR4 receptor. Some cancer cells also upregulate this receptor, particularly those that are predisposed to bone metastasis (Mendoza and Khanna, 2009). Tissues, including the lung, liver and bone, upregulate CXC12 in the mature pre-metastatic niche (Kaplan *et al*., 2006 a). There are multiple mechanisms that contribute to this upregulation including production by mesenchymal cells dues to hypoxia or tissue damage and as a consequence of TIMP-1 production from the primary tumour (Feller *et al*., 2012; Kruger, 2015). This then causes cancer cells to migrate chemotactically in site directed metastasis

Other secreted components prime distant sites to support metastatic cancer cells such as MMP-9 and MMP-2. Both of these proteases are secreted from cells in the lung before metastatic cells enter the tissue (Kaplan *et al*., 2006 a). They cleave ECM components (e.g. MMP-2 cleaves collagen IV) contributing to the remodelling of pre-metastatic tissues (Psaila and Lyden, 2009). This supports the invasion of tumour cells and bone marrow derived cells (BMDCs) to these tissues, which they back further by in some cases acting as chemotactic factors (Zoccoli *et al*., 2012).

A number of factors are secreted directly from the primary tumour such as TGFβ and TNFα. The later of these is an important component of a number of pre-metastatic niche's including the liver and lung (Kruger, 2015). As described above, this inflammatory cytokine depresses immune function by interfering with cytotoxic T cell function (Psaila and Lyden, 2009). This plays an important part in creating a hospitable environment for the establishment of secondary tumours. TGFβ can also induce the expression of S100A8 and S100A9, which are predominantly expressed in the lung pre-metastatic niche (Psaila and Lyden, 2009; Raffi and Lyden, 2006).

These inflammatory proteins are chemoattractants and lead to the secretion of the inflammatory cytokine TNFα via the protein SAA (Lukanidin and Sleeman, 2012). The importance of their role in site directed metastasis has been shown *in vivo* as when they are inhibited, metastasis specifically to the lung decreases (Raffi and Lyden, 2006).

Changes in the architecture of the ECM is another important change in the pre-metastatic niche directed by cytokines from the primary tumour. Fibronectin is one component that is upregulated and it is one of the first changes that can be observed at distant sites after the set-up of a primary tumour (Kaplan *et al*., 2006 b). This stromal protein is secreted by a number of cells including fibroblasts and fibroblast-like cells, which proliferate and secrete fibronectin due to PIGF signals from the primary tumour (Kaplan *et al*., 2006 a). It plays a number of important roles acting as a directive signal for arriving BMDCs and as a scaffold for adhesion of BMDC's and later tumour cells (Feller *et al*., 2012). Another ECM component important in the pre-metastatic niche is collagen type IV. As with fibronectin it supports the adhesion and recruitment of BMDCs. It is cross linked by LOX, a cytokine secreted by hypoxic primary tumour cells (Zoccoli *et al*., 2012). This protein has been shown to have important roles in regulating the ECM of the pre-metastatic niche, localising with fibronectin and oxidising specific lysine residues of collagen IV (Sceneay *et al*., 2013).

A number of changes to the endothelium are also evident in the pre-metastatic niche. First, there are changes to the expression of adhesion proteins mediating the attachment tumour cells and BMDCs, supporting extravasation. E-selectin and P-selectin have been shown to be upregulated (Psaila and Lyden, 2009), as are VCAM and ICAM due to the effects of CXCL12 (Feller *et al*., 2012). Changes to endothelial cells are also produced during the activation of the angiogenic switch by secretion of pro-angiogenic factors such as VEGF and PIGF by the primary tumour (Sceneay *et al*., 2013).

VEGF does not just facilitate the formation of a pro-angiogenic environment activating mature endothelial cells, it also plays an important part in the mobilisation of BMDCs to the pre-metastatic niche. A number of secreted factors in the pre-metastatic niche play an important role in determining the role that the immune system plays in the pre-metastatic niche. The secreted factor G-CSF, for example, mobilises $Ly6G^{+}Ly6C^{+}$ granulocytic myeloid cells to the liver pre-metastatic niche (Kruger, 2015). One of their most important functions is the trafficking of BMDCs to the niche. These cells will then secrete factors themselves and support the development of the pro metastatic phenotype.

1.2.3 Bone Marrow Derived Cells in the Pre-Metastatic Niche

As described previously there is a complex relationship that is both antagonistic and supportive between the immune system and cancer. This relationship extends to the pre-metastatic niche. The arrival BMDCs to the niche is one of the most

significant steps and the cytokines secreted from the tumour will determine the immune profile of pre-metastatic organs. These cells, of which there are many types then secrete factors themselves along with fibroblasts co-opted to support niche progression. This leads to the arrival of more immune cells and creates an environment that supports the invasion, adhesion, survival and growth of metastatic tumour cells.

A critical element in the creation of a pre-metastatic niche was discovered by Kaplan *et al*., in 2005. They discovered that bone marrow derived cells predict tumour metastasis arriving at pre-metastatic sites well before tumour cell. This was shown to be a site specific phenomenon as different tumour types would induce the mobilisation of $VEGFR1^+$ BMDCs to specific tissues. At the pre-metastatic tissue these BMDCs then formed clusters of cells that attached to upregulated fibronectin via VLA-4 ($\alpha_4\beta_1$). These BMDC clusters were observed not just in spontaneous metastasis transgenic mouse model but also human pre-metastatic tissue. They showed that mature VEGFR1 clusters, containing BMDCs, fibroblasts and fibronectin, supported tumour adhesion and growth. The importance of these clusters was further shown when they were disrupted with an anti-VEGFR1 antibody blocking cancer metastasis. With the importance of these BMDC clusters established they discovered that the primary tumour plays an important role in this process showing that media conditioned by a tumour can induce cluster formation. This, is likely due to factors secreted by the primary tumour, some of which were investigated by Kaplan *et al*. including Id3 and VEGF-A. The clusters themselves also released factors supporting tumour metastasis. One example of this was an increase in CXCL12 in the clusters supporting the homing of tumour cells to the niche. This occurs as the clusters mature, recruiting more cells, such as $VEGFR\text{-}2^+$ endothelial progenitor cells, and the BMDCs themselves gain functional heterogeneity. The BMDCs recruited to these clusters included haematopoietic progenitors with maturational heterogeneity (for example some express CD11b). This article has been the foundation of much pre-metastatic niche research.

Further defining the BMDC population in the pre-metastatic niche has been an important part of understanding it. An important component of this population are $CD11b^+$ myeloid cells, many of which are described in Figure 1.4. This is a heterogeneous population including macrophages, monocytes, neutrophils and mast cells (Joyce and Pollard, 2009). These cells are attracted by a number of elements in the pre-metastatic niche such as the cytokines S100A8 and S100A9 which can be produced by endothelial cells upon stimulation by microvesicles (Sleeman, 2012). Another important part of myeloid influx is the remodelling of ECM by Lox crosslinking of collagen IV. This provides a scaffold for $CD11b^+$ encouraging their recruitment (Erler *et al*., 2009; Smith and Kang, 2013). These cells then secrete MMP-2 which cleaves collagen IV leading to further remodelling and the production of chemoattractive peptides which encourages the migration of more $CD11b^+$ BMDCs in a positive feedback loop (Sleeman, 2012; Peinado *et al*., 2011).

Of the myeloid cells one population that has been described in the pre metastatic niche is the $CD11b^+/Gr1^+$ BMDCs which are, again, attracted by S100A8 and

S100A9 (Sceneay *et al*., 2013). These markers are generally used to define the myeloid derived suppressor cells (MDSCs) which is a heterogeneous population of immature myeloid cells with immunoprotective capabilities (Mantovani and Sica, 2010; Joyce and Pollard, 2009). They have been shown in the pre-metastatic niche by a study using media conditioned by hypoxic tumour cells rich in VEGF-A, MMP-9 and CCL2. This caused an influx of certain BMDC populations into a pre-metastatic niche. The cellular subsets included $CD11b^{+}/Ly6C^{med}/Ly6G^{+}$, which defines the granulocytic subset of MDSCs, and immature NK cells with no cytotoxic activity (Sceneay *et al*., 2012). Monocytic MDSCs ($CD11b^{+}/Ly6C^{med}/Ly6G^{+}$) are also found in the pre-metastatic niche and together these populations support angiogenesis and suppress T cell activity (Smith and Kang, 2013; Sceneay *et al*., 2013)

In the study of the pre-metastatic niche and the immune system, a number of similarities have been found with the primary tumour and one example of this is the importance of TAMs. This is another population of cells which, like MDSCs, are derived from the myeloid lineage. These cells have been shown to circulate after the set-up of the primary tumour and can be found in distant organs where along with a VEGFR1+ environment they provide a fertile site for metastasis (DeNardo *et al*., 2008). These cells, as with other myeloid cells the influx of these cells is encouraged by ECM components such as versican and fibrin clots (Kruger, 2015; Kitamura *et al*., 2015). Once at the pre-metastatic niche it has been proposed that they perform a similar function as that in the primary tumour, creating a tumour supportive environment by encouraging a number of processes such as angiogenesis (Joyce and Pollard, 2009).

Apart from the cytokines and angiogenic functions of immune cells the pre-metastatic niche creates an immunoprotective environment. This is a particularly important role for BMDCs in the pre-metastatic niche. There are a number of immunosuppressive cells involved in this role, including MDSCs, which have been mentioned and $CD4^{+}$ T_{reg} cells, one of the few non-myeloid populations shown in the pre-metastatic niche (Kitamura *et al*., 2015). These two groups inhibit the cytotoxic T cell and NK cell mediated immune response (Sleeman, 2012). NK cells are important component of the immune system that can target tumour cells for destruction. There an increased number of immature NK cells in the pre-metastatic niche with no cytotoxic function suggesting that NK cell maturation is compromised in the niche (Sceneay *et al*., 2012). It is possible that this is one mechanism of function for MDSCs and T_{reg} cells. It has also been proposed that MDSCs reduce the expression of IFN-γ expression in the pre-metastatic niche due to MDSCs. This aligns with a Th2 cytokine shift that supports an anti-inflammatory phenotype (Yan *et al*., 2010). Furthermore, the MDSC population has been shown to produce TNFα which increases T_{reg} numbers in niche (Kitamura *et al*., 2015). This co-operation supports the creation of environment that protects metastatic cells from anti-tumour components of the immune system.

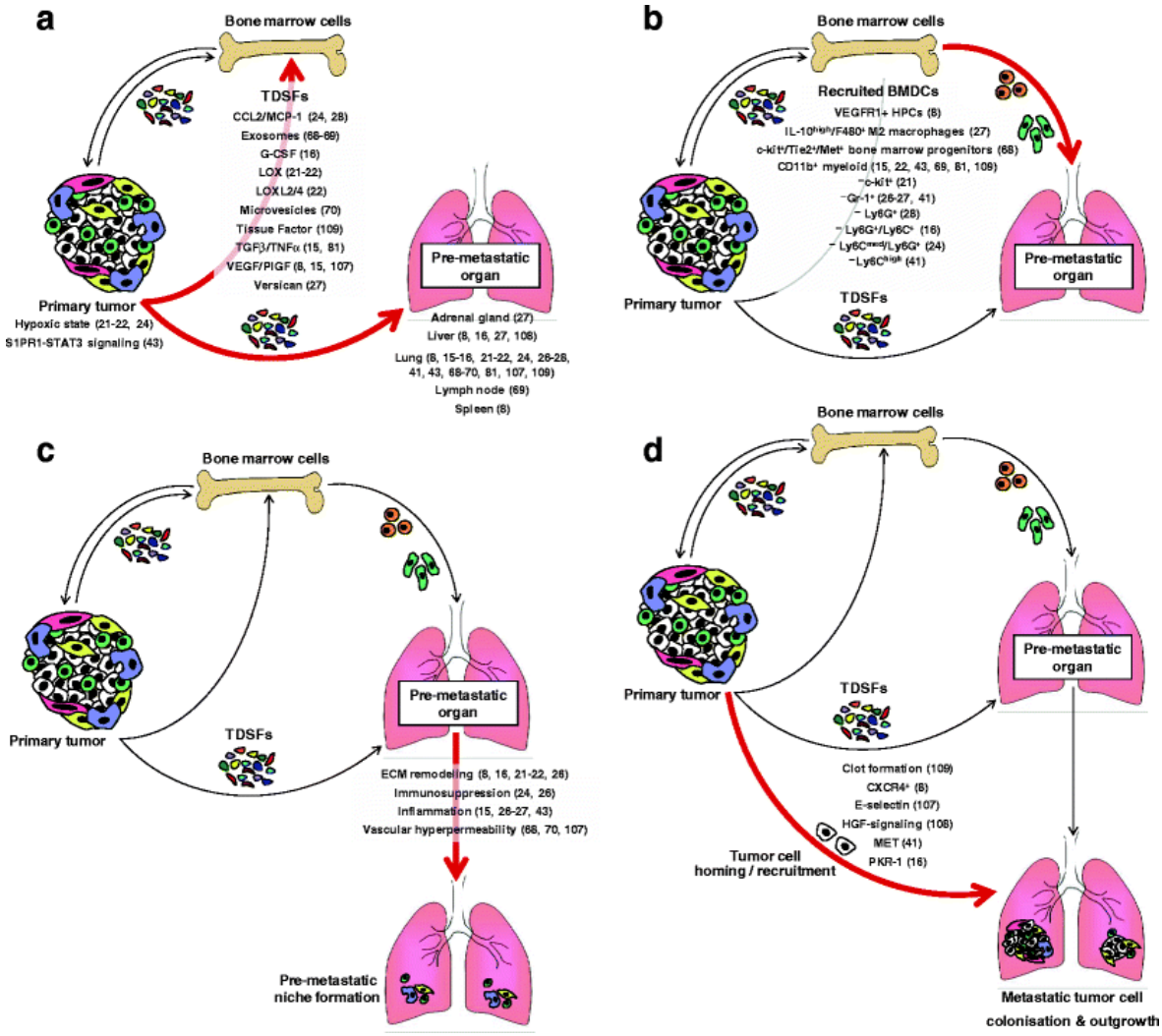

Figure 4: The tumour secreted factors, cytokines and BMDCs involved in creating a pre-metastatic niche and how they support the colonisation and growth of metastatic tumour cells (Sceneay *et al.*, 2013)

The preliminary results describe a co-operation between primary tumour and secondary environment that is dependent on cytokines and the influx of BMDCs, with a particularly important role for myeloid cells. These BMDCs also release cytokines leading to the influx of further BMDCs in a positive feedback mechanism. This all leads to a tumour supportive environment that will encourage metastatic growth. This environment provides potential therapeutic options for preventing metastasis however there is still much to be discovered.

1.3 Integrins

1.3.1 Structure and Activation

Integrins are transmembrane proteins with roles in cell processes such as adhesion that means they are important in a number of pathological diseases such as cancer. Understanding the structure and how the integrin is activated can lead to an appreciation of how this family of proteins plays a role in such diseases.

Integrins are heterodimeric proteins that consist of non-covalently associated α and β subunits (Luo and Springer, 2006). The integrin family subsists of type 1 transmembrane glycoproteins with cytoplasmic, transmembrane and intracellular domains. They have a long ectodomain with a globular head that plays an essential part in integrin ligand binding and usually a short intracellular domain (Arnaout *et al*., 2005).

The family of integrins contains 18α and 8β subunits that come together to for 24 αβ integrins shown in Figure 1.5. Half of the alpha subunits contain a von Willebrand Factor type A subunit (αI domain) that facilitates ligand binding. These 9 αI domain containing α subunits are indicated in Figure 1.5 by asterisks and for 9 of the 24 integrins (Arnaout, 2005).

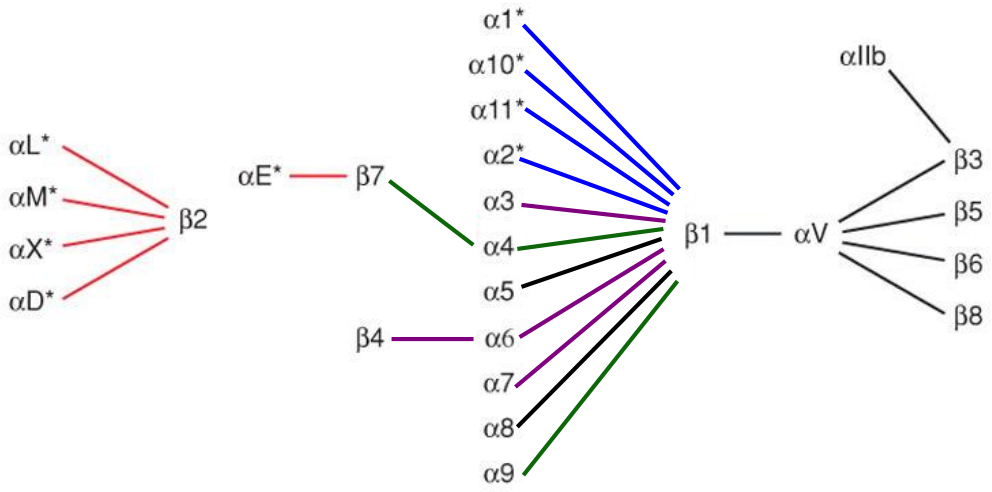

Figure 5: The groups within the integrin protein family (Adapted from Luo et al., 2007)

Red - leukocyte integrins, Black - RGD recognising integrins, Blue - collagen binding integrins, Green - a4/a9 cluster, Purple - laminin binding integrins

The αI domain is the major ligand recognition site for integrins that have it and contains a metal ion dependent adhesion site (MIDAS) (Anderson *et al*., 2014). This motif on the α subunit has multiple binding conformations that are influenced by the binding of a ligand. Upon the binding of a ligand to the αI domain the MIDAS moves to an open conformation shifting a bound metal ion, such as Mn^{2+}, so that ligand affinity increases. In the second, closed conformation, the metal ion is moved away from the binding sites used in the open conformation, consequently decreasing ligand binding efficiency (Luo *et al*., 2007; Liddington and Ginsberg, 2002).

There are three families of α subunits that do not have the αI domain, the laminin receptors, RGD receptors and the α4/α9 cluster shown in Figure 1.5 (Anderson *et al*., 2014; Campbell and Humphries, 2011). In these integrins a second domain that is found directly on the β subunit, the βI/αI-like domain, acts with the β propeller on the α subunit as the major ligand recognition site (Liddington and Ginsberg, 2002). The β propeller also regulates the ligand binding activity of the α subunit in αI domain containing integrins (Anderson *et al*., 2014).

