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Understanding how the host gut microbiota influences distant immunological niches

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Abstract

The host gut microbiota has long been associated with improved health, however these associations have been historically hard to evaluate objectively. In the last decade, the advent of high throughput sequencing has enabled the field to examine the specific members of this ecosystem that are contributing to health. This has seen adoption of probiotic based treatments in several disorders including asthma, allergy and gastrointestinal diseases. However, an entirely unexpected association has been that between the gut microbiome and cancer. Modulation of the gut microbiota has been shown to influence primary tumour growth across multiple diseases; however, despite breast cancer being the most common in the western world, there have not been any studies addressing the role of the microbiome. This thesis intends to fill this gap in the field's knowledge and determine the mechanistic role of the gut microbiota in breast cancer immune responses. We have shown that antibiotic induced dysbiosis accelerates primary tumour growth. However, to our surprise this appears to be driven by metabolic changes rather than immunological modulation. Additionally, we have shown that by supplementing the microbiota with species from a probiotic genus of bacteria, we can improve anti-cancer immune responses by modulating intratumoural cytokine production. Finally, we have shown that the microbiome also plays a role in guiding and controlling metastatic breast cancer by influencing the metastatic niche. Overall, these findings have demonstrated a key role for the microbiome in breast cancer growth and progression. Our data suggests that antibiotic use in BC patients should be examined closely and re-evaluated to avoid comprising treatment efficacy. Furthermore, the use of probiotics show potential for clinical use but should be followed up with robust mechanistic studies before clinical trials are considered.

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1. Introduction

1.1. Preface to the Introduction

The clinical potential of the gut microbiome is only beginning to be realised. Its role in guiding development and function of the immune response is well characterised, but until the last decade, technological limitations prevented a mechanistic understanding of these processes. The ‘omics revolution brought about radical changes in the way microbiome research is conducted and the complex interrelationships between host and microbiota are finally being fully appreciated. Over the last five years, these interactions have led to new avenues in disease research, particularly in the field of cancer. Whilst still in its embryonic stages, compelling associations have been made between a healthy, diverse microbiome and treatment efficacy in several cancers. However, one disease which has been neglected is breast cancer. Despite being the world’s most common cancer, the impact of the microbiome on anti-cancer immune responses in this ubiquitous disease remain uncharacterised. This thesis intends to begin unravelling these associations in an attempt to gain a full mechanistic understanding of the gut microbiome’s potentiation of breast cancer immune responses. The following introduction will summarise the field’s current position, describing the processes involved in development and maintenance of a healthy immune system, how immunity contributes to controlling malignancy and what is currently known about the contributions of the gut microbiota to these processes.

1.2. Breast Cancer

1.2.1. Breast Physiology

Homo sapiens belong to the taxonomic class of Mammalia, so named because of the presence of mammary glands, milk producing tissues used to nourish their young. These glands usually take the form of breasts, an epithelial appendage containing a branched network of lobes and ducts that gradually remodel from infancy to form a functional organ.

At birth, both male and female humans have developed a network of ~15-20 lobules *in utero* and development continues from infancy until 2 years of age [1]. Shortly after birth, loss of maternal estrogens stimulate production of prolactin in the infant, resulting in remodelling events that can lead to transient milk production [2]. Following this, production of the infant's own hormones results in further breast tissue development. This generally continues for longer in females due to increased serum concentrations of estradiol [3]. Early remodelling gradually diminishes and from 2 years onward the human breast remains quiescent until puberty [4].

From puberty, breast development in males and females diverges. The male breast remains quiescent due to increases in testosterone production however the female breast begins extensive remodelling resulting in morphological and functional changes to breast tissue [5]. The pubertal stages of breast growth are documented in detail by Tanner & Marshall [6], however to summarise, increases in estrogen drive production of breast tissue resulting in initial formation of a breast bud, which over time results in formation of the adult breast and nipple (Figure 1.1A&B). At a cellular level, the epithelial structures that form the lobules elongate to form a network across much of the breast tissue. This process is driven by the proliferation of mammary stem cells that are located amongst the cap cells of the terminal end buds [7], [8] (Figure 1.1C). The extension across the breast is a consequence of both elongation and sub-ducting of the epithelial structures, processes that are thought to be driven by estrogen and progesterone, respectively [9]. After completion of pubertal development, the breast enters quiescence until pregnancy. The requirement for lactation initiates another period of expansion and differentiation known as the pregnancy lactation cycle (PLC). After breast feeding is complete, the breast returns to quiescence, but will cycle through expansion again with each subsequent pregnancy [10]. This regular remodelling has been suggested as a short-term risk factor for development of breast cancer, however pregnancy has been associated with reduced risk of BC in the long-term [11][12].

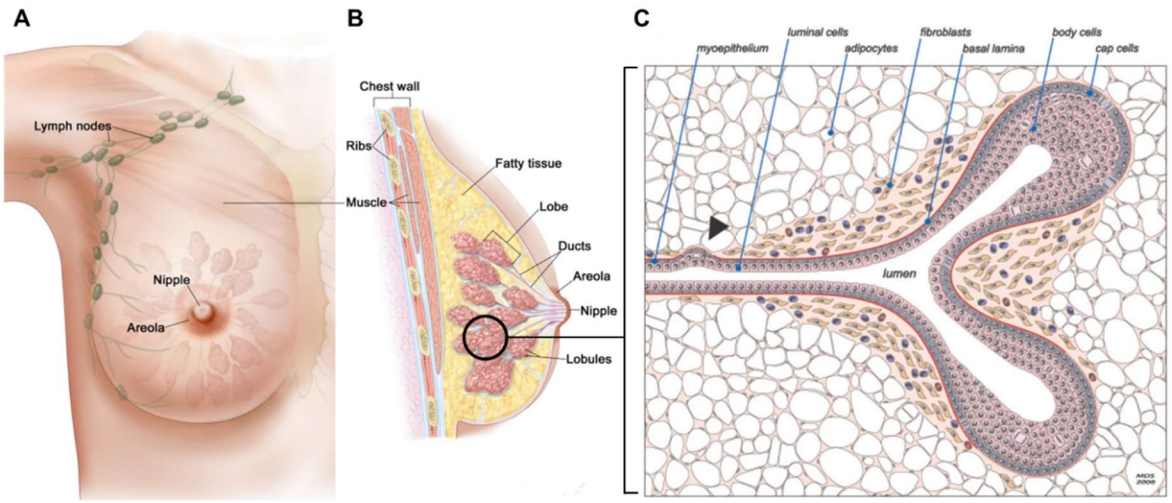


Figure 1.1 – Physiology of the normal adult breast: Tissue consists of several lobes made up of branching structures **A&B)** These structures are lined by a multi-layered, secretory epithelium that are responsible for milk secretion and delivery to the nipple **C)** Adapted from [13] & [14].

1.2.2. Epidemiology

Breast cancer (BC) is the most common cancer in the UK, accounting for 15% of all cancer cases and with a rising incidence rate, (BC incidence increased by 6% in women in the 10 years between 2005 and 2015), it represents a serious societal and fiscal burden to most westernised countries [13]. The reasons for this increase are currently unclear, however, the main correlative factor appears to be age. The median age of the UK has increased from 38 to 40 since 2002 [14] which is an important consideration given the impact age has on development of BC. The risk of BC increases exponentially between the ages of 35 and 50, increasing from 63.1 to 283.5 per 100,000 [15] so an aging population is likely to have increased rates of BC. However, there are several environmental factors that may also be playing a role, including gradually increasing rates of obesity, use of hormone replacement therapies in menopausal women, and improvements in screening technologies [16], [17]. There are also genetic associations with BC, most commonly mutations in the BReast CAncer (BRCA) susceptibility genes. The BRCA 1 and 2 genes encode DNA repfair proteins which have key tumour suppressor roles in breast tissue. Mutations in these genes result in cells losing the ability to repair double stranded DNA breaks leading to significant genetic instability [18]. The risk of developing BC for women with mutations in either BRCA gene is approximately 75% [19], therefore genetic screening after a familial history of BC is becoming clinically routine [20]. Many women who carry a BRCA mutation choose to have

a partial or double mastectomy as this has been shown to greatly reduce their lifetime chance of developing BC [21]. Improving knowledge of BC pathogenesis and epidemiological factors appears to be producing dividends and despite increases in BC incidence, mortality is falling year on year and has fallen by 17% between 2005 and 2015 [22]. Most likely, this is a result of more robust preventative measures and improvements in treatment efficacy, particularly in understanding molecular subtypes of the disease [16].

1.2.3. Types of Breast Cancer

The primary method of BC diagnosis is needle aspiration biopsies. The tissue is histologically analysed to determine the extent of cellular abnormality. This is graded based on morphological parameters such as nuclear pleomorphism and tubule formation along with mitotic counts in an area of interest. The grading ranges from I to III, the latter being cells that are poorly differentiated and rapidly dividing and is associated with poor prognosis [23]. From here, the disease will be determined to be invasive or non-invasive, the latter group of diseases are described as *in situ* and whilst are not malignant, they represent an increased risk of developing breast cancer. The former is designated by irregular cells that have broken into and started to invade the surrounding fatty and connective tissues in the breast and is what is commonly referred to as breast cancer. These are further categorised according to the structural feature of the breast tissue from which they originate. The most common being invasive ductal carcinoma (IDC), originating from the epithelial cells which line the ducts of the glands and is the most common form accounting for ~80% of all breast cancer diagnoses. The other major subtype is invasive lobular carcinoma (ILC), accounting for 10-15% of breast cancer diagnoses, there are also several other rare types which will not be discussed but are reviewed in [24]. Distinction between ductal and lobular carcinoma is typically achieved by histological analysis, ILC has a less cohesive appearance when compared to IDC and is commonly accompanied by a loss of E-cadherin expression. The consequence of this is that whilst IDC and ILC often present in comparable prognostic parameters, the latter has a marginally worse disease free and overall survival rate [25]. This may be due to the increased propensity of ILCs to become multi-focal, likely due to an increased migratory potential in the absence of E-cadherin. With regards to treatment, there is some suggestion that ILCs may derive less benefit from chemotherapies than IDCs, however this has not been conclusively proven [26].

Histological analysis also paved the way to a more personalised approach to treatment in BC. During the mid-1900s, seminal work by William McGuire it was determined that the amount of estrogen receptor (ER) in tumours correlated with outcome [27]. Furthermore,

after clinical trials in the 70s, inhibition of estrogen signalling via tamoxifen was shown to have a clinical benefit in some BC patients [28]. These findings, along with similar studies involving progesterone receptor (PR) and human epidermal growth factor receptor (HER)-2 led to histological analysis becoming common practice in BC diagnosis. However, this paradigm was challenged during the early 2000s after Perou *et al* and Sørlie *et al* conducted pioneering gene expression analysis of over 100 tumours. They were able to cluster tumours into five molecular subtypes which could be loosely defined by their expression of hormone receptors [29], [30]. This work formed the basis for today's breast cancer diagnostic methods which aim to assign patient samples to one of the five 'intrinsic subtypes'. This has led to a stratification of BC subtypes into four main categories: Luminal A & B, HER2 enriched and Triple Negative, each with different treatment strategies and prognoses. Whilst there is some blurring of the lines between these subtypes, clinically the classification system has been demonstrated to improve treatment efficacies and has been adopted by many healthcare systems internationally [31].

More recently, gene expression profiling has started to be utilised in BC diagnosis, tests such as Oncotype DX and Prosigna (PAM50) offer clinicians further information regarding a patients tumour that informs them regarding the patient's risk of distant recurrence. Through measuring the expression of up to 50 genes, these tests generated a score that reflects the likelihood of a tumour metastasising. Furthermore, these tests can transcriptomically allocate tumours to either of the intrinsic subtypes without the risk of observer bias by the pathologist [32]. Ultimately, these further stratifications have shown great promise and improve not just outcomes but also quality of life for patients. A recent study has concluded that any tumours presenting as ER+ with an Oncotype DX score of less than 10 will no longer require chemotherapy, preventing patients from having to undergo unnecessary detrimental treatments [33].

1.2.3.1. Intrinsic Subtypes

Luminal Type

Luminal type cancers arise from the inner epithelial cells of the mammary gland ducts. They account for around 70% of all BC cases and can be broken down into either Luminal A or B. Both subtypes can display either ER, progesterone receptor PR, or both, however only Luminal B can overexpress HER2 [34]. Most luminal cancers are Luminal A which are commonly both ER+ and PR+ and have the most positive prognosis of any subtype. They frequently present as a low-grade tumour and have a low recurrence rate compared to others [34]. Luminal B tumours are frequently ER+ but are often associated with lower levels

of PR. Unlike Luminal A tumours, it is possible that they will display HER2 overexpression. Because of the overlapping receptor status of the two types, Luminal B is often distinguished from A by a higher number of Ki67+ cells. The presence of Ki67 suggests a highly proliferative cell and for this reason Luminal B tumours are often more aggressive and have an overall worse prognosis than Luminal A across several association studies [35], [36].

HER2 Enriched

HER2 enriched tumours are a less common subtype and represent 15-20% of all BC cases [37]. Under normal conditions, HER2 functions as a growth factor receptor in breast tissue, driving cell proliferation and survival. However in BC, amplification of the Her2/*neu* oncogene results in overexpression of HER2 and increased surface levels of the receptor drive aberrant cell growth [38]. This amplification and consequent overexpression has been shown to drive proliferation and malignancy in cultured mammary cells [39]. HER2+ tumours tend to present with a moderate grade, some lymph node involvement and with a relatively high Ki67 ratio which is suggestive of a more aggressive tumour type [40]. For these reasons, HER2+ BC tends to have a relatively poor prognosis, however they are sensitive to growth factor inhibitors such as trastuzumab which significantly improves patient outcomes [37], [41]. This will be discussed in more detail in 1.2.5.

Basal Like

Basal-like BCs originate from the cells of the outer layer of the epithelium and represent around 15% of all BC cases [42], [43]. This cancer type is characterised by expression of cytokeratins and is often triple negative, i.e. lacking any hormone receptor or HER2 overexpression, meaning they are refractory to hormone/growth factor based therapies [44]. Additionally, they are generally more invasive than luminal phenotypes and exhibit a high level of proliferative markers [44]. This high proliferation rate leaves the tumours vulnerable to chemotherapeutic agents and in fact, TN basal-like BC responds very well to chemotherapy. However, prognosis remains poor. This is frequently attributed to an increased risk of relapse in TN basal-like BC subsets and treating this subtype of BC remains a clinical challenge [45].

1.2.3.2. Beyond intrinsic subtypes – Integrative clustering

Whilst the use of the previously discussed BC subtypes has been generally useful for allocating patients to treatment, gene expression studies suggest that they far from represent the full diversity of BC subsets. Following the completion of the METABRIC study which aimed to classify tumours not just by their expression profiles, but also by changes in

genetic copy number, a further diversity in BC subgroups was determined. These groups, known as integrative clusters (IntClusts), are made up of 10 distinct gene expression and copy number alteration profiles that span all the previously described intrinsic subtypes [46]. Of these, seven are dominated by ER+ tumours, compared to the only two intrinsic subtypes which are predominantly ER+, highlighting the lack of resolution in intrinsic phenotyping. Importantly, each IntClust has a distinct prognosis with 3, 4, 7 and 8 representing the clusters with the best prognosis. Clusters 3 and 4 have very little to no copy number aberrations (CNAs) whilst 7 and 8 typically only have changes to chromosomes 1 or 16. These groups exhibit high rates of *PIK3CA* mutation and a conversely low occurrence of *TP53* mutation. The same pattern is present in reverse in the ER+ IntClusts with a comparatively worse prognosis (1, 2 and 6) suggesting mutations in DNA repair mechanisms are a potent driver of poor outcomes in BC [47]. Interestingly, despite cluster 2 having an intermediate 5-year survival rate (78%), the 10-year survival rate falls dramatically to 51%, a change that is not seen in any other IntClust. This highlights the clinical importance of further stratification of BC subtypes. Patients of IntClust 2 could be subjected to more intensive surveillance than those of other subtypes in order to improve outcomes [47]. Furthermore, IntClust 4 can also be separated by their ER expression. Whereas IntClust 4 ER+ tumours exhibit CNAs similar to that of 3, 7 or 8, the IntClust4 ER- tumours have a mutation profile that more closely resembles IntClust 10, a cluster which has a poor prognosis. However, these IntClust 4- tumours are characterised by a high expression of immune related genes, particularly those expressed by T cells [48]. This gives further credence to the theory that functional immunotherapies in BC require better molecular characterisation and will be discussed in more detail in a later section. However, whilst integrative clustering has promising applications, it is not yet clinically feasible due to the cost of such extensive sequencing.

1.2.4. Disease progression

Breast cancer is staged according to the TNM (Tumour, Node, Metastasis) model, which considers tumour size, the number and locality of any affected lymph nodes and evidence of metastasis to other organs. The stages are summarised in Table 1.1. The earliest stage, also known as ductal carcinoma *in situ* (DCIS) describes a non-invasive accumulation of pre-cancerous cells. The other 4 stages all describe cells that have become locally invasive, usually forming a tumour. Generally, stage increases with tumour size, however size is not the primary determinant as larger tumours may be of a lower stage if no lymph nodes are involved. Instead, increasing stage is determined by the extent of spread, i.e. the more nodes involved, the higher the stage. Stage IV, or advanced BC, is described as any

disease where the cancer has spread to distant organs irrelevant of tumour size or lymph node involvement [49]. Staging of breast cancer has a significant impact on prognosis. The five-year survival of a patient presenting with Stage 1 or 2 cancer is over 80%. This is severely reduced at later stages of the disease. Only 15% of patients presenting with stage IV disease will survive for 5 years [50]. Fortunately, with the advent of modern screening programs many BCs are detected early leading to gradual decreases in patient mortality over the past 10 years.

Table 1.1 - Clinical staging of breast cancers, adapted from [49]

Stage	Tumour Size	Lymph Nodes	Metastases
0	Non-Invasive cells	No	No
I	<2cm	Cells	No
II	2-5cm	1-3 Axillary Nodes	No
III	>5cm	4-10 Axillary Nodes	No
IV	Any	Any	Yes

1.2.5. Treatment approaches

Common treatment regimens for BC include a combination of surgery, chemotherapy and radiotherapy and differs depending on the tumour's receptor status, node involvement and the patient's family history. The most common treatment approach is surgery. Most women (81%) receive surgery, this can either be a total mastectomy or breast conserving surgery to remove the tumour whilst maintaining healthy breast tissue. Neoadjuvant therapies are uncommon in BC but may be indicated if a patient's tumour is initially too big or complicated to remove by surgery. There is however some evidence to suggest that this approach offers no long term benefit to survival as the increased exposure of the breast tumour to the treatment increases the likelihood of metastasis [51], [52].

Adjuvant chemotherapy is very common in BC and will usually be started as a soon as clinically possible after surgery. One of the most common and effective chemotherapy regimens is the Docetaxel, Doxorubicin & Cyclophosphamide (TAC) combination. Before the development of taxanes such as Docetaxel for use in BC, patients were treated with the FAC regimen (Using 5-fluoruracil instead of Docetaxel) [53]. However, the TAC regimen was shown to significantly reduce the risk of recurrence and death when compared to FAC [54], [55] in BC and is now the adjuvant chemotherapy recommended by the National

Institute of Clinical Excellence (NICE) [56]. In some cases, adjuvant radiotherapy is also indicated for BC patients (particularly in patients who have had breast conserving surgery) and has been shown to significantly improve recurrence rates in these patients [57], [58]. However, this is also occasionally recommended for patients who have had a full mastectomy, particularly patients who are at risk of local recurrence; defined by patients who have over four local lymph nodes involved [59].

As alluded to in Section 1.2.3, some breast tumours may be responsive to hormone or growth factor-based therapies. Depending on the molecular subtype, these will usually be given alongside chemotherapies [53]. If a tumour is ER+ then the use of estrogen antagonists such as Tamoxifen are indicated to inhibit estrogen dependent mitogenic signalling [60]. Inclusion of Tamoxifen in treatment regimens for ER+ disease reduces the recurrence rate by 30% over 15 years, however there is no benefit in ER- disease [61]. In some cases, such as in post-menopausal women, it is recommended to instead use aromatase inhibitors [62]. Aromatase inhibitors such as letrozole block estrogen production by adipose tissues but not by the ovaries, therefore are of limited use in pre-menopausal women [63]. But, when used according to their indication, have been clinically successful with response rates similar to or above that of tamoxifen [62], [64], [65]. Many ER+ tumours will eventually acquire resistance to estrogen based therapies, this is often a consequence of altered ER presentation on the cell surface, or upregulation of other growth factor receptors such as HER2 [66]–[68]. In the latter case, it may be possible to resensitise tumours to estrogen therapies through administration of selective HER2 inhibitors such as Trastuzumab [69].

Trastuzumab is a function blocking antibody that recognises the HER2 receptor and prevents downstream signalling that would otherwise result in aberrant cell division and proliferation [41]. It has been shown to be efficacious in treating HER2 enriched BCs, with disease free survival and overall survival being increasing by 60% and 66%, respectively, when compared to treatment with chemotherapy alone [70]. Like estrogen based therapies, its use is also susceptible to acquired resistance through several mechanisms such as epitope masking, enzymatic cleavage and upregulation of alternative growth factor signalling pathways [71]. To overcome this, the use of small receptor tyrosine kinase inhibitors has been suggested in order to block other signalling pathways such as mTOR. This is in addition to using other biologic inhibitors of HER2 such as pertuzumab to use an alternative HER2 binding site and prevent dimerisation with HER3 [41], [72].

1.3. The Immune System

Sometime around 10,000 BCE, the microorganism *Variola major* emerged as a human pathogen. The disease it causes, small pox, has decimated human populations throughout history. An outbreak during the 18th century killed 400,000 people a year and for those who survived, morbidity was severe, with a third of cases leading to blindness [73]. But, In 1796 an English physician, Edward Jenner, exposed James Phipps to a similar but far less severe disease, cow pox, becoming the first recorded demonstration of vaccination [74]. Fast forward to 1980 and the World Health Organisation declares small pox eradicated, thanks in no small part to that moment in 1796. Not only did Edward Jenner's work lead to the eradication of a fatal disease, he also kick-started the study of the immune system, now known as immunology. Immunology can largely be split into two arms, the innate and adaptive immune responses. The former based on recognition of molecular patterns leading to an immediate inflammatory response and the latter being a more refined response based on generation of antibodies against specific antigens (Ags). This chapter intends to summarise the cells and processes involved in both responses and their role in conferring immunity to pathogenic challenges.

1.3.1. Haematopoiesis

Haematopoiesis is the production of all blood cells from a series of gradually lineage restricted progenitors. Beginning during the early stages of embryonic development, the first wave of primitive haematopoiesis generates erythrocytes for tissue oxygenation [75]. However, further waves of primitive haematopoiesis result in generation and settlement of haematopoietic stem cells (HSC) in the bone marrow (BM) leading to the beginnings of adult, definitive haematopoiesis and production of mature immune cells [75]. This initial HSC recruitment to the BM has been shown to be driven by CXCL12 production by BM stromal elements resulting in HSCs infiltrating and occupying a niche in the vicinity of sinusoidal vessels [76]. The niche is thought to be supported by the contribution of several perivascular elements, including mesenchymal stem cells (MSCs) and endothelial cells via continued production of CXCL12 and stem cell factor (SCF) [77]–[79]. These HSCs give rise to long-lived, lineage specific progenitor cells that differentiate into the mature effector cells of the immune system through several cell fate crossroads (Shown in Figure 1.2). Adult haematopoiesis predominantly occurs in the BM and myeloid differentiation can occur to completion in this compartment. However, lymphoid cell production requires transport to

exogenous organs such as the spleen or thymus. This will be explored in detail in a later section.

The mode by which HSCs 'decide' which cell lineage to follow is not well understood and is a controversial issue in the literature. Two competing models have been suggested. The stochastic model suggests that cell fate is controlled by a Galton board-esque mechanism. As the cell passes through each differentiation stage, its fate is controlled by the differential regulation of specific transcription factors (TFs) [80], [81]. The inherent randomness is driven by reciprocal regulation of these TFs, such as that seen by PU.1 and GATA-1. Expression of PU.1 is required for differentiation of HSCs and multipotent progenitor cells (MPP) to the common myeloid (CMP) and lymphoid (CLP) progenitor cells [82], [83]. However, expression of PU.1 is regulated by the GATA-1 TF and vice versa. The reciprocal interaction of these two proteins results in a molecular tug of war. If PU.1 becomes the dominant TF then cell fate is directed towards the immune repertoire and if GATA-1 wins, cells are directed towards megakaryocytes and erythrocytes [84]–[86]. How one TF becomes dominant is not fully understood and may in part be explained by the deterministic model which states haematopoietic progenitor cell fate is controlled according to demand by haematopoietic growth factor production such as the colony stimulating factors (CSFs).

Elevated serum levels of CSFs are seen during infection and individual members have been shown to drive production of macrophages, neutrophils and other granulocytes separately. These will be covered in more detail in Section 1.3.2 [87]. Additionally, several Interleukins (ILs) have been shown to drive generation of haematopoietic progenitors. Production of lymphoid lineages is dependent on the presence of IL-7 as IL-7 KO animals show impaired steady state generation of lymphoid populations [88]. The generation of lymphoid cells is partially rescued by administration of exogenous Bcl-2, an anti-apoptotic protein, which increases T-cell populations but not B cells. This suggests IL-7 is required for T-cell survival during maturation, but not for differentiation of the CLP into T-cell lineages [89]. It is, however, required for B cell differentiation as preventing apoptosis does not restore B cell populations. This is because IL-7 signalling also upregulates the transcription factor Early B cell Factor (EBF) 1 which is essential for B cell lineage differentiation [90].