The αI-like domain acts in a similar way to the αI domain, containing a MIDAS motif that enables the β subunit to directly bind a ligand (Zhang *et al*., 2012; Luo 2006). It also has distinct open and closed conformations that allosterically regulate ligand binding. The β propeller ligand binding site is shielded by the α1-like domain when the protein is in a low affinity conformation. When a stimulus is present, a shift is induced so the β propeller ligand binding site is open (Hynes, 2002).

1.3.2 Integrin Signalling and Activation

Integrins signal in a bidirectional manner, capable of transmitting an intracellular signal extracellularly and vice versa. These signalling pathways are known as inside-out and outside-in signalling.

In inside out signalling intracellular signals cause changes to the β cytoplasmic tail that lead to the activation or inactivation of the integrin. The different activation states lead to the binding of different cytoplasmic ligands that compete with each other for the same domains. This is controlled by the phosphorylation of NPxY motifs found on the cytoplasmic tail that ligands bind to via phosphotyrosine binding (PTB) domains. When the NPxY domain is phosphorylated it is inactive and proteins such as Dok1, which have a higher affinity for the phosphorylated NPxY domain bind to it. When the β tails are unphosphorylated and active, talin is able to bind to the tail setting up protein complexes (Anthis 2011; Oxley 2008). These cytoplasmic ligands interact with the β cytoplasmic tail disrupting its association with the α cytoplasmic tail allowing activation to take place. During activation there are conformational changes in the ectodomain that increases the affinity of the integrin to its extracellular ligands (Ginsberg and Shattil, 2005).

These conformational changes are an essential part of controlling the activation state of an integrin. There are two current models of activation, the deadbolt model and the switch blade model. These models of activation are based on conformational changes between low affinity and high affinity states (Campbell and Humphries, 2011).

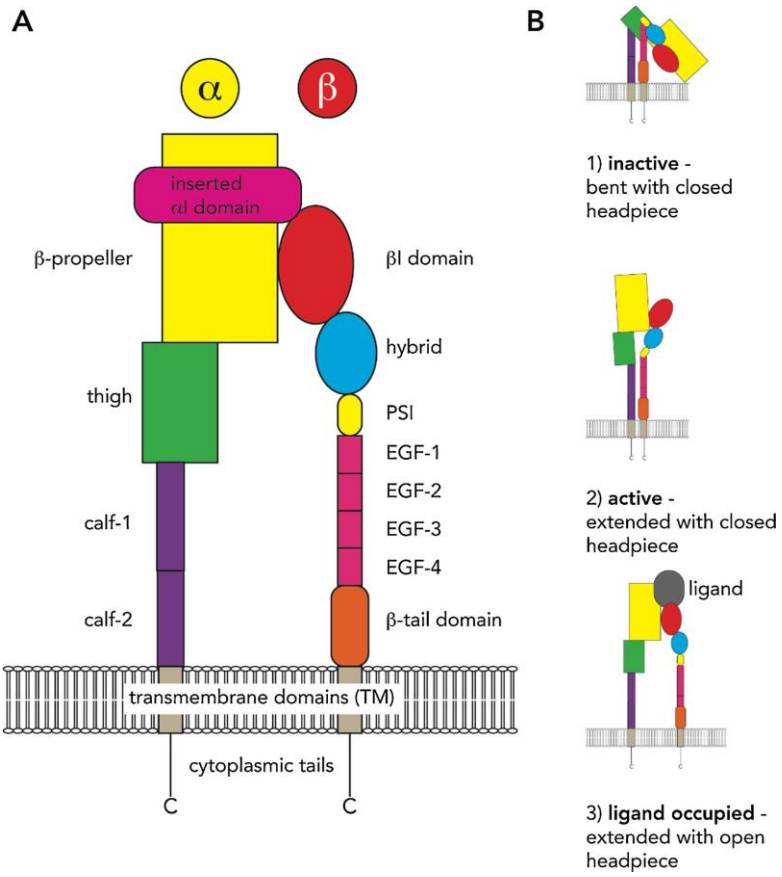

Figure 6: The domains and activation states of integrins (Anderson *et al.*, 2014)

The two models debate the conformation change that occur in the integrin subunits during activation. Originally three integrin conformational states were proposed that relate to the activation state which are shown in Figure 1.6. The first of these is the bent or inactive state, which the association of the integrin tails maintains. In this conformation the α1 domain, β1 domain and the β propeller are bent closely to the cytoplasm. The second is the active state where the integrin is extended but the headpiece is closed. The third is the ligand occupied state with an open headpiece where conformational changes in the ligand binding site have occurred and the cytoplasmic tails and the transmembrane domains have separated (Ye *et al*., 2011). The deadbolt model on the other hand, suggests that less dramatic conformational changes occur and that once activated the integrin maintains its bent state but a change occurs that allows the ligand binding domains to be exposed (Mehrbod *et al*., 2013; Takada *et al*., 2007; Takagi *et al*., 2002). Despite there being evidence that suggest that integrins can bind ligands in the bent state current electron microscopy evidence seems to favour the switchblade model (Campbell and Humphries, 2011; Anderson *et al*., 2014) with some data seeming to contradict the deadbolt model (Zhu *et al*., 2007). Historically this has been one of the main focuses of integrin structure and activation but since this structural change only seems to apply to some integrins there has been attention on regulation of integrin activation by structural changes in the headpiece (Zhu *et al*., 2013).

The bi-directionality of an integrin means that signals can be propagated in a second manner, from the extracellular to the intracellular environment. In outside-in signalling ligands from the extracellular matrix bind to the head domain of the integrin which leads to intracellular signalling (Anthis and Campbell, 2011). Apart from the models described above, these signals are transduced by conformational changes such as the separation of integrin tail domains (Takada *et al*., 2007), and integrin clustering that is enabled by oligomerisation of the transmembrane domains of an integrin (Ginsberg *et al*., 2005).

The transduction of an extracellular signal allows protein signalling complexes to develop at the cytoplasmic β tail which leads to the activation of intracellular signalling pathways. A protein family that plays an essential role in mediating integrin signalling is the Src family Protein Tyrosine Kinases (SFKs). SFKs bind directly to the β tail regulating the activation of kinases such as Focal Adhesion Kinase (FAK) via phosphorylation influencing cell migration and growth factor signalling (Harburger and Calderwood, 2009; Klinghoffer *et al*., 1999).

1.3.3 The Signalling and Functions of Integrins

The bidirectional signalling of integrins, means that integrins play a number of roles connecting the intracellular environment with the extracellular. These functions are both mechanical and chemical with ligands including extracellular matrix and cytoskeletal components and signalling molecules.

The different integrins recognise a number of components of the ECM as ligands including fibronectin, collagen and laminin (Albelda and Buck, 1990). The intracellular tail of the integrin then forms attachments to the cytoskeleton, generally actin (Delon and Brown, 2007; Hotchin and Hall, 1995). This means that mechanical stresses in the external environment can be transmitted leading to reorganisation of the actin cytoskeleton and consequently the cell shape and polarity (Schwartz and Ginsberg, 2002).

The connections that integrins form between cells and the extracellular matrix also facilitates their role in adhesion complexes and lamellopodia (Delon and Brown, 2007). Generally, these are focal adhesions connecting to the actin cytoskeleton with the exception being $\alpha_6\beta_1$ that connects to intermediate filaments in hemidesmosomes (van der Flier and Sonnenberg, 2001). Integrin adhesion and motility interactions are relevant to a number of cellular processes dependent on the locality and time of express. One example of integrin functions is the role of β_2 in inflammation where it facilitates leukocyte adhesion in extravasation. In inflammation the integrins on leukocytes and their receptors on the endothelium are activated allowing leukocytes to adhere to the endothelial layer before extravasation (Hynes, 1992).

The formation of complexes at the β cytoplasmic tails of integrins does not only facilitate the connections to cytoskeletal components. As mentioned previously,

talins and kindlins (Moser *et al*., 2009) play an important role in setting up these complexes separating the cytoplasmic domains of the α and β subunits. This allows the recruitment of further proteins after clustering including the IPP complex, containing integrin linked kinase, PINCH, and parvin, Paxillin, Vinculin, FAK and Src (Legate *et al*., 2006). These then lead to the activation of a number of pathways downstream of integrins (Danen and Yamada, 2001) and the signal that is transmitted is influenced by the ligand, ligand state and interactions with the ECM. The major signalling pathways that are influenced include the small GTPases, PI3K/Akt, and JNK/ERK MAP kinase pathways (Sieg *et al*., 2000; Moreno-Layseca and Streuli, 2014).

Integrin signalling has a number of functions including the influence of the fate of cells, particularly anchorage dependent cells (where viability is controlled by adhesions, by interactions with the ECM). Changes in integrin interaction can lead to mitogenic (growth) signalling or apoptotic signalling (Danen and Yamada, 2001; Stupack and Cheresh, 2002).

Integrins can affect the growth and motility of cells by their interactions with growth factor signalling pathways. This occurs in a number of ways, first by *trans*-activation of growth factor receptors, and second by the influence of components of growth factor signalling pathways. It has been suggested that growth factor receptors can cluster with integrins with FAK acting as a “receptor-proximal bridging protein” (Sieg *et al*., 2000). This is thought to lead to phosphorylation affecting a number of growth factor pathways including PDGF, FGF, HGF and EGF (Schwartz and Ginsberg, 2002). There is also a lot of crossover between pathways downstream of integrins and growth factor receptors. A number of integrin signals are involved in proliferative signalling but the ERK and AKT pathways in particular plays a role in cell cycle control. The activation of these proteins by adhesion pathways contribute to cyclin D1 production allowing cells to proceed from G1 to S phase (Moreno-Layseca and Streuli, 2014). This is just one example of how anchorage via integrins mediates progression through the cell cycle.

It has also been suggested that in the absence of a ligand integrins can cause cells to enter apoptosis. There are two distinct mechanisms that integrin blockade can be a part of, anoikis and Integrin Mediated Death (IMD). Anoikis is a form of apoptosis cause by detachment of the cell from the ECM. As adhesion receptors integrins play a role in this process and a number of signalling pathways related to integrins have been implicated in this process such as the PI3K pathway (Frisch and Screaton, 2001). It has been shown *in vitro*, that blockade of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ can lead to anoikis in endothelial cells (Maubant *et al*., 2006). On the other hand, the second mechanism of apoptosis, IMD, is proposed to be caused directly by unligated integrins. In this process caspase-8 is recruited and activated giving integrins a direct role in cell survival (Stupack *et al*., 2001).

Overall integrin signalling plays role in wide a number of cell processes that dictate cell fate. The specific functions, however, of each of the members of this protein family is determined by the localisation and ligand binding affinity of each integrin

(Takada *et al*., 2007). As integrins are expressed in most tissues they have function in many processes, both homeostatic and pathological.

1.3.4 Integrins and Cancer

One pathological process that many members of the integrin family have been shown to have a role in is cancer. These functions have been shown to be highly complex, playing parts in a number of the hallmarks of carcinogenesis including metastasis, angiogenesis, and cell proliferation. A number of integrins are expressed by cells in the immune system giving this protein family links to the relationship between cancer and the immune system. There are also changes to the expression of integrins within cancer cells themselves. Some integrins are up-regulated, $\alpha_v\beta_3$, while some, such as the potential tumour suppressor $\alpha_2\beta_1$, are downregulated by cancer cells (Desgrosellier and Cheresh, 2010).

Those members of the integrin family upregulated by cancer cells have been shown to be linked with a more invasive and metastatic phenotype (Keely, *et al*., 1998). This is conferred by a number of factors including the signalling pathways and adhesive properties mentioned above. The anchorage related signalling pathways that control both growth and survival play important roles in the proliferative potential of tumour cells (Liotta and Kohn, 2001). Through these integrins can control the growth of tumour playing anti-tumorigenic roles that must be overcome. While integrins can convey signals that encourage cell survival when ligated, when they have no ligand their signalling can lead to cells entering apoptosis pathways (Desgrosellier and Cheresh, 2010). As well as survival signals, when connected to stromal components they encourage the progression of cells through the cell cycle through their coupling to growth factor receptors. In tumour cells this dependence on the adhesion for cell cycle progression diminishes their proliferative potential. One example of this is $\alpha_5\beta_1$ which in the absence of its fibronectin substrate diminishes proliferation of tumour cell lines leaving them unable to progress to S phase of the cell cycle (Varner and Cheresh, 1996). Tumours must overcome these pathways before gaining the anchorage-independent growth that characterises them.

The protein family also confers a number of tumour supportive properties however, in some cases enhancing oncogenic signalling, and integrin adhesion to the ECM can facilitate a more aggressive cancer phenotype by supporting invasion and migration. The focal adhesion complexes that integrin clustering is part of can facilitate the metastasis of tumour cells in a number of ways. First integrins can support invasion of tissues at the site of the primary tumour and at metastatic sites by their adherence properties (Hood and Cheresh, 2002). Second, integrins play important roles in cell migration through their links to the actin cytoskeleton supporting the formation of stress fibres. This then supports the migration of integrin expressing cancer cells to and from tissues to transport systems in metastasis (Keely *et al*., 1998; Jin and Varner, 2004).

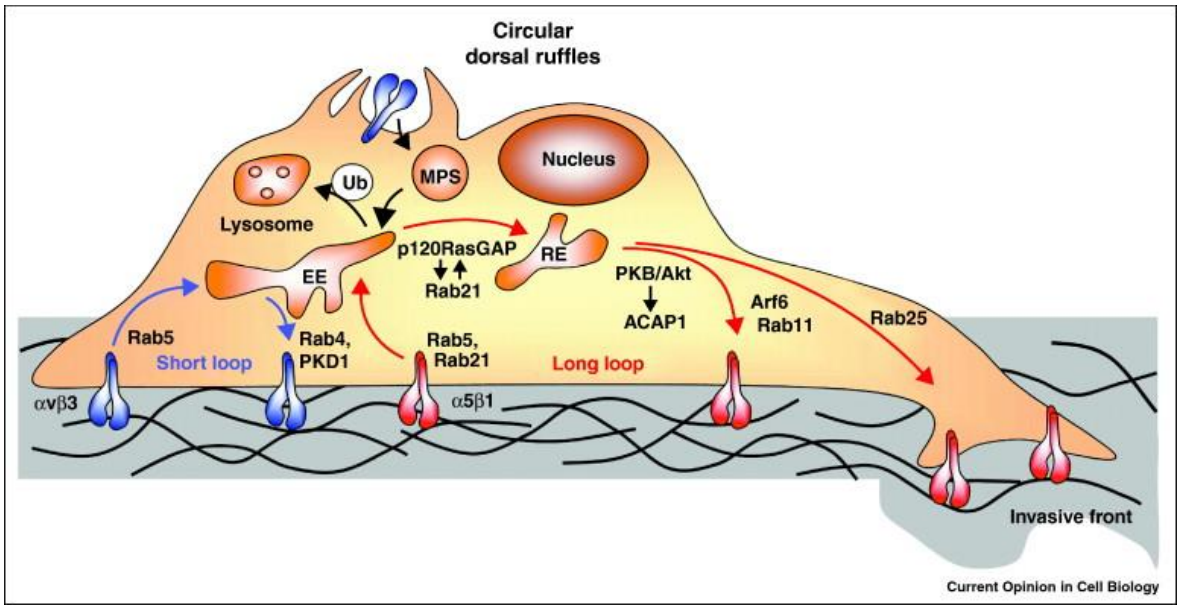

Figure 7: The invasive and migrational roles of integrins based on their attachments between the cell and the extracellular matrix (Margadant *et al.*, 2013)

Integrins expression in cancer biology is not just confined to the tumour cells themselves, but also extends to cells in the tumour niche including bone-marrow derived cells and endothelial cells. Integrins expressed by immune cells play a number of roles. They can play important roles in the trafficking of leukocytes to tumours by facilitating the adhesion of these cells to the endothelium. One example of this is $\alpha_4\beta_1$ which regulates tumour invasion by myeloid cells and consequently the colonisation of the tumour by macrophages (Jin *et al*., 2006). Integrins on both tumour cells ($\alpha_v\beta_3$) and platelets ($\alpha_{IIb}\beta_3$) are also essential for the formation of bridges between these cell types increasing the chance of survival of metastatic cells in circulation and promoting tumour cell arrest (Felding-Habermann *et al*., 2001; Desgrosellier and Cheresh, 2010).

The expression of integrins by another tumour niche component, endothelial cells, has also been of particular interest in cancer research because of their support of angiogenesis. The integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are thought to play direct parts in angiogenesis supporting the migration, proliferation and survival of endothelial cells (Jin and Varner, 2004). These roles in increasing tumour angiogenesis have led to the study of these integrins as potential therapeutic targets. A second role comes from the regulation of endothelial cell interactions with pericytes by $\alpha_v\beta_5$. In tumours this interaction can be faulty leading to unstable vessels which are poor drug delivery systems (Desgrosellier and Cheresh, 2010).

1.4 Integrin $\alpha_v\beta_3$

1.4.1 Structure and Ligands of $\alpha_v\beta_3$ Integrin

As with other integrins $\alpha_v\beta_3$ is a transmembrane protein that is conveys intracellular signals and interacts with the ECM via inside-out and outside-in signalling. This

integrin conforms to the switchblade model existing in three states that regulate the activity of integrins, the bent conformation, extended with closed headpiece and the ligand bound extended conformation.

Discovering the crystalline structure of the integrin $\alpha_v\beta_3$ in the presence of Ca^{2+} and Mn^{2+} (Arnaout *et* al., 2002) was crucial in shedding light on the integrin structure and activation models described above. Of particular importance were studies looking at the interactions of $\alpha_v\beta_3$ integrin with a ligand. A critical part of the $\alpha_v\beta_3$ integrin structure is that instead of the αI domain it has the αI-like domain (Shimaoka and Springer, 2003; Puklin-Faucher and Sheetz, 2009). This is an essential ligand binding domain, found on the β subunit, that interacts with the β propeller, present on the α subunit (Arnaout *et al*., 2002). When the ligand is bound it interacts extensively with these domains, and with the MIDAS motif found in the αI-like domain (Arnaout *et al*., 2007). This is facilitated by conformational changes in the high affinity conformation of $\alpha_v\beta_3$ where the MIDAS motif opens up allowing interactions with the ligand and the β propeller and αI-like domain move closer together (Arnaout *et al*., 2007; Shimaoka and Springer, 2003).

During their bi-directional signalling the different integrins bind to a number of ligands. The ligand specificity is determined by integrin recognition sequences which in the case of $\alpha_v\beta_3$ is the RGD integrin binding site. This is a sequence of three amino acids; Arg-Gly-Asp which is expressed by ligands and recognised by the ligand binding site of $\alpha_v\beta_3$ integrin (Schaffner and Dard, 2003). The RGD motif interacts with $\alpha_v\beta_3$ integrin in a number of ways. The divalent cations in the MIDAS within the αI-like domain co-ordinate with the ligand aspartic acid (Shimaoka and Springer, 2003) and Asp218 of the β propeller on the α_v subunit interacts with the arginine residue (Arnaout *et al*., 2002).

A number of proteins express this RGD sequence including some ECM components. As such integrin $\alpha_v\beta_3$ binds to a number of ECM proteins including von Willebrand Factor, vitronectin, fibrinogen, collagen, fibronectin and osteopontin (Schaffner and Dard, 2003). In this context as with most integrins $\alpha_v\beta_3$ plays a role in focal adhesion complexes. There are also a number of ligands apart from matrix proteins including some membrane receptors (counter receptors) that contain the RGD sequence, facilitating cell-cell interactions.

Apart from the β propeller on the α subunit there are three other domains that form the leg. The β subunit has six leg domains that are linked to the αI-like domain via a hybrid domain. These include four EGF domains linked by disulphide bonds and a β tail domain (βTD) (Arnaout *et al*., 2002; Arnaout *et al*., 2007). These β leg domains are important, particularly the βTD in intracellular integrin signalling supporting the formation of signalling complexes (including FAK and Src) and attachments to the actin cytoskeleton (Arnaout *et al*., 2002; Puklin-Faucher and Sheetz, 2009). When talin binds to the β subunit leg it can initiate inside-out integrin activation and signalling leading to tail dissociation and an increase in the angle between the hybrid domain and αI-like domain in a switchblade like movement (Puklin-Faucher and Sheetz, 2009; Shimaoka and Springer, 2003). Intracellular

binding of talin will also lead to integrin clustering, an important step in the formation of focal adhesions (Puklin-Faucher and Sheetz, 2009; Margadant *et al*., 2011).

As with other integrins, these interactions control the survival, morphology, growth and in some cases differentiation of cells.

1.4.2 Signalling of $\alpha_v\beta_3$ Integrin in Endothelial Cells

The main functions of the endothelial cells are as a transport system and barrier. This plays roles in the migration of cells from one organ to another and the formation of new blood vessels. Studying the cell specific roles of proteins has been an essential part of dissecting the functions of proteins in homeostasis in disease. This is because of the importance of location and time of expression when painting a picture of the function of proteins. The integrin $\alpha_v\beta_3$ is expressed by a number of cell types including smooth muscle cells, monocytes, platelets and endothelial cells (Shimaoka and Springer, 2003). Its role in endothelial cells in particular has been studied because of the highly complex part it plays in tumour angiogenesis. This in turn has led to its study as a therapeutic target.

Signalling of $\alpha_v\beta_3$ integrin can affect the behaviour of endothelial cells. As described above, integrins can cluster with growth factor receptors and crosstalk with their pathways. In endothelial cells one important example is the co-operation between $\alpha_v\beta_3$ and VEGFR-2. This controls adhesive and migratory properties of endothelial cells in specific scenarios including VEGF induced angiogenesis (Weiss and Cheresh, 2011). The receptor complexes that these two transmembrane proteins form have been shown by co-immunoprecipitation (Soldi *et al*., 1999). The importance of this complex has also been shown as disrupting it via cytoplasmic mutation of the β_3 subunit will abrogate the migration and adhesion of endothelial cells (Plow *et al*., 2014). This collaboration is particularly evident when $\alpha_v\beta_3$ is interacting with its receptor vitronectin bringing about an increase in VEGFR2 phosphorylation and its signalling response. This in turn leads to an increase in endothelial cell migration and proliferation (Soldi *et al*., 1999). This example of inside out signalling is dependent upon kindlin-2 binding to the β_3 cytoplasmic tail via the amino acids Arg-Gly-Thr (Liao *et al*., 2015). It causes the activation of VEGFR-2 signalling pathway including FAK, which enables motility and proliferation and RasMAPK which is linked to proliferation (Munoz-Chapuli *et al*., 2004; Karkkainen and Petrova, 2000).

There are also some $\alpha_v\beta_3$ signalling pathways, which can govern the survival and proliferation of endothelial cells. These are also commonly linked to the interactions that $\alpha_v\beta_3$ facilitates with the ECM. First, there is evidence that $\alpha_v\beta_3$ induces survival by suppressing the apoptotic protein p53 and one of the products it induces, the cell cycle inhibitor $p21^{WAF1/CIP1}$ (Strömblad *et al*., 1996). Second the integrin can enhance survival by increasing the expression of the anti-apoptotic protein Bcl-2 in comparison to Bax (Munoz-Chapuli *et al*., 2004; Soldi *et al*., 1999). These roles

have been supported by evidence that antagonists of $\alpha_v\beta_3$ integrin lead to an increase in apoptosis of endothelial cells (Soldi *et al*., 1999).

Conversely, it has been suggested that when $\alpha_v\beta_3$ integrin is unligated its signalling can lead to apoptosis in endothelial cells in two main ways. One theory is based on evidence that the apoptotic protein caspase-8 interacts directly with the cytoplasmic domain of the β_3 subunit. It is suggested that when $\alpha_v\beta_3$ integrin interacts with an extracellular ligand, caspase-8 remains bound and active but when the integrin is unbound caspase-8 is activated allowing it to induce apoptosis (Sheppard, 2004; Stupack *et al*., 2001). The second theory also suggests that unligated $\alpha_v\beta_3$ will lead to the activation of caspase-8 but this time through another pathway. When the integrin is unligated protein Kinase A is activated, a process shown to be suppressed when $\alpha_v\beta_3$ interacts with ligands. When this apoptotic pathway is activated the angiogenic process is disrupted showing the importance of integrin adhesion in the formation of new blood vessels (Kim *et al*., 2002).

Most research into $\alpha_v\beta_3$ functions in endothelial cells has focused on how it affects cell behaviour. There is also some evidence however that $\alpha_v\beta_3$ might play a role in trans-endothelial migration. One example is evidence that expression of $\alpha_v\beta_3$ by endothelial cells can facilitate platelet adhesion. It was shown that activating the endothelium increased $\alpha_v\beta_3$ expression and platelet adhesion and that addition of a cyclic peptide selective for $\alpha_v\beta_3$ disrupted this interaction (Gawaz *et al*., 1997).

Overall studies of these functions have led to suggestions that $\alpha_v\beta_3$ has a role in the remodelling of the vasculature supporting an angiogenic phenotype in endothelial cells. This is a complex role, however, that depends on the substrate and the cellular context of expression. To study these roles in detail a number of endothelial cell specific $\alpha_v\beta_3$ integrin knockout mouse models have been created. One of these is harnessed in this study, the Tie-1Cre mouse model which knocks out the β_3 subunit and confines Cre recombinase activity to endothelial cells using the Tie-1 promoter (Gustafsson *et al*., 2001). This is just one example of models available to study $\alpha_v\beta_3$ in endothelial cells.

1.4.3 The Role of $\alpha_v\beta_3$ Integrin in Cancer

The integrin $\alpha_v\beta_3$ has a number of roles supporting tumour progression. Apart from its role in angiogenesis it can also be involved in migration of immune cells. It is also known to be highly expressed in a number of cancers and, via its interactions with the extracellular matrix, $\alpha_v\beta_3$ supports invasion. This has been linked in multiple studies to a poor prognosis, supporting the metastatic properties of cancer cells. In an aging population great importance has been placed on finding therapeutic targets for anti-cancer therapies and $\alpha_v\beta_3$ is one example.

As described in section 1.1.3 the migration of endothelial cells and their eventual proliferation is essential for sprouting angiogenesis. The role of $\alpha_v\beta_3$ integrin in these processes, and in endothelial survival has led to suggestions of a role for this integrin

in angiogenesis. This has been supported by evidence that $\alpha_v\beta_3$ is upregulated by angiogenic endothelium (Munoz-Chapuli *et al*., 2004; Sheppard, 2004). The expression of this integrin can also be induced by pro-angiogenic factors (Jahroudi and Greenberger, 1995). One example is Del-1 which can induce pro-angiogenic gene transcription when bound to $\alpha_v\beta_5$ integrin. This will induce the expression of the angiogenic transcription factor Hox-D3 transcription factor which in turn increases the expression of $\alpha_v\beta_3$ (Sheppard, 2004).

The importance of this process in tumour progression has led to studies looking at $\alpha_v\beta_3$ specifically in relation to tumour angiogenesis. The interactions of this integrin with VEGFR-2 control the activation of these receptors with cross talk dependent on Src. This increases VEGF-A induced angiogenesis which is particularly prevalent in the tumour (Weiss and Cheresh, 2011). This pro-angiogenic role in the tumour is supported by studies that look at the effect of $\alpha_v\beta_3$ antagonists on angiogenesis. One example is the use of cilengitide, a cyclic RGD peptide, in tumours which can decrease blood vessel growth and increase endothelial cell apoptosis further proving the angiogenic properties of $\alpha_v\beta_3$ (Liu *et al*., 2008; Weiss and Cheresh, 2011).

The role that $\alpha_v\beta_3$ plays in angiogenesis is highly complex however which is evident from studies using β_3 knock out mice. Studying mice deficient in the β_3 subunit led to an unexpected increase in pathological angiogenesis in the mouse. This provided a pro-tumorigenic environment and led to enhanced tumour growth. Since there were also elevated levels of VEGFR-2 in these mice a compensation mechanism exists revolving around VEGFR-2 (Reynolds *et al*., 2002). This has shown a far more intricate function for $\alpha_v\beta_3$ in angiogenesis that would need to be dissected before $\alpha_v\beta_3$ antagonists could be used in clinical settings.

Another major role that $\alpha_v\beta_3$ plays in tumorigenesis is down to its expression by cancer cells themselves. This integrin can be upregulated by a number of cancers including glioblastomas and breast cancers (Weiss and Cheresh, 2011). This increase in expression leads to an advantage in metastasis supporting the migration and invasion of cancer cells. In the case of breast cancer, it is also proposed to support homing to the bone because of its ability to recognise specific components of the bone ECM such as bone sialoprotein (Sloan and Anderson, 2002). It is recruited to the migrating edge of cells in a process shown in Figure 1.7 supporting movement along the ECM (Margadant *et al*., 2013). After migration $\alpha_v\beta_3$ plays a role in the formation of another set of specialised actin protrusions that govern invasion, invadopodia (Huttenlocher and Horwitz, 2011), also shown in Figure 1.7. In fact, the expression of $\alpha_v\beta_3$ has been shown to be linked to a trans-endothelial migratory phenotype which can be blocked with $\alpha_v\beta_3$ antibodies (Bauer *et al*., 2007).

This integrin can also support tumour growth through a cancer cell specific role in a population of cancer cells which I have not yet discussed - cancer stem cells. These are a population of cells within the tumour that self-renew, differentiate and due to these characteristics have the potential to form tumours when transplanted. By eliminating this population of cells it is thought that the chance of tumour recurrence

in patients would decrease but this has not yet led to viable therapeutic options (Yu *et al*., 2012). The integrin $\alpha_v\beta_3$ is thought to have some influence in this subpopulation as β_3 expression can be a marker of this population (Desgrosellier and Cheresh, 2010). This role has been studied leading to contradictory results. One paper showed that $\alpha_v\beta_3$ activates stem-like properties including anchorage independence, and tumour initiative potential by complexing with KRAS and RalB to activate TBK1/NFκB (Seguin *et al*., 2014). On the other hand, it has been suggested that when bound to vitronectin $\alpha_v\beta_3$ will lead to the downregulation of stem cell genes and nuclear localisation of β-catenin. They suggest that through this ligation cancer stem cells differentiate allowing the formation of new tumours; blocking the interaction between vitronectin and $\alpha_v\beta_3$ blocks tumour formation (Hurt *et al*., 2010). Whatever the function that $\alpha_v\beta_3$ plays in cancer stem cells, it further supports the direct role that $\alpha_v\beta_3$ performs in cancer biology.

Overall the evidence shows that $\alpha_v\beta_3$ can be involved in supporting cancer progression in a number of ways.

1.5.2 Previous Data

As described previously, although $\alpha_v\beta_3$ has shown promise as a potential therapeutic target, when used in clinical trials, $\alpha_v\beta_3$ therapies have led to indeterminate results. To dissect the multifaceted role of proteins with extensive and complex roles, cell and tissue specific knockout mouse models can be exploited.

It has been mentioned that the role of $\alpha_v\beta_3$ in endothelial cells has been a focus of research in this laboratory. Steri *et al*., 2014, carried out a number of studies looking at the role endothelial $\alpha_v\beta_3$ plays in the primary tumour using cell-specific knock out mice. It was shown that acute depletion of endothelial β_3 integrin inhibits tumour growth and angiogenesis.

There is a role for tumoural $\alpha_v\beta_3$ supported by clinical evidence correlating the expression of $\alpha_v\beta_3$ by tumours with a worse prognosis (Sloan *et al*., 2016). With a role in the primary tumour established the study was extended to examine the effect of depleting endothelial β_3 integrin on the formation of secondary tumours.

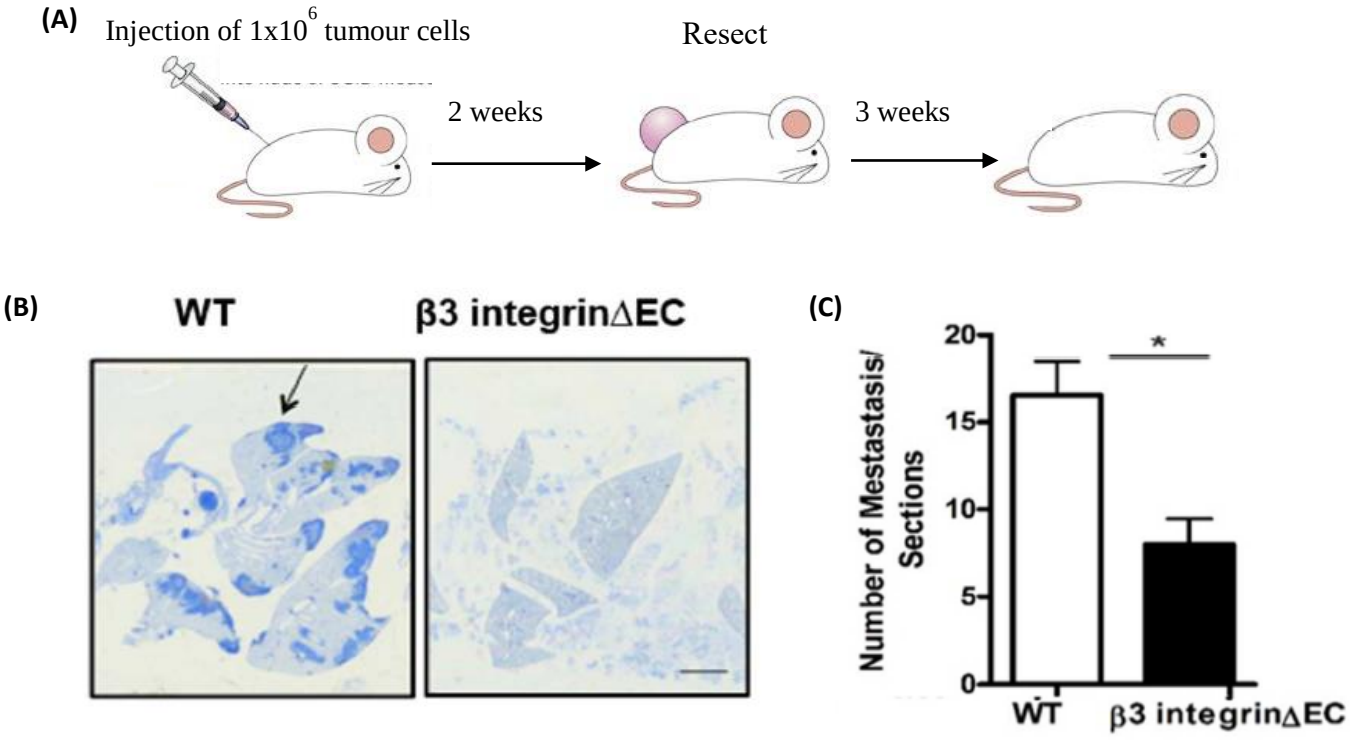

Figure 8: Constitutive knock-out of β_3 integrin in endothelial cells decreases lung metastasis

(A) $1x10^6$ CMT19TF1 cells were injected subcutaneously into $\beta_3$flox/flox/Tie1 Cre and WT (Cre-negative) mice and a primary tumour was grown for 2 weeks. The tumour was resected and the mice were then left for another 3 weeks to allow macrometastases to develop **(B)** Microscope images from H&E staining of lungs for the identification of tumour metastasis **(C)** The number of metastases on the stained sections were then counted (n=3 for each genotype, Mean + SEM from 3 independent experiments, * $p<0.05$)

A long term cell specific knock out mouse model of β_3 depletion in endothelial cells was used, the β_3 flox/flox/Tie1-Cre mouse model. In metastasis the primary tumour plays a very important role in allowing the secondary tumour to seed and develop at its metastatic site. As such the primary tumour was set up in a metastatic model shown in Figure 1.8A using CMT19TF1 cell line, a lung carcinoma cell line that spontaneously metastasises to the lung when grown subcutaneously (Steri, 2015). This model of spontaneous metastasis has been exploited and adapted throughout this study. Steri also performed experimental metastasis studies (performing tail vein injections of tumour cells, eliminating the set-up of the primary tumour) to compare with these results. This showed no difference in the number of metastasis in lungs β_3 flox/flox/Tie1-Cre and Control mice. This shows that the primary tumour is essential in the metastatic advantage that $\alpha_v\beta_3$ confers.