In myeloid lineage determination, the story is more complex as there appears to be a great deal of cytokine redundancy. Administration of exogenous IL-3 has been shown to amplify myeloid populations and increases in serum IL-3 levels are seen during infection, resulting in a subsequent increase in circulating monocytes [91], [92]. However, IL-3 KO animals have no obvious defects in haematopoiesis during steady state [93], [94]. Does this suggest the deterministic and stochastic models of haematopoiesis can be reconciled? Perhaps steady state haematopoiesis is driven by stochastic events and during challenge the immune system switches to deterministic immune programming to respond to pathological demands? The answer is not clear, however several technologies, such as single cell sequencing, have been advancing the haematopoietic field, allowing for discrete snapshots of progenitor cell expression to be profiled in detail [95]. Perhaps with these advances in technologies the two concepts may one day be unified.

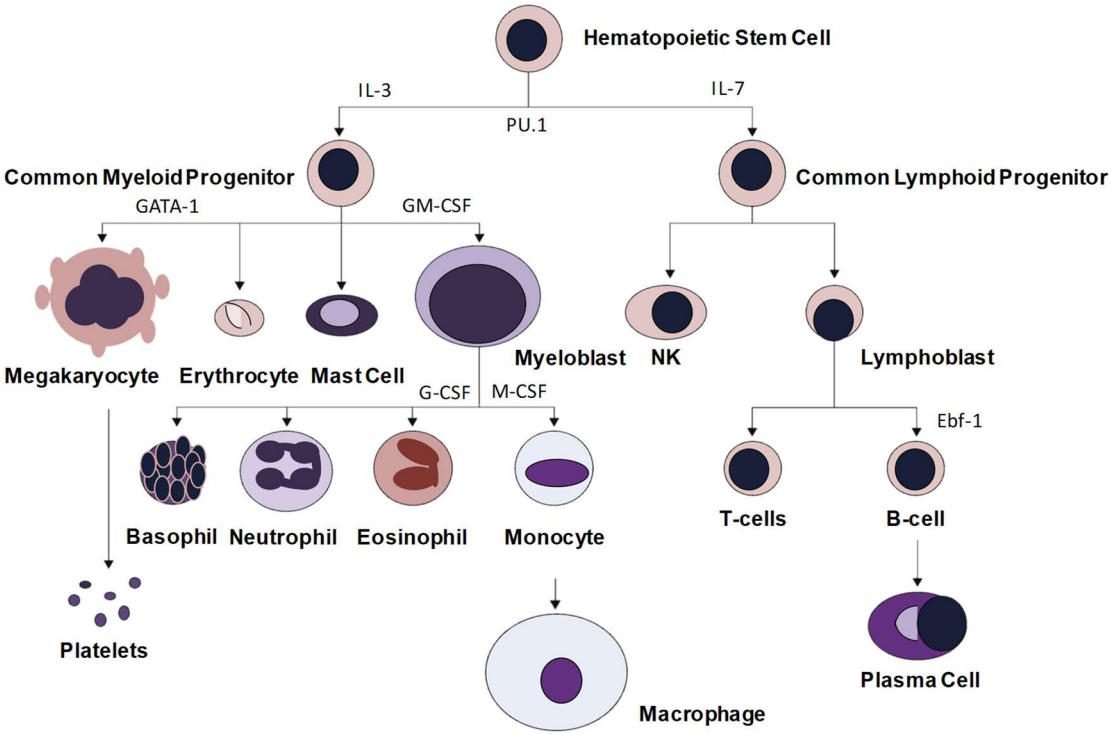


Figure 1.2 – Schematic of haematopoietic lineages: HSC differentiation states are defined by exposure of progenitor cells to cytokines and upregulation of transcription factors. This schematic describes the different pathways HSCs and the specific progenitors can take to produce mature leukocytes, megakaryocytes and erythrocytes. Also shown are transcription factors and cytokines discussed in text that are known to play a role in lineage determination. Abbreviations: CSF – Colony Stimulating Factor, (GM)-CSF – Granulocyte/Macrophage, (M)-CSF – Macrophage, (G)-CSF – Granulocyte, NK – Natural Killer. Adapted from [100].

1.3.2. Myeloid Cells

Myeloid cells form the basis of the innate immune system. Originating in the BM, myeloid cells migrate into the blood stream where they travel to resident tissues [96]. Here, they may terminally differentiate, such as in the case of monocytes and macrophages to generate tissue specific myeloid cell populations such as Kupffer cells in the liver [97]. In their resident tissue, myeloid cells are the primary phagocytic cell type. They patrol the tissue and engulf debris and foreign materials. If the materials are recognised as pathogenic, the myeloid cells become activated and begin production of cytokines which drive inflammatory processes, incite HSC differentiation and initiate the adaptive immune programme [98], [99]. This chapter will summarise the distribution, characteristics and function of several myeloid cell types.

1.3.2.1. Monocytes

Monocytes make up ~10% of all leukocytes and their production occurs mostly in the BM, where myeloid progenitor cells diverge from granulocytes and dendritic cells (DCs) to become monocytes under the control of Macrophage Colony Stimulating Factor (M-CSF) [100], [101]. From here, monocytes migrate out of the BM and begin circulating in the vasculature, where they are predominantly found. Monocytes were once thought to be purely a developmental stage in the generation of tissue resident macrophages and DCs as they have been shown to differentiate to both easily in culture [102], [103]. Whilst this statement is true, it does not paint the full picture. Monocytes are incapable of recapitulating the full repertoire of DC subsets suggesting there are other DC progenitor cells (to be discussed in a later section) [104]. Also, whilst monocytes will readily differentiate into macrophages *in vivo*, this appears to be limited to occurring during immunological responses and does not constitutively occur in the replenishment of tissue resident macrophages during the steady state [105]. Instead, these populations are maintained by self-renewal and as such, whilst they resemble BM derived monocytic (BMDM) macrophages, they in fact display expression profiles that are distinct to the tissue they inhabit [106], [107]. This begs the question, what is the function of circulating monocytes during steady state?

Recently, new roles for monocytes have been uncovered. Steady state monocytes can be split into two populations which are defined by their Ly6C status. Non-classical, $Ly6C^-$ monocytes are confined to the vasculature, only extravasating at sites of tissue injury [108]. Whilst classical, $Ly6C^+$ monocytes have been shown to constitutively extravasate into steady state tissues. Here they may differentiate into macrophages, or they can acquire

expression of MHCII and migrate to nearby lymphoid organs without differentiation [109]. It is thought that, monocytes that start upregulating MHCII are directly engaging in Ag presentation, a role previously thought to be exclusive to macrophage differentiated monocytes [110].

However, the classical role of the monocyte is to home to the site of tissue inflammation, extravasate and differentiate. This process is summarised in Figure 1.3. This process is initiated by chemokine release at the site of injury. Production of C-C motif Chemokine Ligand (CCL)-2 and 7 can be initiated by any nucleated cell in response to tissue injury or bacterial infection respectively. These interact with C-C Chemokine Receptor (CCR)-2 and 7 on the monocyte to initiate migration [111]–[113]. Additionally, monocytic CCR1+5 can be stimulated by a variety of epithelial derived ligands in response to aseptic tissue injury [114]. Monocyte homing is undertaken by a process of rolling along the vasculature, driven by reciprocal interactions between the monocytes and endothelial cells via selectins. Monocytes express L-selectin at their cell surface and via interaction with endothelial P-selectin glycoprotein ligand-1 (PSGL-1), are captured and secured onto the vasculature [115]. Additionally, this process can be mediated by P & E selectins expressed at the surface of inflamed endothelial cells via interaction with CD44 and E-Selectin Ligand-1 (ESL-1) on the surface of monocytes [116]. Monocytes complete their journey by arresting at their extravasation site through activation of integrins. This is achieved by Intercellular Adhesion Molecule-1's (ICAM-1) interaction with the integrin Lymphocyte function-associated antigen-1 (LFA-1) [117]. Arrest is mediated by increased LFA-1 affinity as a result of conformational changes induced by chemokine concentration [118]. These strong associations stop the rolling process allowing the monocyte to extravasate paracellularly in a Platelet Endothelial Cell Adhesion Molecule (PECAM)-1 dependent manner [119]. At the basal side of the endothelium the monocyte encounters inflammatory Pathogen Associated Molecular Patterns (PAMPs) and begins the process of differentiation into a macrophage. Macrophages have a diverse activation repertoire depending on the source of inflammation and this will be discussed in the following sections.

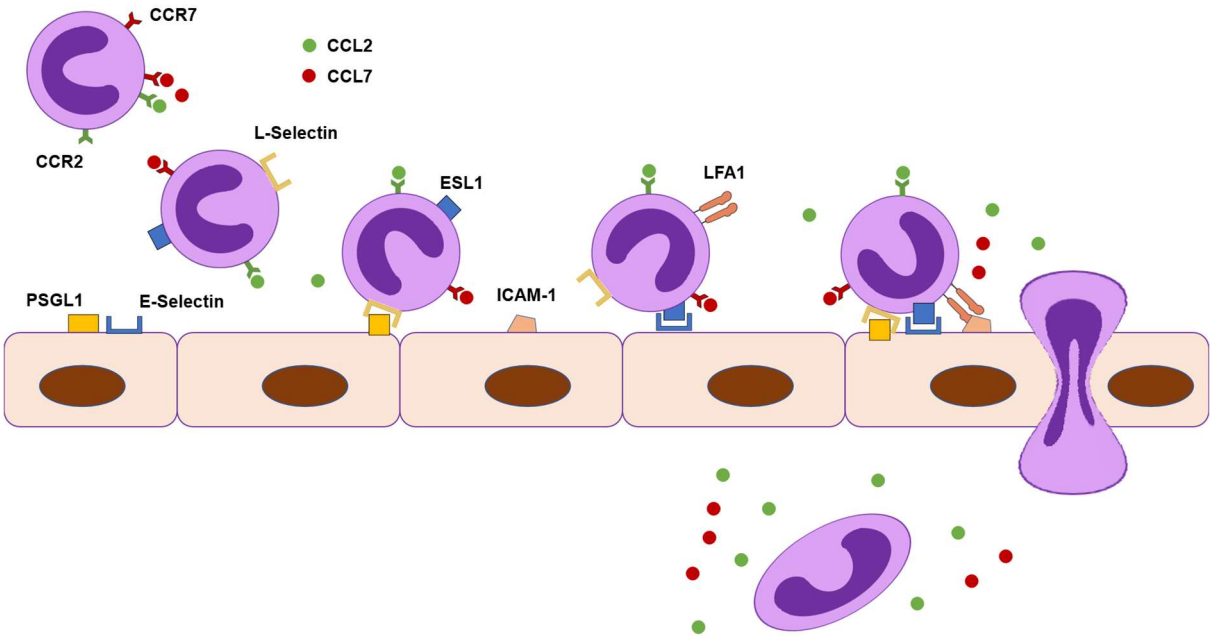


Figure 1.3 – The process of Monocyte rolling: Upon encountering chemokines, monocytes will begin migration to the source by concentration gradient. Chemokine signalling pathways upregulate L-selectin on the monocyte and inflamed endothelium shows upregulated expression of the E&P selectins. These capture the monocyte and initiate rolling in an ICAM-1 dependent manner. The monocyte arrests at the site of inflammation, extravasates and is free to gather Ag for presentation at lymphoid organs or differentiate into a macrophage. Adapted from [786].

1.3.2.2. Macrophages

Macrophages are the canonical phagocyte of the immune arsenal, discovered by Elie Metchnikoff, a discovery for which he was awarded the Nobel prize along with Paul Ehrlich in 1908. Macrophages play a nuanced role in both tissue homeostasis and inflammation, the understanding of which is still an active area of research [120]. During steady state, the primary role of macrophages is clearance of apoptotic cells. This is achieved by resident populations of terminally differentiated macrophages present in several organs. The many faces of the tissue resident macrophage are summarised in Table 1.2. These cells were once thought to be replenished by BMDM migration and differentiation, however it is now clear that these macrophages are self-renewing and inhabit their respective tissues during embryogenesis [106].

Table 1.2 – Functions of tissue resident macrophages

Tissue	Specific Function
Bone	Bone resorption [121]
Lung	Secreting antimicrobial compounds, recruitment of neutrophils during infection [122]
Thymus	Phagocytosis of apoptotic lymphoid progenitors [123]
Spleen	Sensing of circulatory Ags [124]
Liver	Clearance of bacterial and cell debris from bloody, removal of aged erythrocytes [97], [125]
Gut	Maintenance of intestinal homeostasis, sensing of bacterial Ags [126]

In contravention to the generation of tissue resident macrophages during steady-state, the predominant source of macrophages during inflammation is through recruitment and differentiation of monocytes as described previously. The activation states of macrophages are broadly described by the M1/M2 paradigm. Whilst this is helpful for conceptualisation of macrophage function, it represents digitalisation of an analogue system and does not capture the full complexities of macrophage activation states. Nonetheless, this nomenclature is helpful and as such will be used to loosely describe macrophage function. The M1/M2 polarisation repertoire was originally coined by Mills and colleagues who identified a macrophage subset that was 'alternatively activated' and capable of eliciting tissue repair [127]. The determination of M1/M2 polarisation is based on how macrophage subsets metabolise Arginine. M1 macrophages break Arginine down into Citrulline and cytotoxic Nitric Oxide (NO) via Nitric Oxide Synthase (iNos), whereas M2 macrophages use Arginase to produce Urea and Ornithine which has been shown to promote tissue repair [128]. The M2 phenotype was later elaborated on by Mantovani *et al*, who suggest that macrophages exist somewhere in a spectrum of polarisation and categorise M2 macrophages further depending on their cytokine production and immune modulatory effects [129].

Classically activated or M1 macrophages are the prototypic, mononuclear phagocyte. Their potent production of cytotoxic NO is designed to rapidly kill pathological cells. Activation of macrophages towards an M1 phenotype is driven by stimulation with Interferon gamma (IFN-γ) and Lipopolysaccharide (LPS) via Interferon Gamma Receptor 1/2 and TLR-4 respectively [130], [131]. This induces upregulation of several pro-inflammatory cytokines, including IL-1β, Tumour Necrosis Factor (TNF)-α and IL-6 which promote immune cell

infiltration into the inflammatory site [132]. Additionally, M1 macrophages can activate naïve CD4+ T-cells through their production of IL-12 and IL-23 [133], [134]. The former directs T-cells towards a T helper 1 (T_H1) phenotype which through production of IFNγ potentiates the cytotoxic activity of macrophages and CD8+ T cells [135]. The production of IL-23 pushes naïve T cells towards a Th17 phenotype, characterised by production of pro-inflammatory IL-17. This cell type also produces Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), a known polariser of macrophages to the M1 phenotype resulting in a positive inflammatory feedback loop [134], [136], [137]. Dysregulation of this feedback loop has been suggested to play a role in several autoimmune pathologies [138].

Alternatively, activated, or M2 macrophages, are generally considered to be immune inhibitory, classified as such by their high production of IL-10, a highly immunoregulatory cytokine [139]. The M2 phenotype has been broken down further into M2a, b and c depending on the function of the cell [140]. The M2a polarisation state, also known as the wound healing macrophage, is induced by exposure to IL-4 and IL-13 that results in upregulation of IL-10 and IL-1 receptor antagonist (IL-1RA), both of which are anti-inflammatory [141]. Production of CCL2 is also upregulated in M2a macrophages and has been shown to polarise CD4 cells to a Th2 phenotype and is associated with increased humoral immunity, the mechanisms of which will be discussed in later sections [142]. M2b macrophages differ from the normal M2 phenotype in that they do not upregulate Arginase and therefore Orthinine production, however, they retain the high production of IL-10 that is ubiquitous across all subsets [129]. Macrophage polarisation to this orientation is driven by exposure to immune complexes such as complement and opsonised elements in addition to LPS [143]. Whilst responding to similar stimuli as M1 macrophages, they only partially recapitulate the phenotype. Polarisation to M2b results in production of a similar repertoire of pro-inflammatory cytokines to M1, but they do not produce NO and are therefore not cytotoxic. Additionally, they do not produce IL-12 and are therefore are not capable of eliciting Th1 responses [143]. Instead, M2b macrophages have been shown to upregulate CCL1 production which in turn interacts with CCR8 on CD4+ T cells to drive Th2 polarisation [144]. M2c macrophages retain the arginase mediated production of Ornithine as seen in M2a, but their polarisation is initiated by IL-10 [129]. Also referred to as ‘Deactivated’ macrophages, this arm of the spectrum is highly immune regulatory and upregulates production of tissue repair factors such as TGF-β, processes that are closely associated with the pro-tumorigenic properties of M2 macrophages [145], [146].

Despite broadening of the macrophage spectrum by devolution of M2 phenotypes, there is still contention in the field surrounding activation states. There is also no unification of

macrophage nomenclature with some groups choosing to adopt the 'Classical, Alternative, Type II and Deactivated' model while some classify macrophages by their activating cytokines [147]. This likely reflects the lack of understanding the field currently has regarding the complexities of macrophage activation and whilst the M1/M2 paradigm remains, the lines are continuing to be blurred.

1.3.2.3. Neutrophils

Shock and awe is a military campaign that was brought to the fore by Colin Powell during the Gulf War. It calls for rapid exhaustion of the enemy by an overwhelming and intimidating force. In the immune arsenal, this role is performed by the neutrophil. Composing ~50% of all leukocytes in the body, the neutrophil mobilises rapidly to sites of inflammation and begins producing huge amounts of cytotoxic factors and cytokines [148]. Often described as the immune system's first responder, neutrophils originate in the BM from committed CMPs. Their differentiation is primarily under the control of GM-CSF and in KO animals or humans with inactivating mutations in GM-CSF, severe neutropenia is observed [149], [150]. Neutrophil differentiation is in part controlled by IL-6 and IL-3 *in vivo,* however there is a degree of redundancy as KO animals have normal steady state neutrophil production [151], [152]. Post-mitotic neutrophils are retained in the BM by interactions between CXCR4 and CXCL12 on stromal cells [153]. Neutrophil mobilisation is induced by exposure to G-CSF which in turn down regulates production of CXCR4 and allows for transcellular migration into the vasculature [154], [155]. From here, under homeostatic conditions roughly 50% of neutrophils remain as circulatory cells, whilst the other half will extravasate into tissues and become part of the marginated pool, though both can be mobilised rapidly during inflammatory conditions [156]. The half-life of post-mitotic neutrophils is ~5 days. Aged neutrophils begin upregulating CXCR4 and return to the BM. Here they apoptose, are phagocytosed by stromal macrophages inducing G-CSF release and mobilisation of new neutrophils resulting in a homeostatic cycle of neutrophil replenishment [157], [158].

The lifecycle of a neutrophil changes dramatically during periods of stress and inflammation. Increases in pro-inflammatory cytokines such as G-CSF, IL-8 and CXCL1 drive increased mobilisation of BM neutrophils [159], [160]. Neutrophil mobilisation is incredibly rapid and numbers of circulating cells can increase by 10-fold within a matter of hours [161]. Release of pro-inflammatory cytokines also drives neutrophil migration, particularly CXCL1 which is a potent neutrophil chemoattractant [162]. Neutrophil migration occurs in much the same way as monocyte attraction, with capture by selectins and integrin dependent rolling to the site of extravasation (see Figure 1.3). Transmigration of neutrophils occurs through slightly

different mechanisms however. Neutrophils start secreting factors that increase vascular permeability and migrate paracellularly by rearrangement of their LFA-1, ICAM-1 adhesion complexes rather than the PECAM-1 dependent migration seen with monocytes [163]–[165].

Once at the basolateral surface of the endothelium, neutrophils will activate fully. This occurs in stepwise fashion and neutrophils are commonly partially activated whilst extravasating after exposure to locally produced, inflammatory cytokines [166]. This initial activation primes neutrophils for response to further activation signals which leads to rapid full activation and initiation of effector functions when neutrophils reach the site of inflammation [167]. The primary role of the neutrophil is phagocytosis of infectious agents and other pathological materials. Once activated, neutrophils also release their granular payload which includes cytotoxic agents such as NADPH oxidase produced reactive oxygen species and iNos produced NO [168]–[171]. However, neutrophils also have a more nuanced role in activation of downstream immune pathways. Through the release of several pro-inflammatory cytokines neutrophils can promote the infiltration of macrophages, NK cells and other immune cells. For example, activated neutrophils secrete CCL3 which promotes dendritic cell chemotaxis and maturation, potentiating their ability to present Ag [172]–[175]. They also initiate components of the cellular adaptive immune response by activating naïve CD4+ T cells and polarising them towards Th1 and Th17 phenotypes resulting in production of an inflammatory environment [176].

After neutrophils have reached their phagocytic capacity they initiate apoptosis and are themselves phagocytosed by macrophages. This process is essential for resolution of inflammation. Engulfment of exhausted neutrophils in this manner polarises macrophages towards the M2 phenotype resulting in upregulation of IL-10 [132]. Production of this immunoregulatory cytokine suppresses inflammation and brings the acute phase of immunity to an end. Thus, neutrophils are both the arbiters and arbitrators of the immune response, with the ability to aggressively drive inflammatory processes and the power to resolve inflammation and return to steady state.

1.3.2.4. Dendritic Cells

Dendritic cells are the best characterised of the professional antigen-presenting cells (APCs), which is no surprise given their role revolves almost entirely around presentation of Ags. This section discusses the differentiation, maturation and function of DCs inhabiting lymphoid tissues. Maturation of non-lymphoid associated DCs will be discussed in a later

section and will be specific to gut DC maturation. Lymphoid tissue associated DCs begin life, like all other cells of the immune system in the BM [177]. The predominant precursor is the CMP, however there is evidence that suggests CLPs may also give rise to DCs, but given the CMP outnumbers the CLP by 10:1, almost all DCs are derived from the CMP during steady state [178]. The CMP gives rise to an intermediate progenitor, the Macrophage-DC progenitor (MDP) which whilst partially fated can still give rise to monocytes [179]. A further differentiation step leads to generation of the Common DC Progenitor (CDP), a fully committed DC progenitor, before development of the pre-DC [180]. From here, pre-DCs migrate out of the BM and transit to lymphoid tissues where they begin maturation in a fms-like tyrosine kinase (Flt)-3 dependent manner [181]–[183]. Mature DCs can be categorised as classical DCs (cDCs) and non-classical DCs, the latter being largely outside the scope of this thesis but will be briefly discussed with respect to Ag sensing in the gut in a later section. cDCs are found in all lymphoid tissues and most non-lymphoid tissues and can be split into two populations based on their function and cell surface markers: the lymphoid specific $CD8\alpha^+$ and their non-lymphoid equivalents $CD103^+$ or $CD11b^+$ DCs [184]–[187].

The former is the predominant cDC type found in lymph nodes (LNs) and whilst they are well studied, their precise role in the LN is not fully understood [188]. The LN also contains a number of migratory DCs that are thought to be transporting their Ag cargo for profiling by T cells [189]. Resident $CD8\alpha^+$ DCs play some role in cross presentation of Ags when the migrating DC is unable to elicit T cell responses itself [190]. They have also been shown to regulate lymphocyte trafficking into the LN by modulation of vascular permeability [191]. On the other hand, $CD11b^+$ DCs are not particularly well studied, but their role in lymphoid tissues is better defined. The complication with $CD11b^+$ DCs is that despite further subdivision into Endothelial cell-Selective Adhesion Molecule-1 (ESAM-1) hi and lo populations their heterogeneity is yet to be fully described [187], [192]. However, the function of these two splenic populations is only partially understood. Both are localised in the splenic marginal zone where they sense blood-borne Ags, but the ESAM-1^{hi}, $CD11b^+$ subset of cDCs appear to be the primary APCs in this zone [193]. This is highlighted in targeted depletion studies, where notch signalling is impaired in splenic stromal cells resulting in reduced numbers of ESAM-1^{hi}, $CD11b^+$ DCs and severely impaired CD4+ cell recruitment and activation [194]. These cDCs are also poor producers of cytokines suggesting their role is promotion of humoral rather than cellular immunity [195]. However, the reverse is true for ESAM-1^{lo}, $CD11b^+$ DCs, Whilst they do not participate as readily in CD4+ T cell recruitment, they are willing producers of pro-inflammatory cytokines and polarise CD4+ cells to a Th1 phenotype [196]. This suggests a potential “detector” and

"presenter" system amongst DCs, where one subset, in this case the ESAM-1^{lo} cells, produce a beneficial cytokine milieu for the "detector" cell to elicit adaptive immune responses [197]. This results in effective induction of adaptive immune repertoires in response to antigens being presented in secondary lymphoid organs. Therefore, lymphoid resident DCs are master regulators of adaptive immune responses.

1.3.3. Lymphoid Cells

Lymphoid cells are the primary effector cells of the adaptive immune response. Their activity is generally dependent on recognition of Ags being presented by other cell types. Whilst production of lymphoid cells occurs from the same HSC as myeloid cells, their lineage fating splits at a very early stage of development. Progenitor cells from the HSC upregulate Flt3R expression by interaction with their ligands on the surface of BM stromal cells which is accompanied by a concomitant increase in IL-7R expression, an essential regulator of lymphoid progenitor cell fating [198], [199]. From here, the cells will split into T cell, B cell or NK committed progenitors, the latter being a result of exposure to c-kit ligands, which will be discussed in more detail later [200]. Differentiation of B cells is known to be dependent on upregulation of the TF EBF1 which upregulates several genes required for B cell function including CD19 and the B cell receptor (BCR) [201], [202]. The differentiation factors required for T cell fating are not currently understood, however Notch-1 signalling is thought to play an essential role [200]. After generation of a committed precursor, maturation of both B and Tcells follows a similar regime. In order to become a functional B or T cell, a functional BCR or T cell receptor (TCR) must be produced. This is accomplished by VDJ recombination, the process of random rearrangement of immunoglobulin genes or the TCR gene by recombination activating genes (RAG) 1/2 enzymes [203]. The complexities of this process are outside the scope of this thesis, but the key stages are summarised in Figure 1.4. The end product is a highly specific receptor that can recognise an incredibly diverse set of ligands and is key to the specificity of the adaptive immune response [204]. Due to the randomness of TCR and BCR development and the huge number of permutations, it is inevitable that some recombination events will generate inactive, or more worryingly, self-recognising receptors. This is controlled by positive and negative selection of populations during maturation of each cell type in the thymus or BM for T and B cells respectively. These maturation events will be discussed in later sections but lead to generation of a naïve T or B cells ready for education and initiation of effector functions. These activation processes and the relevance of T and B cell functions will be discussed in the following sections.