The primary tumour was grown to $1cm^3$, according to the restrictions of the Home Office Licence, before it was resected. The mice were then left for another three weeks to allow metastatic tumours to grow before being sacrificed. Paraffin sections from lungs were used for H&E staining to visualise metastases, shown in Figure 1.8B. The number of metastatic tumours per section were then counted and the

results of this are presented in Figure 1.8C. The experiment shows a decreased number of spontaneous lung metastases in endothelial β_3 depleted animals compared to controls, showing that when $\alpha_v\beta_3$ integrin is depleted long term in endothelial cells there is a less supportive environment for a successful metastasis to occur.

1.6 Project Aims

The experiments presented in Figure 1.8 establish a pro-metastatic role for $\alpha_v\beta_3$ in endothelial cells. As described above endothelial cells can have a number of roles in cancer; they are a barrier for tumour cells and immune cells during metastasis, they can release a number of factors that support tumour growth and they play a part in the angiogenic switch. This and the importance of the primary tumour in this metastatic advantage were taken into account to decide on two main points of focus for the project.

The aim of this project was to evaluate two options in relation to $\alpha_v\beta_3$ and its effect on metastasis:

1. That the primary tumour vasculature might be “leaky”; have increased permeability leading to an increased number of circulating tumour cells
2. That there may be changes in the pre-metastatic niche meaning $\alpha_v\beta_3$ may play a role in developing an environment more hospitable for metastatic tumour growth.

2. Method

2.1 *In vitro* Cell Lines and Growth

2.1. CMT19T F1

This cell line was based on the mouse lung carcinoma CMT19T cell line derived from the CMT167 cell line. These cells were selected after one round of spontaneous metastasis to increase their metastatic potential to the lung.

2.1.2 B6-LV1

This was a breast carcinoma cell line given by the Weilbaecher lab which spontaneously metastasis to bone and lung obtained through multiple rounds of *in vivo* metastatic selection.

2.1.3 Maintenance of Cell Culture

Cells were grown in DMEM high glucose media at 37°C in a 5% CO_2 humidified atmosphere in gelatine coated flasks. Cells were maintained through passaging when they reached ~ 80% confluency washing with x1 PBS and 0.25% Trypsin:EDTA to detach cells.

2.1.4 Preparation for Injection

At~ 80% confluency cells were detached with 0.25% Trypsin:EDTA and collected in falcon tubes for counting. Following this cells were centrifuged at 1200 x g for 15 minutes before the supernatant was removed and the cells were washed in PBS. CMT19T F1 cells were then resuspended in PBS to allow injections of $1x10^6$ cells/100µl and kept on ice before use. B6-LV1 cells were then resuspended in 1:1 Matrigel/PBS mixture to allow injections of $1x10^6$/50µl.

2.2 Cre Genotyping

2.2.1 Isolation of DNA from Tissues

Tissue from the ear or tail was digested in 100µl tail lysis buffer overnight at 56°C. Then 100µl of isopropanol was added to each sample to precipitate the DNA and the samples were centrifuged at 2500 x g for 20 minutes. The pellet was dried for 30 minutes at 56°C, resuspended in 200µl TE Buffer and left at RT overnight.

2.2.2 PCR

Each PCR sample contained 9.1µl of MegaMix Blue Reagent, 0.08µl of forward primer and 0.08µl of reverse primer (both at 100 µM starting concentration), and 0.8µl isolated DNA. These went through the following reaction 95°C for 4 minutes followed by 35 cycles of denaturation (95°C for 1 minute), annealing (57.5°C for 45 seconds) and extension (72°C for 1 minute). The programme finished with an elongation step at 72°C for 10 minutes.

2.2.3 Electrophoresis

A 1.8% agarose gel (1.8g agarose, 98ml dH_2O, 2ml 50x TAE Buffer, 5µl 10mg/ml Ethidium Bromide) was used for DNA electrophoresis. Amplified DNA samples were loaded with 1x Loading Dye and a 1Kb DNA Ladder and separated for about 1 hour at 100V. The gel was then imaged under UV light using a UV Gel Image Capture System Box.

2.3 *In vivo* Studies

2.3.1 Injections

Mice aged between 6-8 weeks were immobilised and injected subcutaneously (CMT19T F1) or into the mammary fat pad (B6-LV1) with cells prepared as described.

2.3.2 Tumour Resection

The tumour size was measure regularly via callipers until it reached $1cm^3$, at about week 3. At this point mice were anaesthetised with isoflurane inhalant and the tumour was surgically resected.

2.3.3 Primary Tumour Permeability Assay

B6-LV1 Tumours were grown for 14 days. Tail vein injections of 200µl 1% FITC-dextran (x1 PBS) were performed. After 30 minutes, animals were perfused with PBS and the primary tumours were harvested. They were placed in formamide at 37°C for 48 hours and 200µl of this solution was analysed by fluorescence (excitation 490nm and emission 520nm). Obtained OD values were normalised against the weight of the tumour.

2.3.4 Sacrifice and Dissection

Mice were euthanised by cervical dislocation and lungs were inflated by tracheal injection and removed sterilely for later analysis.

2.4 Immunohistochemical Analysis

2.4.1 Embedding

Lungs were inflated with 5% gelatin extracted and fixed in 4% PFA for 1 hour at 4°C followed by 20% sucrose overnight at 4°C. The lungs were then embedded in 7.5% gelatin:15% sucrose. Blocks were then flash frozen in isopentane cooled by liquid nitrogen and stored at -80°C.

2.4.2 Sectioning

Sections of 10µm were taken at -20°C using a CryoStat (Leica Biotech) and stored at -80°C.

2.4.3 Staining

Sections were warmed in PBS to dissolve gelatin before blocking with 5% Goat Serum/0.1% Triton/PBS. The sections were then left for 30 minutes at RT in a moist chamber. The blocking solution was replaced with FITC-conjugated anti-CD45 diluted 1/500 in 5% Goat Serum/PBS and left in a moist chamber overnight at 4°C. Slides were washed in 1x PBS for 5 minutes three times and mounted with Flouromount, containing DAPI.

2.4.4 Microscopy

A Zeiss AxioPlan 2ie Microscope was used to take a defined region (encompassing the lung) of 5x images of DAPI and CD45 staining with a 10% overlap that were stitched by AxioPlan software.

2.4.5 Analysis

A Macro on FIJI was used with the following method. The region of interest was defined using a freehand tool and a threshold value, Triangle, was set. to eliminate

any background and focus on the staining of interest. Finally, parameters were set to filter objects according to circularity and size. The values were normalised against the ROI size to gain comparable values.

2.5 Flow Cytometry

2.5.1 Sample Preparation

Lung lobes were removed and rinsed in 70% ethanol and minced until homogeneous. This was digested with 0.1% Collagenase I/ PBS (supplemented with 1mM $CaCl_2$/1mM $MgCl_2$) at 37°C for 1 hour. A single cell suspension was then created by filtering cells through a 70µm mesh. These were collected in FACs tubes with 1ml red blood lysis buffer and incubated at RT for 1 minute. The cells were then centrifuged at 1000 x g for 5 minutes and resuspended in FACS buffer.

2.5.2 Staining

Samples were stained with four antibodies diluted 1:200 in FACS buffer; FITC anti-CD45, PE anti-Ly6C, PerCP-Cy5.5 anti-Gr-1, and APC anti-CD11b and incubated on ice for 20 minutes. Samples were then washed twice in PBS, and resuspended in 2% PFA/PBS and stored at 4°C until acquisition.

2.5.3 Acquisition

Samples were run using a BD Accuri C6 flow cytometer collecting $1x10^7$ events per sample. Single stained controls were also run to allow later compensation.

2.5.4 Analysis

Compensation was performed using single stained fluorescence controls to account for spill-over into other channels. An FSC-A/SSC-A plot was then used to remove cell debris and gate onto live cells. A quadrant plot was then used to gate onto the myeloid population ($CD45^+$/CD11b) followed by a scatter plot to differentiate between PMN and Monocyte populations of MDSCs.

2.6 Cytokine Array

2.6.1 Sample Preparation

Lung lobes were removed and homogenised in x1 PBS containing Halt Protease Inhibitor and Triton X-100 was added to a final concentration of 1%. Samples were then frozen at -80°C overnight after which they were thawed and centrifuged at for 5 minutes at 1200 x g to remove cell debris. The protein content was then quantified via a Bio-Rad DC Protein Assay Kit using BSA standards of known concentrations. Then 5µl of each sample was added to 200µl Reagent B and 25µl (50:1 Reagent A:Reagent S) and incubated at RT for 15 minutes. Absorbance was then measured at 750nm and the standards were used to create a standard curve used to obtain protein concentrations for unknown samples. Samples were then pooled (7 of each tumour bearing genotype) to allow equal protein volume (57µg) from each samples up to 200µg.

2.6.2 Immunoblotting

An immunoblotting procedure was then followed with the reagents and according to the instructions supplied by the manufacturer of the kit.

2.6.3 Imaging and Analysis

Blots were imaged using a FUJIFILM Luminescent Image Analyser LAS-1000plus for up to 10 minutes taking pictures at 30 second intervals. The densities of each spot was then analysed using FIJI analysing two lanes at once using one as a control to produce comparable values. An average of reference spots was then taken and values were normalised to these.

2.7 Western Blot Analysis

2.7.1 Sample Preparation

Lungs were prepared and quantified as described in 2.6.1. Samples were prepared to allow 35µg of protein in 1x Reducing Buffer and 1x Sample Buffer with 0.1% Tween-20 x1 PBS up to 20µl. These were then incubated at 95°C for 5 minutes and centrifuged at 10000 x g for 1 minute.

2.7.2 SDS-PAGE Electrophoresis

Prepared samples were loaded into a 12% polyacrylamide gel with Spectra™ Multicolour Broad Range Protein Ladder. The gel was then run in Running Buffer at 100V until samples reached the bottom of the gel.

2.7.3 Transfer

Protein was then transferred to a nitrocellulose membrane in Transfer Buffer at 4°C for 1 hour and 15 minutes at 115V.

2.7.4 Immunoblotting

The nitrocellulose membrane was blocked (5% Milk Protein in 0.1% Tween-20 PBS) for 30 minutes at RT and washed three times in 0.1% Tween-20 PBS for 5 minutes. The membrane was incubated with primary antibodies diluted 1:1000 (5% BSA in 0.1% Tween-20 x1 PBS) overnight at 4°C. It was then washed three times in 0.1% Tween-20 PBS for 5 minutes. Secondary antibodies diluted 1:2000 (5% BSA in 0.1% Tween-20 x1 PBS) were then added and the membrane was incubated for 1 hour at RT. Finally, the membrane was washed three times in 0.1% Tween-20 x1 PBS for 5 minutes.

2.7.5 Imaging and Analysis

The membrane was incubated for 1 minute with ECL reagents and imaged with a FUJIFILM Luminescent Image Analyser LAS-1000plus taking images at 20 second intervals. The image was then quantified with FIJI normalising results against a loading control.

2.8 Statistical Analysis

Data was analysed by Student's *t*-test and ANOVA using the GraphPad Prism v6.01. *P* values <0.05 were considered significant and error bars on graphs show mean +SEM.

2.9 Reagents, Buffers and Kits

2.9.1 Buffers and Solutions

DMEM High Glucose

DMEM high glucose+Glutamax (Invitrogen), 10% Fetal bovine serum (FCS), heat inactivated (HyClone, Invitrogen), 2 mM L-Glutamine (Invitrogen), 100 U/ml Penicillin/ 100 µg/ml Streptomycin (Invitrogen)

Tail Lysis Buffer

0.2% SDS, 100 mM Tris-base, 200 mM NaCl, 5 mM EDTA, 0.5 mg/ml proteinase K

TE Buffer

10 mM Tris-base, 1 mM EDTA

x50 TAE Buffer

242 g Tris-base, 57.1ml acetate (100% acetic acid), 100 ml 0.5 M EDTA, up to 1 litre dH_2O

FACS Buffer

x1 PBS, 1% Fetal bovine serum, 10mM HEPES (1M stock solution, Invitrogen)

12% Acrylamide Gel

A) Stacking

5% Acrylamide Solution (30%, Protogel, National Diagnostics (Hessle, UK)), 0.1% SDS, 0.1% ammonium persulphate, 0.5% TEMED, dH_2O

B) Resolving

12% Acrylamide Solution, 0.1% SDS 3ml, 0.1% ammonium persulfate, 0.5% TEMED, dH_2O

Running buffer

25 mM Tris-base, 192 mM glycine, 0.1% SDS, dH_2O

Transfer buffer

25 mM Tris base, 192 mM glycine, 20% methanol, dH_2O

2.9.2 Reagents

Matrigel (0.7% solution): BD Bioscience

MegaMix Blue Reagent: Cambio (Cambridge, UK)

FITC Dextran (low MW 4300): Sigma-Aldrich

1Kb Plus DNA Ladder: Invitrogen

Fluoromount-G with DAPI: eBioscience (Hatfield, UK)

Red blood lysis buffer (10X): eBioscience (Hatfield, UK)

Halt Protease Inhibitor: Invitrogen

NuPAGE Sample Reducing Agent (10X): Invitrogen

NuPAGE LDS Sample Buffer (4X): Invitrogen

Spectra^{TM} Multicolour Broad Range Protein Ladder: ThermoFisher Scientific (UK)

2.9.3 Kits

Bio-Rad DC Protein Assay kit: Bio-Rad Laboratories (Hertfordshire, UK)
Proteome Profiler Mouse XL Cytokine Array ARY028: R&D Systems (Abingdon, UK)
ECL kit: Bio-Rad Laboratories (Hertfordshire, UK)

2.9.4 Antibodies

2.9.4.1 Flow Cytometry
FITC anti-CD45 (Immunohistochemistry) – Clone A20: eBioscience (Hatfield, UK)
PE anti-Ly6C – Clone HK1.4: eBioscience (Hatfield, UK)
PerCP-Cy5.5 anti-Gr-1 – Clone M1/70: eBioscience (Hatfield, UK)
APC anti-CD11b – Clone RB6-8C5: eBioscience (Hatfield, UK)
2.9.4.2 Western Blot
anti-GAPDH – 14C10, # 2118: Cell Signalling Technology
anti-CCL21 – ab9903: abcam
anti-CCL6 – ab196521: abcam
anti-P-Selectin - # AF737: R&DSystems (Abingdon, UK)

2.9.5 Primers

Tie1-Cre PCR
Forward primer: 5'-GCC TGC ATT ACC GGT CGA TGC AAC GA-3'
Reverse primer: 5'-GTG GCA GAT GGC GCG GCA ACA CCA TT-3'

2.9.6 Equipment

PROTRAN Nitrocellulose Membrane: ThermoFisher Scientific (UK)
UV BioDoc-It Imaging System: UVP (Cambridge, UK)
Accurie C6 flow cytometer: BD Bioscience
FUJIFILM Luminescent Image Analyser LAS-1000plus: Fujifilm UK (Bedford, UK)
UV/Vis Spectrometer Microplate Reader: BMG Labtech (Aylesbury, UK)
Zeiss AxioPlan 2ie Microscope
CryoStat (Leica Biotech)

3. Results

3.1 Studying the Interactions of the Endothelium with Tumour Cells

The first step of the metastatic cascade that endothelial cells play a role in is intravasation, where they act as a physical barrier between the tumour and a transport system. Changes to this barrier will affect the chance of cancer cells gaining access to a transport system. With a change to the number of cancer cells in the vascular system the chance of metastatic cells reaching a metastatic site and successfully colonising it increases (Criscuoli *et al*., 2005).

There is evidence that FAK, a protein influenced by $\alpha_v\beta_3$, can affect the permeability of the vasculature by targeting vascular endothelial cadherin (Chen *et al*., 2012). Increasing the permeability of the vasculature influences the intravasation and extravasation of cancer and cancer supportive cells, consequently encouraging metastasis (Jean *et al*., 2014).

To study the potential of $\alpha_v\beta_3$ integrin to influence permeability an *in vivo* study of the permeability of the primary tumour vasculature was carried out.

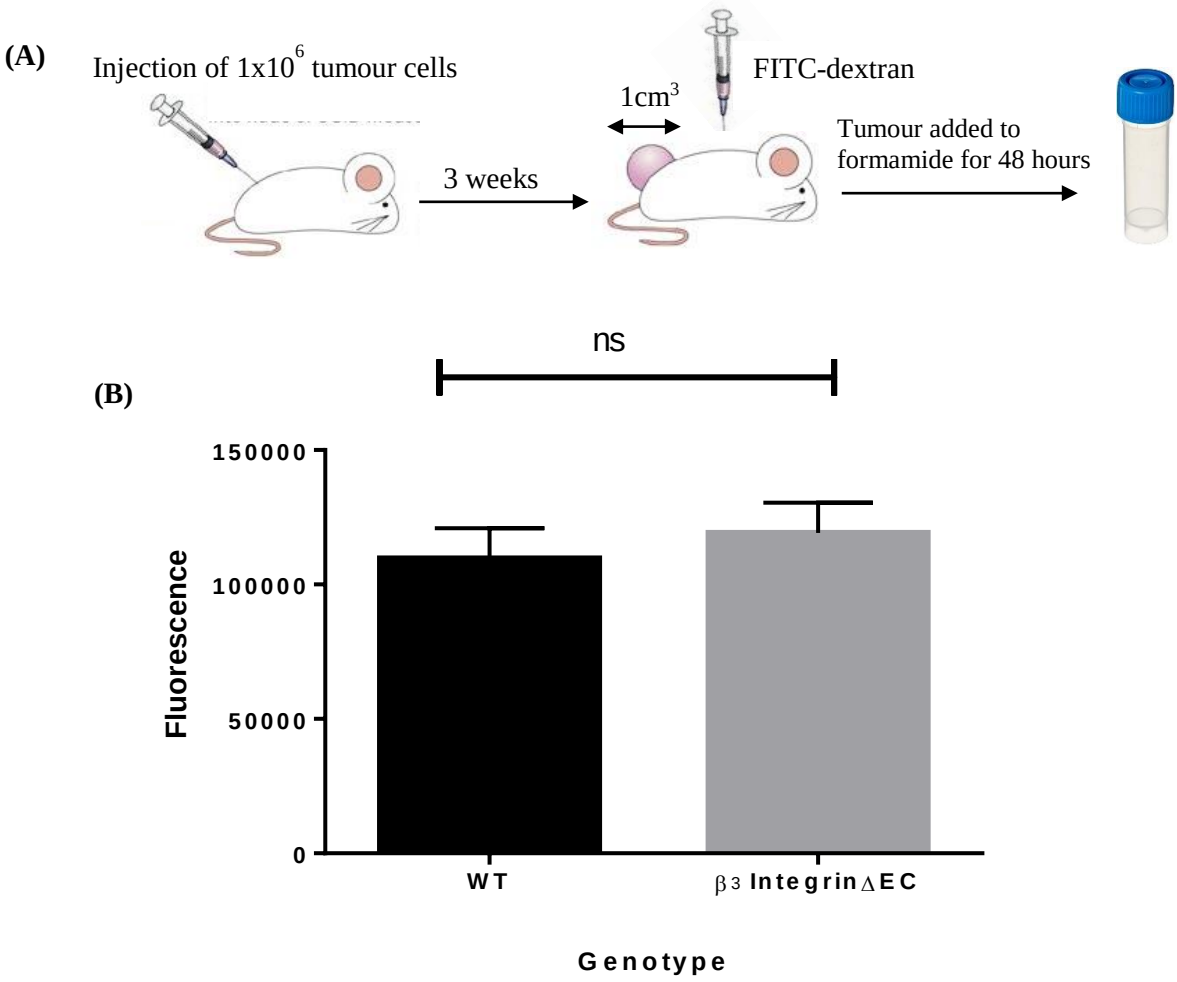

Figure 1: The permeability of the tumour vasculature *in vivo* is not affected by knocking out $\alpha_v\beta_3$ in endothelial cells.

(A) B6LV1 tumours grown for 3 weeks in WT (Cre-negative) and $\beta_3$flox/flox/Tie1-Cre (β_3IntegrinΔEC) mice. FITC-dextran was injected intravenously and circulated for 30 minutes before sacrifice. Tumours were left for 48 hours in formamide to extract FITC and fluorescence was quantified. **(B)** The fluorescence was normalised against the tumour weight and the mean is shown in a bar graph (WT n=6, β_3IntegrinΔEC n=5; Mean + SEM; ns= no significance).

A breast cancer cell line (B6LV1) that spontaneously metastasises to breast and lung was used for this experiment, shown in Figure 3.1A. Tumours were grown for three weeks before mice were sacrificed for the experiment. Low molecular weight FITC-dextran, which is small enough to leak out of permeable vessels, was injected via the tail vein. The mice where left for 30 minutes for the FITC-dextran to circulate before the primary tumours were harvested. These were left in formamide for 48 hours to extract the FITC-dextran before fluorescence was quantified. Results were then normalised against the weight of the primary tumour and are presented in Figure 3.1B.

Figure 3.1B shows that $\alpha_v\beta_3$ has no significant effect on the permeability of the primary tumour vasculature. This was supported by studies from Steri (2015; unpublished) that looked at a potential increase in intravasation/dissemination and consequently metastasis by identifying the numbers of tumour cells. Previous experiments showed that the numbers of circulating tumour cells were not significantly affected by a knockout of β_3 integrin in endothelial cells.

3.2 Cytokine Profile of the Pre-Metastatic Niche

The pre-metastatic niche is the set-up of an environment in distant organs that leads to preferential metastasis to said organ. The formation and development of this niche is reliant upon signals from the primary tumour. The importance of the primary tumour in the metastatic phenotype in endothelial β_3 integrin knock out mice, shown in the previously described experiments, may be explained by a role for $\alpha_v\beta_3$ integrin in influencing the pre-metastatic niche.

Since it is secreted factors and cells from the primary tumour and bone marrow that are the essential elements for establishing the pre-metastatic niche, transport systems play an important role in the set-up. As such, endothelial barriers will control the movement of niche components to 'soil' tissues. One of the ways that the pre-metastatic niche is manipulated is by cytokine release. For example, VEGF, which has a well-documented role in supporting the homing of bone marrow components to tissues is known to play a significant role in niche generation (Kaplan *et al*., 2005). Cytokine presence is then propagated by the immune cells that begin to colonise the pre-metastatic environment.

To assess how the cytokine profile of the pre-metastatic niche is affected by acute depletion of endothelial β_3 integrin a cytokine array was used. Tumours were grown in seven mice from each genotype, WT (Cre-negative) and β_3 flox/flox/Tie-1Cre, for

three weeks to allow time for the pre-metastatic niche to be set up. Lungs from these mice and three control non-tumour bearing β_3IntegrinΔEC mice were lysed, quantified, and pooled so that there were equal quantities of protein from the individual samples in each pool. They were then used for a cytokine array that detected 111 mouse cytokines. The array was analysed using FIJI™ using one consistent line to get around the arbitrary assignment of units. The results of the arrays were then normalised against an average of the three reference spots.

Each of the 111 cytokines detected by the array was researched, looking for potential roles in cancer development and the immune system. The relevant cytokines have been grouped into six categories and are presented in graphs in Figure 3.2 with explanations of their roles in tables below.

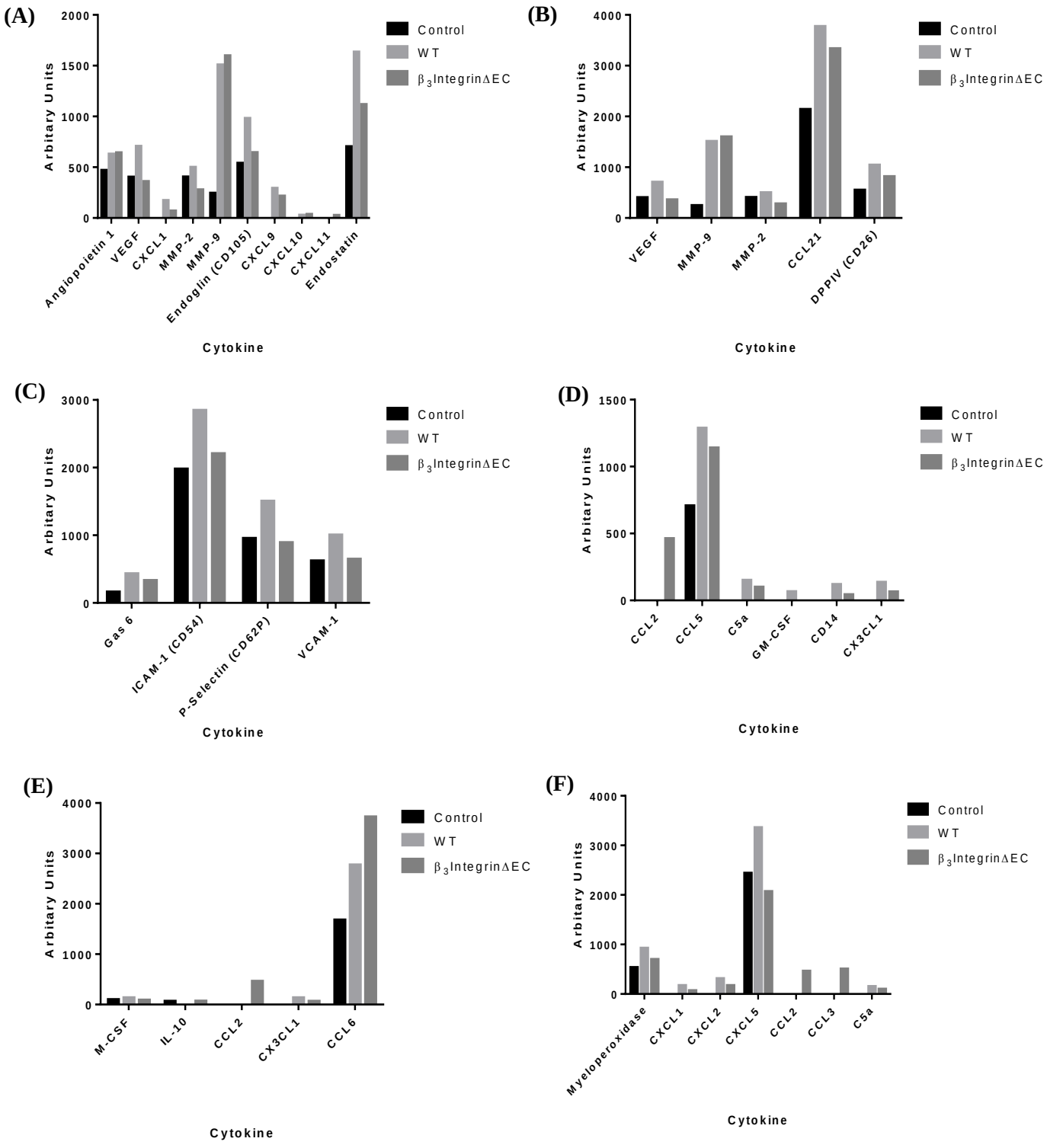

Figure 2: The effect of depleting endothelial cell $\alpha_v\beta_3$ on the cytokine profile of the lung pre-metastatic niche.

Untreated lungs (Control) and pre-metastatic lungs from $\beta_3$flox/flox/Tie1-Cre and WT (Cre-negative) mice with tumours that had been grown for three weeks were digested. The tissue lysates of the same genotype (Control n=3, WT n=7, β_3IntegrinΔEC n=7) were pooled into one sample that was used in a cytokine array. The array was analysed relative to reference points using ImageJ. The results were then assembled into groups according to the different functions of the cytokines. **(A)**Angiogenic markers **(B)** Pre-metastatic niche markers **(C)** Leukocyte recruitment markers. Myeloid cells have an important role in the

development of the pre-metastatic nice; **(D)** Monocyte recruitment markers **(E)** Macrophage recruitment markers (**F)** Neutrophil recruitment markers.

The angiogenic potential of an environment is defined by the balance of pro to anti-angiogenic factors. A number of angiogenic factors, including angiostatin, angiopoietin 1 and VEGF are shown in Figure 3.2A and the roles of these factors are shown in Table 1. Of these cytokines, a few (both pro and anti angiogenic) are much more greatly expressed by WT mice (VEGF, endoglin and endostatin) with most being almost equally expressed by WT and β_3IntegrinΔEC but lower in non-tumour bearing mice. Overall the expression patterns of the cytokine array do not seem to suggest any particular shift to a pro or anti angiogenic phenotype.

Table 1: Angiogenic factors in the pre-metastatic niche

	Cytokine	**Function**
Promoting Factors	VEGF (VEGF-A)	First known as a vascular permeability factor this cytokine has been shown to be essential for the vascularisation of the tumour stimulating the proliferation, migration and survival of endothelial cells (Ferrara *et al*., 2003; Neufeld *et al*., 1999; Bergers *et al*., 2000).
	Angiopoietin 1	Influences angiogenesis via its interaction with Tie2 promoting endothelial cell survival and in the later stages of vascular remodelling inhibiting vessel permeability and stabilising blood vessels (Suri *et al*., 1996; Kwak *et al*., 1999; Gavard *et al*., 2008).
	MMP-2,-9	These proteases break down the basement membrane facilitating the movement of endothelial cells and playing an important role in vascular remodelling (Carmeliet and Jain, 2000; Zheng *et al*., 2006). MMP-9 in particular is important for tumour angiogenesis by its stimulation of VEGF secretion and when genetically ablated, angiogenesis is reduced (Bergers *et al*., 2000).
	CXCL-1	A tumour promoting chemokine secreted from tumour and endothelial cells, it promotes the influx of angiogenesis supporting neutrophils and supports endothelial cell motility and proliferation (Miyake *et al*., 2013; Scapini *et al*., 2004).
	Endoglin	A marker of tumour vasculature, endoglin is expressed by proliferating endothelial cells and plays an important role in forming a mature and stable vasculature in mice (Dallas *et al*., 2008; Ten Dijke *et al*., 2008.
Inhibitory Factors	CXCL-9,-10,-11	The production of these angiostatic cytokines is linked with a reduction in tumour angiogenesis (Liu *et al*., 2011). *In vitro* this effect has been shown to be mediated by the expression of the receptor CXCR3B on the endothelium (Strieter *et al*., 2006)
	Endostatin	An endogenous inhibitor of angiogenesis and anti-tumorigenic cytokine, endostatin inhibits the migration and promotes apoptosis of endothelial cells (Dhanabal *et al*., 1999; Yamaguchi *et al*., 1999; O'Reilly *et al*., 1997).

There are a number of cytokines which have been studied as pre-metastatic niche related or metastasis supportive cytokines that were a part of this cytokine array.

These are presented in Figure 3.2B and more in depth descriptions of their roles are described in Table 2.

While there mostly seems to be a trend towards an increase in expression of these cytokines in tumour bearing mice only a few show notable differences in expression in WT compared to β_3IntegrinΔEC. The almost doubled expression of VEGF in tumour bearing WT mice, combined with its role in the formation of pre-metastatic cluster of haematopoietic progenitor cells (Kaplan *et al*., 2005) makes VEGF of interest. As does the particularly high expression and tumour cell homing role of CCL21 (Krieg and Boyman, 2009).

Table 2: The functions of cytokines related to metastasis

Cytokine	Function
VEGF	This factor is secreted by the primary tumour and recruits VEGFR1$^+$ HPCs to tissues. These form cellular clusters critical to pre-metastatic niche formation (Kaplan *et al*., 2005).
MMP-9	When VEGFR1$^+$ HPCs enter a tissue and from pre-metastatic clusters they secrete MMP-9. This contributes to pre-metastatic niche formation by degrading the basement membrane and allowing the invasion of further cells which define the niche (Kaplan *et al*., 2005; Kaplan *et al*., 2006).
MMP-2	This is another protease secreted by BMDCs that are recruited to the pre-metastatic niche (Sceneay *et al*., 2013).
CCL21	The receptor for this chemokine, CCR7, is highly expressed by metastatic breast cancers and mediates the homing of these metastatic tumour cells to specific tissues (Strieter, 2001).
DPPIV	Expressed on the lung endothelium, DPPIV has been shown *in vivo* to facilitate the invasion of metastatic breast cancer cells by acting as an adhesion receptor in the lung (Johnson *et al*., 1993; Cheng *et al*., 1998).

Cytokines and chemokines play important roles in the immune system, co-ordinating the homing of leukocytic populations to different tissues. Some are involved in the movement of specific leukocyte sub-populations but others control general leukocyte movement and the expression of these is grouped into Figure 3.2C. Most of the proteins below are involved in the extravasation of circulating leukocytes to peripheral tissues. They are not cytokines themselves, but their expression is influenced by surrounding cytokines.

In Figure 3.2C there seems to be a correlation between depleting β_3 integrin in endothelial cells and decreasing the expression of these leukocyte recruitment proteins. Since many of these proteins facilitate adhesion to the endothelium (as described in Table 3) and are expressed by endothelial cells these changes in expression in β_3IntegrinΔEC mice is of particular interest.

Table 3: The leukocyte related functions of cytokines

Cytokine	Function
P-Selectin	This transmembrane protein is expressed by endothelial cells and plays a part in the trafficking of leukocytes to sites of inflammation (Muller, 2013). This role is facilitated by P-selectin's interactions with selectin ligands on leukocytes such as P-selectin glycoprotein ligand-1 (Norman *et al*., 1995). The importance of this function has been shown in P-selectin knock out mice which display faulty leukocyte rolling and transendothelial migration (Mayadas *et al*., 1993).
VCAM-1	Another protein that supports adhesion of leukocyte populations to the endothelial layer, it interacts with the leukocyte integrin VLA-4 allowing the tethering and rolling of leukocytes across the endothelium (Elices *et al*., 1990).
ICAM-1	The presence of ICAM-1 is upregulated on the endothelium by pro-inflammatory cytokines (Lawson and Wolf, 2009). It interacts with LFA-1 on all leukocyte populations to support the adhesion and eventual migration of leukocytes to inflamed tissues (Makgoba *et al*., 1988).
GAS-6	It plays a role in the sequestering and extravasation of leukocytes and when knocked out in mice the influx of leukocytes to sites of inflammation decreases (Tjwa *et al*., 2008; Laurance *et al*., 2012).

The protein expression shown in Figure 3.2C may suggest an increased influx of leukocytes to the pre-metastatic niche in WT (Cre negative) mice. A number of the cytokines in the array control the movement of specific populations of immune cells that have tumour supportive roles. Some of these are described in Figures 3.2D-F.

The recruitment of monocytes from the circulatory system to the primary tumour is a recognised part of tumour biology. Part of the myeloid lineage, they are pre-cursors to a number of populations including macrophages and monocyte-derived dendritic cells (Richards *et al*., 2013). The presence of these cells has also been used to identify the existence of a pre-metastatic niche (Batista *et al*., 2014).

The roles of all the cytokines associated with monocytes are described in Table 4 but the cytokine of particular interest in Figure 3.2D is CCL2. The chemokine is one of the most well-known monocyte recruitment proteins. It plays an important role in the infiltration of monocytes into the primary tumour and via monocytes, will support the extravasation of tumour cells at metastatic sites (Bonapace *et al*., 2014; Qian *et al*., 2011). Despite this obviously pro-tumorigenic role, when β_3 Integrin is depleted in endothelial cells and metastasis reduces, its expression increases.

Table 4: Cytokines related to monocyte homing

Cytokine	Function
CCL2	Chemokine for monocytes which expresses CCR2, the CCL2 receptor. In CCL2 deficient mice monocyte recruitment to inflamed tissue is diminished (Qian *et al*., 2011).
CCL5	This is another potent monocyte chemokine via the CCR5 receptor and when this axis is disrupted monocyte infiltration decreases (Soria and Ben-Baruch, 2005).
C5a	Complement component 5a acts as a chemotactic agent for monocytes and upon binding its receptor C5L2, stimulates the release of cytokines (Manthey *et al*., 2009).

GM-CSF	Administering this to humans has been shown to increase the number of monocytes in circulation (Hamilton, 2002).
CD14	This is a monocyte membrane bound protein that can be used as a marker for the presence of monocytes (Schütt, 1999; Simmons *et al*., 1989).
CX3CL1	Expressed by endothelial cells CX3CL1 interacts with CX3CR1 on monocytes facilitating their adhesion to the endothelium as part of an inflammatory response. In secreted from it will also act as a monocytic chemokine (Imaizumi *et al*., 2004; Ancuta *et al*., 2006; Imaizumi *et al*., 2000).

Part of the significance of monocytes in cancer biology is that they are pre-cursors to the macrophage lineage. After monocytes are recruited from circulation they will differentiate into macrophages. This differentiation is controlled by a range of cytokines including a number of those described in Table 5. Macrophages can have distinct roles in inflammation which is controlled by their polarisation to M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotypes. In tumours macrophages tend to shift to a tumour associated, M2c phenotype (Sica *et al*., 2006). One cytokine that is presented in Figure 3.2E and plays an important role in this process in IL-10. Its expression pattern, however, does not suggest that it plays a role in the endothelial β_3 integrin mediated metastatic phenotype.