Number of gene segments in the three human immunoglobulin loci			
Segment	Light chains		Heavy chain
	κ	λ	H
Variable (V)	34–38	29–33	38–46
Diversity (D)	0	0	23
Joining (J)	5	4–5	6
Constant (C)	1	4–5	9

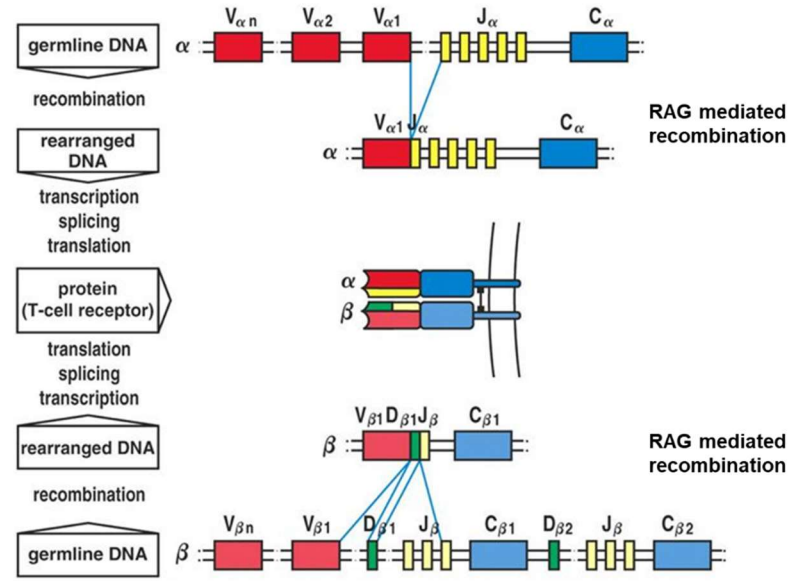


Figure 1.4 – Diversity generated by VDJ recombination: Through recombination of multiple gene segments split across the Variable, Diversity and Joining regions of TCR and Ig genes, the complete repertoire of joining specificities is encoded. The recombination of these genes is random and driven by RAG1 recognition of recombination signal sequences and creation of a single stranded nick. This reaction introduces a double stranded break, removal of the non-coding ends and joining of the coding ends. This process occurs multiple times to join all gene elements until a functional TCR or Ig is formed. The numbers of each V, D and J component result in an extremely high potential diversity, as many as $5x10^{13}$ different permutations are possible. Adapted from [787], [788].

1.3.3.1. T Cells

T cells are broadly separated depending on their TCR heterodimer and can present either the well-studied αβ TCR or the non-classical γδ TCR [205]. Both have distinct functions and play different roles in the adaptive immune response. Differentiation of both linages occurs in the thymus and the cellular decisions required for commitment to the latter will be discussed in a later section. Production of αβ TCR presenting T cells has already been briefly summarised in 1.3.3 and occurs through recombination events in the α and β subunit genes to produce highly diverse repertories of TCR specificities [206]. The TCR associates with CD3 on the surface of T cells to form the TCR complex capable of MHC recognition [207]. From here, αβ T cells are further defined as being helper or cytotoxic by presentation of the TCR co-receptors CD4 or CD8, respectively [208]. The decision to follow either lineage occurs in the thymus where lymphoid progenitor cells enter the thymus and quickly differentiate into double negative (DN) T cell precursors. These precursors pass through four defined stages (DN1-4), during which time rearrangement of their α and β TCR subunits occurs [209]. After DN4, T cell precursors upregulate both CD4 and CD8 and, via interaction with thymic epithelial cells, present self-Ags via MHC I and II. Any T cells incapable of MHC recognition are rejected, as are any cells that recognise the Ag too strongly and therefore are at risk of generating autoimmune reactions [210]. After this stage, fating towards CD4 or CD8 occurs resulting in downregulation of one receptor and generation of single positive (SP) naïve T cells. The molecular processes that underpin this selection are not fully understood and there is some argument between stochastic or deterministic models of T cell differentiation. However, fating towards $CD4^+$ populations are driven by upregulation of the TF GATA3 and $CD8^+$ by Runx3 [211]. This decision occurs in the cortex of the thymus and once complete, the SP T cell precursors move to the medulla for further selection events to ensure correct MHC I or II recognition depending on CD8 or CD4 presentation, respectively [212]. T cell precursors that pass this selection process are free to migrate to the periphery as naïve T cells and are ready for activation.

1.3.3.1.1. T helper Cells

Helper T cells are the master orchestrators of the immune response, capable of inducing or suppressing innate immune cell activation/recruitment, directing cytotoxic T cell responses, and eliciting antibody production by B cells. The diverse toolset of T_H cells is reflected by their extensive polarisation repertoire, these are summarised in Figure 1.5. Activation of T helper cells is a result of CD4+ T cells recognising their cognate Ag/MHC II complex via the TCR/CD3 complex [207]. This process also requires signalling by co-stimulatory receptors such as CD28 which binds to CD80 or CD86 on APCs and upregulates T cell

survival/proliferation factors such as NF-κB and OX40. Signalling via these factors induces clonal expansion of T_H populations capable of mounting an Ag specific immune response [213], [214]. After activation, polarisation to a particular T_H subset is largely controlled by the cytokine milieu to which the cells are exposed [215]. The subsets of T_H cells, their polarisation signals, and functions are summarised below.

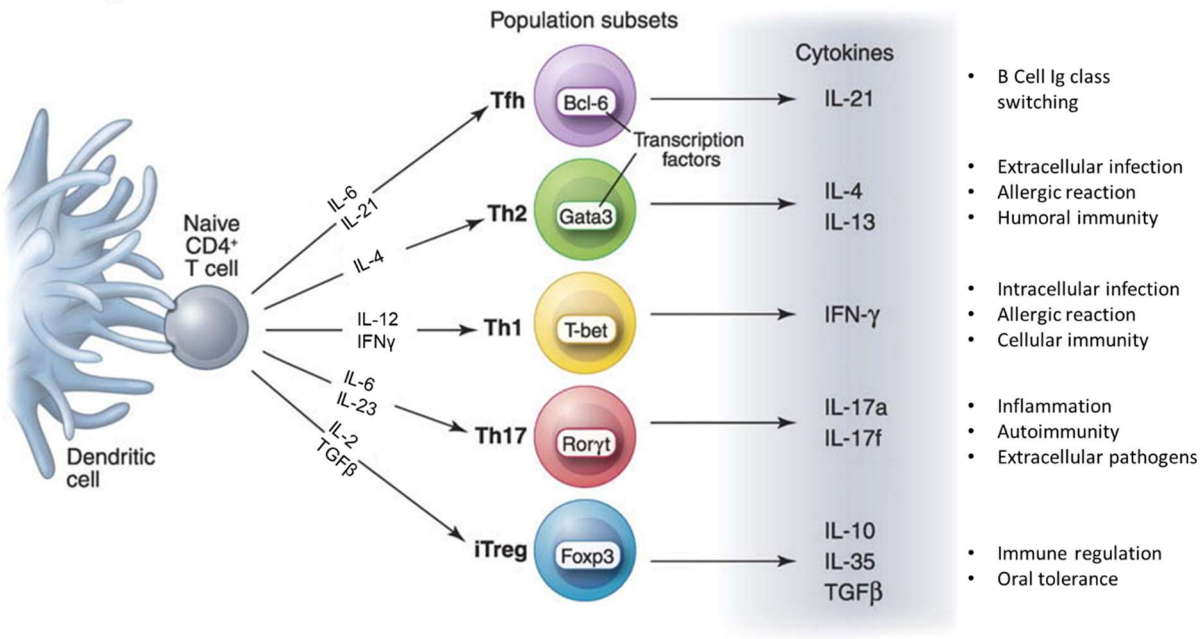


Figure 1.5 – Diversity of T_H activation states: Activation of naïve CD4+ T cells results in generation in several phenotypic subsets depending on the cytokine milieu. These activation states can participate in virtually all arms of immunity, T_H1, T_H2 and T_H17 cells all have critical roles in host defence, Tfh cells assist B cells with antibody production and T_{regs} can promote tolerance to commensal organisms and resolution of inflammation. Adapted from [216].

T_H1 / T_H2 Cells

The distinction between CD4+ T_H cells was first reported in the late 80s with the observation of a T_H population with a distinct cytokine profile. These subsets, coined T_H1 and T_H2 are identified by their production of IFNγ and IL-4, respectively, and now play well understood roles in immunity [217], [218]. Interestingly, their polarisation is driven by CD4 T_H cell exposure to the cytokines that each subtype produces i.e. IFNγ generates T_H1s and IL-4 T_H2s respectively suggesting T_H polarisation is enhanced by positive feedback loops [219], [220]. Both cell types have distinct roles in immune responses. T_H1s are highly effective against intracellular pathogens through potentiation of macrophage cytotoxicity by TNFα secretion and resistance to viral infections by IFNγ [221], [222]. The function of T_H2s is

diametrically opposed to that of T_H1s as their main function is clearance of extracellular pathogens, particularly helminth infections, through upregulation of eosinophilic cytokines such as IL-5 [223].

T_H17 Cells

The primary role of T_H17 cells is the production of pro-inflammatory cytokines and their presence has been associated with several inflammatory and autoimmune disorders. Characterised by their expression of IL-17, they were first identified as a T_H subset distinct to T_H1 or 2 cells in 2005 and have received significant attention ever since [224], [225]. Their programme of differentiation from naïve T_H cells is controlled by exposure to several cytokines including TGF-β and IL-6 but is most strongly associated with the presence of IL-23 in a process involving upregulation of the transcription factor RORγT [226]–[229]. Once differentiated, the effects of T_H17 on immunity are largely a result of IL-17 production. The IL17s are a family of 6 cytokines (A-F), the functions of which will be discussed in detail in a later section, however T_H17 cells can produce A and F and as a result have been shown to be potent inducers of neutrophil recruitment [230]. However, the functions of T_H17 cells are not limited to IL-17 as they have been shown to secrete several other cytokines. Production of IL-21 is responsible for mediating the T_H17 equivalent of the autocrine positive feedback loops seen in T_H1 and T_H2 cells [231], resulting in rapid expansion of T_H17 populations. Additionally, IL-21 has been shown to induce B cell proliferation and maturation, with IL-21 KO animals exhibiting significantly impaired humoral immune responses [232]. Furthermore, T_H17 cells also produce IL-22, which interestingly does not directly interact with immune cells as they do not express the receptor. Instead, it mediates its effects by signalling via epithelial cells to upregulate anti-microbial defence mechanisms and initiate production of parenchymally produced inflammatory cytokines [233], [234]. These abilities give T_H17 cells the capacity to potently initiate inflammatory immune responses and, as such, they are frequently associated with inflammatory diseases such as psoriasis and rheumatoid arthritis [235].

T regulatory (T_{reg}) Cells

As the supervisors of the immune response, T regulatory cells potently suppress immune responses to prevent immune mediated tissue injury. Discovered in 1995 by Sakaguchi *et al*, the presence of T_{regs} has become a hot topic due their double edged nature [236]. The earliest marker used to identify T_{reg} was CD25, however this population was found to still be extremely heterogenous [237]. Later, characterisation of T_{regs} was narrowed down by expression of the Forkhead box P3 (FoxP3) TF, now known to be essential for generation of T_{regs} from progenitors [238]. Primarily developing in the thymus, T_{regs} can also be

generated peripherally in a manner that is specific to gut associated lymphoid tissues, the mechanisms of which will be discussed in a later section [239], [240]. Whilst development of T_{regs} in different sites results in distinct functional profiles, both are dependent on the presence of IL-2. In fact, animals deficient for IL-2 exhibit significantly lower T_{reg} numbers, resulting in a predisposition to autoimmune disorders [241]. This alludes to the key function of T_{regs}: suppression of immune responses. The requirement for T_{regs} in the immune repertoire is highlighted when they are artificially depleted. When T_{reg} depleted animals are challenged with diphtheria toxin, uncontrolled expansion of almost every immune lineage is initiated. This leads to the eventual death of the animal within 10-14 days, a poignant example of the physiological importance of T_{regs} [242]. Mediation of immune regulation is accomplished by T_{regs} by several mechanisms, but principally through production of IL-10, a potent immunoinhibitory cytokine, the actions of which will be discussed in detail in 1.3.6 [243]. In addition, T_{regs} can modulate immune responses by catalysing the production of Adenosine from ATP by its ectoenzymes CD39 and CD73. Depletion of stromal ATP levels in this manner results in inhibition of ATP dependent DC maturation and blunting of any resultant adaptive immune activation [244]. Similarly, T_{regs} can prevent activation of CD8+ T cells by sequestration of their activating cytokines such as IL-2. As alluded to earlier, IL-2 is required for T_{reg} activation and is internalised by T_{regs} after receptor binding, resulting in reduced stromal availability and prevention of CD8+ T cell mediated cytotoxicity [245]. The consequences of these potent immune inhibitory effects are heavily context dependent. Whilst the presence of T_{regs} has been shown to be beneficial in inflammatory disorders such as colitis, they have been shown to facilitate immune escape by cancer cells resulting in more aggressive tumours with increased mortality rates and are therefore a major focus of current translational immunology [246], [247].

1.3.3.1.2. Cytotoxic T Cells

Cytotoxic T cells (CTLs) are the hit squads of the adaptive immune response, gathering in large numbers upon instruction by APCs. They then move out and carry out the killing of infected or damaged cells. First discovered in the 70s as a cytotoxic subdivision of lymphocytes, they were later found to be Ag guided by interaction with MHCs, a reaction that results in highly specific cytotoxicity [248], [249]. The education of CTLs occurs in secondary lymphoid organs, where during steady state, they patrol T cell zones and frequently interact with APCs, predominantly DCs, remaining in a state of quiescence until recognition of a pathogenic Ag [250]–[252]. After discovery of a cognate MHC/Ag complex, the lifestyle of the CTL changes dramatically. They undergo extremely rapid clonal expansion. It is estimated a single, naïve CTL can undergo 19 rounds of division within 6

hours, resulting in a 100,000-fold expansion [253]–[255]. This proliferation is driven principally by TCR induced upregulation of Erk signalling, but other co-stimulatory receptors such as the TNF receptors i.e. OX40 and CD28 mediated Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signalling also play key roles [256]–[258]. During activation, CTLs differentiate into two states, the short-lived effector cell (SLECs), responsible for cell killing, and the memory CD8+ T cell, ready to respond at short notice if the Ag is encountered again [259]. This heterogeneity in the proliferating T cell pool has not yet been completely explained. Some argue that these differences arise due to varying activation states depending on co-stimulatory receptor activation and environmental cytokine exposure. For example, whilst IL-2 is required for CTL proliferation, high levels of IL-2 signalling have been shown to drive SLEC differentiation [260], [261]. A contradictory argument states that the heterogeneity is stochastic, and merely a result of asymmetrical cell division, during which the machinery required for differentiation into SLECs or memory CTLs is randomly distributed around the cell, and a bias to one or the other results in differential activation states [262].

Regardless of the mechanisms of differentiation, the outcome for each CTL type is unchanged and well understood. The memory population remains in secondary lymphoid organs and will expand rapidly again when recognising their cognate Ag on APCs, reducing CTL response time by around a third [263]. The SLEC population mobilises from secondary lymphoid organs to the inflammatory site. This is primarily controlled by upregulation of the chemoattractant receptor CXCR3 during their activation and is activated by its ligands, CXCL9, 10 & 11 [264]. Attraction of CTLs to infected cells is largely associated with production of CXCL9 & 10 by parenchymal cells, a process that is potently driven by T_H cell cytokine production [265], [266].

At the site of infection, CTLs recognise MHC I presented Ag by non-APCs and initiate their killing repertoire, release of granzymes and induction of apoptosis by interaction of CTL FasL with the target cell's Fas receptor [267]. The ability of CTLs to induce target cell death is incredibly robust and CTLs have been shown to induce cell death within minutes *in vitro*, killing cells serially and simultaneously in an almost relentless fashion [268]. Fortunately, T cells are equipped with inherent regulatory mechanisms to prevent catastrophic immune responses. Production of IL-10 by T_H cells and T_{regs} results in effective inhibition of CTL responses by reduced proliferation and reduction of IL-2 production to prevent continuous CTL activation [269]. Additionally, CTLs are equipped with intrinsic inhibitory pathways resulting in T cell exhaustion [270]. Usually only seen during chronic infection or malignancy, T cells lose their cytotoxic capabilities over time. The exhausted T cell is

characterised by production of the receptor PD-1, a receptor that initiates regulatory signalling pathways in CTLs [271], [272]. Upregulation of PD-1 has been shown to result in reduced cancer cell killing and inhibiting PD-1 signalling has received considerable attention in immunotherapies [273]. This will be discussed in detail in a later section.

1.3.3.2. Natural Killer Cells

Natural killer cells (NKs) represent a unique element of the lymphoid cell lineage in that they participate in the innate immune response [274]. Making up 5-10% of all circulating lymphocytes they represent a relatively common population of cytotoxic mononuclear cells [275]. Their origins are predominately in the BM, however peripheral NK cell development has been observed [276]. Originating from the CLP, commitment to NK precursors is dependent on BM stromally derived IL-15 and in studies using IL-15 KO mice, numbers of circulating NKs are significantly depleted [277], [278]. Once a committed NK precursor, the immature NKs begin upregulation of functionally essential receptors (NKG2D, CD244, CD122, NK1.1 etc) in a stepwise fashion before finally producing a mature NK [276]. This final stage is characterised by the ability to produce IFNγ and perform perforin mediated cytotoxicity. From here, NKs migrate out and are functionally prepared for activation [279]. Some NKs home to secondary lymphoid tissues, where they await activation by mature, Ag presenting DCs and begin producing IFNγ. This potentiates T cell responses in secondary lymphoid organs, contributing to immunity [280], [281]. However, most are found as circulating cells, where upon immunological challenge, they will exert potent cytotoxic effects [282]. Recognition of pathological cells by NKs is dependent on a balance of stimulatory and inhibitory receptor recognition. The primary example of this is MHC I presentation. By interaction with MHC class I molecules on other cells, NKs can determine the cell as self and killing is prevented. However, many infected and cancerous cells will repress MHC I to prevent Ag presentation to CD8+ T cells. Whilst this is effective, NKs will no longer accept the cell as self and initiate killing in a process known as the missing self hypothesis [283], [284].

However, to prevent erroneous killing of healthy, self-cells, NK mediated killing is also controlled by several other receptor interactions. For example, if the NK receives more stimulatory than repressive signals, target cell killing is initiated. This occurs in a similar fashion to CTLs e.g. administration of perforins, deposition of granular contents, and induction of apoptosis by Fas/FasL interactions [285]. The independence of NKs from MHC directed cytotoxicity makes them rapid and effective killers, however this can occasionally lead to issues. For example, erroneous NK killing has been shown to strongly contribute to β cell elimination in the pancreas, leading to development of type I diabetes [286]. This is

important to consider given NKs have attracted significant attention as potential immunotherapeutics, for example in cancer, where in some situations, they have been shown to effectively induce tumour regression in pre-clinical models [287]. The contributions of NKs to anti-tumour immunity will be discussed in detail in a later section.

1.3.3.3. B Cells

The humoral immune response targets pathogens for destruction by innate and adaptive immune components alike. Through generation and secretion of antibodies, B cells can label pathogenic Ags, resulting in rapid detection by other immune cells. Generation of a diverse array of antibodies is a result of the previously discussed recombination of BCR gene segments V, D & J. It is estimated that these recombination events are capable of generating Abs that can recognise over 5×10^{13} different Ags [288]. This process occurs in B cell precursors inhabiting the bone marrow and is essential for generation of a mature BCR. The consequence of such extraordinary diversity is the certainty of self-recognition [289]. This is partially tempered by negative selection of B cell clones in the BM prior to exit. Any B cells found to be self-reactive are destroyed or, will undergo a second round of BCR gene arrangement in an attempt to generate a functional receptor [290]. Once the B cell has passed selection and its BCR is complete, signalling via the BCR represses recombination machinery to prevent further recombination events and the B cell is free to leave the BM [291].

Peripheral B cells can be broken down into two subsets. Firstly, there is the B-1 lineage, produced early in life, migrating out to peripheral organs of neonates. In adulthood, these populations are maintained by self renewal [292]. The B-2 lineage are bone marrow derived throughout life and represent the canonical B cell. The B-2 cells circulate to peripheral organs and eventually enter secondary lymphoid organs where they take residence in the B cell niche of the spleen: the marginal zone (MZ) [293]. Homeostasis of the B cell niche is maintained via supply and demand. Stromal cells in the MZ secrete B cell survival factors such as B lymphocyte stimulator (BLyS), which through interaction with its receptor promotes B cell survival by upregulation of NF-κB signalling. The availability of BLyS in the niche is limited and once the number of B cells results in low availability, infiltration is prevented and the niche maintained until levels of BLyS dramatically change, i.e. during infection [294], [295]. From here, B-2 cells are available to participate in humoral immune responses and can be activated in two ways. First, by T cell independent mechanisms through interactions with Ags via their TLRs. This results in a short-term production of unspecific IgM dominated Abs. These IgMs can recognise several common prokaryotic

produced molecules such as LPS and result in rapid generation of humoral immunity [296], [297].

The second brand of B-2 cell activation is dependent on T cell activation and is initiated by MHC II stimulation. This results in large scale proliferation of B cells to produce germinal centres, areas of the spleen with clonally expanded B cell populations [298]. To improve Ag specificity, T cell dependent B cell activation also upregulates production of Activation Induced Deaminase (AID) and initiation of somatic hypermutation. Through generation of point mutations in the Ig V region, AID can produce B cell variants with differential Ab specificities [299]. By competitive selection of these variants based on Ag affinity, a pool of B cells that can produce highly specific Igs is formed. These cells are known as plasma cells [300]. After immune challenge, plasma cells home back to the BM and are ready to participate again if their cognate Ag is recognised [301], [302]. This process provides specific immunity that is maintained over the life time of the organism and is responsible for the immunity conferred by vaccines.

1.3.4. The Interleukins

At the second international lymphokine workshop, held in Ermatingen Switzerland, 1979, the term Interleukin was born. Derived from the latin Inter, 'between' and Leukin, 'white', an enormous area of scientific research and a cornerstone of immunology was created [303]. At the time, the international community was feverishly trying to determine the functional properties of the first interleukin, Interleukin-1. Today, over 30 interleukins have been identified, each with diverse and context dependent functions. Largely produced by cells of the immune system, interleukins play a role in almost every aspect of immunity [304]. As a result, many have already been discussed, however two deserve special attention in this thesis due to their pivotal role in the phenotypes described. Over the next few sections, the diversity, structure and function of interleukin 10 and 17 will discussed in detail.

1.3.4.1. Interleukin-10

Many ILs are involved in activation and orchestration of immune responses, but how does the immune system control itself? The answer in most cases is IL-10. The prototypic cytokine of its namesake IL family, it was originally identified as an immune regulatory cytokine produced by T_H2 cells [305]. It is now understood to be primarily produced by T_{reg} cells but can also be produced by several other cell types including some myeloid cells such as monocytes, macrophages and DCs in response to pro-inflammatory cytokine signalling [306]. The IL-10 family, and IL-10 itself, is defined by the presence of multiple alpha helical structures in its tertiary configuration [307]. In humans and mice, it exists as a homodimer

of around 20kDa and shares 72% sequence homology across both species, allowing for murine IL-10 to interact with the human receptor [308], [309]. The receptor for IL-10 (IL-10R) is a tetrameric receptor consisting of two α and β subunits. Whilst the latter is expressed ubiquitously, IL-10Rα is only expressed in leukocytes meaning the effects of IL-10 are limited to immune populations as IL-10 binds with high affinity to IL-10Rα, but not the β subunit [310], [311]. However, IL-10Rβ is still required for downstream signalling as β subunit KO animals exhibit defective IL-10 signalling and a concomitant predisposition to autoimmune disorders [312].

After ligand binding, signal transduction is performed by Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathways. Receptor activation results in Jak1 phosphorylation and subsequent phosphorylation of STATs 1, 3 and 5, although much of IL-10's function appears to be STAT3 dependent [313]. Activation of STAT3 has been shown to induce numerous transcriptional repressors of pro-inflammatory genes, particularly interfering with NK-κB gene induction, a known TF for several pro-inflammatory pathways [314]. Interestingly, IL-6 signalling is also STAT3 dependent, yet has opposing functions to that of IL-10 [315]. A complete explanation of this paradox is not yet understood; however, some groups have suggested this is due to temporal control of STAT3 activation. Whilst IL-10 maintains prolonged STAT3 activation, IL-6 only results in transient activation. This theory is supported by experiments where exogenous IL-10 is administered to DCs that have an artificially truncated STAT3 response. Under these circumstances, IL-10 begins to function in an IL-6 like manner resulting in upregulation of inflammatory cytokine production [316].

Aside from repressing inflammatory cytokine expression, activation of IL-10 signalling has also been shown to directly inhibit immune processes in T cells. IL-10R activation leads to phosphorylation of another Janus kinase, Tyk2 which when activated phosphorylates the phosphatase Tyrosine-protein phosphatase non-receptor type 6 (PTPN6) leading to dephosphorylation of the T cell costimulatory receptor CD28 and inhibition of T cell responses [317], [318]. Additionally, IL-10 inhibits T cell function by repressing their DC mediated activation. Signalling through IL-10R on DCs results in downregulation of MHC II expression and inhibition of the T Cell costimulatory receptor CD80 [319].

The potent immune regulatory effects of IL-10 result in effective suppression of immune responses, particularly T cell mediated immunity. Functionally though, IL-10 has a reputation for being a double-edged sword when it comes to pathophysiology. In some cases, such as asthma it has a demonstrable protective effect. Levels of IL-10 are

significantly reduced in the airways and alveoli of asthma sufferers compared to healthy individuals [320]. Additionally, some inflammatory airway immunotherapies have been shown to be dependent on T_{reg} produced IL-10 for efficacy [321]. However, its role in other pathologies is somewhat less well defined, especially in cancer where IL-10 is quite the enigma. Prototypically, IL-10 production in tumours was thought to drive tumorigenesis by suppression of immune responses. This makes sense logically, as anti-tumour immune responses are essential for effective cancer cell killing and the inhibitory effects of IL-10 may impair this process [322]. In fact, high serum levels of IL-10 have been demonstrated as a negative prognostic factor in several meta-analyses across different cancer types, including breast cancer [323], [324]. However, recently a different picture has been painted for IL-10's place in cancer pathogenesis - a more nuanced role dependent on inherent tumour inflammation. In tumours with pathologies that result in overt inflammatory responses, administration of IL-10 is protective [325]. More so, IL-10 has been shown to modulate CD8+ T cell functions and improve their cytotoxicity [326]. The mechanisms behind this are unclear, but it may be a consequence of IL-10's ability to increase IFNγ production by CTLs. Increased IFNγ in the tumour microenvironment results in upregulation of MHC I molecules and increases T cell mediated killing [326].

To summarise, the diverse roles of IL-10 in health and disease make it an intriguing member of the interleukins. Elucidation of its exact role in cancer remains to be seen and will likely be heavily context dependent. This gives credence to further studies in order to fully understand IL-10's properties with a view to its eventual exploitation in immunotherapies.

1.3.4.2. Interleukin-17

Since the discovery of IL-17A (AKA CTLA-8) in 1993 there have been 5 further members of the IL17 family discovered so far [327]. The family is characterised by a high proportion of cysteine residues in their C-terminus, resulting in formation of a cysteine knot feature [328]. Whilst not all family members are produced by immune cells, they all play a role in modulation of immune responses. The properties of all members of the IL17 family are summarised in Table 1.3, although very little is known about the functions of B, C or D. However, only IL-17A&C are of relevance to this thesis and therefore are the only isoforms to be discussed in detail.

Table 1.3 - Properties and functions of IL17 isoforms [304], [329]

Name	Receptor	Cellular Origin	Function
IL-17A	IL-17RA	T_H17, γδ T cells, CD8+ T cells, NKs	• Secretion of inflammatory cytokines • Neutrophil recruitment
IL-17B	IL-17RB	GI cells, neurons	• Proinflammatory
IL-17C	IL-17RE	GI cells, CD4+ T Cells, Macrophages, DCs	• Proinflammatory
IL-17D	?	Skeletal muscle, lung, adipose, lung, brain	• Proinflammatory
IL-17E/ IL-25		T_H2 cells, eosinophils, basophils	• Eosinophil recruitment • B cell maturation
IL-17F	IL-17RA/ IL-17RC	T_H17, basophils, mast cells, monocytes	• Secretion of inflammatory cytokines • Neutrophil recruitment

IL-17A

As a potent inducer of inflammation and the first identified member of the IL17 family, IL-17A has received a great deal of research attention and is well characterised. Production of IL-17A is predominantly by T_H17 cells, but has also been seen by CD8+ T cells, NKs and γδ T cells [330], [331]. Its most common form is as a homodimer, but has also been shown to dimerise with IL-17F, though this dimerisation is not well characterised [328], [332]. Signalling by IL-17A is exerted by interactions with IL-17RA, a ubiquitously expressed receptor that is enriched in many leukocyte populations including CD4+ T cells and macrophages [333]. The primary goal of IL17A signalling is initiation of pro-inflammatory cytokine expression. The presence of IL-17A has been shown to effectively upregulate several cytokines such as IL-6, IL-8, CXCL1 and G-CSF in an NF-κB dependent manner [334]. Upregulation of the latter is a key initiator of neutrophil chemoattraction and IL-17A has been demonstrated to strongly induce neutrophil infiltration [335]. This ability makes it a key regulator of early immune responses and therefore it is essential for host defence mechanisms. Animals with IL-17RA defects are highly susceptible to infection by many pathogenic microorganisms including *Klebsiella pneuomoniae* and *Candida albicans* [335], [336]. Whilst protective during infection, upregulation of IL-17A has been associated with driving tumour growth. In breast cancer, upregulation of IL-17A promotes resistance to chemotherapies by upregulating Erk signalling [337]. Additionally, the presence of IL-17A in hepatocarcinoma has been shown to promote tumour growth by immune modulation. Expression of the chemokine CXCL5 is upregulated in an IL-17A dependent manner

resulting in infiltration of innate immune inhibitory cells: Myeloid Derived Suppressor Cells (MDSCs) which have been shown to be tumorigenic [338]. Therefore, suppression of IL-17A may offer a potential therapeutic avenue in cancer immunotherapies. This idea is supported by experiments that show inhibition of IL-17A by siRNA suppresses tumour growth in pre-clinical models of colon adenocarcinoma and melanoma [339]. There are currently two biologic IL-17A inhibitors approved for use by the FDA for psoriasis and rheumatoid arthritis [340]. Whether either of these drugs could be repurposed for use in cancer remains to be seen.

IL-17C

Very little is known about IL-17C, in fact its functional receptor was only identified recently which will hopefully lead to more insight regarding its role in immunity [341]. It is the second largest of the IL-17 family at 40kDa and shares 83% sequence similarity with its mouse homologue [304]. Its production is predominantly by epithelial cells, particularly in the GI tract and has been implicated in host defence during GI infections by upregulation of inflammatory cytokines and anti-microbial peptides via NF-κB signalling pathways [341]. Production of IL-17C has also been shown to potentiate autoimmune disorders. In models of glomerular inflammation, IL-17C signalling is essential for pathophysiology via promotion of T_H17 cell function [342]. Additionally, in models of colitis induced by imiquimod, IL-17C was shown to drive inflammation. However, in dextran sulphate sodium models, IL-17C upregulation was protective [343]. This suggests that IL-17C is capable of complex, context dependent responses that are yet to be elucidated.

1.4. The Anti-Cancer Immune Response

In his 1909 paper, ‘Über den jetzigen Stand der Karzinomforschung’, or in English, ‘About the current status of carcinoma research’, immunologist Paul Ehrlich considered how, despite the frequent occurrence of cellular transformations leading to unchecked cell division, many animals develop very few cancerous lesions [344]. His answer was, the immune system. He theorised that immune cells can act as sentinels, perusing cells and tissues looking for any unsavoury characters and eliminating them. Despite not proving it experimentally, Ehrlich had stumbled upon what is known as cancer immunosurveillance.

It took some time for Ehrlich’s theory to catch on. During the 50s, work by Frank Macfarlane-Burnet and Lewis Thomas on graft-versus-host immune responses and adaptive immunity led to the first observations of antigen dependent tumour immunity [345]. Both independently speculated that lymphocytes were the mediators of this cancer immunity, however at the time, the technology did not exist to properly characterise these responses. Years later, early work investigating cancer immunosurveillance used thymectomy and athymic nude mice to deplete lymphoid populations. Whilst a susceptibility to virally induced tumours was observed, groups found no differences with chemically induced or spontaneous cancers [346]. This early work was criticised for having relatively low numbers of biological replicates and for looking over a limited time span. However, a later study by Rygaard and Povlsen, using 10,800 nude mice showed no differences in spontaneous tumour formation over a period of up to 7 months, apparently disproving the cancer immunosurveillance hypothesis [347].

For almost 25 years the field lay dormant, so much so that the landmark review published on the millennium by Hanahan and Weinberg, ‘The Hallmarks of Cancer’ made no mention of immune mediated contributions to tumorigenesis [348]. However, in the early 90s, the field began to stir. Targeted KO of IFNγ and perforin genes showed that mice were predisposed to 3'-methylcholanthrene (MCA) induced tumour formation, proving that the immune system has some contribution in the regulation of tumorigenesis in chemically induced models [349], [350]. The seminal paper came in 2001 after the development of RAG-2 KO animals. These animals are unable to undergo VDJ recombination and are therefore severely immunocompromised due to lacking almost all lymphoid cells [351]. These animals were susceptible to rapid induction of sarcoma after MCA injection and developed significantly more spontaneous tumours than their wild-type compatriots, proving for the first time that the immune system prevents formation of spontaneous cancers [352].

Since these findings, the field has gone on to elucidate many of the mechanisms underpinning cancer immunosurveillance and in the 2011 revision of Hanahan and Weinberg's 'The Hallmarks of Cancer', tumour inflammation and escape of immune surveillance were added as critical drivers of tumorigenesis [353]. Now, over 100 years after Ehrlich's famous words, albeit with only moderate success, the first immune mediated therapies are starting to feed into the clinic. This chapter will summarise the underlying mechanisms of tumour immunosurveillance, exploring what makes it a success, what causes it to fail, and discussing how it can be exploited in a therapeutic setting.

1.4.1. Immunogenic elimination of malignant cells

Tumour immunosurveillance is not easy to observe *in vivo*. Current technologies do not allow for *in vivo* detection of eliminated malignant cells. However, by studying patients who are immunocompromised, there is compelling evidence for its existence in humans. In meta-analyses of patients with familial disorders or those who are receiving immunosuppressive drugs after organ transplant, significantly higher levels of malignancy are observed across almost all cancer types [354]. But, how does the immune system detect and eliminate early malignancies? The current dogma of immune interaction with tumours is summarised in Figure 1.6, however generally it is thought to consist of induction of adaptive immunity through presentation of danger signalling. However, due to difficulties in observing immunosurveillance *in situ,* mechanistic insights have been inferred from experiments using immunocompromised animals; particularly with respect to incidence of spontaneous cancer. The background cancer rate in mice is very low, fewer than 5% of the population including aged animals will develop cancer. However, immunocompromised animals have significantly increased rates of cancer incidence. For example, animals deficient in IFN-γ production present with significantly elevated rates of B cell lymphoma [355]. Given that signalling via IFNs plays such an important role in guiding cytotoxic T cell activity, it was hypothesised this was due to inefficient CTL activity. This has been experimentally confirmed by observations of higher cancer incidence in perforin KO animals. Perforin is one of the key mediators of CTL mediated cell killing and the rate of B cell lymphoma increases from 0-6% in WT to 40-60% in the absence of perforin [356]. This suggests that T cell mediated cytotoxicity plays an indispensable role in the anti-cancer immune response. This principle is supported by clinical data that shows clonal expansion of tumour antigen specific CD8+ T cells in patients with several cancers [357]. But how do early malignant cells elicit these adaptive immune responses? Efforts to determine the primary APC of early tumour growth have strongly implicated the concerted effort of DCs and IFN signalling in the initial steps of anti-tumour immunity. Early malignant cells have

been shown to drive type I IFN production in models of MCA sarcoma. This increased IFN production drives activation of CD8+ DC subsets, which in turn cross-present tumour antigen to CD8+ CTLs. This interaction strongly induced clonal T cell expansion and facilitated tumour elimination [358]. Whilst this study demonstrated that Interferon Alpha and Beta Receptor Subunit (IFNAR)-1 deficiency in CD8+ DCs prevented T cell mediated cytotoxicity of tumour cells, it left unanswered which of the type I IFNs were responsible for this effect. Furthermore, the cellular source of the type I IFNs was yet to be elucidated. These findings were elaborated on by Fuertes *et al.* who used a B16 melanoma model to show that CD11c+ DCs were primarily responsible for upregulation of type I IFNs. Significantly, they found the most upregulated was IFN-β and abrogation of IFN-β signalling prevented CD8+ T cell mediated tumour cell killing [359]. Together, these papers suggest that DC priming and their subsequent production of IFNs to guide T cell responses is a key mediator of early anti-cancer immunity. However, it is not yet clear how DC activation is induced in response to malignant cells. One theory is the recognition of death related signals. During malignant progression, necrotic cell death is common. This results in presentation of damage associated molecular patterns (DAMPs) designed to alert the immune system to viral infection or aseptic tissue injury [360]. In models of viral infection, the C-type lectin domain family 9 member A (DNGR-1) on DCs has been shown to recognise intracellular ligands that are exposed during cell death. This, in turn, drives activation of CD8+ CTLs via antigen cross presentation by CD8+ DCs, a process that mirrors the activation steps described by Fuertes *et al.* and Diamond *et al*. above [361]. Whilst recognition of these DAMPs in malignant cell mediated DC activation has not yet been probed, it presents a potentially interesting avenue of investigation. In addition to the cytotoxic activities of adaptive immune cells, cells of the innate immune system, such as NKs, also participate in anti-cancer immunity. Rather than antigen dependent cytotoxicity, NK mediated tumour cell killing is dependent on recognition of tumour associated molecular patterns (TAMPs). However, because the cytotoxic actions of NKs are antigen independent, they do not need to undergo clonal expansion. Therefore, elimination of malignant cells by NKs is extremely rapid and it has been suggested that they represent the initial responder to neoplastic cells. Several danger signals have been observed that mediate NK tumour cell killing. These signals are recognised by an array of stimulatory receptors on NKs, the best studied in the context of cancer being NKG2D. Expressed on all NKs, this receptor recognises MHC I related ligands, many of which are not expressed by normal cells making them ideal for identification of malignancy [362]. In mice, malignant expression of Retinoic acid early inducible 1 (Rae-1) acts as a potent NKG2D ligand [363]–[365]. Surface expression of rae-1 is induced by DNA damage through Stimulator of Interferon Genes (STING) mediated signal transduction, enabling NKs to eliminate potential malignant cells

at very early stages of dysplasia [366]. Additionally, NKs can recognise non-self, biological entities and induce cell death. A primary example of this is the detection of MHC class I molecules on the candidate cell. Many malignancies will downregulate MHC I expression to avoid presentation of danger signals to CTLs. However, the absence of surface MHC I is acutely recognised by NKs, resulting in cytotoxicity due to detection of a 'non-self' entity [282]. These mechanisms have recently been applied to BC. Despite its reputation for being a non-immunogenic cancer, Tu *et al.* demonstrated that NK recognition of reduced MHC I expression confers a selective pressure on BC cells. Using orthotopic E0771 models, the authors demonstrate that MHC I downregulation is prevented in WT animals. However, in animals with Ly49 deficient NKs, MHC I expression can reduce BC cell numbers. Adoptive transfer of tumours from Ly49 deficient animals to WT animals resulted in rapid tumour elimination, mediated by NKs [367]. This suggests that in murine models of BC, NKs can mediate anti-tumour cytotoxicity. But, tumour cells respond to this selective pressure by maintaining their expression of MHC I to avoid detection by NKs. This subversion of the immune response occurs in numerous tumour models and is known as immunoediting. In response to selection pressures, tumours can change their gene expression to avoid detection by the immune response. This principle will be discussed in detail in the following section.

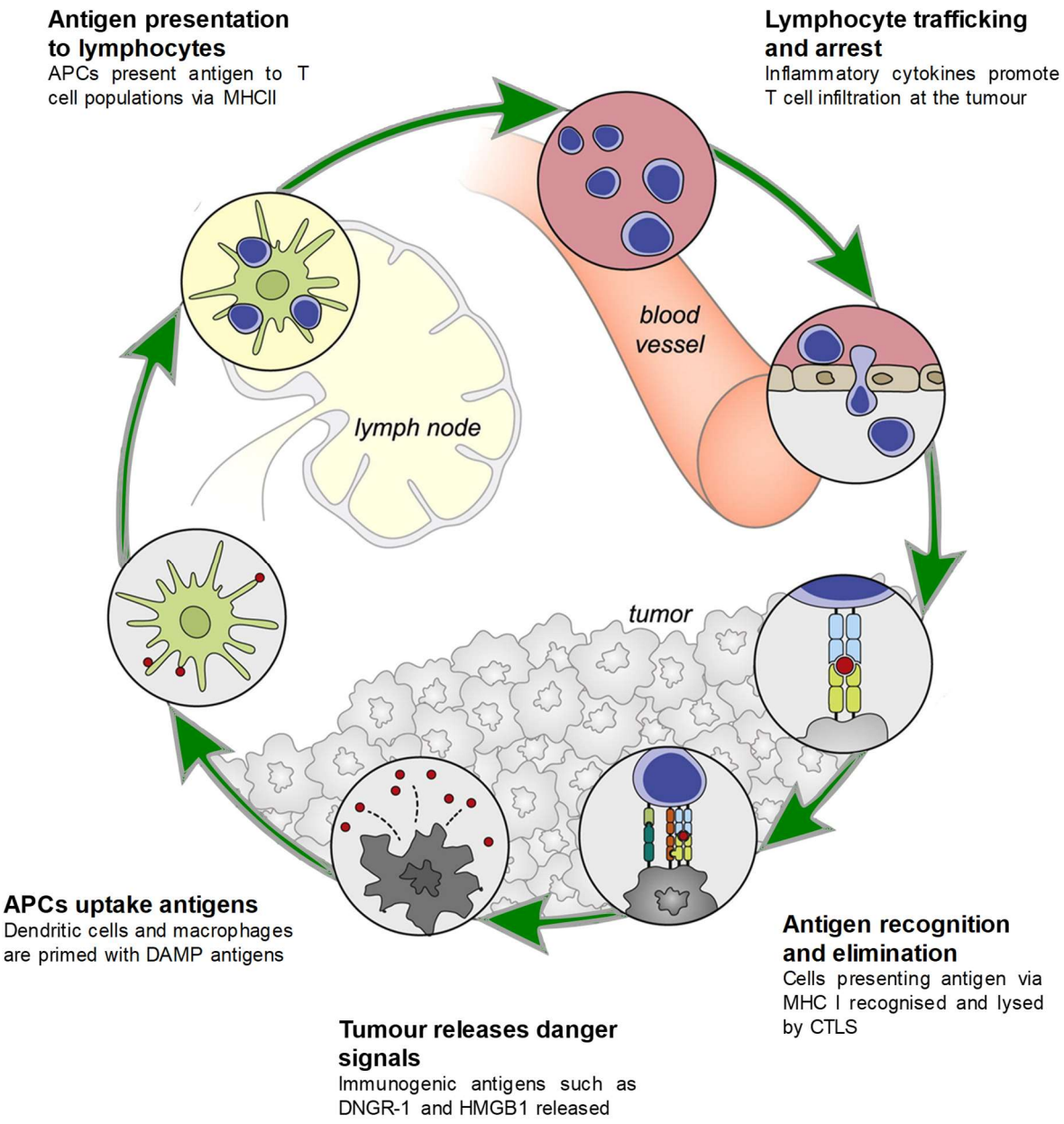


Figure 1.6 – The anti-tumour immunity cycle: Figure summarising the key steps in induction of anti-tumour immunity. Tumours present molecular patterns that indicate tissue injury through immunogenic cell death. These antigens are recognised by cognate APCs and transported to nearby lymph nodes for interrogation by lymphocytes. In the LN, antigen loaded APCs are exposed to multiple T cells clones. If the antigen is recognised by the cognate T cell receptor, rapid clonal expansion of these T cells is initiated. The T cells are mobilised and recruited to the tumour site by pro-inflammatory cytokines such as CXCL12. In the tumour microenvironment, T cells interrogate resident cells until their cognate antigen is encountered, either directly or by presentation via MHC I. This induces TCR signalling and in the presence of other stimulatory signals results in T cell mediated cytolysis of tumour cells and elimination of the tumour. Adapted from [789].

1.4.2. Immunoediting and Escape

Whilst it is likely that there are many occasions in the human lifetime where neoplastic cells are effectively removed by the immune response, some mutations result in a growth rate that matches the immune elimination rate. At this point, the tumour enters the stage of immunoediting known as equilibrium. This is typically the most prolonged stage of the tumoural immune evolution and can occur over many years, often before the tumour is detectable [368]. The earliest observations of immune equilibrium were a result of challenging animals after adoptive transfer of tumour antigen sensitised T cells. Implantation of the previously encountered tumour cells resulted in a period of immune driven tumour dormancy. This was demonstrated by inhibition of IFN-γ signalling which alleviated the CTL mediated constraints on tumour growth [369]. This work was elaborated on in MCA induced models of sarcoma. Some animals did not present with palpable tumours, but small cellular masses were present. Upon treatment with CD8 targeting antibodies, intratumoural CTLs were depleted and the cellular masses went on to form tumours [370]. These findings mirror what is occurring in tumours under natural conditions. Their growth is limited by immune regulation until they can find a way to overcome the challenge by suppression of immune effector cells. Tumours achieve this through their genomic instability which results in an incredibly heterogenous cell population. Through Darwinian processes and under the selection pressures of the immune response, non-immunogenic cells are gradually selected through attrition of their immune susceptible counterparts. Eventually, non-immunogenic cells predominate the tumour and growth escapes equilibrium, the tumour is finally allowed to grow [371]. This section will summarise the mechanisms by which tumours are known to subvert the immune response and discuss the key cellular and molecular players in these processes.

The predominant methods used by tumours to subvert immunity are summarised in Figure 1.7 and one of these ways is through recruitment and expansion of inhibitory immune cell populations. Several tumours are known to recruit macrophages and high numbers are a negative prognostic factor. Increased macrophage recruitment is frequently a result of upregulated CCL2 secretion by tumour cells and other stromal components [372]. However, recruitment alone is not the key driver of the macrophage's positive contribution to tumour growth. Macrophages exhibit a spectrum of polarisation, broadly defined as M1 or M2, the latter being demonstrated to have potent pro-tumour effects. In order to promote M2 macrophage polarisation, immunoedited tumours have been shown to upregulate TGF-β production [373]. M2 polarised TAMs primarily inhibit immune mediated cytolysis by production of Arginase-I. This results in depletion of L-arginine in the tumour

microenvironment and improves cell survival by inhibition of several immune effectors [374]. However, as alluded to in Section 1.3.2.2, it can be difficult to functionally separate M2 macrophages from MDSCs. Both are highly immunosuppressive in the tumour microenvironment and produce high levels of Arg1. However, the number of both cell types have been demonstrated to expand in immunoedited tumours [375]. The mechanisms of MDSC expansion at the primary tumour are not well understood and appear to be context dependent. However, like macrophages, CCL2 appears to play a central role. Abrogation of CCL2-CCR2 signalling significantly impairs MDSC recruitment and their associated immunosuppression in multiple tumour models [376]. Once at the tumour site, MDSC activation occurs in response to pro-inflammatory cytokines, initiating their immunosuppressive repertoire. For example, exposure to IL-1β and IFN-γ have been shown to initiate expression of NOS2 and Arg1 in MDSCs respectively. This has significant consequences on the TME. Production of ROS by NOS2 potently inhibits T cell activation. Furthermore, high levels of Arg1 results in a low availability of L-arginine for metabolism by T cells. Together, these effects result in T cell apoptosis and suppression of T cell mediated responses in the tumour [377], [378]. Additionally, MDSCs are capable of inhibiting innate cytotoxicity by modulation of NKs via membrane bound TGF-β1. NKs cultured in the presence of MDSCs no longer responded to IFN-γ mediated activation and were no longer able to lyse tumour cells *in vivo* [379]. Furthermore, the presence of MDSCs has been shown to promote the infiltration and expansion of T_{regs}, another highly immunosuppressive cell population. The processes that control this aspect of MDSC function are not fully understood. However, production of TGF-β1 and Arg1 by MDSCs has been heavily implicated. In MCA sarcoma models, T_{reg} differentiation was induced by MDSCs exposed to IFN-γ through increased TGF-β1 and IL-10 production. Abrogation of both TGF-β and IL-10 signalling resulted in reduced T_{reg} accumulation and inhibited tumour growth [380]. Additionally, suppression of Arg1 activity in MDSCs has been shown to prevent the associated expansion of existing T_{reg} populations in B cell lymphoma. However, it is not yet clear how Arg1 mediates this effect [381].

Accumulation of T_{regs} also occurs via MDSC independent processes during immunoediting, particularly through exposure to lymphokines. In BC, this is at least partly controlled by production of CXCL12, a potent lymphocyte chemoattractant. In patient tumours with high numbers of intratumoural T_{regs}, there was a strong positive correlation with CXCL12 positivity [382]. Consistent with these findings are results that suggest the use of CXCR4 antagonists, the receptor for CXCL12, results in reduced T_{reg} number and improved anti-tumour immunity [383]. The immunosuppressive function of T_{regs} largely revolves around interruption of adaptive immunity. This is chiefly controlled by production of secreted factors

such as IL-10 and TGF-β1, the effects of which are summarised in 0.However, T_{regs} also mediate some of their inhibitory functions by direct cell-cell contact. For example, T_{regs} have been shown to induce downregulation of CD80 and CD86 presentation at the surface of DCs resulting in impairment of their ability to present antigen [384]. Furthermore, T_{regs} express the inhibitory protein, Lymphocyte Activation Gene (LAG)-3 at their surface. Through interaction with MHCII on DCs T_{regs} induce inhibitory signalling pathways that suppress DC maturation [385]. T_{regs} have also been shown modulate immune checkpoint proteins. Presentation of CTLA-4 at their surface polarises DCs to a suppressive phenotype by induction of indoleamine 2,3-dioxygenase (IDO) expression [386]. Additionally, the presence of T_{regs} has been positively correlated with increased expression of PD-L1, the ligand for the inhibitory T cell receptor, PD-1 and can have profound effects on tumour immunity [387], [388].