Mostly the expression of these macrophage associated cytokines in Figure 3.2E is low but CCL2 and CCL6 have interesting expression patterns in conflict with their published roles. Due to the monocytic recruitment properties of CCL2 it also correlates with the number of macrophages. Once again it reportedly plays a tumour and metastasis supportive role which stands at odds with its expression only in the lungs of tumour bearing β_3IntegrinΔEC mice. This oddity is shared by CCL6 which has a role in macrophage homing and is also most strongly expressed by tumour bearing β_3IntegrinΔEC mice.

Table 5: Cytokines that regulate macrophage infiltration and function

Cytokine	**Function**
M-CSF	In a tumorigenic environment M-CSF can recruit macrophages, supporting their migration and survival (Mantovani *et al*., 2002). It can also stimulate the differentiation of monocytes to macrophages (Martinez *et al*., 2006).
IL-10	This interleukin plays an important part in macrophage polarisation, supporting an M2c phenotype. These are known as tumour associated macrophages (Mantovani *et al*., 2005; Martinez *et al*., 2008).
CCL2	*In vivo* CCL2 can act as a macrophage chemokine via the receptor CCR2. In tumour the levels of CCL2 positively correlate with the number of TAMs (Conti and Rollins, 2004; Zhang *et al*., 2010).
CX3CL1	It has been shown *in vitro* and *in vivo* that CX3CL1 can recruit CX3CR1 expressing macrophages (Reed *et al*., 2012; Truman *et al*., 2008).
CCL6	Produced by a number of tumours it is most commonly a chemokine that facilitates homing of macrophages, an ability shown *in vitro* and *in vivo* (Coelho *et al*., 2007).

Neutrophils are another subset of leukocyte with both pro-tumorigenic and anti-tumorigenic properties in the primary tumour microenvironment in a similar way to polarised macrophages and T cells (Gregory and Houton, 2011). As part of the polymorphonuclear leukocyte (PMN) family, they have been described as determinants of a pre-metastatic soil (Batista *et al*., 2014).

Of the neutrophil chemoattractants described in Table 6 CXCL5 was expressed most strongly with a trend suggesting that β_3 integrin in endothelial cells could support the influx of neutrophils. Contrary to this both CCl-2 and -3, which are involved in the recruitment of PMN cells to tissues, are expressed by tumour bearing β_3IntegrinΔEC mice but not by other mice. This recruitment of BMDCs is thought to support tumour growth so this expression pattern does not adhere to expectation. This could suggest a complex profile of PMNs with a different function in the pre-metastatic niche of the different genotypes.

However, with these chemoattractants not influencing the infiltration of just one population of immune cells it is hard to picture how these expression patterns might affect immune cells in the pre-metastatic niche.

Table 6: Cytokines controlling PMN and Neutrophil infiltration

Cytokine	Function
Myeloperoxidase*	As a recruiter of neutrophils myeloperoxidase supports the adherence of these leukocytes to the wall of the endothelium eventually leading to tissue invasion (Klinke *et al*., 2011).
CXCL-1,-2*	Macrophages secrete these CXCR2 ligands, and they are critical in mediating the recruitment of neutrophils to tissues (De Filippo *et al*., 2013; Scapini *et al*., 2004).
CXCL-5	Similarly to CXCL-1 and -2, CXCL-5 is a chemoattractant for neutrophils via the receptor CXCR-2. This effect has been demonstrated both *in vitro* and *in vivo* (Song *et al*., 2013; Vieira *et al*., 2009).
CCL-2,-3*	These are important mediators of neutrophil recruitment, promoting their extravasation into tissues and when depleted neutrophil transmigration decreases (Reichel *et al*., 2012; Reichel *et al*., 2009).
C5a	Exposure of neutrophils to this chemoattractant induces the formation of pseudopodia and migration (Wright *et al*., 2010).

*Also shown to be involved in PMN recruitment

Overall there seems to be an increased expression of cytokines in tumour bearing mice in comparison to control mice, suggesting that the primary tumour is affecting this secondary environment. The method used for this cytokine array, however, meant that it was not possible to show statistical significance of individual cytokines. Therefore, certain cytokines were chosen (based on antibody availability, expression pattern and role in pre-metastatic niche development) to be studied individually. To do this the pools were deconstructed and each tissue lysate sample was analysed via Western blot shown in Figure 3.3A. The expression of the proteins was quantified using FIJI and as an extra layer of accuracy, the results were normalised using the loading controls.

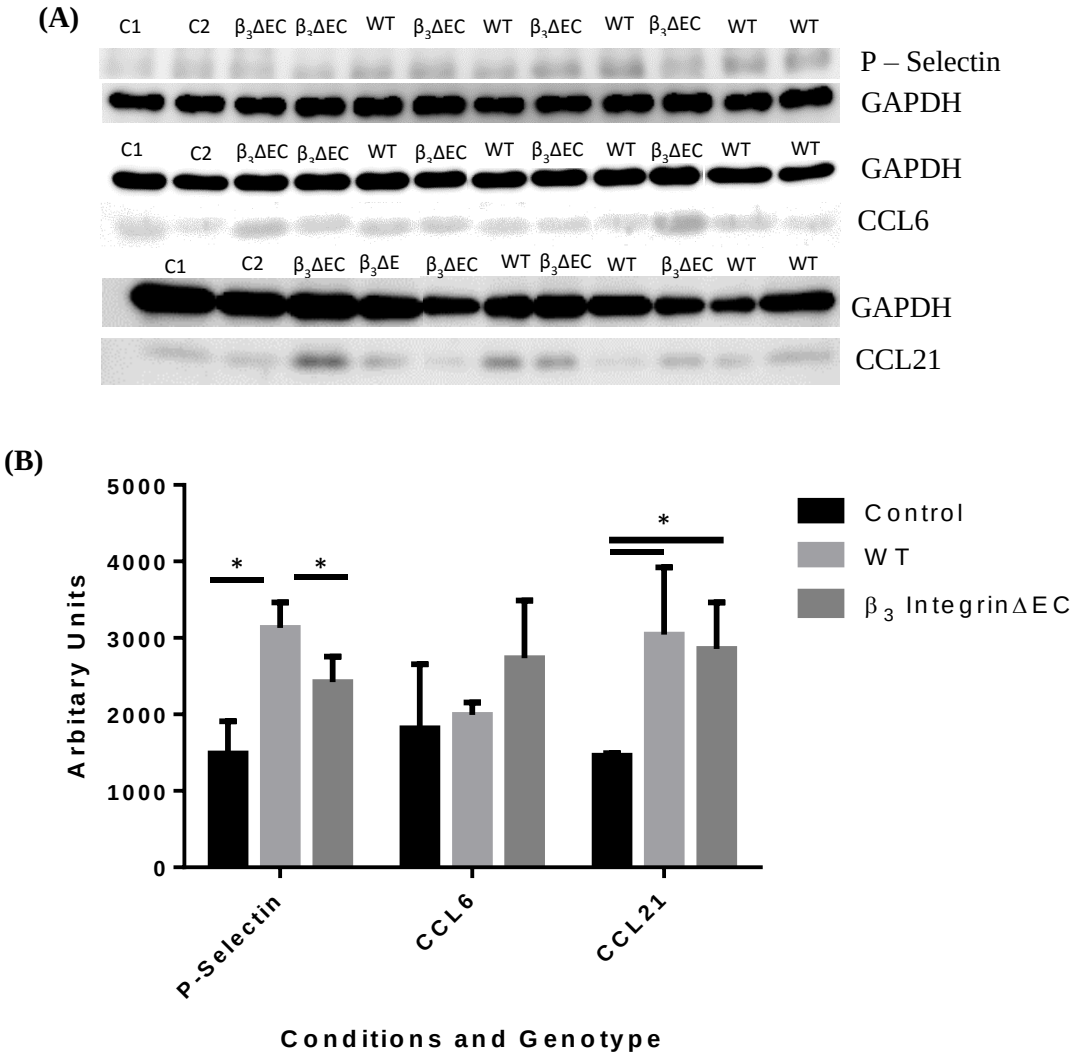

Figure 3: Pools were deconstructed to study the significance of cytokines of particular interest.

Tissue lysates from the lungs of control mice and pre-metastatic lungs from β_3IntegrinΔEC and WT (Cre-negative) mice were studied for the presence of P-selectin, CCL6, CCL21 and a loading control, GAPDH (Control n=2, WT n=5, β_3IntegrinΔEC n=5). **(A)** The loading control was used as a point of reference to normalise the results. **(B)** The levels of cytokines relative to the loading control was quantified using ImageJ (Mean + SEM; * $p<0.05$).

All these cytokines follow similar expression patterns to those described in the cytokine array which might support the trends suggested by the array. Of these three cytokines, however, only two of them have changes of expression in the different conditions and genotypes that is statistically significant following densitometric analyses.

The first is P-selectin which plays an important role in the adhesion of leukocytes to the endothelium. The expression of this protein is significantly higher in β_3 integrin positive tumour bearing mice in comparison to the control non tumour mice. There is also a significant decrease in the expression of this protein in the pre-metastatic niche when β_3 integrin is depleted in endothelial cells. This suggests that there could be changes to populations of leukocytes in the pre-metastatic niche.

There is also a significant increase in the expression of CCL21 in the tumour bearing mice compared to the control mice. As described in Table 2, when this protein is expressed in the pre-metastatic niche it can support the homing of metastatic cancer cells to this supportive environment. While there is no significant change in the expression of this protein with β_3 integrin depletion the increased expression in tumour bearing mice could be taken as support of the presence of a pre-metastatic niche and the trends that the cytokine array suggests.

3.3 Leukocyte Populations in the Pre-Metastatic Lung

The significant increases of P-selectin expression in WT tumour bearing mice in comparison to the others lead me to study how overall populations of leukocytes might be affected in the pre-metastatic lung. CD45 (a pan leukocyte marker) staining was utilised for this in flow cytometry and immunostaining of lung sections.

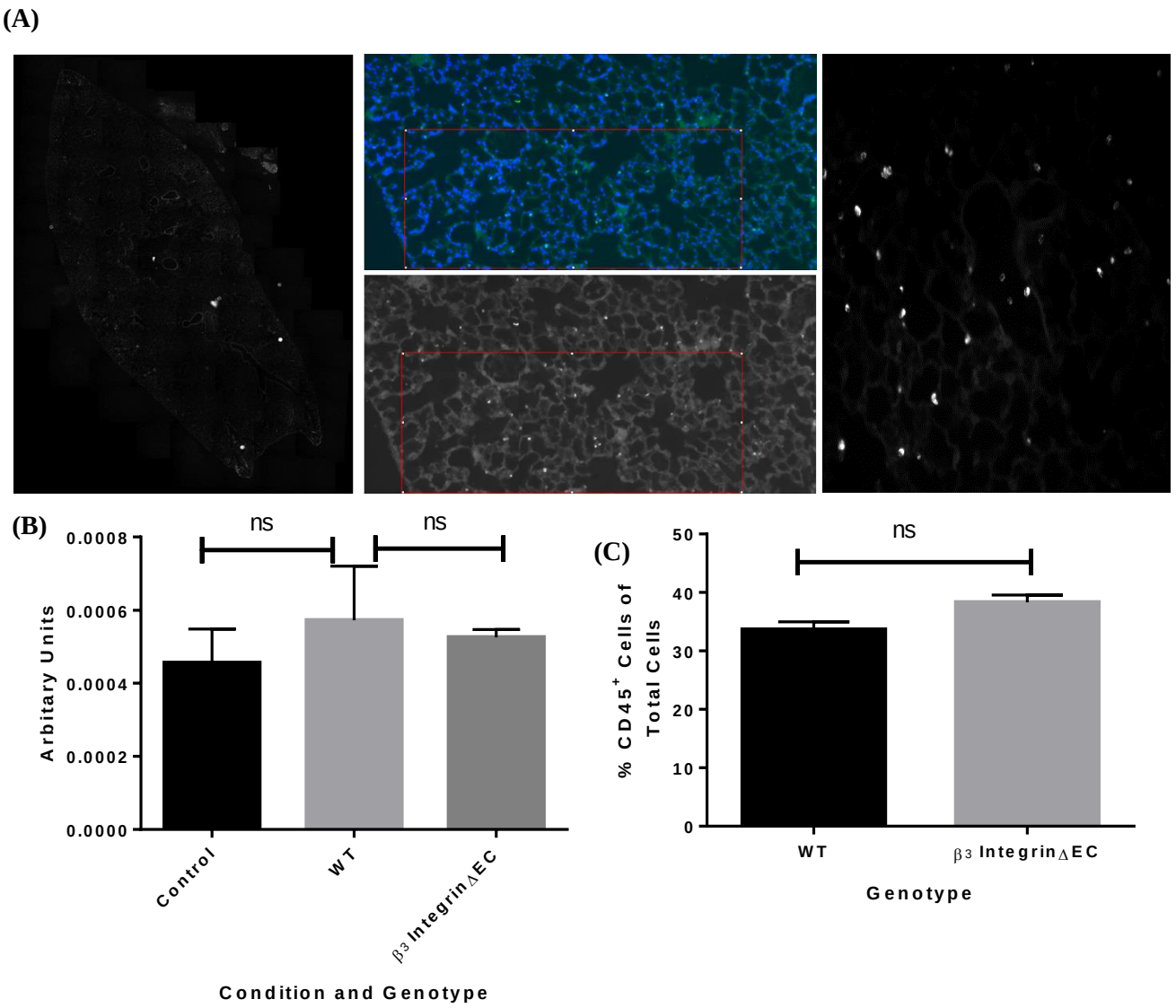

Figure 4: Overall populations of leukocytes in the pre-metastatic niche are not affected by the depletion of $\alpha_v\beta_3$ in endothelial cells.

(A) Cryosectioned control and WT (Cre-negative) and $\beta_3$flox/flox/Tie1-Cre (β_3IntegrinΔEC) pre-metastatic lung were stained with CD45-FITC (green) and DAPI (blue). Images were collected and stitched using Zeiss Axioplan to prepare images of whole lung sections. **(B)** ImageJ was used to quantify the staining which was then normalised against the size of the lung section (Mean + SEM; Control n=2, WT n=3, β_3IntegrinΔEC n=3; ns= no significance). **(C)** Flow cytometry using pre-metastatic WT and $\beta_3$flox/flox/Tie1-Cre was used to find the number of $CD45^+$ cells (WT n=4, β_3IntegrinΔEC n=4).

The pre-metastatic mouse model was once again exploited in this experiment using the CMT19T-F1 cell line. Primary tumours were grown for 3 weeks (to about $1cm^3$)

before mice were sacrificed allowing time for the pre-metastatic niche to develop. In figures 4A and B the lungs were inflated and embedded in gelatine before freezing in liquid nitrogen cooled isopentane. The samples were then cryosectioned and stained with DAPI (blue) and CD45-FITC (green), shown in Figure 3.4A. To obtain an idea of the presence of CD45 in the pre-metastatic niche that was as accurate as possible the whole lung sections were analysed. To do this images were taken and then stitched together to get images of the whole lung, shown in Figure 3.4A. FIJI was then used to quantify the CD45 staining in the lung using a macro based on particle analysis, to keep consistency, eliminating background based on threshold, circularity and size. These results were normalised against the size of the lung section and are presented in Figure 3.4B. This figure shows no significant change in CD45 staining, and therefore leukocyte presence, in the pre-metastatic lung with β_3 integrin depletion however these are again preliminary results and could be repeated.

This result is supported by the analysis of CD45 in the pre-metastatic lung using flow cytometry. Once again using the CMT19T-F1 cell line for the pre-metastatic niche model the numbers of $CD45^+$ cells in the pre-metastatic lung was analysed using flow cytometry gating to remove dead cells and cell debris. This also showed that β_3 integrin depletion has no significant effect on leukocyte infiltration.

While Figures 3.4B and C show some small changes in the expression of CD45 in the pre-metastatic lung none of these are significant. Overall, the infiltration of leukocytes to the pre-metastatic lung does not seem to be affected by depletion of β_3 integrin in endothelial cells. Since there is no general leukocytic change to the pre-metastatic niche, individual leukocyte lineages were studied.

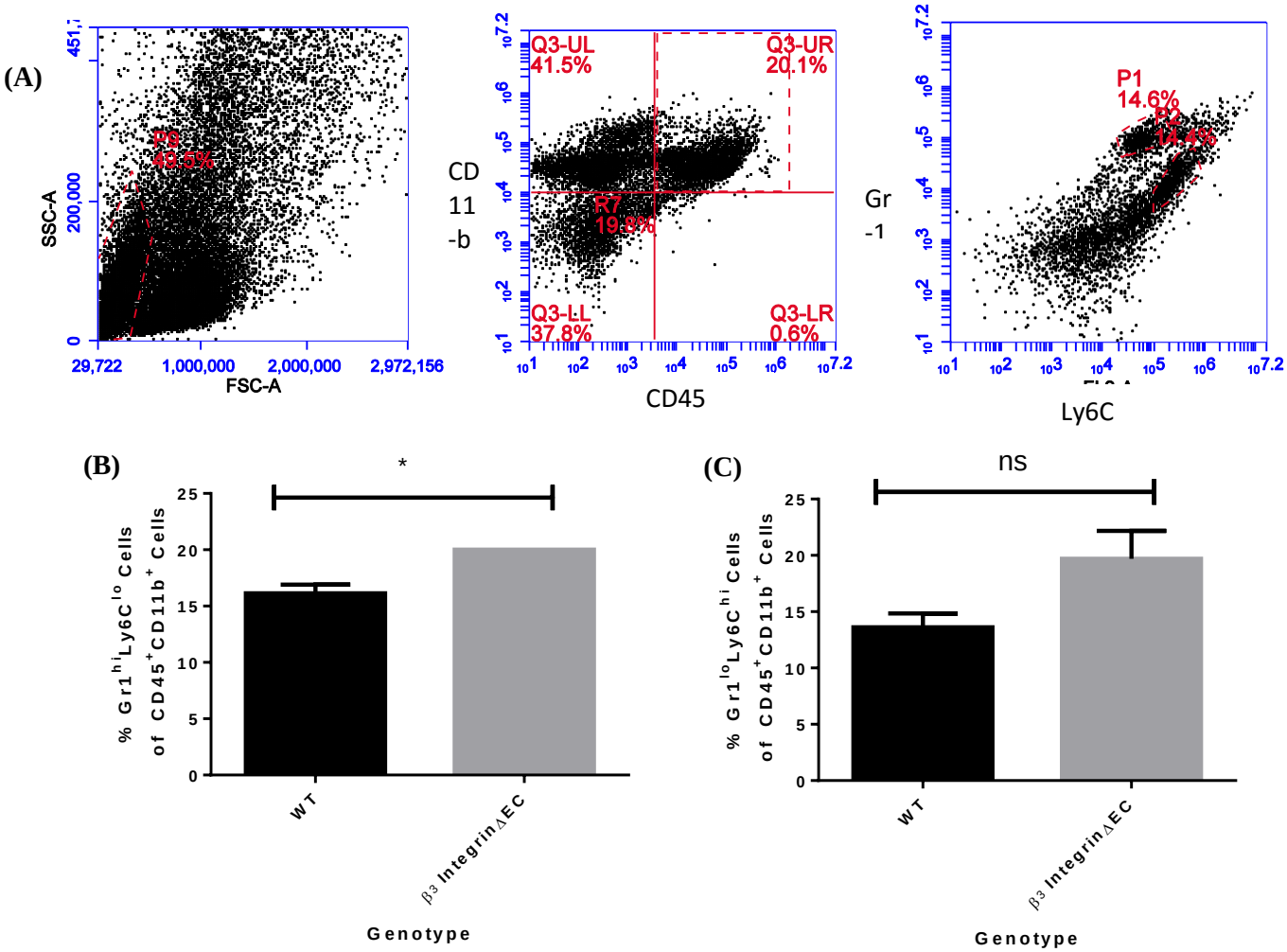

Figure 5: Knocking out $\alpha_v\beta_3$ in endothelial cells increases the number of PMN cells but not monocytes in the pre-metastatic lung.