PD-1 expression is induced in activated T cells and is physiologically required to self-regulate cytotoxic responses to avoid tissue damage. Disruption of peripheral tolerance by knockout of PD-1 results in accumulation of autoreactive T cells and development of several autoimmune diseases [389]. Additionally, during viral infection, expression of PD-1 is upregulated in order to limit clonal expansion of pathogen specific T cell clones and resolve inflammation [390]. These processes are mirrored in anti-tumour immunity, activated tumour antigen specific T cells upregulate PD-1 in order to maintain homeostatic conditions [391]. However, during the process of immunoediting, tumour cells can increase their expression of PD-L1, acting as a molecular shield against T cell mediated lysis [392]. Induction of PD-1 signalling results in attenuation of stimulatory signals from the TCR and CD28, resulting in reduced cytokine production, cell cycle arrest and induction of apoptosis [393]. Additionally, PD-1 signalling in CD4+ T cells can induce their differentiation into inducible T regulatory cells (iT_{regs}) which can further impair T cell responses by expression of cytotoxic T-lymphocyte-associated protein (CTLA)-4 [394], [395]. In fact, suppression of CTLA-4 mediated signalling has also garnered significant attention with respect to its use in immunotherapy. Expression of CTLA-4, much like PD-1, is induced in activated T cells and is constitutively expressed by T_{regs}. As a homologue of the stimulatory receptor CD28, CTLA-4 binds to CD80 and CD86 presented by APCs, but with a much higher affinity, preventing T cell activation and driving inhibitory signalling processes similar to those seen in PD-1 activation [396]. Presentation of CTLA4 may also extrinsically suppress T cell responses by modulation of APCs. CTLA-4 can capture the co-stimulatory ligands CD80/86 from interacting DCs by transcytosis, therefore suppressing their ability to activate other T cells [397]. Furthermore, CTLA-4 signalling on T_{regs} drives IL-10 production and suppresses CD80/86 expression in DCs [398]. Both T cell regulatory processes have a profound impact

on the tumour microenvironment. In several pre-clinical studies, PD-1 and CTLA-4 mediated immune suppression has been shown to inhibit anti-tumour immunity. Furthermore, inhibition of these processes by biologic inhibitors has demonstrated astounding results in both pre-clinical models and patient trials. Importantly, these clinical benefits have been demonstrated in multiple cancers and immune checkpoint therapies are now clinical practice in a number of pathologies. The use of immune checkpoint inhibitors, the extent of their clinical success and the emerging challenges associated with their use will be summarised in the next section.

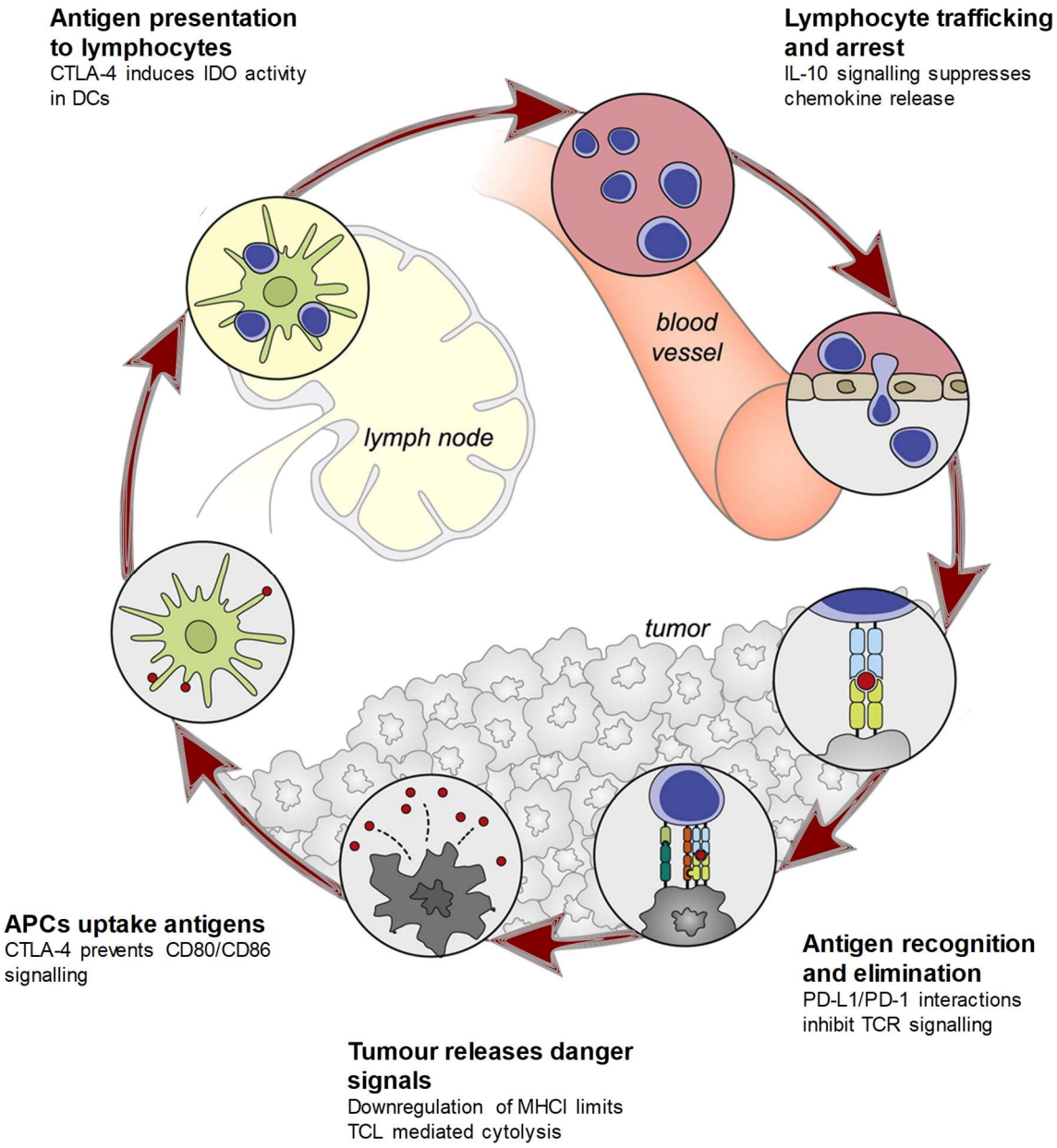


Figure 1.7 – Subversion of anti-tumour immunity: Figure summarising the methods by which tumours can evade immunity and prevent cytolysis. Presentation of associated danger signals can be prevented by downregulation of MHC I expression or antigen manipulation. Furthermore, some tumours can suppress MHC I expression without completely eliminating it, therefore avoiding NK mediated death. Through expression of CTLA-4, tumour cells can prevent DC activation by sequestration of their activating receptors CD80 and CD86, therefore preventing DC mediated antigen presentation to lymphocytes. Additionally, CTLA-4 mediated signalling can induce IDO expression and production of tryptophan metabolites by DCs, inhibiting their activation of T cells. Through recruitment of inhibitory cell populations such as Tregs and MDSCs, tumours can prevent T cell recruitment via inhibitory cytokine such as IL-10. Finally, by upregulating PD-L1, tumours can induce CD8+ TCL senescence, thereby avoiding cytolysis. Adapted from [789].

1.4.3. Potential Therapeutic Approaches

During the nineteenth century, the surgeon William Coley lost a 17-year-old patient to metastatic bone cancer. Distressed after watching her gradual decline, Coley became determined to find new ways to approach cancer treatment. After trawling through the case notes stored in New York Hospital, Coley came across the case of Fred Stein. During treatment for his inoperable sarcoma, Stein developed erysipelas, an infection now known to be caused by *Streptococcus pyogenes*. Astonishingly, Stein's tumour began regressing and when Coley managed to locate the patient in 1891, his tumour had been completely cured. Unknowingly, what Coley had uncovered was a reawakening of the immune response leading to rapid immunological destruction of the tumour and 'Coley's toxin' continued to be used in cancer treatment into the 1960s [399]. Sadly, the use of immunotherapy fell by the wayside for much of the 20th century until the immunosurveillance renaissance of the early 2000s. Since then, several immunotherapeutic approaches have made it into the clinic. This section will summarise the current state of anti-cancer immunotherapies and discuss promising preclinical studies which may contribute to the next generation.

Early efforts to control tumours using immunotherapy focused on vaccinations, but despite initially promising results, the field failed to make major advances. However, in 2010 the first cancer vaccine, Sipuleucel-T, was approved by the FDA for use in metastatic prostate cancer. The mechanism of action is not fully understood, however patient DCs are collected and cultured in the presence of GM-CSF conjugated to prostatic acid phosphatase (PAP). The DCs are re-administered to patients resulting in increased cytotoxicity of PAP expressing cells and improved overall survival [400]. Despite this, the cancer vaccination field is yet to find its feet and no further treatments have seen FDA approval. Instead, focus has shifted to adoptive T cell therapies. The technology works in much the same was as Sipuleucel, however instead of DCs mediating the effects, T cells are isolated and expanded *in vitro.* Early attempts to bring this technology to the clinic were based on *in vitro* antigen sensitisation of cultured T cells with antigen pulsed DCs or tumour fragments [401]. Whilst some approaches demonstrated clinical potential, particularly in melanoma, none gained FDA approval due to lack of efficacy. However, in 2010 Kochenderfer *et al.* published impressive pre-clinical data using chimeric antigen receptor (CAR) T cells with specificity to the B cell receptor CD19 [402]. These T cells express engineered TCRs which bind in an MHC independent manner to specified antigens. In this case, the anti-CD19 CAR T cells could recognise and rapidly destroy B cells. In subsequent clinical trials, the treatment achieved remarkable response rates in B -cell acute lymphoblastic leukemia. These studies

led to CD19 targeting CAR T cells becoming the first adoptive T cell therapy to be approved by the FDA in 2017 and was later approved for use in refractory B cell lymphomas in May 2018 [403]–[405]. Whilst CAR-T therapies are technologically impressive and clinically outstanding in certain cases, they suffer from many shortfalls, the most obvious being cost. A course of CAR-T therapy costs in excess of $500,000 when accounting for leukapheresis and other associated costs [406]. Additionally, CAR-T therapies are still susceptible to immunoregulation by inhibitory signalling molecules such as PD-1 and CTLA-4. Work is currently underway to determine if CAR-Ts can be further modified to express extracellular PD-1 or CTLA-4 domains fused to the stimulatory CD28 cytoplasmic domain, making their ligand interactions stimulatory instead of inhibitory [407]. Additionally, some clinical trials are planned to combine CAR-T therapies with the already available biologics available to block inhibitory signalling [408]. These drugs were amongst the earliest developed immunotherapies and are the cornerstone of current immunotherapy. Known as immune checkpoint inhibitors (ICI), their mechanism of action is dependent on alleviation of inhibitory signalling pathways in T cells. Through interaction with their ligands, PD-1 and CTLA-4 can potently suppress T cell activation, impair cytokine production and reduce their cytotoxic potential. Development of biologics to target these pathways was initiated during the late 90s, when CTLA-4 blocking antibodies demonstrated pre-clinical efficacy in melanoma. After a raft of clinical trials throughout the 2000s, the anti-CTLA-4 antibody, ipilimumab was finally approved by the FDA in 2011. In its phase III trial, ipilimumab increased overall survival in patients with previously treated metastatic melanoma by 4 months, a feat which no drug had previously achieved. Since then, several new classes of immune checkpoint inhibitors have been approved by the FDA, most prominently the anti-PD-1 biologics nivolumab and pembrolizumab. After outstanding results in phase 1 clinical studies, where objective response rates (ORRs) of 30-40% were seen across non-small cell lung carcinoma (NSCLC), melanoma and renal-cell cancer, pembrolizumab achieved the FDA's breakthrough therapy designation for metastatic melanoma and NSCLC. Shortly after, pembrolizumab received its first approval for use in advanced melanoma and has since been approved for use in over a dozen cancers. This rapid adoption of immunotherapies and the breadth of their efficacy is testament to their potential in cancer therapeutics. However, checkpoint inhibitors are not immune to the challenges seen in conventional cancer treatments. Whilst ICIs demonstrate durable responses, with many patients still responding up to 5 years after starting therapy, acquired resistance is beginning to emerge with longer treatment regimens. The mechanisms of which are largely unknown and must be extrapolated from pre-clinical data, however several theories have been proposed. In pre-clinical models, PD-1 based therapies reinvigorate exhausted CD8+ T cells, improving their effector functions and driving T cell mediated destruction of tumours. However, to

maintain clinical response, expansion of memory T cells is also required. Whilst this occurs in many patients, expansion of memory T cells is limited in some, resulting in relapse after the conclusion of treatment [409]. The reasons for this are currently unclear, but there are suggestions that exhausted T cells may enter an epigenetic senescence that limits their plasticity and impairs their transition into memory T cells [410]. Additionally, further immunoediting processes have been shown to mediate ICI resistance. An analysis of patients who have relapsed following anti-PD-1 therapy revealed loss of function mutations in JAK1 or 2 signalling, rendering cells insensitive to IFN-γ. This resulted in a downregulation of MHC I and impaired CTL mediated tumour cell elimination [371]. Interestingly, this immunoediting appears to be a result of selection pressures exerted by exposure to IFN-γ. Across multiple pre-clinical models, the presence of IFN-γ in the tumour microenvironment generated clones which were resistant to CTLs via downregulation of IFN-γ mediated signalling [411]. Furthermore, high throughput screening designed to identify ICI augmenting targets highlighted significant results after deletion of PTPN-2, through enhancement of IFN-γ signalling processes [412]. Therefore, modulation of IFN-γ signalling may provide avenues to offset acquired resistance programs in ICI based therapies. Importantly, some patients' tumours just do not respond to checkpoint inhibitors. The factors that contribute to innate resistance are not understood. Some tumours can be susceptible to ICIs, but just lack the necessary ingredients without intervention. Attempts to alter the microenvironment to improve ICI efficacy have been partially successful and the host microbiota appears to play a surprising, but significant, role. The contributions of the microbiome will be discussed in detail in Section 1.5. However, other tumours remain stubborn with respect to immunotherapies. One example is breast cancer, where despite a number of trials, no ICIs have been approved for clinical use. The challenges surrounding use of ICIs in BC, and the future direction of immunotherapies in the disease will be discussed in the next section.

1.4.4. Immunotherapy in Breast Cancer

Historically, BC has been considered a non-immunogenic disease, therefore immune mediated therapies were thought to be largely irrelevant. However, over the last decade, further stratification of BC subtypes has led to new strategies in the immunological management of BC. Importantly, evaluation of immune infiltrates as a prognostic marker revealed that immune contributions are not equal across all molecular subtypes. In HER2+ and TNBC, lymphocyte infiltrate correlates positively with a favourable prognosis. However, no such association exists in patients with ER+ disease [413]. Additionally, whilst tumoural expression of PD-L1 is a negative prognostic marker across all subtypes, TNBC have higher rates of PD-L1 positivity when compared to other subtypes [414]. The reasons for these differences are unclear, however may be related to the mutational load of each subtype. Mutational load is known to correlate positively with tumour immunogenicity due to increased quantities of mutated antigens for recognition by adaptive immune cells [415]. Furthermore, analysis of mutational load in BC subtypes revealed HR+ tumours had significantly reduced mutational load when compared to HER2+ or TN tumours [416]. This may also explain why TNBC is more likely to present as PD-L1 positive. It is possible that immunoediting has already occurred and enriched for immunosuppressive clones. This has been demonstrated in other cancers, but is yet to be experimentally validated in BC [417]. However, the immunophenotypic differences revealed between subtypes has led to specific targeting of immunotherapies towards HER2+ and TNBC. Furthermore, the apparent susceptibility of HER2+ and TNBC to immunotherapies is clinically advantageous. As discussed earlier, whilst HR+ tumours respond well to current hormone based treatment strategies, HER2+ and TNBC remain difficult to treat. Therefore, these two molecular subtypes have been the primary focus of immunotherapy in BC to date. So far, PD-1 based therapies have shown the most clinical potential. In a recent phase Ib study, pembrolizumab was administered to TNBC patients. An ORR of 18.5% was achieved, which, whilst significantly lower than the response rates seen in classically immunogenic cancers such as melanoma (~30%), these results show that immunotherapy has some potential in BC [418], [419]. Impressively, similar response rates are seen in patients with metastatic TNBC and a recent study has reported a 23% ORR in patients who have yet to receive any systemic chemotherapy. However, in a tandem cohort of patients who had received at least one systemic therapy prior to initiating immunotherapy, the response rate fell to <5% [420], [421]. The reasons for this are not clear, however may be related to selection pressures exerted by previous treatments leading to enrichment of immune refractory clones. An improved response rate may be achieved in dual therapy with CTLA-4 targeting biologics. This has already seen success in melanoma, where dual treatment with pembrolizumab

and ipilimumab improved response rates from 30% to over 60% [422]. Similar studies in BC are underway but the results are yet to be published (NCT02453620). Disappointingly, despite encouraging results with PD-1 inhibitors, studies with CTLA-4 inhibitors alone have been less successful. A phase I study assessing the use of tremelimumab alongside exemestane initially demonstrated promising results in metastatic BC. Whilst no objective response was seen, 42% of patients had stable disease for at least three months following treatment. Furthermore, increased numbers of peripheral, activated CD4 and CD8+ T cells were seen in most patients, indicative of an improved anti-tumour response [423]. However, in a subsequent study, where patients with TNBC or ER+ metastatic BC were recruited, only 3 of 18 patients responded. Critically, none of the 11 recruited ER+ patients responded and the trial was not taken further [424]. This highlights the need to specifically target certain molecular subtypes in order to maximise the potential for immunotherapeutic response.

In addition to the current frontline immunotherapeutics, some alternative strategies have shown some clinical potential in BC. A recent study employed the use of activated T cells armed with a bispecific antibody targeted to both CD3 and HER2. These T cells have HER2 specific cytotoxic effects and have been demonstrated to eliminate HER2+ BC cells *in vitro.* A phase I trial in patients with metastatic BC of varying HER2 status yielded positive results. Stable disease was achieved in 59% of patients and those with HER2+ disease had an overall survival of 57.4 months, compared with ~30-40 months achieved in patients treated with trastuzumab in separate trials [425], [426]. Phase II trials are underway, but the results are yet to be published (NCT01147016).

More recently, Zacharakis *et al.* have demonstrated the ability to induce complete remission in metastatic BC patients using adaptive transfer of autologous, tumour antigen specific T cells. Patient TILs were cultured *in vitro* from tumour fragments in the presence of high doses of IL-2 to stimulate T cell expansion. Alongside this, whole exome sequencing of the breast lesion was conducted to assess the extent of somatic mutation in the tissue. After identification of 62 nonsynonymous mutations, the TIL pools generated *in vitro* were screened against the tumoural mutations and reactive clones selected against four mutant proteins. Re-administration of these TILs, alongside IL-2 agonists and PD-1 inhibitors resulted in a 51% reduction in tumour burden within 6 weeks. Incredibly, after 22 months, the tumour load had been completely eradicated. This effect was accompanied by persistent antigen specific T cell responses in peripheral blood isolated lymphocytes [427]. Whilst only demonstrated in one patient, this is a remarkable clinical response and suggests that personalised immunotherapeutic strategies may be the key to unlocking their potential in the management of BC. Crucially, this study highlights what is likely the missing piece of

the BC immunotherapy puzzle, personalisation of treatment. As has been shown with current conventional BC treatments, specificity of treatment is key to ensuring good response rates. The heterogeneity seen in the immunogenicity of patient tumours suggests this will also need to be undertaken for immunotherapy. However, the current understanding of the tumoural markers that are indicative of response to immunotherapy are not yet clear. Muddying the field further, are the recent findings that highlight the importance of the host microbiota in directing immunotherapeutic efficacy. This may require further stratification of BC patients, not just by their tumoural markers, but also the composition of their microbiota. These concepts will be discussed in detail in the next chapter, but likely represents a significant challenge to successful immunotherapy in BC.

1.4.5. Metabolic Regulation of Anti-Tumour Immunity

Whilst the major focus of controlling anti-tumour immunity has been on tumour-immune cell interactions, an appreciation also needs to be held for the indirect actions of tumour cells on immune output. An emerging field is a consequence of the merging of two fields; the metabolic dysregulation of tumours and the reliance of immune cells on metabolic pathways for proper function. The impact of metabolism is perhaps best understood in T cells, particularly in the transition from resting naïve T cells to activated, effector cells. During quiescence, T cells predominantly utilise oxidative phosphorylation as an energy source. However, during activation T cells must switch to using glycolysis in order to sustain their rapid clonal expansion [428]. After expansion, T cells will once again revert to predominantly using oxidative phosphorylation and interestingly, the intricacies of this reversion has been shown to influence the transition from T effector to T memory cell, however this is currently poorly understood [429]. The metabolic transition of T cells during activation draws close parallels with the metabolic reprogramming of malignant cells. Described by Otto Warburg, tumours cells have been observed to consume huge amounts of glucose despite the presence of oxygen in order to satisfy their energy and biosynthetic demands. As described, this is at odds with the requirements of tumour infiltrating lymphocytes and competition for glucose in the tumour microenvironment can lead to repression of T cell activities through inhibition of IFN-γ production and cytotoxic programmes [430]. In addition to glucose competition, increased rates of glycolysis by tumour cells can also inhibit immune cells through production of lactate. Exposure of T cells to lactate results in inhibition of IFN-γ production, suppression of CD8+ T cell cytotoxicity and reduced numbers of TILs. This effect has been shown to be reversible by genetic targeting of lactate dehydrogenase A (LDHA), an enzyme which catalyses the production of lactate from pyruvate and therefore represents a potential therapeutic intervention [431], [432]. In addition to the effect on TILs,

dysregulated glucose metabolism in tumours has been shown to interfere with myeloid cell function. Lactate uptake by macrophages stimulates vascular endothelial growth factor (VEGF) and Arg1 expression, hallmarks of the M2, protumorigenic macrophage. This effect has been shown to drive tumour growth in BC and its suppression by inhibition of downstream signalling reverses the negative impacts on tumourigenesis [433].

In addition to dysregulation of glucose metabolism, reprogramming of both amino acid and lipid metabolism has also been shown to influence immune function in tumours. The former is best understood with respect to glutamine metabolism. Many tumours upregulate glutaminolysis to provide the proteins and nucleotides required for growth, resulting in depletion of glutamine in the TME. As described in previous sections, glutamine is required by T cells for expansion and has also been shown to play a role in determining the activation state of T helper cells [434]. Depletion of glutamine or disruption of its metabolic pathways in CD4+ T cells lead to increased expression of FoxP3, the transcription factor responsible for Treg differentiation, a T cell subset which has been implicated in tumour promotion [435]. Tumours also exhibit dysregulated lipid metabolism and has been shown to manifest either as an increase in β-oxidation to satisfy the cell's energy requirements or more frequently lipid biosynthesis is aberrantly activated. Under physiological conditions, most cells do not produce lipid *de novo* and instead satisfy their lipid requirements through utilisation of free fatty acids (FFAs) from the blood. However, cancer cells require large amounts of lipoprotein and cholesterol for membrane production and therefore have been shown to reprogram in order to activate these anabolic pathways. This results in accumulation of these components in the TME and has been associated with impaired anti-cancer immunity [436]. Uptake of oxidised lipids by dendritic cells results in their accumulation, similar to that of LDL uptake by macrophages during atherosclerosis. This has been shown to limit antigen cross presentation in DCs and inhibit the anti-tumour immune response [437]. Furthermore, uptake and metabolism of cholesterol by T cells has been shown to inhibit their proliferation and effector function in CD8+ cells. Interestingly, this effect is reversible by inhibition of the cholesterol esterification enzyme ACAT1 [438].

Whilst metabolic regulation of immunity clearly plays a role in determining anti-cancer immune responses, the question still remains, what is the best way to target it? The obvious answer is through pharmacological inhibition, but it may be possible to utilise alternatives, such as by modulation of the microbiota. Microbially derived metabolites have been shown to confer protective effects on the host, particularly with respect to butyrate's effect on colon physiology which will be discussed in detail later. Additionally, other microbial metabolites have been shown to regulate distant immunological processes. Production of

desaminotyrosine (DAT) by the microbiota has been shown to promote type I interferon responses in the lung and is protective in the context of influenza infection [439]. Whether these effects are also relatable to tumour immunity is still to be determined, however the effect of the microbiota on metabolism and immunity will be discussed in the next section.

1.5. The Host Microbiota

The epithelial surfaces of almost every multicellular organism are populated by an ecosystem of microbes [440]. Their presence, alluded to by Theodor Escherich upon isolation of *Eschericia coli* from the stool of young children, led to the subsequent identification of hundreds of microbial species inhabiting the human body. Whilst the functional roles of some were identified by association, much of the microbiome's potential lay dormant. That is, until the 'omics revolution of the early 21st century. A field that was once reliant on trial-and-error isolation techniques was thrown wide open by the advent of next generation sequencing technologies and the huge databases of genomic information that they produced [441]. Finally, microbiologists could gain a detailed understanding of not just which microbial species inhabit the human body, but what their functional implications are. Large scale studies such as the human microbiome project have shone light on a plethora of physiological microbial functions from neurobiology to cancer, a list that just seems to keep expanding. This section will focus on the gut microbiome, discussing its role in maintaining tissue homeostasis and how dysfunction can lead to pathology.

1.5.1. Development of the gut microbiota

The gut microbiome is home to an unimaginable diversity of microbes, in fact, the number of bacterial cells on a human match that of the host's own cells [442]. But, where do all these microbes come from given that the human foetus, under normal conditions, is thought be sterile *in utero?* [443]. Admittedly, this paradigm is currently being challenged, with the suggestion that the gut may be partially colonised by amniotic bacteria. However, there is still no incontrovertible evidence to counteract this claim [444]. Instead, it's thought that colonisation begins almost immediately after birth, both from environmental exposures and through contact with the vaginal canal. From here, the microbiome enters a highly plastic state, rapidly increasing in functional diversity until three years of age. Unless extrinsically disturbed, this microbiome remains stable into adulthood, highlighting the importance of proper microbial development in infants [445]. This section will summarise how the adult human microbiome is formed and describe the key functional components of the gut microbiota at different time points through infancy.

Initial colonisation of the gut microbiome is predominantly achieved by vertical transition from the mother, marked by transient occupation by bacterial species that are highly abundant in the vaginal canal. However, this initial colonisation does not occur in babies delivered by caesarean and can lead to improper development of the microbiota. For

example, vaginally delivered infants show a microbiome rich in *Lactobacillus* whereas infants delivered by caesarean show higher levels of *Staphylococcus* [446], [447]. These differences have been suggested to drive lifelong alterations in the eventual diversity of the adult microbiome and have been associated with impaired immune development [448]. Therefore, elective caesarean is being actively discouraged amongst medical professionals, along with promotion of breast-feeding due to the recent awareness of how breast-feeding guides proper development of the gut microbiota. In fact, initiation of milk consumption causes the first major shift in the infant microbiome. The environmental bacteria acquired during birth are gradually replaced with members of the *Bifidobacterium* genus. In fact, in many infants these species of bacteria predominate the microbiome until weaning [449]. This predomination of the *Bifidobacteria* genus is likely a result of the nutritional niche created by human breast milk oligosaccharides such as fucosyllactose (FL). Colonisation of the gut by FL utilising *Bifidobacteria* during breast feeding and their subsequent production of acetate has been shown to have protective effects for the infant host [449]. However, colonisation by *Bifidobacteria* is impaired in formula fed infants leading to over colonisation of other microbial members such as *Clostridium difficile* [450]. Whilst in most cases this accumulation in infants is asymptomatic, the presence of *C.difficile* has been shown to impair proper development of the microbiota, particularly when the infant shifts to solid foods, a change that brings about the second major overhaul of gut microbial species [448].

Under normal conditions, the weaning process brings about the second major change in the gut microbiome of infants. Likely driven by the abundances of complex carbohydrates, the number of *Bifidobacterium* species begin to decline and are replaced with species of the Bacteroidetes and Firmicutes phyla [451]. Consumption of solid food creates a range of nutritional niches for bacterial species to occupy. These are gradually filled between years 1-3 of growth, marking a switch from vertical bacterial transmission to horizontal transmission from other humans and the environment, leading to an individually unique signature of bacterial colonisation [452]. The composition and distribution of the adult gut microbiota will be discussed in the following section.

1.5.2. Composition of the adult gut microbiota

The adult gut microbiome whilst remaining stable with age, exhibits significant spatial and temporal differences. It is estimated the human gut contains up to 35,000 different bacterial species that together make up the over 10 million non-redundant genes that contribute to the human microbiome [453], [454]. These bacterial species are distributed along the entire

length of the gastrointestinal tract with gradually increasing abundance, the communities present in each GI segment are summarised in Figure 1.8. Starting with the oesophagus, which has the lowest bacterial load with only 10^1-10^2 colony forming units (CFU)/ml [455]. It shares most of its diversity with the oropharyngeal sites of microbial colonisation. The microbiota of the oesophagus is dominated by Firmicutes of which the most abundant species are of the *Streptococcus* and *Prevotella* genera [456]. Characterisation of the oesophageal microbiome has been challenging owing to the low number of bacteria at this site. As such, the functional relevance of these bacteria is not fully understood. However, dysbiosis may be associated with development of certain oesophageal malignancies [457]. As such, screening of the oesophageal microbiome has been suggested as a prognostic factor but is not yet in clinical practice.

The next most abundant site of microbial colonisation is the stomach, with a bacterial density of approximately 10^3-10^4 CFU/ml [442]. Study of the gastric microbiome received intense attention after Barry Marshall's fated self experiment demonstrating that *Helicobacter pylori* (*H.pylori)* is a key driver in the development of peptic ulcers and eventually stomach cancer [458]. The stomach exhibits extraordinary diversity given its apparently inhospitable environment and contains over 100 different phylotypes distributed across 8 phyla, the most abundant of which are the Firmicutes [459]. Mirroring findings in the oesophagus, the most prominent bacterial species in the healthy stomach were of the *Streptococcus and Prevotella* genera, however it is difficult to distinguish whether these are truly resident to the stomach or just a result of swallowed species from the oesophagus [460].

Interestingly, this diversity is completely ablated when individuals are positive for *H.pylori*, the contribution of each phylum skews considerably towards Proteobacteria and *H.pylori* becomes the most abundant bacterial species [461]. More so, over 80% of the individuals tested in this study were positive for *H.pylori* and based on subsequent large scale screening studies, it's estimated that over 50% of the population is infected with *H.pylori,* yet gastric cancer only occurs in 1-3% of infected individuals [462]. This suggests the contributions of the microbiome to stomach pathologies are not fully understood and further mechanistic studies are required.

Following on from the stomach is the small intestine, where the microbial inhabitants are understudied, largely due to difficulties in sample acquisition at these sites. However, it's estimated that the small intestine contains roughly the same number of CFU/ml as the stomach [442]. The profile of the duodenum is dominated by Firmicutes and Actinobacteria,

of which *Streptococcus* and *Actinomyces* are the most abundant genera [463]. Functionally, the microbiome of the duodenum has a high number of fatty acid metabolism genes and interestingly, genes associated with fatty acid catabolism are enriched in obese individuals suggesting the duodenal microbiome may play a role in controlling obesity [463]. The microbiome of the jejunum is highly understudied and was only recently fully metagenomically profiled [464]. Like the duodenum it is predominated by Firmicutes, particularly *Streptococcus*, however a larger contingent of Bacteroidetes are noted, likely due to increased mucosal content of the jejunum compared to the duodenum or ileum [465]. The final part of the small intestine, the ileum, contains the highest bacterial abundance. With a CFU/ml of ~10^8, the ileum rivals the bacterial abundance observed in the colon. For this reason, it was assumed that there is significant overlap between the ileal and colonic microbiomes. However, recently Villmones *et al*. characterised the ileal microbiome through surgically acquired samples and found a microbial profile that is distinct from that of colon. Whilst the ileal microbiome is still dominated by Firmicutes, individuals from the Bacilli class predominate, whereas in the colon, the predominant class of bacteria are Clostridia [466]. These findings are important to consider when investigating host-microbiome immune interactions. The ileum exhibits the highest density of Peyer's patches (PPs) across the GI tract, containing almost 50% of all PPs [467]. Therefore, the bacterial species present here are the dominant contributors to microbial guided immune education, a process that will be discussed in detail in later sections.

Finally, the large intestine contains the highest number of microbes in the human body, the colon alone has been estimated to contain $3.8x10^{13}$ bacteria, only one order of magnitude less than the total number of cells in the human body [442]. The ease of sampling the large intestinal microbiome by fecal collection has led to it becoming the prototypic site used to describe the human microbiota. The main components of the large intestinal microbiome, like that of the small intestine are members of the Bacteroidetes and Firmicutes phyla [468]. However, unlike the observations at other sites, the ratios of each phyla are highly variable in the colon. In some cases, Bacteroidetes have been observed at higher ratios than Firmicutes [469]. This ratio has generated some discussion with respect to obesity. A decrease in Bacteroidetes and concomitant increase in Firmicutes in the colon has been associated with obese individuals [470]. However, this association is yet to be mechanistically established.

After these phyla, the next most abundant is Actinobacteria, mainly comprised of members of *Bifidobacteria*, a genus of gram-positive, obligately anaerobic bacteria that have received considerable attention for their probiotic potential [468]. As discussed earlier, the presence

of *Bifidobacteria* species in the infant microbiome correlates with enhanced gut health and immune development. Whilst the number of *Bifidobacteria* in the adult gut microbiome is decreased, they have been shown to play numerous key roles in maintenance of gut health [471]. These will be partially discussed in the next sections.

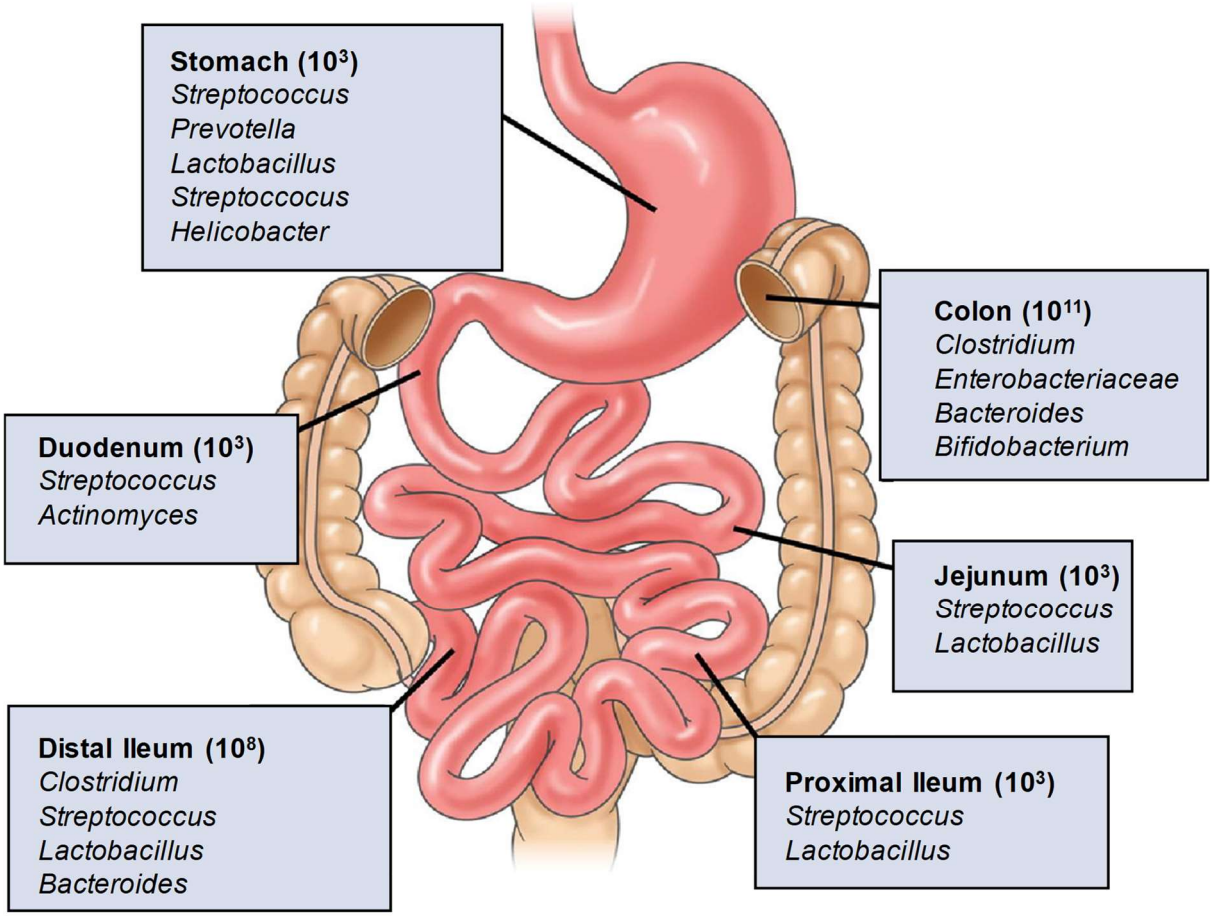


Figure 1.8 – Microbial composition of the GI tract: The GI tract displays high spatial diversity and despite sampling difficulties, the bacterial composition at each site is at least partially known. The diagram above summaries the most abundant genera at each point in the GI tract. Adapted from [508].

1.5.3. Physiological roles of the gut microbiota

The formation of the adult microbiota and the process of colonisation is likely a result of co-evolution of humans with their bacterial companions [472]. This coexistence has led to a mutual dependence, where bacteria are rewarded with nutrition in exchange for performing several physiological functions. The advent of Germ-Free mice has allowed the

physiological role of the microbiota to be probed in detail and it is now known that the microbiome is required for proper development of the gastrointestinal and immune system [473]. This section will discuss these roles and the members of the microbiome response for their mediation.

Maintenance of Barrier Function

Forming part of the innate immune system, the epithelial barrier in the gut prevents commensal bacteria from escaping the lumen. Consequences of a leaky epithelial layer range from generation of chronic inflammatory responses, such as that seen in inflammatory bowel diseases, to systemic, life-threatening infection. Several members of the gut microbiota have been implicated in promoting gut barrier function and have been shown to modulate epithelial cell function in two ways. Firstly, by direct interaction with TLRs on the surface of epithelial cells. Signalling through TLR2 by bacterially derived lipopeptides maintains barrier function during inflammation by upregulation of epithelial survival factors [474]. Additionally, some soluble proteins have been shown to mediate similar effects. *Lactobacillus rhamnosis* produce p40, which has been shown to prevent epithelial apoptosis as a result of inflammation and confers resistance to DSS induced colitis [475].

Additionally, the microbiome plays a pivotal role in GI development. Proper development of the gastrointestinal system is dependent on generation of an effective bloody supply and this process appears to be driven by interactions with commensal gut bacteria. Germ free (GF) animals do not form a comprehensive villus capillary network, resulting in reduced villus surface area and impaired peristalsis. The consequences of which are malnutrition and stunted growth of GF animals. This impairment is partially recovered when the microbiome is reconstituted with samples from healthy animals or by administration of *Bacteroides thetaiotaomicron*, a member of the microbiome that has been implicated in several beneficial processes [476], [477].

Nutrient Availability

The main nutrient used by members of the gut microbiota is complex carbohydrates, many of which cannot be directly utilised by humans. However, the metabolic products of many gut microbes can be utilised by humans and form a key component of human nutritional intake. In fact, GF mice must consume 30% more calories than their eubioitic compatriots in order to maintain their bodyweight [478]. These nutritional benefits of the gut microbiome are primarily mediated by production of short chain fatty acids (SCFAs) and is achieved by a group of enzymes termed the Carbohydrate-active enzymes of which the glycoside hydrolases (GHs) belong to [479]. The GHs are a major part of complex carbohydrate

metabolism and the human genome is known to encode 95 members of the family [479]. However, in comparison to the 260 GHs encoded by the *Bacteroides thetaiotaomicron,* the human contribution represents a drop in the ocean when considering the whole of the gut microbiome [480].

Interestingly, aside from improving caloric bioavailability from food, microbial enzymes have also been shown to modify polyphenols to make them bioavailable or bioactive. Polyphenols are found in numerous plant and fruit products and their consumption is considered to be beneficial. However, many polyphenols are highly glycosylated and not immediately bioavailable for humans [481]. Many members of the gut microbiome, including members of the *Bacteroides* and *Bifidobacteria* genera have been demonstrated to remove sugar moieties from numerous polyphenols enabling their absorption and utilisation by the host [482], [483].

Immunomodulation

The gut microbiota plays essential roles in the proper formation, co-ordination and maturation of the immune system. This occurs throughout development but also continues into adulthood, where control of immune responses by the microbiome is thought to play a role in many pathologies. The next section will summarise these interactions in detail.

Pathogen Protection

Aside from their impacts on systemic immune education, gut microbial species can also elicit local events that protect the host from pathogenic colonisation. Production of lactic acid by *Lactobacillus* has been shown to permeabilise gram negative bacteria and sensitise pathobionts to elimination by host lysozyme [484]. Additionally, several bacterially produced factors have been shown to regulate intestinally produced immune factors. Colonisation of GF animals with a mature microbiome results in significant upregulation of RegIIIγ, a C-type lectin that is capable of direct antimicrobial activity [485]. Also, the presence of certain members of the microbiome directs upregulation and class switching of Ig production in the gut. Through sensing of bacteria via TLRs, gut epithelial cells upregulate factors that lead to B cell class switching from IgM to IgA production [486]. Production of IgA is a key component of mucosal immunity, preventing pathogens from accessing epithelial receptors required for their invasion [487].

1.5.4. Microbial education of the immune system

The gut contains a rich diversity of immune cells and the gut associated lymphoid tissues (GALT) contain over 70% of all human leukocytes. These populations play essential roles in immunogenic tolerance but can also direct maturation of the developing immune system. Moreover, recent evidence has suggested that exposure to certain bacterial species may potentiate protective immune responses, particularly in the context of cancer [488]. This section will summarise how the microbiome contributes to immune function and how dysbiosis may lead to development of pathologies.

1.5.4.1. Structure and composition of gut associated lymphoid tissues

The GALT is the collective term for all the tissues involved in mucosal immunity. Starting from the immune component of the lamina propria, through to the unique PP and the draining mesenteric lymph node, the GALT has a diverse array of specialised functions and its structure is summarised in Figure 1.9. Cellular members of the GALT encompass virtually every immune cell type and interestingly, many of them display unique profiles compared to other gut resident members of their lineage. This section will briefly describe the structure of these systems and discuss the different cells that contribute to mucosal immunity.

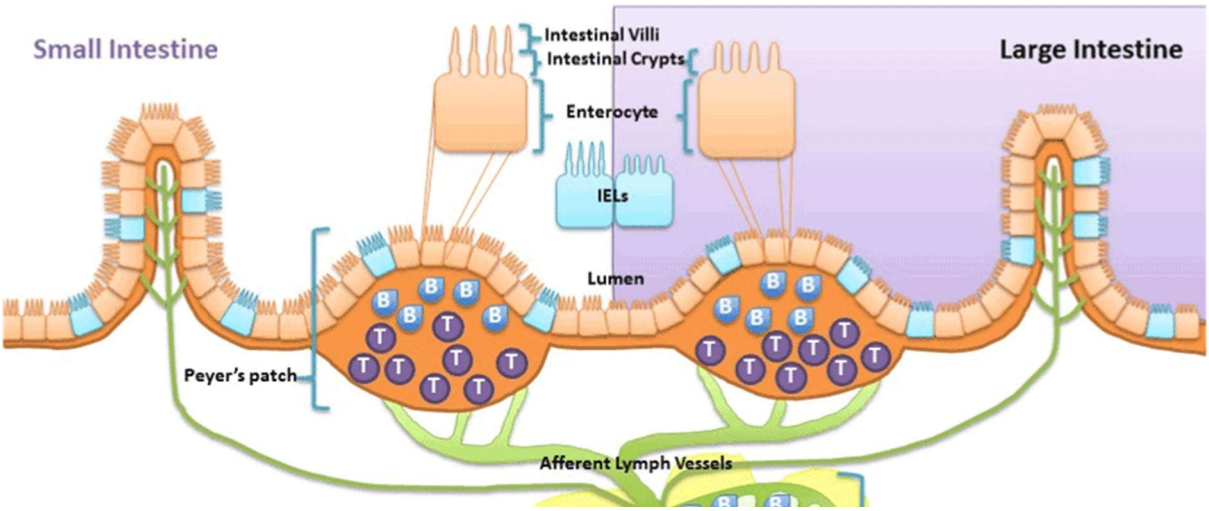


Figure 1.9 – Structure and distribution of GALT: The GALT covers three main sites of leukocyte activation and expansion. At the forefront are the cells of the lamina propria and Peyer's patches, which are constitutively exposed to gut luminal Ags. The APCs in these sites can immediately interact with T and B cells resulting in clonal expansion and modulation of local immune responses. Alternatively, APCs, particularly DCs can migrate out of the GI tissues via lymphatics to the draining mesenteric lymph nodes. Here, they interact with an extremely high density of lymphoid cells and can influence both GI and systemic immunity. Adapted from [526].

The gut lumen is lined with a specialised epithelium formed by enterocytes. Underlying this is a layer of connective tissues known as the lamina propria which contains a diverse set of immune cells including macrophages, neutrophils and several T cell subsets [489]. The role of these cells is primarily host defence to pathogens by upregulation of antimicrobial factors, phagocytosis and presentation of Ag for continuation of immune responses. The LP also contains populations of $CD103^{+}$ DCs which have been shown to migrate from the mucosal surfaces to the mLN via lymphatics and have been heavily associated with maintaining commensal tolerance [489]. Whilst effective at directing immune responses, the cells of the lamina propria do not constitutively sample antigens. This role is instead performed by cells inhabiting stretches of specialised follicle associated epithelium (FAE) which are present across the entire length of the GI tract. However, in some areas congregations of FAE organise into structures known as Peyer's patches. As alluded to earlier, the ileum contains a diffuse network of these specialised lymphoid tissues. Lacking a robust mucus layer, facilitating direct interactions with gut resident microbes, they play an essential role in sampling of microbial antigens and their structure is summarised in Figure 1.10 [490]. The FAE which is in direct contact with the gut lumen, directly below this the sub epithelial dome (SED) which has a high concentration of APCs and the deepest layer is a collection of B cell follicles surrounded by an interfollicular region (IFR) populated by T cells [491]. Antigen sensing is undertaken by two distinct mechanisms, both of which have heavy DC involvement.

Firstly, the FAE is equipped with highly specialised cells, microfold cells (M cells), which uptake microbes and antigen without disruption of barrier function. The luminal contents are transcytosed and deposited in a specialised invagination on the basal side of the epithelium that is enriched with APCs, particularly DCs [492]. Consistent with their role in gut Ag sensing, PPs harbour an extremely diverse array of DC subtypes, the full repertoire is highlighted in Table X. Two of the most important are the DN CD11c+ DCs and the lysoDCs. The latter, were named due to their high levels of lysozyme production and reside proximal to M cell invaginations in the SED. Their primary role is phagocytosis of microbes after M cell transcytosis and presentation of their associated Ags to other immune cell types. They are capable of eliciting pro-inflammatory responses by production of IL-6 and TNFα and have been shown to potentiate Ag uptake by other DCs through production of IL-22 binding protein (IL-22BP) [493], [494]. They also form an integral component of the PP antigen sensing machinery. By transcellular extension of dendrites through M cells, they can uptake luminal antigens and present them to basal immune cell populations through increased MHCII production [495].

The other critical DC component of the PP are the DN CD11c+ DCs, which are generally found across all layers of the PP. Their wide distribution has led to the suggestion that they play a role in shuttling Ag between the SED and the IFR for sensing by T cells [496]. Consistent with this role, they play an important role in retaining CD4+ T cells in the IFR and have been shown to be essential for their activation [497], [498]. Additionally, they have been demonstrated to lymphatically migrate out of the PP to the mLN, one of the distal sites of mucosal immunity.

Here, migratory DN DCs from the PP and $CD103^{+}$ DCs derived from the lamina propria pass through highly populous regions of B and T cells and initiate clonal expansion of T cell subsets [489], [499]. Generally, commensal Ags induce the proliferation of T_{regs} that are critical for the maintenance of oral tolerance. These expanded lymphoid populations will traffic back to the gut mucosal surfaces by recirculation, begin producing IL-10 and prevent commensally initiated inflammatory responses in order to maintaining gut barrier function [500], [501].

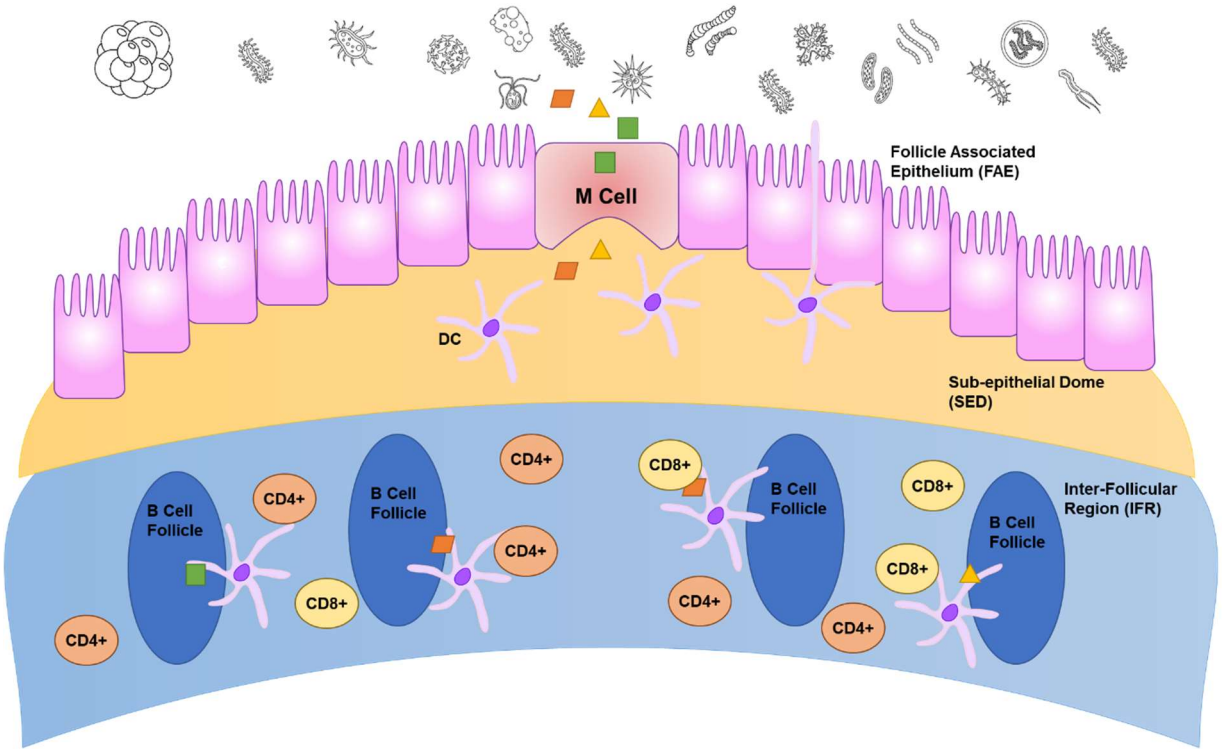


Figure 1.10 – Structure of a Peyer's patch: Antigen import across the follicle associated epithelium (FAE) is mediated by M cell transcytosis or paracellular extension by dendritic cells. Antigen is initially sensed by DCs resident in the sub-epithelial dome (SED) before migration into the distal interfollicular region (IFR). Here, DCs are free to activate naïve T and B cells, driving their clonal expansion and class switching of B cells. Adapted from [529] & [538].