Pre-metastatic lung cells from WT (Cre-negative) and $\beta_3$flox/flox/Tie1-Cre (β_3IntegrinΔEC) mice were suspended and analysed using flow cytometry (Mean + SEM; WT n=3, β_3IntegrinΔEC n=3; ns = no significance, * $p<0.05$). **(A)** The first gate was used to remove dead cells and cell debris; the second gate was used to gate the $CD45^+CD11b^+$ populations. **(B)** The P1 gate identifies the PMN cells ($C45^+CD11b^+Gr1^{hi}Ly6c^{lo+int}$) and the mean numbers are shown in a bar graph **(C)** The P2 gate identifies Monocytes ($C45^+CD11b^+Gr1^{lo+int}Ly6c^{hi}$) in the lung with mean percentages represented in a bar graph.

There are a number of myeloid subsets that infiltrate the tumour microenvironment including polymorphonuclear leukocytes and monocytes playing pro-tumorigenic roles (Elpek *et al*., 2014). It has been suggested that changes to the numbers of these cellular subsets in tissues can indicate the formation of a pre-metastatic niche (Batista *et al*., 2014).

To study these two fractions of $CD45^+CD11b^+Gr1^+$ myeloid cells a series of gating was necessary (shown in Figure 3.5A). First dead cells and cell debris were gated out, secondly the $CD45^+CD11b^+$ fraction was gated onto, and thirdly the $Gr1^{hi}Ly6c^{lo+int}$ – PMN (P1) and $Gr1^{lo+int}Ly6c^{hi}$ – monocyte (P2) fractions were identified. These fractions as a percentage of $CD45^+CD11b^+$ in the pre-metastatic lung are presented in Figures 3.5B and C.

Though the numbers of monocytes in the pre-metastatic lung increase in β_3 knockout mice this change is not statistically significant. There is, however, a small but significant increase in the numbers of PMN cells that colonise the pre-metastatic niche brought about by acute depletion of β_3 integrin in endothelial cells. This phenotype is supported by the expression patterns of CCL-2 and -3 in the cytokine array. Since this is associated with an increase the number of metastases the results seem to suggest that this fraction may include cells with anti-tumorigenic functions, such as N1 or G1 cells.

4. Discussion

4.1 Methodology

4.1.1 Mouse Models

This study has used a model that depletes $\alpha_v\beta_3$ in endothelial cells via *tie-1* directed expression of Cre and floxing the β_3 gene. The role of $\alpha_v\beta_3$ is not isolated to endothelial cells however and there are studies looking at which tissues are effected by Tie1 directed Cre expression. To study this the *tie-1*-Cre model was crossed with ROSA26R-LacZ Cre reporter mice which leading to LacZ staining with Cre expression. This showed that apart from a strong expression of the promoter in the endothelium, post-natal neural and haematopoietic cells also expressed LacZ (Gustafsson *et al*., 2001). The latter of these, however, is not surprising given that endothelial cells share a common progenitor with haematopoietic cells known as the hemangioblast (Vogeli *et al*., 2006).

The integrin $\alpha_v\beta_3$ can act as an adhesion molecule, supporting migration, or as a signalling receptor in the haematopoietic lineage which has been shown to affect the pre-metastatic niche. One study showed that knocking out β_3 in macrophages supported tumour growth by increasing polarisation of macrophages to an M2 phenotype by controlling STAT1/6 signalling. This study used the B6LV1 cell line (renamed PyMT-BO1) which will be discussed further in section 4.1.2 (Su *et al*., 2016). Since platelets also express *tie-1* (Tsiamis *et al*., 2000) this model may also lead to the depletion of the integrin $\alpha_{iib}\beta_3$ in these cells effecting the binding of tumour cells to platelets during circulation supporting metastasis. As such using this model could interfere with cancer related studies by depleting non endothelial β_3 integrin. Gustafsson *et al*. (2001), show that the expression of $\alpha_v\beta_3$ in the haematopoietic lineage does diminish in adulthood. Due to the roles $\alpha_v\beta_3$ plays in non-endothelial cells (such as haematopoietic cells) in cancer progression a number of experiments were performed to confirm this. This gives further confirmation that $\alpha_v\beta_3$ depletion is isolated to endothelial cells and platelets are not affected in the *tie-1*-Cre model. On the other hand, Steri (2015; unpublished) showed that while $CXCR4^+$ cells, an important subset in the pre-metastatic niche had no significant change in β_3 expression, the endothelial expression of β_3 does significantly decrease in association with a number of haematopoietic markers including CD45 and Ly6G. This suggests that there is further need for confirmation that Cre expression (and therefore β_3 depletion) is confined to endothelial cells.

In order to confirm there is no non-endothelial depletion of β_3 integrin a number of approaches could be used. Bone marrow reconstitution could be performed (as has been done in a study looking at the effect of $\alpha_v\beta_3$ on vascular leakage (Su *et al*., 2012)). Performing metastasis studies after replacing the bone marrow of β_3IntegrinΔEC mice with WT bone marrow could fully eliminate the possibility that depletion of haematopoietic β_3 causes any phenotypic changes. Alternately a tamoxifen inducible knockout of $\alpha_v\beta_3$ in endothelial cells, β_3 flox/flox/Pdgfb-

iCreERT2 is available which does not express $\alpha_v\beta_3$ in the bone marrow. This would act to not only confirm results but also allows time specific deletion of endothelial β_3 integrin providing a model more akin to a therapeutic setting.

4.1.2 Pinning Down the Development of the Pre-Metastatic Niche and Modelling it in Mice

Mouse models are an essential part of translating research into a therapeutic intervention. An important part of developing models of the pre-metastatic niche is pinning down the exact point at which the niche develops. This prevents confusion of the pre-metastatic niche with the distinct, early metastatic niche which is influenced by the presence of tumour cells.

The seminal article by Kaplan *et al*. (2005) used a spontaneous metastatic model to document the time it took for the pre-metastatic niche to develop and tumour cells to enter the niche. They showed that BMDCs started migrating to the lung twelve days after tumour implantation. At day fourteen clusters of BMDCs formed in the niche at future metastatic site. At day eighteen metastatic tumour cells associated with these clusters and these individual tumour cells grew to form micro metastases at day 23. This showed the progress of the future metastatic site from a pre-metastatic niche to an early metastatic niche. This cannot be used across all spontaneous metastasis models however, as different cell lines and mouse models lead to a different metastatic process with different timescales and involve different cytokines and potentially BMDCS.

The model I used for the pre-metastatic niche was developed by Steri (2015) and based on her model of spontaneous metastasis. The *tie-1* Cre model (on a C57BL6/129Sv mixed genetic background) was used along with the CMT19T F1 mouse cell line. This cell line was based on the CMT19T lung carcinoma cell line derived from CMT167 cells. To gain a cell line with a greater metastatic potential (CMT19T F1) the CMT19T cells were put through one round of *in vivo* selection of lung metastatic cells. These were injected subcutaneously (ectopically) to gain allograft primary tumours which would spontaneously metastasise to the lung. Since only this cell line has been used during this study only the lung pre-metastatic niche has been studied. To expand this further the PyMT-BO1 (used by Su *et al*., 2016) breast carcinoma cell line could be used which metastasises to both bone and lung. This cell line is particularly novel offering a spontaneous metastatic breast carcinoma model to bone.

Using this model of spontaneous metastasis Steri (2015) measured that at day twenty cells began to colonise metastatic tissues. It is at this point in this metastatic mouse model that the primary tumours were resected. Based on this data, mice were sacrificed at this time to allow the study of the effects of endothelial β_3 depletion on a developed and mature pre-metastatic niche.

There are issues with this model however as the interactions between the stroma and tumour are essential in cancer development. These subcutaneous injections are ectopic transplants that are not in the organ from which the cell line is originally derived. An important part of modelling cancer in mice is modelling all the stages of metastasis and ectopic transplants cannot fully represent the tumour environment (Saxena and Christofori, 2013). This is particularly significant when modelling the pre-metastatic niche which relies on signals from the primary tumour and its environment. It is also unclear whether reactions to therapeutic interventions in ectopic models will be the same as in orthotopic models (transplants in the tissue of origin) showing the shortcomings of these models (Francia *et al*., 2011).

As mentioned before the time line established by Kaplan *et al*. (2015), does not traverse all pre-metastatic niche models. Variation among mice and cell lines changes how a tumour will develop and metastasise, therefore changing the timings of the pre-metastatic niche. As such, quantifying the time frame of the pre-metastatic niche must be done with each model. This makes the process of following these results up in a conditional knock out model (as confirmation of the role of endothelial β_3 integrin in metastasis) harder.

Even within a spontaneous model based on an orthotopic cell transplant there can be issues. One example is the use of cells line which by growing *in vitro* means that by passing them, cells can undergo changes and adapt to a 2D culture. They can also represent a specific clone rather than a heterogeneous population (Saxena and Christofori, 2013; Khanna and Hunter, 2005). This can lead to issues with replicating results and does not accurately model a human tumour. Another example is that by carrying out injections of cell this can cause an inflammatory response and may not model all the interactions between the environment and the tumour that have been shown to be essential (Khanna and Hunter, 2005).

One way to create more refined models of metastasis that better demonstrate all the stages of the metastatic process would be to use genetically engineered mouse models of metastasis. This would generate orthotopic tumour without the need for injection in an immunocompetent environment better reflecting the interactions between the tumour and the host (Francia *et al*., 2011). One example of a genetically engineered model is the MMTV-PyMT breast cancer model (Khanna and Hunter, 2005). These can be refined even further thanks to the advent of temporal and tissue specific knock out models such as MMTV-Cre; $Trp53^{fl/fl}$ which metastasises to the lung and liver (Saxena and Christofori, 2013). This can be extended to studying the pre-metastatic niche and should lead to a better translation between research and therapeutic interventions.

Despite these options there are still issues, especially with modelling the pre-metastatic niche, such as the fact that mouse organ tropism does not necessarily mimic human metastasis (Saxena and Christofori, 2013). This raises the question of whether a mouse model can ever truly mimic human disease? The inherent physiological difference between mice and humans may mean the answer is no and

as such perhaps other models with more human physiological and anatomical similarities such as dogs should be explored (Khanna and Hunter, 2005).

4.1.3 Quantifying Immunohistochemistry

Immunohistochemistry has many uses in identifying the expression of proteins by cells and tissues particularly in the field of pathology. Historically immunohistochemistry has been analysed by eye; choosing frames of an image and counting cells exhibiting staining or assessing the extent of staining by eye (e.g. grading via H&E staining). This is open to bias however, as the eye can be drawn to specific regions and since the results do not take into account the whole section results may not be accurate. A significant part of my time was devoted to finding a method I was able to use to quantify whole sections of the lung pre-metastatic niche.

To quantify whole lung section, images of a lung section were taken at a magnification of x5, and stitched via Zeiss Axioplan software to get a complete image of the lung section, shown in Figure 3A. To reduce human error as much as possible an ImageJ macro with a standardised method was used. First the region of interest (ROI) was defined using a freehand tool to focus on the section itself and remove unnecessary parts of the image. This is used by a number of other quantification methods (Kokolakis *et al*., 2008; Arqués *et al*., 2012). A threshold value was then set using a standardised algorithm, Triangle, to eliminate any background and focus on the staining of interest. ImageJ provides a number of filters that can be used for different situations e.g. a greater intensity of staining (Jensen, 2013). Finally, parameters were set to filter objects according to circularity and size. The values obtained from this analysis were then normalised against the ROI size to gain comparable values.

An important part in this method is the normalisation of the staining value against the size of the ROI. There are a number of other methods that can be used to eliminate variation caused by differences in section size. One example is the division of the integrated density value (IDV) from a merged protein and nuclear stained image by the number of cells (gained from the nuclear staining value) to gain a value for protein expression vs cell number (Arqués *et al*., 2012). This method may produce a more accurate result as it uses the cell number instead of the size of the section to normalise values against and get comparable results. It also allows a wider application than the dot blot analysis (which identifies cells expressing markers by size and circularity) I have used.

I have aimed to use whole sections in this analysis but there are now developments that allow the creation of a 3D image of tissues using microscopy. Quantification of this would produce an even more accurate picture of the presence of specific cells/expression of protein in tissues. Examples of quantification of 3D images are now described in the literature (Hirashima and Adachi, 2015) however this is an expensive procedure that requires significant expertise - given the limited timeframe

I had, balanced against cost, I felt the chosen method was a satisfactory compromise that would allow me to define areas for future focus.

4.2 Endothelial Cells as a Barrier in Metastasis

4.2.1 Permeability of the Endothelium

The endothelium acts as a barrier to the movement of cancer cells in the metastatic cascade at both primary and secondary tissues. As such, changes to the permeability of the endothelium can increase the number of tumour cells entering and leaving the vasculature, thereby increasing the success of metastasis. There is some evidence that signalling from $\alpha_v\beta_3$ integrin could play a role in the process by inducing changes in endothelial permeability however findings are contradictory.

There are suggestions that $\alpha_v\beta_3$ integrin negatively regulates permeability induced by VEGF-A, which affects adherens junctions by manipulating VE-cadherin. It has been shown that β_3 integrin null mice have an increased sensitivity to VEGF-A mediated changes to endothelial permeability, which is put down to elevated levels of Flk-1. This suggests that $\alpha_v\beta_3$ integrin might enhance barrier function and therefore play an anti-metastatic role (Robinson *et al*., 2004).

On the other hand, evidence from $\alpha_v\beta_3$ null mice suggests a pro-metastatic role for this integrin via permeability (Weis and Cheresh, 2005). The global deletion of this molecule contradicts the anti-metastatic phenotype of the *tie-1*Cre $\beta_3$fl/fl model (presented in this dissertation). Part of this contradiction may, in fact, be due to the very fact that the study uses a global knockout of $\alpha_v\beta_3$ integrin whereas our results study the role of $\alpha_v\beta_3$ in endothelial cells specifically. The difference in mouse models may also lead to the contradictory results surrounding the role of $\alpha_v\beta_3$ in permeability, allowing the possibility of an endothelial specific pro-metastatic permeability. This is supported by evidence that in endothelial cells $\alpha_v\beta_3$ co-operation with VEGFR-2 plays an important role in VEGF-A signalling (Soldi *et al*., 1999; Mahabeleshwar *et al*., 2006).

There are a number of proteins that are activated by $\alpha_v\beta_3$ co-operation with VEGFR-2 that have a role in endothelial barrier function. One example is FAK which translocates to adherens junctions in endothelial cells upon stimulation by VEGF-A and enhances permeability by phosphorylating VE cadherin (Chen et al 2012; Jean et al 2014). Another example is c-Src which can also be stimulated by VEGF-A signalling, disrupting the association of VE cadherin with β catenin, leading to an increase in permeability and consequently metastasis (Criscuoli *et al*., 2005; Weis *et al*., 2004). This is supported by the suggestion that $\alpha_v\beta_3$ signalling in endothelial cells disrupts VE cadherin and consequently adherens junctions through phosphorylation of FAK and c-Src leading to an increase in permeability (Alghisi *et al*., 2009).

The results in section 3.1 suggest that the barrier function of endothelial cells is not affected by depletion of β_3. This has been backed up by the fact that there are no changes in the numbers of circulating tumour cells in WT mice in comparison to β_3 integrin ΔEC mice (Steri, 2015; unpublished). This has only been studied using one method however and others could be used to confirm this result such as the Miles assay which uses Evans Blue to colour tissues with permeable vessels allowing the study of multiple tissues (Radu and Chernoff, 2013). Changes in permeability should also be investigated at the secondary site however as the primary tumour can influence the secondary site during pre-metastatic niche formation. This is an early event in the formation of the niche (Maru, 2015) supporting myeloid cell infiltration and later the extravasation of tumour cells. Potentially this could be supported by the increase in VEGF-A in the pre-metastatic niche of the lungs of WT mice suggested by the cytokine array. This result (suggesting an increase in VEGF-A) is preliminary however and experimental repeats would be necessary to determine if this is statistically significant.

4.2.2 Adhesion of Cells to the Endothelial Layer Mediated by $\alpha_v\beta_3$

An important part of trans-endothelial migration is the binding of the cell to the endothelial layer, a step which is relevant to both BMDCs and tumour cells. In some cases, metastatic growth starts upon adhesion to the endothelium in the secondary site growing into the tissue. By supporting the influx of BMDCs and tumour cells to the secondary site, adhesion is an important step in metastasis. When expressed by the endothelium $\alpha_v\beta_3$ certainly supports interactions with the ECM but could it also mediate cell-cell interactions as it does when it is expressed by certain myeloid populations and cancer cells?

The vasculature of the primary tumour undergoes changes that differentiate it from the endothelium of the pre-metastatic niche. As such evidence suggesting primary tumour endothelium does not influence intravasation should not rule out the possibility of interactions between tumour cells and endothelial $\alpha_v\beta_3$ at the secondary site. There are a number of $\alpha_v\beta_3$ counter receptors expressed by tumour cells including L1 and PECAM-1. There is some evidence that the first of these can mediate interaction between tumour cells and endothelial $\alpha_v\beta_3$. A study showed that hypoxia, a state that can stimulate metastasis, increased the expression of $\alpha_v\beta_3$ in endothelial cells and antibodies targeting $\alpha_v\beta_3$ inhibited tumour cell adherence (Niu *et al*., 2007).

In certain inflammatory situations it has also been suggested that endothelial $\alpha_v\beta_3$ can regulate leukocyte attachment to the endothelium. For example, there is evidence that $\alpha_v\beta_3$ is upregulated when endothelial cells switch to an inflammatory phenotype (possibly due to inflammatory molecules) supporting the recruitment of certain leukocyte populations (Bechah *et al*., 2009). Another example is PECAM-1, which can interact with $\alpha_v\beta_3$, can be expressed by myeloid populations supporting leukocyte adhesion to the endothelium (Piali *et al*., 1995). This could potentially translate to a role for $\alpha_v\beta_3$ in the infiltration of immune cells into secondary organs supporting pre-metastatic niche formation.

These results are based on very specific examples but could translate to the pre-metastatic niche which can be considered an inflammatory site (Maru, 2015) and is in part induced by hypoxia. If changes to the BMDC populations in the pre-metastatic niche are identified these areas of study could lead to an explanation other than that suggested by the cytokine array (a significant increase in P-selectin in WT mice over control and β_3 integrin ΔEC mice).

4.3 Immune Cells in the Pre-Metastatic Niche

4.3.1 Changes in Myeloid ($CD45^+/CD11b^+$) Pre-Metastatic Niche Components with Depletion of endothelial β_3

The importance of BMDC mobilisation to the formation of the pre-metastatic niche has been demonstrated a number of times but as mentioned previously the haematopoietic signature of the niche is still being dissected. The experiments identifying changes to myeloid components of the lung pre-metastatic niche in this study have been based on a paper that studied how haematopoietic homeostasis can determine the success of metastasis. Two $CD11b^+/Gr1^+$ myeloid subsets were studied which are frequently referred to as MDSCs, the granulocytic (or polymorphonuclear (PMN)) subset, which is indicative of haematopoietic changes and the monocytic subset (Batista *et al*., 2014). In our studies only the PMN cells change significantly in the lung pre-metastatic niche increasing with depletion of β_3 integrin in endothelial cells.

The MDSC population is derived from myeloid progenitor cells which differentiate into immature myeloid cells before entering circulation. Upon migration to peripheral organs these cells, depending on the environment of the tissue, will differentiate into mature myeloid cells (e.g. macrophages or neutrophils) or become an activated but immature subset known as MDSCs (Goedegebuure *et al*., 2011; Yang *et al*., 2013). This MDSC population also includes progenitors of granulocytes, macrophages and dendritic cells and when cultured these cells will differentiate into mature myeloid cells (Gabrilovich *et al*., 2012; Youn *et al*., 2012). As with other leukocyte populations, MDSC accumulation in the pre-metastatic niche is a response to a number of tumour derived factors including CCL2 and CXCL5 (Gabrilovich *et al*., 2012). The cells are then activated by factors such as TGFβ, which can be found in the pre-metastatic niche, leading to the stimulation of a number of signalling pathways e.g. NFκB (Gabrilovich and Nagaraj, 2009). After activation they undergo expansion colonising the niche. This is again stimulated by tumour derived factors such as M-CSF and VEGF (Condamine *et al*., 2015).

These cells have a prominent role in the pre-metastatic niche with a greater representation of the granulocytic over the monocytic subset. They play a number of roles in the niche regulating tumour invasion, angiogenesis and creating an immunosupportive environment for tumour cells (Talmadge and Gabrilovich, 2013). Of particular significance is the immunosuppressive role of MDSCs, thought to be

reliant upon factors such as arginase-1 and iNOS (Youn and Gabrilovich, 2010). MDSCs cause the activation and expansion of T_{reg} cells and inhibit $CD8^+$ T cell function so that they no longer respond to stimulation by antigens (Gabrilovich and Nagaraj, 2009). There has been debate over the immunosuppressive role of the G-MDSC sub population however recently several papers have offered proof of T_{eff} suppressive functions (Raber *et al*., 2014; Gabrilovich *et al*., 2012).

Overall it is generally accepted that these cells play roles that support metastasis which is at odds with their increase in a model with decreased metastases. Despite this contradiction the trend is supported by a number of cytokines in the array such as CCL2 which recruits G/PMN-MDSCs and Mon-MDSCs to the pre-metastatic niche (Mitchem and DeNardo, 2012). As such this population of cells may be playing an alternative, anti-tumorigenic role in the niche that would decrease metastasis.

In a number of leukocyte populations, a hetero-functionality has been observed due to a phenotypic polarisation induced by secreted factors. This supports an anti- or pro-inflammatory phenotype shown to be particularly significant in tumour biology. One example, the M1 (pro-inflammatory)/M2 (anti-inflammatory) functional axis, has been briefly described previously but this anti and pro-tumour polarisation does not only extend to macrophages. It is also evident in T cells and neutrophils and recently there have been suggestions of MDSC polarisation of both granulocytic and monocytic subsets (Yang, 2013).

This is associated with the close relationship of MDSCs with mature myeloid cells, in fact some characterise G-MDSCs to be an immunosuppressive population of neutrophils (Coffelt *et al*., 2016). There is evidence that in certain inflammatory situations MDSCs, as with neutrophils are though to exhibit an N1 or N2 phenotype. At the tumour G-MDSCs can differentiate into to a tumour associated neutrophil (N2) phenotype, also referred to as G2 and though it is not clear what proportion of TANs are derived from G-MDSCs (Fridlender *et al*., 2012; Fridlender *et al*., 2009). As with neutrophils this possible polarisation/differentiation is suggested to be dependent upon TGFβ with its presence causing a G2/N2 shift in comparison to the G1/N1 shift with TGFβ antibodies (Yang *et al*., 2013; Yang, 2013). This polarisation of MDSCs from G2 to G1 leads to a reduction in their immunosuppressive functions. By repolarising the cells to an anti-tumour phenotype it could be possible to use them as an anti-tumour immunotherapy (He *et al*., 2016).

This is the work of small n numbers and only one experiment therefore repeats would be necessary before relying upon this result. This also means that the change to the monocytic fraction should not be discarded with these cells also increasing with β_3 depletion. The polarisation of G-MDSCs is still a theory with little to no evidence backing it up however Mon-MDSC polarisation is better established. As with G-MDSCs, Mon-MDSCs can differentiate to a TAMs when they enter the tumour micro-environment. This population of cells can polarise to an M2 phenotype with immunosuppressive properties e.g. M2 MDSC cause an increase in T_{reg} function and decrease T_{eff} function while the M1 MDSC phenotype leads to the opposite (Yang *et al*., 2013; Ma *et al*., 2011). Experiments have also been carried

out that repolarise Mon-MDSCs from an M2 to M1 phenotype leading to a loss of their immunosuppressive function (He *et al*., 2016). This, again, could be a promising therapeutic strategy in the future.

4.3.2 Mature Myeloid cells in the Pre-Metastatic Niche and the Influence of Cytokines

The greatest proportion of the pre-metastatic niche seems to be MDSCs however there are also a number of other myeloid cells, some of which have been mentioned in Figure 3.2. At the late stage of the pre-metastatic niche that this study focuses on a diverse range of cytokines would be expected and this is reflected in the cytokine array.

There are a number of studies showing potential roles for the mature classes of myeloid cells which have been mentioned (e.g. neutrophils and macrophages). In pancreatic cancer, for example, MIF from the primary tumour leads to the secretion of TGFβ followed by fibronectin production. This lead to the arrest of bone marrow derived macrophages (identified by F4/80) and, to a lesser extent, neutrophils in the development of the pre-metastatic niche supporting metastasis (Costa-Silva *et al*., 2015). Another study showed that neutrophil accumulation in pre-metastatic lungs increased in *Ifnar1*$^{-/-}$ mice and was associated with an increased success of tumour metastasis. It was suggested that this was due to an increase in CXCR-2 expression by neutrophils and G-CSF in serum allowing neutrophils to support extravasation and proliferation of tumour cells (Wu *et al*., 2015).

As shown in these studies cytokines are an important part of determining the influx of these bone marrow derive myeloid cells and a number of cytokines involved in this process have been studied in the cytokine array Figure 3.2. There is a trend suggesting an increase of chemokines associated with neutrophil influx in WT mice compared to control and β_3IntegrinΔEC mice. This trend is reflected by myeloperoxidase, CXCL-1, -2 and -5 and by complement component 5a. There are two outliers of this trend however, CCL-2 and -3, which are only expressed by β_3IntegrinΔEC mice. The cytokines associated with macrophage infiltration in this array seem to tell a different story however, particularly CCL6 which shows a 1.4-fold increase in β_3IntegrinΔEC mice compared to WT. This trend and increase is reflected in the western blot, but it is shown to be insignificant. CCL-2, a cytokine which also supports macrophage infiltration and is known as macrophage chemoattractant protein 1, echoes this trend. This cytokine is particularly important as there are a number of studies that show it supports the formation of the pre-metastatic niche. Secreted by the primary tumour this chemokine can encourage the mobilisation and expansion of MDSCs (Sceneay *et* al., 2013; Sceneay *et al*., 2012). This function is therefore greatly at odds with its expression by β_3IntegrinΔEC mice only.

Overall the cytokine array shows a distinct presence of inflammatory and tumour supportive cytokines associated with tumour injection. The two genotypes then vary

greatly, expressing some cytokines equally and others at different levels. Apart from confirming the trends suggested in this array of cytokines such as VEGF-A by further experiments a number of other cytokines could also be studied in the pre-metastatic niche. One example is TGFβ which modifies the niche and may also suggest functional changes of neutrophils. Based on this both neutrophils and macrophages could be used to identify changes in the pre-metastatic niche.

4.3.3 Immunomodulation in the Pre-Metastatic Niche

As has been described, one of the most important functions of MDSCs in the pre-metastatic niche is to provide an immunosuppressive environment that supports tumour growth. This is done by increasing T_{reg} function and preventing the activation of T_{eff} cells that will specifically target cancer cells. The main focus of this study, as with many studies of the pre-metastatic niche has been how β_3 depletion could potentially support tumour metastasis via the immune system. This system is not only supportive however and can mount a response against a tumour. This is evident in the elimination relationship of the tumour with the immune system during immunosurveillance. As such an anti-tumour immune response could be explored in relation to endothelial expression of $\alpha_v\beta_3$ integrin.

The roles of T lymphocytes, NK cells and APCs in the recognition of tumours as non-self and consequent elimination have been described in section 1.1.4. Markers identifying the cells could be used to study how these populations change in the pre-metastatic lung using immunohistochemistry (as has been done with leukocytes and MDSCs in section 3). Examples of such markers include $CD8a^+$ for dendritic cells, $CD4^+/CD45RA^-$ T cells (eliminating the T_{reg} population), and $CD8^+/CD107a^+$ to identify degranulating cytotoxic T cells (Miyara *et al*., 2009; Apetoh *et al*., 2015; Gajewski *et al*., 2013).

Part of this anti-tumour function can be determined by cytokines that are found in an inflammatory environment. These signals are particularly important in the context of this study because of the role they play as messengers from the primary tumour setting up the pre-metastatic niche. Since there is no overall leukocyte increase any changes in T lymphocytes in the niche will probably be a specific mechanism such as cytokines which induce specific functional changes in immune cells. Some of these have been mentioned, including IFNγ, CXCL-9, -10, and -11 (which are all involved in T_{eff} function). Identifying the expression of these cytokines in the pre-metastatic niche could shed light on any change in the function of the immune system with endothelial $\alpha_v\beta_3$ depletion.

4.4 Exosomes and the Pre-Metastatic Niche

A more recent development in the understanding of the pre-metastatic niche has been the discovery of exosomes (or microvesicles) as messengers conveying information from the primary tumour. The development of a number of cancers is associated with exosome production allowing communication with other tissues to

create a tumour supportive environment (Zhang and Wang, 2015). In this vein these messengers have also been shown to be able to influence the cells of secondary tissues contributing to the formation of the pre-metastatic niche.

Exosomes are small membrane bound vesicles created by cells that carry RNA and functional proteins. They can travel through the transport systems reaching distant tissues or move to nearby cells before fusing with them and transferring their load. This allows both distant and nearby cell to cell communication that can affect the cellular phenotype (Alderton, 2012). Tumour cells secrete exosomes into the surrounding microenvironment and the blood stream allowing them to communicate with surrounding and distant tissues, an advantage to tumour development. The number of exosomes that tumour cells secrete is in fact linked to the stage and invasiveness of a cancer (Peinado *et al*., 2011; Peinado *et al*., 2012).

The secretion of the messenger vesicles by tumour cells into the blood stream led to interest in exosomes in regards to the priming of the pre-metastatic niche. It was suggested that the vesicles where a way of carrying tumour secreted factors (an important part of niche initiation) to the niche. A number of papers have now shown evidence to support this theory. One study injected mice with exosomes derived from tumour serum leading to an increased likelihood of metastasis. Analysis of the vesicles showed that they carried a number of pre-metastatic niche factors such as S100A9 and S100A9. They then studied changes of BMDC behaviour showing that exosomes educated BMDCs mobilising them leading to an increased number at pre-metastatic tissues (Peinado *et al*., 2012). Another study, also mentioned above, showed that pancreatic cancer could induce formation of a liver pre-metastatic niche by exosomes expressing MIF. Upon fusing with Kupffer cells the exosomes caused a change in the architecture of the tissue leading to the influx of BMDCs, a critical point in pre-metastatic niche formation (Costa-Silva *et al*., 2015).

There are now a number of examples showing that exosomes secreted from the primary tumour can educate the pre-metastatic niche. There is also some evidence that part of this process involves a change in the phenotype of endothelial cells. As mentioned, an increase in permeability is an early event in the formation of the niche and it has been suggested that exosomes can play a part in this. They showed that exosomes released from breast cancer cells expressed miR-105 and fused with endothelial cells transferring this microRNA. Injection of these tumour derived exosomes into mice led to an increase of miR-105 in distant pre-metastatic organs followed by reduced endothelial expression of ZO-1 (which regulated endothelial adheren junctions) and a decrease in barrier permeability. This was accompanied by an increase in metastasis that was blocked by anti-miR-105 suggesting that miR-105 released by tumour exosomes increased permeability of endothelial cells in the pre-metastatic niche (Zhou *et al*., 2014).

For exosomes to induce changes in cells they must bind and fuse with them before unloading the proteins and RNA that they transport. Since $\alpha_v\beta_3$ is a transmembrane adhesion molecule it may be possible that it supports the adhesion and fusion of exosomes expression proteins with RGD motifs to endothelial cells. This could

potentially support the formation of the pre-metastatic niche and provide a metastasis supportive role for $\alpha_v\beta_3$. One study presented evidence suggesting that exosomes expressing ADAM15 bound to tumour $\alpha_v\beta_3$ in an RGD dependent manner leading to a decrease in tumour growth (Lee *et al*., 2012). Another study showed that dendritic derived exosomes bound to cells via their expression of $\alpha_v\beta_3$ integrin (Pitt *et al*., 2014).

With the emergence of exosomes as an important mediator of primary tumour signals to the pre-metastatic niche this could be an interesting area to study as an explanation for the pro-metastatic effects of endothelial $\alpha_v\beta_3$.

4.5 The Pre-Metastatic Niche and Therapeutic Intervention

As metastasis is the cause of 90% of cancer related deaths (Sceneay *et al*., 2013) cancer research has entered a mind-set similar to Paget's, that the focus of research should be understanding the metastatic process. Part of this has and will be understanding the pre-metastatic niche. There are now a number of researchers starting to look into how the pre-metastatic niche can be harnessed in therapeutic interventions reversing its phenotype as an environment receptive to metastasis.

One strategy has been to selectively target cells within the BMDC clusters that form in the pre-metastatic niche. It has been suggested that selectively targeting $VEGFR1^+$ BMDCs to prevent migration could enable a double hit targeting both the primary tumour environment (which also has $VEGFR1^+$ HPCs) and pre-metastatic niche (Peinado *et al*., 2011). Alternatively, MDSCs could be targeted to reduce their immunosuppressive functions thereby enhancing anti-tumour immunity. One way to do this would be to induce MDSC differentiation, lowering their numbers and reducing their pro-tumour effects. This can be done with al-*trans* retinoic acid which induces their differentiation in to APCs or with a low dose of IFNγ (Talmadge, 2007; Zoccoli *et al*., 2012). It may also be possible to reverse the supressive effect of MDSCs on T cells by targeting proteins such as ARG1 and NOS2. In mice this has been shown to increase the number of anti-tumour T cells (Talmadge, 2007). Targeting the immunosuppressive function of MDSC's could then be used in combination with immunotherapies, increasing their effectiveness (Ko *et al*., 2009).

Another way of translating knowledge of the pre-metastatic niche into therapeutic interventions has been to target exosomes (which play an important part in educating the niche to support tumour cells). It is thought that of the many pathways cells can use for the secretion of exosomes, tumour cells use a mechanism based on Rab27a. Knocking down expression of this protein has resulted in a decrease in neutrophil mobilisation and metastasis in a breast cancer model and reduced metastasis and BMDC recruitment in a melanoma model (Vader *et al*., 2014). Another thought has been to use exosomes to enhance the anti-tumour immune response by priming T cells with exosomes carrying antigenic material. Clinical trials of this theory have been met with little success currently, possibly due to molecules

within the exosomes that have immunosuppressive properties (Peinado *et al*., 2011).

Better understanding of the pre-metastatic niche may not only lead to new therapies but also to an earlier detection of cancer metastasis, another important part in the survival of cancer patients. The detection of myeloid cluster could lead to an earlier identification of a patients' inclination to metastasis than is currently possible. By identifying this proclivity towards metastasis at an earlier stage this may also lead to earlier therapeutic interventions, increasing the chance of patient survival (Psaila and Lyden, 2009).

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