1.5.4.2. Development

The pivotal role of the microbiome in initial immune development has been highlighted by studies using GF mice. In the absence of a diverse microbiome, GF mice display extensive structural and functional defects in their immune system. The formation of PPs is severely impacted, with GF mice displaying malformed and infrequent PPs [502]. They also exhibit lower luminal production of IgA and reduced numbers of CD4+ T cells in the lamina propria [503], [504]. Interestingly, these effects extend beyond development of mucosal immune structures and impact secondary lymphoid organs. Both the spleen and mesenteric lymph nodes have poorly formed B and T cell zones in GF animals leading to reduced serum levels of IgG and impaired activation of most αβ T cell subsets [505], [506]. Therefore GF animals are severely immunocompromised leaving them unable to resolve infections that are routine for SPF animals [507]. Additionally, these immune disturbances are associated with promotion of allergic and autoimmune diseases. For example, GF mice exhibit dysregulated expansion of invariant NKT (iNKT) cells in both the colon and lung and have increased sensitivity to allergens [508]. This sensitisation is reversible upon monocolonisation with *Bacteroides fragilis (B.fragilis)* or inoculation with *B.fragilis* derived sphingolipids, but only when the intervention is administered to neonates [509].

GF status has also been associated with susceptibility of animals to anaphylaxis in response to food allergens. In contrast to the reduced IgA and IgG production seen in GF animals, production of IgE is significantly increased [510], [511]. This is important given IgE is a key activator of mast cells which potently drive allergic reactions [512]. These increases in IgE induced mast cell activity in GF animals leaves them vulnerable to initiation of severe allergic reaction in response to peanut allergens. An effect that can be reversed by cohousing GF animals with eubiotic, SPF mice [513]. However, similarly to the effects on iNKT induced allergy, this is only effective when given to neonates and does not ameliorate symptoms when given to adult mice. This is suggestive of an essential role of the early microbiome in regulating immune responses.

This mechanistic data from animals has been in part reinforced by association studies in humans. Upregulation of IgE has been shown to potentiate allergic diseases in humans and the microbiome of infants with IgE mediated immune disorders has been profiled by Cahenzli *et al*. This study found that upregulation of IgE is associated with distinct microbial profiles, particularly increases in species of *Anaerobacter* and reductions in *Bacteroides* [514]. Additionally, dysbiosis induced by environmental factors has been associated with underdeveloped immunological responses. As discussed earlier, infants delivered by C

section show an initial dysbiosis in response to limited exposure to maternal bacterial species. This later resolves, with convergence of microbial signatures between those born vaginally and by C section [515]. Despite this, infants born by C section show impaired immune cell development, with reduced levels of circulating IgG, IgM and lower mucosal IgA, lower levels of IFNγ production and blunted CD4+ T cell responses to pathogens [516]–[518]. Concerningly, these differences have been shown to persist into adulthood when mice are delivered by C section and the impacts of these changes on adult humans are still to be elucidated [519]. However, lack of microbial exposure in early life is a key component of the hygiene hypothesis, which states that allergic diseases are increasing because humans are gradually living cleaner lifestyles [520]. Whilst increased levels of hygiene have been overwhelmingly beneficial for human health, perhaps we are beginning to tip the balance. Perhaps humans are becoming too clean and in turn, eliminating beneficial microbes essential for proper growth.

1.5.4.3. Homeostasis

In addition to its prominent role in immunological development, the gut microbiome is known to contribute to production of several immune cell types during homeostasis. Promotion of inflammation is essential for robust responses to pathogenic challenge. As discussed earlier, the microbiome can potentiate the production of locally produced antimicrobial factors that prevent colonisation and invasion of the GI tract by pathogens. Interestingly though, it's also capable of directing systemic immune responses by modulation of innate and adaptive immune processes. The presence of segmented filamentous bacterial (SFB) species in the gut microbiome has been shown to initiate the expansion of T_H17 cells. Inoculation of GF animals with a cocktail of SFB drove the upregulation of IL-6 and IL-23, key initiators of T_H17 differentiation, conferring resistance to colonisation by the pathogenic *Citrobacter rodentium* [521]. The microbiome has also been shown to potentiate the immune response to infections of distant organs. Depletion of the microbiome by antibiotics results in impairment of viral immunity in mice challenged with influenza. This phenotype was rescued by rectal administration of exogenous TLR agonists and was particularly associated with TLR2 & 9 activation. Mechanistically, this was shown to be a result of decreased DC migration from the lung to secondary lymphoid organs due to decreased systemic levels of IL-1β. The lack of DC mediated antigen presentation in draining LNs resulted in blunted T and B cell responses and susceptibility to infection [522]. Furthermore, in addition to the microbiome's ability to guide adaptive immune responses, it also potentiates innate responses. Microbially produced peptidoglycans can be detected in serum and the BM and potentiate the activity of neutrophils. Addition of exogenous peptidoglycan increases the ability of neutrophils to respond to infection by *Streptococcus*

pneumoniae and *Staphylococcus aureus* resulting in enhanced immunity to these opportunistic pathogens [523].

The microbiome also plays a more nuanced role in controlling inflammation by encouraging the expansion of immunoregulatory cell types. Administration of *Bacteroides fragilis* has been shown to drive differentiation of CD4+ T cells into T_{regs} through production of the TLR2 agonist polysaccharide A (PSA) [524]. Some members of the *Bifidobacteria* genera have also been shown to induce similar effects on T_{reg} expansion. Supplementation with *Bifidobacteria bifidum* has been shown to significantly increase IL-10 producing T_{regs} *in vivo* [525]. Furthermore, these effects can be driven by supplementation with *Bif* membrane vesicles alone, eliminating the need for administration of live bacteria [526]. This *Bif* mediated IL-10 production and subsequent immunosuppressive effect has garnered significant attention for the use of *Bif* in treatment of autoimmune disorders [527]. The potential for probiotic based immunotherapies and the differential contributions of the microbiota to pathology will be discussed in the next section.

1.5.4.4. Pathology

Due to the diverse immunomodulatory roles of the microbiome discussed in earlier sections, it has a directly conflicting duality in its function. Promotion of immunity and the concomitant upregulation of inflammation may be beneficial in the resolution of immunity, but chronic inflammation is implicated in the pathogenesis of several diseases. This section will summarise how these opposing roles can result in complex roles for the microbiome in disease and how it can be the problem and the solution.

Promotion of inflammation has been associated with several members of the microbiome, but interestingly is not limited to driving inflammation in the GI tract. Studies of autoimmune arthritis has shown that certain members of the microbiome are directly pathogenic. In GF animals, autoimmune arthritis is significantly reduced, however this effect is lost when the microbiome is reconstituted with that from a healthy individual. Interestingly, similar effects are seen upon monocolonisation with SFB and result in increased populations of T_H17 cells marked by upregulation of IL-17 production [528]. Additionally, gut microbes can directly exert autoimmune effects. Translocation of *Enterococcus gallinorium* from the gut to the liver initiates systemic autoimmunity, resulting in a lupus like autoimmunity which can be reversed by treatment with systemic antibiotics [529]. But, perhaps the best studied contribution of the gut microbiome to pathogenesis is in inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. These diseases affect 300,000 people in the UK alone and their incidence is increasing worldwide every year [530]. Characterised by chronic

inflammation of the GI tract as a consequence of impaired epithelial barrier function, resulting in increased basal infiltration of bacterial species and a positive feedback loop of inflammation [531]. Initiation of IBDs are associated with genetic predisposition and diet but also with antibiotic use suggesting pathogenesis may be related to outgrowth of pathobionts in the gut [532]. This hypothesis is supported in human association studies that have shown some bacterial species, such as *Escherichia coli* to be enriched in IBD patients [533], [534]. However, a unique bacterial origin is yet to be mechanistically proven in humans. Much of the understanding of IBD pathogenesis comes from mouse studies and the microbiome has been shown to be essential for development of colitis as GF animals are not susceptible to IBD induced by T cell adoptive transfer [535], [536]. Mechanistically, this has been associated with induction of T_H17 cells by bacterial species [537]. Although this is yet to be narrowed down a single species in preclinical models, as discussed earlier, SFB are potent initiators of IL-17 production and represent a potential culprit [528].

But, it is not all doom and gloom for the microbiome. In addition to the pathogenic effects of some species, others have been shown to control pathogenesis and ironically the answer to some of the potential ill effects of the microbiome in IBD is supplementation with other bacterial species. As discussed earlier, supplementation with *B. fragilis* drives expansion of T_{regs} and promotes immunoregulatory effects. Administration of these bacteria in models of IBD show marked resolution of inflammation and improved disease outcomes [538]. Interestingly, *B. fragilis* is a common component of the normal microbiome and its reduced abundance is associated with IBD in humans [539]. But what causes some individuals to lose *B. fragilis* and why do some patients retain *B. fragilis* and still develop IBD? The answer is likely a result of push and pull between several microbial constituents rather than the individual contributions of any one species requiring a carefully balanced microbiota for maintenance of homeostasis.

But where and how can this balance be struck? The solution is likely underpinned by a clichéd adage used to describe a healthy, balanced diet; everything in moderation. The reality is, that a diverse microbiome encompassing a wide variety of functional genes seems to result in the best outcomes. Compromising this balance, as discussed in this section, can diminish the host's wellbeing. In fact, very recently a link has been made between the host microbiome, the immune system and initiation of malignancy and will be explored in detail in the next section.

1.5.5. The Microbiota and cancer

The association between the microbiota and cancer was originally thought to be that of causation, in fact there are currently ten microbes on the IACR's list of group 1 carcinogens [540]. Of these ten, six of them are viral and the most well-known of these associations is between human papillomavirus (HPV) and cervical cancer. The prevalence of HPV in the western world is extremely high with 8 out of 10 people being infected with the virus at some point in their lives [541]. With 14 million new cases each year, there was significant concern amongst the medical community when the link between HPV and cervical cancer was uncovered. In fact, it's estimated that HPV infection is implicated in almost 100% of all cervical cancers and has also been associated with causing throat, vaginal and anal cancers [542]. The worst offenders are the HPV 16 and 18 strains, whose integration in the host genome initiates expression of the viral oncoproteins E6 and E7 [543]. These strains alone account for over 70% of all cervical cancers. In the mid-2000s, comprehensive vaccination campaigns were undertaken by several western countries and almost 90% of girls aged 11-13 received the vaccine in the UK [544]. These vaccine is almost 100% effective and has led to a steep decrease in HPV infection rates, particularly against HPV 16 and 18 [545]. It is still too early to tell how effective the HPV vaccine has been with respect to reduction of cervical cancer cases as the vaccination program is only celebrating its 10th birthday this year. However, a recent study conducted in the US has observed a 50% reduction in the numbers of young women presenting with early cervical malignancies [546].

Aside from virally induced malignancy, many bacterial species have also been implicated in driving carcinogenesis. The best studied is the induction of gastric carcinoma by *H.pylori*. Tumorigenesis is initiated by insertion of CagA, a bacterially produced protein, via type IV secretion systems directly into the epithelial cells of the stomach. Here, CagA activates β-catenin resulting in expression of numerous proliferative, anti-apoptotic and proangiogenic genes, a perfect cocktail for driving malignant transformation [547]. Whilst not included in the 10 group 1 carcinogens, several other bacterial species have been shown to drive carcinogenesis by similar mechanisms suggesting a broad set of commensals are capable of inducing tumour growth [548].

In additional to direct tumorigenic activities, some bacterial species have been shown to drive tumorigenesis by modulation of immune responses, particularly in colorectal cancers (CRC). Inflammation associated with IBDs has been shown to potentiate malignant transformation of enterocytes, particularly the upregulation of IL-17A production seen during

IBDs [549]. Additionally, individual members of the commensal microbiome have been implicated in driving tumorigenesis. The bacterium, *Fusobacterium nucleatum (F. nucleatum),* which is part of the eubiotic microbiome increases in abundance in patients with CRC [550]. Furthermore, in preclinical models of CRC, the presence of *F. nucleatum* promotes tumorigenesis by driving myeloid immune cell infiltration and induction of an inflammatory intratumoural milieu [551].

Whilst the evidence that suggests some microbes are carcinogenic is incontrovertible, these associations are usually a result of local interactions. Given it is now well established that the microbiome can initialise and direct systemic immune responses and that the immune response plays such an integral role in controlling tumour growth, it is reasonable to presume that the microbiome may also play a role in directing distant anti-cancer immune responses.

This potential association was first probed in 2013 by Iida *et al.*, and turned the scientific viewpoint of microbes in cancer on its head. The group was interested in how immunostimulant, cytosine-phosphorothioate-guanine nucleotides (CpG), a putative chemotherapeutic may be modulating the host microbiome. Administration of CpG generates rapid TNFα associated myeloid infiltration into tumours which is followed by activation of tumour specific CD8+ T cells and associated cytotoxicity of tumour cells. Whilst this treatment regimen provided effective tumour control in eubiotic animals, surprisingly, if the animals were treated with an antibiotic cocktail (ABX), the efficacy of CpG was completely abrogated. This effect was accompanied by a reduction in tumour infiltrating, TNFα producing leukocytes suggesting the microbiome conferred the capability of certain immune populations to participate in tumour killing [552]. For the first time, the microbiota had been shown as an essential component in anti-cancer immunity.

These exciting findings were published at the same time as a study by Viaud *et al.,* whose study focused on the use of cyclophosphamide (CTX), a potent inducer of immunogenic cell death and is occasionally indicated for use in BC. The effects of CTX are driven by induction of T_H1 and T_H17 cells and a depletion of immunoregulatory T_{regs} resulting in an inflammatory intratumoural milieu. However, an adverse consequence of CTX induced T_{reg} depletion is dysfunction of oral tolerance and many patients will develop mucositis, an inflammation of the GI epithelium that is often treated with ABX. However, at the time, the impacts of ABX induced dysbiosis had never been evaluated. The authors found that treatment with ABX potently inhibited the anti-tumour effects of CTX treatment across several tumour models. They attributed the loss of efficacy to reduced intratumoural T_H1

and T_H17 infiltration and interestingly, the negative effects of ABX administration were completely reversed when the animals' microbiome were reconstituted with that of a healthy individual [553]. These findings had huge clinical implications, suggesting that the use of ABX alongside chemotherapies may be strongly interfering with their treatment and impacting outcomes.

These principles were later extended to immune checkpoint inhibitors, the same group found that GF or ABX treated mice did not respond to anti-CTLA4 treatments whereas eubiotic SPF exhibited effective tumour suppression. Depletion of the microbiome was accompanied by reduced intratumoural DC infiltration and an inability to promote IL-12 dependent T_H1 polarisation. Strikingly, these effects were mirrored when administering fecal microbiota transplants (FMT) using samples taken from human patients undergoing anti-CTLA4 treatment. Animals which were reconstituted with feces from responders also responded, however animals that were given non-responder feces were completely unresponsive to anti-CTLA4 therapy [554]. Incredibly, for the first time an association between the human microbiome and differential responses to chemotherapy in human patients was shown. Very recently, the same group again probed these relationships, but using a longitudinal study of human responders and non-responders. This time, the study focused on patients receiving anti-PD-1 therapy and of the 249 patients recruited, they found that 69 had received ABX in the 2 months prior to, or after their treatment. Using this data, they found that use of ABX is a key predictive factor in non-response to anti-PD-1 therapies. Furthermore, use of ABX was associated with reductions in progression free and overall survival. Upon sequencing the fecal microbiome of these patients, the commensal *Akkermansia muciniphila* (*A. muciniphila)* had increased abundance in responding patients relative to non-responders, suggesting it may be responsible for potentiation of immunotherapy. To probe the potential mechanisms by which *A. muciniphila* may impact the anti-cancer immune response, FMTs were administered to GF animals using patient feces. As seen in earlier studies, feces from human responders potentiated the effects of immunotherapy in animals and vice versa for non-responders. This potentiation was associated with significant increases in CXCR3+ effector T cells seen in secondary lymphoid organs and the tumour microenvironment, suggesting the microbiome is essential for T cell activation in anti-tumour immunity [555]. For the first time, a direct association has been made between treatment efficacy and the quality of the gut microbiome in human patients. Considering these findings, it would be advantageous to include screening of the microbiome when designing treatment regimens for patients. However, this is not yet feasible with the current costs of next generation sequencing.

There have also been suggestions in the literature that administration of probiotic bacteria may be beneficial to cancer outcomes, even in patients with an already healthy microbiome. Sivan *et al.* observed that administration of *Bifidobacteria* spp. to eubiotic mice resulted in significant reductions in tumour volume. Additionally, it was demonstrated that these probiotics potentiated anti-PD-1 immunotherapy. These effects are a result of *Bif* mediated increases in DC maturation resulting in intratumoural DCs upregulating the CD8+ costimulatory molecules CD40 and CD70 and the CD8+ T cell chemoattractant, CXCL9. Through these processes, administration of *Bif* improves CTL mediated killing of cancer cells and induces tumour regression [556]. Whilst encouraging, these findings have not yet been fully translated to humans. Recently, the same group profiled the microbiomes of melanoma patients receiving anti-PD-1 therapies and found enrichment of some *Bifidobacteri*um spp. in the fecal microbiome of responders vs non-responders [557]. However as yet, the efficacy of probiotic administration has not been assessed by clinical trial.

1.6. Research Aims and Objectives

Following recent observations in preclinical models of other cancers, a critical role of the microbiota in guiding anti-cancer immune responses has been demonstrated. This project intends to build on the current literature by characterising the influence of the gut microbiota in breast cancer. We intend to undertake this characterisation using physiologically relevant preclinical models via orthotopic implantation of mammary carcinoma cells. The major objective of this project is to determine if disturbance of the microbiota via antibiotic use has any impact on breast cancer growth or development. Furthermore, this project intends to probe the relationships between individual microbes and the anti-cancer immune response in breast cancer; particularly with respect to Bifidobacteria. In order to achieve these goals, the major aims of this project are as follows:

1. Characterise the role of the microbiota in primary tumour growth via its depletion by administration of a harsh antibiotic cocktail

2. Determine if antibiotic administration may impact primary tumour growth in a clinical setting through use of patient relevant antibiotics

3. Investigate whether administration of probiotic bacterial species has an impact on primary tumour growth

4. Characterise the mechanistic contributions of the microbiota by examining the key cellular and molecular players that are modulated during primary tumour growth

5. Follow tumour progression to metastasis in order to probe the role of the microbiota in this process, particularly with respect to establishment of the early metastatic niche.

2. Materials and Methods

2.1. Animals

2.1.1. Antibiotic Administration

Animals were treated with antibiotics 3 times weekly by oral gavage (200µl in water). Animals were treated with either an antibiotic cocktail consisting of 1mg/ml Amphoteracin B (Sigma-Aldrich, St-Louis, Missouri, USA), 25mg/ml Vancomycin (Sigma), 50mg/ml Neomycin (Sigma), 50mg/ml Metronidazole (Sigma) with drinking water being supplemented with 1mg/ml Ampicillin (Sigma) or 14mg/ml Cephalexin (Sigma). Antibiotic treatment began 5 days prior to tumour cell injection and was maintained throughout animal experiments.

2.1.2. Probiotic Administration

Animals were treated either once or three times weekly with a probiotic gavage of up to five bifidobacteria strains in 200ul PBS. Treatment with bifidobacteria commenced two or five days prior to tumour cell injection and was maintained throughout the experiment. All isolates were obtained from the Hall lab and species/strains were as follows: *breve* ssp. UCC2003, *longum* ssp. 8809, *pseudocatulenatum* ssp. 210, *bifidum* ssp. 80, *animalis* ssp. 506.

2.2. *In vivo* tumour growth assays

Syngeneic mouse breast carcinoma (B6BO1[a] or E0771[b]) cells were injected at $1x10^5$ per 50µl of a 1:1 mixture of PBS and Matrigel (Corning Life Sciences, Corning, New York, USA) into the left inguinal mammary fat pad (MFP) of age matched female mice. Tumours were measured in two dimensions (Length x Width) every two days from 7 days post injection (DPI) using digital calipers. Upon conclusion of the experiment or once the tumours reached $1000mm^3$ the animals were sacrificed by cervical dislocation and tissues harvested for various downstream analyses. Tumour volume was calculated according to the following formula: length * $width^2$ * 0.52.

[a]B6BO1 cells obtained from Dr Katherine Weilbaecher (Washington University, St Louis, MO, USA)

[b]E0771 cells obtained from Prof Kairbaan Hodivala-Dilke (Queen Mary University, London, UK)

2.3. Immunohistochemical analyses

2.3.1. Frozen tissues

2.3.1.1. Formaldehyde fixed

Tissues were fixed overnight in 4% PFA at 4°C before washing twice in PBS. Tissues were incubated overnight or until the tissue sank in 15% sucrose in PBS at 4°C. Tissues were then embedded in an 8% gelatin solution and moulds were left to set at 4°C overnight. The gelatin blocks were trimmed to size and then snap frozen in isopentane cooled to -50°C in liquid nitrogen vapours. Blocks were stored at -80C until ready to section. Tissue was sectioned at 8µm on a HM550 cryostat (Microm, Bicester, UK), collected on positively charged glass slides (Thermofisher Scientific, Waltham, Massachusetts, US) and stored at -80°C until staining.

Sections were allowed to air dry at room temperature (RT) for 10 minutes before the gelatin embedding medium was melted in 37°C PBS for 10 minutes. Sections were permeabilised with PBS + 0.3% triton X-100 (Thermofisher Scientific) before blocking in PBS/5% goat serum (Sigma) at RT for 30 minutes. Sections were incubated in appropriate primary antibodies (Table 2.1) made up in blocking solution at 4°C overnight in the dark. If using unconjugated antibodies, sections were washed 3x5mins in PBST before incubation in the appropriate secondary antibody for 2 hours at RT. Sections were again washed 3x5mins and briefly dried, one drop of fluoromount + DAPI (eBioscience, Thermofisher) was added to each section, coverslip mounted and sealed with nail polish. Slides were stored at 4°C before imaging. Slides were imaged using an Axioplan 2 fluorescent microscope (Zeiss, Oberkochen, Germany) and analysed using ImageJ software.

2.3.1.2. Fresh

Tissues were submerged in OCT and frozen by floating moulds on 100% isopentane (Sigma) cooled to -80°C by liquid nitrogen vapours. Once solid, blocks were stored at -80°C until ready to section. Once sectioned, tissues were post-fixed in ice cold methanol for 5 minutes, washed three times in PBS and staining continued as in 2.3.1.1.

2.3.2. Formaldehyde fixed paraffin embedded tissue

Tissues were fixed overnight in 4% PFA at 4°C before washing twice in PBS for 30 minutes. Tissues were gradually dehydrated through successively increasing concentrations of

ethanol (30%-100%) before clearing in Histoclear (National Diagnostics, Atlanta GA, USA) and embedding in paraffin. Paraffin blocks were sectioned using a HM355 S microtome (Microm, Bicester, UK) at a thickness of between 5µm and 10µm and mounted onto positively charged glass slides (Thermofisher). Sections were dried o/n at 37°C. Prior to staining sections were rehydrated by washing in Histoclear before incubations in gradually decreasing concentrations of ethanol (100%-50%) with a final wash in dH_2O. Heat-mediated antigen retrieval was performed by boiling sections in sodium citrate buffer (10mM tri-sodium citrate (Thermofisher), 0.05% Tween-20 (Thermofisher), pH 6) for 20 mins. They were then allowed to cool and Immunohistochemical staining was completed as per section 2.3.1.1.

2.3.3. Immunocytochemistry

Cells were incubated on acid washed glass coverslips overnight before fixation in ice cold methanol for 10 minutes. Cells were permeabilised for 10 minutes in 0.1% Triton X-100 before blocking in 5% goat serum for 30 minutes. Primary antibodies were diluted to the concentrations indicated in blocking buffer and applied overnight at 4°C. Cells were washed using PBS + 0.1% Tween-20 and secondary antibody was applied at the appropriate dilution in PBS for 2 hours at room temperature in the dark. Cells were washed again in PBS + 0.1% Tween-20, mounted using fluoromount + DAPI and the coverslips sealed for imaging.

2.3.4. Positive and Negative Staining Controls

To test the function of the antibodies used in our staining, several positive and negative controls were utilised. The use of Endomucin to detect blood vessels in healthy and cancerous tissue has been previously validated in our lab [558] and did not require controls. The spleen was used as a positive control tissue for all T cell staining, but particularly for testing the function of our CD3 antibody. The proper function of our F4/80 antibody was evaluated using our tumour tissues as we have observed ~20-30% F4/80 positivity in the tumours during our flow cytometric analyses. The Estrogen and Progresterone receptor antibodies were tested using mammary glands. All staining was also validated using the relevant IgGs to ensure our results were not caused by non-specific binding.

2.3.5. List of immunostaining antibodies

Table 2.1 – List of antibodies used in immunostaining protocols

Target	Manufacturer	Product Code	Concentration
Primary Antibodies			
Fluoromount+DAPI	eBioscience	E115189	N/A
Endomucin	Santa Cruz	sc65495	1:500
CD3	eBioscience	14-0032-81	1:200
F4/80	Abcam	ab6640	1:200
Estrogen receptor	Abcam	ab32063	1:200
Progesterone receptor	Abcam	ab63605	1:200
Secondary Antibodies			
Anti-Rabbit Alexa 488	Invitrogen	913921	1:250
Anti-Rabbit Alexa 594	Invitrogen	A11012	1:250
Anti-Rat Alexa 488	Invitrogen	A11006	1:250
Anti-Rat Alexa 594	Invitrogen	A21209	1:250

2.4. *In vivo* metastasis experiments

2.4.1. Early-metastatic dissemination

B6BO1 tumour cells were injected into the left inguinal MFP of age matched C57BL/6 female mice. Tumours were allowed to reach 1000mm^3, in early-dissemination experiments animals were sacrificed, for overt metastatic methods, see 2.4.2. For early metastatic experiments, metastatic organs (lung and long bone) were excised, snap frozen in liquid nitrogen and stored at -80.

2.4.2. Development of metastatic lesions

Progression of metastatic lesions was monitoring by In Vivo imaging. Animals were injected I.P. with 100µl 3mg/ml luciferin (Promega, Madison, WI, USA). The animals were left for 5 minutes for luciferin to circulate before being anaesthetised. Anaesthesia was induced under 4% isoflurane and maintained at 2%. Animals were exposed at 30 second intervals using the InVivo Xtreme imaging system (Bruker).

2.4.3. *Ex vivo* luciferase assay

Metastatic organs were homogenised with a scalpel and the homogenate was placed into safe lock centrifuge tubes (Eppendorf, Hamburg, Germany) with acid washed glass beads (Sigma) and 1ml of cell culture lysis reagent (Promega, Madison, Wisconsin, US). Tissue was lysed in TissueLyser LT (Qiagen, Hilden, Germany) for 1 minute at 50hz. Homogenate was centrifuged at 14,000rpm for 15 minutes and supernatant stored at -80°C. Using a black, clear bottomed 96 well plate (Corning) 50µl homogenate was added to each well and 50µl of luciferase assay reagent (Promega) added on top. Plate was imaged immediately using an InVivo Xtreme imager (Bruker, Billerica, Massachusetts, US).

2.5. Cell Isolation and Culture

2.5.1. Breast cancer cell culture

B6BO1 and EO771 cells were cultured in high glucose DMEM (Invitrogen, Carlsbad, California, US) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Invitrogen) and 100 units/mL penicillin/streptomycin (Pen/Strep) (Invitrogen). Cells were maintained at 37°C and 5% CO_2. Tissue culture plastic was coated with 0.1% porcine gelatin (Sigma) in water for 1 hour at 37°C prior to culture.

2.5.2. Primary Splenocyte isolation and culture

Splenocytes were isolated by excision of the spleen under sterile conditions and dissociating through a 70µm cell strainer (Invitrogen) with 2ml RPMI media (Invitrogen). Cells were collected, centrifuged at 300 x g for 5 minutes at 4°C and washed twice with PBS. Cells were plated into a 6 well plate at 10 million cells per well with RPMI supplemented with 10% FBS, 1% Pen/Strep, 10µM β-mercaptoethanol (Invitrogen) and 10mM HEPES (Invitrogen). Cells were maintained at 37°C and 5% CO_2.

2.5.3. Bone Marrow Macrophage isolation and culture

Bone marrow cells were isolated from hindlimb bones under sterile conditions. Both hindlimbs were removed, cleaned of skin and muscle, feet removed and bones separated at the knee. The epiphyses of both the femur and tibia were removed and placed into a 0.5ml spin tube with a small hole which was inserted into a 1.5ml spin tube. The bones were spun at 500 x g for 2 minutes to elute bone marrow. Cells were cultured according to [559], to summarise, cells were culture overnight, washed to remove dendritic cells and cultured

in the presence of M-CSF (Peprotech, Rocky Hill, NJ, USA) for up to 7 days to induce monocyte differentiation into macrophages.

2.6. Flow Cytometry

2.6.1. Cell Isolation

2.6.1.1. Tumour and Lung

Organs were excised from freshly sacrificed animals and tissues were mechanically homogenised using scalpels. Homogenate was incubated in collagenase solution (0.2% Collagenase IV (Invitrogen), 0.01% Hyaluronidase (Sigma) & 2.5U/ml DNAse I (Sigma) in HBSS) for 1 hour at 37°C with regular agitation. Supernatant was passed through a 70µm cell strainer and centrifuged for 5 minutes at 300 x g/4°C. Pellet was washed twice in PBS and stained according to 2.6.2.

2.6.1.2. Bone Marrow

Cells were isolated as in 2.5.3 and stained according to 2.6.2.

2.6.1.3. Spleen, Mesenteric Lymph Node and Peyer's Patches

Organs were excised from freshly sacrificed animals and homogenised through a 70µm cell strainer with 2ml RPMI. Cells were centrifuged at 300 x g for 5 minutes at 4°C and washed once in PBS. Cells were stained according to 2.6.2.

2.6.2. Staining Protocol

Cells were resuspended in 10ml 1X red blood cell lysis buffer (Invitrogen) and incubated for 5 minutes at RT. Cells were washed once in PBS, counted using a haemocytometer (Sigma) and 1 million cells per condition transferred to a 96 well plate for staining. Cells were incubated in a fixable Live/Dead stain (Invitrogen, Thermofisher) for 30 minutes at RT, washed twice and blocked in Fc Block (Miltenyi, Bergisch Gladbach, Germany) made in FACS buffer (1% FBS in PBS) for 10 minutes at 4°C. Cells were resuspended in 100µl antibody solutions (Table 2.2) and incubated at 4°C for 30 minutes in the dark. For cell surface only staining, cells were incubated in 4% PFA for 30 minutes, washed once in PBS and stored at 4°C until analysed. If intracellular staining is required, cells were incubated in FoxP3 fixation/permeabilisation buffer (Thermofisher) overnight at 4°C, washed twice in 1X permeabilisation buffer (Thermofisher), blocked in 5% normal rat serum for 30 minutes at RT and stained in the relevant antibody diluted in 1X permeabilisation buffer for 30 minutes

at RT in the dark. Cells were washed twice in 1X permeabilisation buffer, then finally resuspended in FACS buffer and stored at 4°C until analysed.

2.6.3. Data Collection and Analysis

All data was collected using a Becton Dickinson (BD, Franklin Lakes, NJ, USA) LSR II Fortessa with standard filter sets and five lasers. Data was analysed using FlowJo software (BD).

2.6.4. Gating Strategies

Gating strategies are detailed in Appendix Figure 7.1

2.6.5. List of Flow Antibodies and other reagents

Table 2.2 – List of flow cytometry antibodies and reagents

Target	Conjugate	Manufacturer	Product Number	Clone	Concentration
CD45	PerCP-Cy5.5	eBioscience	45-0451	30-F11	1:400
CD3	APC	eBioscience	17-0031	145-2C11	1:200
CD4	PE	eBioscience	12-0041	GK1.5	1:200
CD8	PE-Cy7	eBioscience	561967	53-6.7	1:400
FoxP3	FITC	eBioscience	48-5773	FJK-165	1:100
NK1.1	Alexa 700	eBioscience	56-5941	PK136	1:200
CD11b	Alexa 700	eBioscience	56-0112	M1/70	1:400
F4/80	PE-Cy5	eBioscience	15-4801	BM8	1:400
Ly6G	APC-Cy7	BD	560600	IA8	1:200
Gr-1	APC-Cy7	BD	47-5931	RB6-8C5	1:200
CD206	PE	Biolegend	41705	C068C2	1:100
MHCII	eFluor450	eBioscience	48-5321	M5/114.1.2	1:200
Live/Dead	FITC	Invitrogen	L34970	N/A	1:200

2.7. Western Blot Analyses

2.7.1. Western blot protocol

Cells and tissues were lysed using an SDS lysis buffer (3% SDS, 60mM Sucrose and 65mM Tris-HCl pH 7.4). Cells were scraped off their plates using a rubber policeman, tissues were briefly mechanically homogenised using a scalpel. Samples were transferred to safe-lock eppendorf™ tubes containing a small quantity of acid washed glass beads (Sigma) and homogenised in a Tissue Lyser LT (Qiagen) for 2 minutes at 50hz. Lysates were centrifuged at 12000 x g for 10 minutes at RT. Protein concentration was quantified using the BioRad DC protein assay (BioRad, Hemel Hempstead, UK). Where possible, 30µg of protein was loaded onto a 10% polyacrylamide gel for SDS-PAGE. Protein was transferred onto nitrocellulose membrane (Amersham, Amersham, UK), blocked in 5% milk skim powder (Oxoid, Thermofisher) in PBS with 0.1% Tween-20 (PBST) (Thermofisher) for 30 minutes at RT. Membranes were incubated with appropriate primary antibodies (Table 2.2) diluted in PBST + 5% goat serum (Sigma) overnight at 4°C. Blots were washed 3X in PBST and incubated for 2 hours at RT with diluted horseradish peroxidase (HRP) conjugated secondary antibodies (Dako, Agilent, Santa Clara, CA, US) diluted in PBST + 5% goat serum. Blots were washed 3X in PBST and incubated for 5 minutes in enhanced chemiluminescence reagent (Pierce, Thermofisher). Chemiluminescence was detected using a Fujifilm LAS-300 darkbox (Fujifilm UK Ltd, Bedford, UK).

2.7.2. List of western blot antibodies

Table 2.3 – List of antibodies used in western blot protocols

Target	Manufacturer	Product Code	Concentration
Primary Antibodies			
HSC70	Santa-cruz	sc7298	1:5000
E-Cadherin	Abcam	ab76055	1:1000
Vimentin	Abcam	ab137321	1:1000
Estrogen receptor	Abcam	ab32064	1:1000
Progesterone receptor	Abcam	ab63605	1:1000
Secondary Antibodies			
Anti-Rabbit HRP	Dako	P0448	1:1000
Anti-Mouse HRP	Dako	P0447	1:1000

2.8. Microbiome Sequencing

2.8.1. Fecal DNA Extraction

Feces was weighed into MPBio Lysing Matrix E bead beating tubes (MPBio, Santa Ana, CA, USA) and extraction was completed according to the manufacturer's protocol for the MPBio FastDNA™ SPIN Kit for Soil but extending the beat beating time to 3 minutes. The DNA recovered from these samples was assessed using a Qubit® 2.0 fluorometer (Invitrogen).

2.8.2. 16s rRNA

2.8.2.1. Library Preparation and Sequencing

Extracted DNA was normalised to 5ng/ul and used in 16S amplicon PCR targeting the V1+2 of the 16S gene using the primers detailed in Table 2.4 and the following PCR cycle:

95°C for 3 minutes, 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds with a final 72°C step for 5 minutes. Primer sequences are detailed below.

PCR products were taken through a round of AMPure XP bead clean-up to remove primers and sent to the Sanger Institute (Cambridge, UK) for sequencing by Illumina MiSeq 2x300bp paired end chemistry in multiplex generating ~100,000 reads per sample. Raw reads were returned to QIB for analysis.

Table 2.4 – V1+2 16S amplicon primers used for sequencing library preparation

Primer Name	Sequence
V1FW_SD501	AATGATACGGCGACCACCGAGATCTACACAAGCAGCATATGGTAATTGTAGMGTTYGATYMTGGCTCAG
V1FW_SD502	AATGATACGGCGACCACCGAGATCTACACACGCGTGATATGGTAATTGTAGMGTTYGATYMTGGCTCAG
V1FW_SD503	AATGATACGGCGACCACCGAGATCTACACCGATCTACTATGGTAATTGTAGMGTTYGATYMTGGCTCAG
V1FW_SD504	AATGATACGGCGACCACCGAGATCTACACTGCGTCACTATGGTAATTGTAGMGTTYGATYMTGGCTCAG
V1FW_SD505	AATGATACGGCGACCACCGAGATCTACACGTCTAGTGTATGGTAATTGTAGMGTTYGATYMTGGCTCAG
V1FW_SD506	AATGATACGGCGACCACCGAGATCTACACCTAGTATGTATGGTAATTGTAGMGTTYGATYMTGGCTCAG
V1FW_SD507	AATGATACGGCGACCACCGAGATCTACACGATAGCGTTATGGTAATTGTAGMGTTYGATYMTGGCTCAG
V1FW_SD508	AATGATACGGCGACCACCGAGATCTACACTCTACACTTATGGTAATTGTAGMGTTYGATYMTGGCTCAG
V1FW_SA501	AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGTAGMGTTYGATYMTGGCTCAG
V2RV_SD701	CAAGCAGAAGACGGCATACGAGATACCTAGTAAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD702	CAAGCAGAAGACGGCATACGAGATACGTACGTAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD703	CAAGCAGAAGACGGCATACGAGATATATCGCGAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD704	CAAGCAGAAGACGGCATACGAGATCACGATAGAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD705	CAAGCAGAAGACGGCATACGAGATCGTATCGCAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD706	CAAGCAGAAGACGGCATACGAGATCTGCGACTAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD707	CAAGCAGAAGACGGCATACGAGATGCTGTAACAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD708	CAAGCAGAAGACGGCATACGAGATGGACGTTAAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD709	CAAGCAGAAGACGGCATACGAGATGGTCGTAGAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD710	CAAGCAGAAGACGGCATACGAGATTAAGTCTCAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD711	CAAGCAGAAGACGGCATACGAGATTACACAGTAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT
V2RV_SD712	CAAGCAGAAGACGGCATACGAGATTTGACGCAAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT

2.8.2.2. Bioinformatic Analysis

At QIB, an in-house PE protocol was used for sequencing analysis. After demultiplexing and quality control of raw paired reads using FASTX-Toolkit [560] (minimum quality 33 for at least 50% of the bases in each read sequence) reads were aligned against the SILVA database (version: SILVA_128_SSURef_tax_silva) [561] and BLASTN (ncbi-blast-2.2.25+; Max e-value 10e-3) [562]. BLAST files were imported into MEGAN6 [563] to create proprietary rma6 files using the following parameters: 100 as maximum number of matches per reads, and “Min Score = 50” and “Top Percent = 10”. All rma6 files of paired read sequences were then normalised and compared using MEGAN6.

2.8.3. Statistical Analysis

To make comparisons between study sets, the samples were normalised to the sample with the lowest number of reads. Principal Coordinate Analysis plotting was performed using Bray-Curtis distances from the 16S MEGAN community profiles.

2.9. Fecal Metabolomics

2.9.1. Sample Preparation

Faecal samples were prepared for 1H NMR spectroscopy by mixing 25mg (FW) of faecal samples with 600µL NMR buffer made up of 0.1 M phosphate buffer (0.51 g Na2HPO4, 2.82 g K2HPO4, 100 mg sodium azide and 34.5 mg sodium 3-(Trimethylsilyl)-propionate-d4 (1 mM) in 200 mL deuterium oxide) with a tube pestle. Sample tubes were vortexed for 5 minutes, then centrifuged at 12,000 x g for 5 minutes at 4°C. Supernatant was transferred to a 5-mm NMR tube for recording.

2.9.2. NMR Conditions

High resolution 1H NMR spectra were recorded on a 600MHz Bruker Avance spectrometer fitted with a 5 mm TCI cryoprobe and a 60 slot autosampler (Bruker, Rheinstetten, Germany). Sample temperature was controlled at 300 K. Each spectrum consisted of 1024 scans of 65,536 complex data points with a spectral width of 12.5 ppm (acquisition time 2.67 s). The noesypr1d presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 3 s) and mixing time (D8 = 0.01 s). A 90° pulse length of 9.6 µs was set for all samples. Spectra were transformed with 0.3 Hz line broadening and zero filling, manually phased, and baseline corrected using the TOPSPIN 2.0 software. Spectra were transferred into AMIX ® software for bucketing and multivariate analysis applied (using Matlab ® Toolbox software). Spectra were transformed with 0.3 Hz line broadening and zero filling, manually phased, and baseline corrected using the TOPSPIN 2.0 software. Metabolites were identified using information found in the literature (references) or on the web (Human Metabolome Database, http://www.hmdb.ca/) and by use of the 2D-NMR methods, COSY, HSQC, and HMBC.

2.9.3. Statistical Analysis

Multivariate statistical analyses (Principal Component Analysis) were carried out using the PLS Toolbox v5.5 (Eigenvector Research Inc., Wenatchee, WA) running within Matlab, v7.6 (The MathWorks Inc., Natick, MA). Autoscaling was applied to the columns of the bucket table. Univariate analyses were carried out on individual variates in Excel (t-tests).

2.10. Mesoscale Discovery Multiplex Arrays

Tissue samples were weighed into a MPBio Lysing Matrix E bead beating tube (MPBio) with 1ml of homogenisation buffer (PBS + 10% FBS (Invitrogen) + cOmplete™ protease inhibitor (Roche)). Tissues were homogenised using an MPBio Fast Prep bead beater at speed 4.0 for 40 seconds followed by speed 6.0 for 40 seconds. Samples were centrifuged at 12,000 x g for 12 minutes at 4°C and subsequently stored at -80°C until analysed. Samples were run on a Mesoscale Discovery (MSD, Rockville, MD, USA) V-PLEX Pro-Inflammatory Panel 1 Mouse Kit according to the manufacturer's instructions. Plate was read using an MSD QuickPlex SQ 120 imager.

2.11. ELISA Assays

Protein was extracted from tumour and intestinal tissue using the protocol detailed in 2.10. Assays were performed using IL17A and IL-10 ELISA kits eBioscience (eBioscience, Thermofisher) according to the manufacturer's instructions. Plates were read using Molecular Devices (San Jose, CA, US) VersaMax plate reader according to manufacturer's instructions.

2.12. Bacterial Culture

Bifidobacteria strains were cultured in brain heart infusion (BHI) (Oxoid, Thermofisher) supplemented with 50mg/L Mupirocin (VWR, Radnor PA, USA) under anaerobic conditions using a Whitley DG250 anaerobic cabinet (DW Scientific, Shipley, UK). Bacteria were subcultured 1:10 every two days for the duration of experiments.

2.13. Probiotic Cocktail Preparation

Bifidobacteria were centrifuged at 3200 x g for 10 minutes at 4°C, washed twice in PBS before final resuspension in 4ml PBS. Each individual strain was serially diluted and plated on BHI agar containing 50mg/L Mupirocin to check the viability and number colony forming units/mL, this data is presented in table Table 2.5. To prepare cocktail, 1ml was taken from each strain resuspension and combined, the cocktail was administered as described in 2.1.2.

Table 2.5 – CFU/ml of Bif spp. In gavage

Bifidobacteria spp & ssp.	CFU/ml
Bifidobacterium breve UCC2003	$7.62x10^{9}$
Bifidobacterium longum 8809	$6.1x10^{7}$
Bifidobacterium pseudocatunelatum 210	$4.86x10^{6}$
Bifidobaterium bifidum 80	$2.19x10^{8}$
Bifidobacterium animalise 506	$4.38x10^{11}$

2.14. Taqman gene expression analysis

2.14.1. RNA Extraction

Frozen tissue was defrosted at 4°C and briefly homogenised using a scalpel. The homogenate was transferred to an MPBio Lysing Matrix E bead beating tube containing RNAbee (AMSBio, Cambridge, MA, USA) and homogenised using a TissueLyser LT. Extraction of RNA was performed using a phenol/choloroform method followed by purification of RNA using the SV Total RNA Isolation System (Promega) according to manufacturer's instructions. RNA concentration and purity was analysed on a Nanodrop 2000 system (Thermofisher) to assess A260/280 and A260/230 ratios. Samples were stored at -80°C until ready to analyse.

2.14.2. Reverse Transcription PCR and Quantitative Real Time PCR

Preparation of cDNA was performed with 1µg of isolated RNA from 2.15.1 using MMLV-Superscript (Sigma) for a final cDNA concentration of 0.5ng/µl. Taqman quantitative real-time PCR (qRT-PCR) was carried out using 5ng cDNA for genes of interest (GOI) and 1ng cDNA for 18s rRNA controls with 8.33µl Taq Mix (PCR Bio, London, UK) and 1.25µl of primer probe mix in a 25µl reaction volume. Reactions were performed using a 7500 Fast Real Time PCR System (Applied Biosciences, Foster City, CA, USA) with the following conditions:

2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. All probes were obtained from Applied Biosciences and are detailed in Table 2.6.

Table 2.6 – List of probes used in Taqman gene expression analysis

Gene Name	Probe ID
18S	Mm03928990_g1
IL17A	Mm00439618_m1
IL17B	Mm01258783_m1
IL17C	Mm00521397_m1
IL17D	Mm01313472_m1
IL17E	Mm00499822_m1
IL17F	Mm00521423_m1

2.15. Scratch Wound Assays

B6BO1 cells were cultured according to 2.5.1 and 200,000 cells were plated into each well of a 6 well dish (Triple Red, Buckinghamshire, UK). Cells were cultured until 100% confluent before three wounds were administered to each well using a sterile P200 pipette tip. Phase contrast images were taken immediately and cells were incubated for 24 hours at 37°C and 5% CO_2 in the indicated concentration of antibiotic before another image of each wound was taken. Wound closure was assessed by pixel comparison of wound size at each time point across three fields per wound.

2.16. Cytokine Arrays

Tumour cytokine expression was assessed using the Mouse XL Cytokine Array (R&D, Bio-Techne, MN, USA) according to the manufacturer's instructions. Membranes were imaged using a FujiFilm LAS-3000 darkbox.

2.17. RNA Sequencing

Whole tumour RNA was extracted as in 2.14.1 Extracted RNA was then quality checked and quantified using a 2100 Bioanalyzer (Agilent) with an RNA 6000 Nano analysis kit (Agilent) and any samples with a RIN value of >8 were considered for use in sequencing. Suitable samples were sent to the Wellcome Trust Sanger Institute for sequencing. All samples were processed by poly-A selection and then sequencing using non-stranded, paired end protocol. Initial processing was performed at Welcome Trust Sanger Institute as follows. Data demultiplexed and adapter removed. Raw reads quality controlled using FastQC (0.11.3, [564]) and trimmed (phred score > 30) using FASTX (0.1.5, [560]). This

was followed by read alignment to mouse reference genome (NCBI Mus musculus GRCm38) using Tophat (2.1.1, [565]) using maximum intron size 500.000 bp and default settings. Aligned transcripts were assembled and quantified using Cufflinks (2.1.0, [565]) (applying standard parameters).

At QIB, read alignment and quantification was performed using Kallisto [566]. The quantified read data was then normalised and differential expression analysis was conducted using DeSeq2 [567].. Transcript IDs were annotated using the Ensembl Biomart database. Significantly up and down regulated genes (p_{adj} <0.05) were used to perform biological process and pathway analysis using DAVID. Biological processes were annotated according to the GO_TERM_BP_ALL database and pathway analysis was performed using KEGG pathways. Significantly enriched pathways were determined by an enrichment score of less than 0.05, however scores of less than 0.1 were also evaluated as non-significantly altered pathways and processes.

3. Treatment with broad spectrum antibiotics results in microbial dysbiosis that compromises the anti-tumour immune response in breast cancer

Treatment with antibiotics (ABX) is an essential component of cancer therapy. As alluded to in chapter 1, several chemotherapies result in gut mucosal inflammation that is resolved after ABX administration [553]. Furthermore, the underlying mechanisms of chemotherapeutic drugs can lead to patients becoming neutropenic and therefore dangerously immunocompromised. These patients are frequently treated with a combination of colony stimulating factors and ABX to prevent opportunistic infection [568]. The same principles are also applied to patients undergoing surgical resection of their tumours, particularly in BC. In women who are undergoing mastectomy, prophylactic administration of ABX is recommended in many cases and in those who are also undergoing breast reconstruction, prophylaxis is essential [53]. Whilst being effective at preventing infection, ABX are frequently used without any clinical understanding of how they may be impacting the patient's disease. Recently, there have been several descriptions in preclinical models of how ABX use may be impairing chemotherapy and immune checkpoint inhibitors [552], [554], [556]. More worryingly, these findings appear to translate to human disease and the use of ABX has been associated with worsened prognosis in some cancers [555]. As yet, the impact of ABX induced microbial dysbiosis has not been evaluated in BC. To address this, we subjected animals harbouring two different BC tumour models to varying ABX regimens. We observed increases in tumour volume across all BC models and ABX regimens. Additionally, increased tumour volume was accompanied by microbial dysbiosis and ineffectual anti-tumour responses. This chapter discusses these findings in detail.

3.1. Characterisation of the murine breast cancer cell lines B6BO1 and EO771 reveals their differential receptor status

The phenotype of BC tumours is an important prognostic tool and often guides the treatment regimen a patient will receive. In addition, the phenotype of a patient's tumour has been shown to influence the composition of the intratumoural immune milieu. When comparing tumour infiltrating leukocyte (TIL) infiltration across tumour subtypes, significantly reduced infiltrate is seen in luminal subtypes when compared to basal cancers. This is likely explained by the differences in receptor status across these tumours; many luminal tumours express one or both of ER or PR, whereas basal tumours are frequently TN [35]. In fact, the presence of ER in tumours has been shown as a key determinant in the extent of tumour infiltrating immune cells. Hormone receptor (HR)+ tumours generally have a lower level of immune infiltrate when compared to TNBC or HER2+ tumours [569]. Additionally, increased numbers of intratumoural leukocytes in the latter subtypes results in incremental improvements to overall survival. However, this effect is not seen in HR+ breast tumours suggesting that, not only do the molecular subtypes influence TIL infiltration, but also regulate differential impacts of TIL infiltration [570]. To this end, it was essential for our analyses to span multiple BC tissue types. We employed the use of two different models, the B6BO1 cell line which is a luminal B cancer derived from MMTV-PyMT animals and the EO771 cell line, isolated from spontaneous, basal tumours in C57BL/6 animals. Whilst the HER2 status was known to be negative according to the collaborators which supplied the cells, the ER and PR status was unknown. Therefore, we undertook Western blot analysis of whole tumour protein lysates to determine their hormone receptor status (Figure 3.1A&B). Both were conclusively ER+ and the EO771 cells were PR-, however the PR status of the B6O1s was unclear. To support our Western blot analysis, we also undertook immunocytochemistry to assess the quantities of each receptor in cultured cells. This analysis reiterated the results of the Western blot and confirmed to us that the B6BO1 cell line is expressing PR (Figure 3.1C).

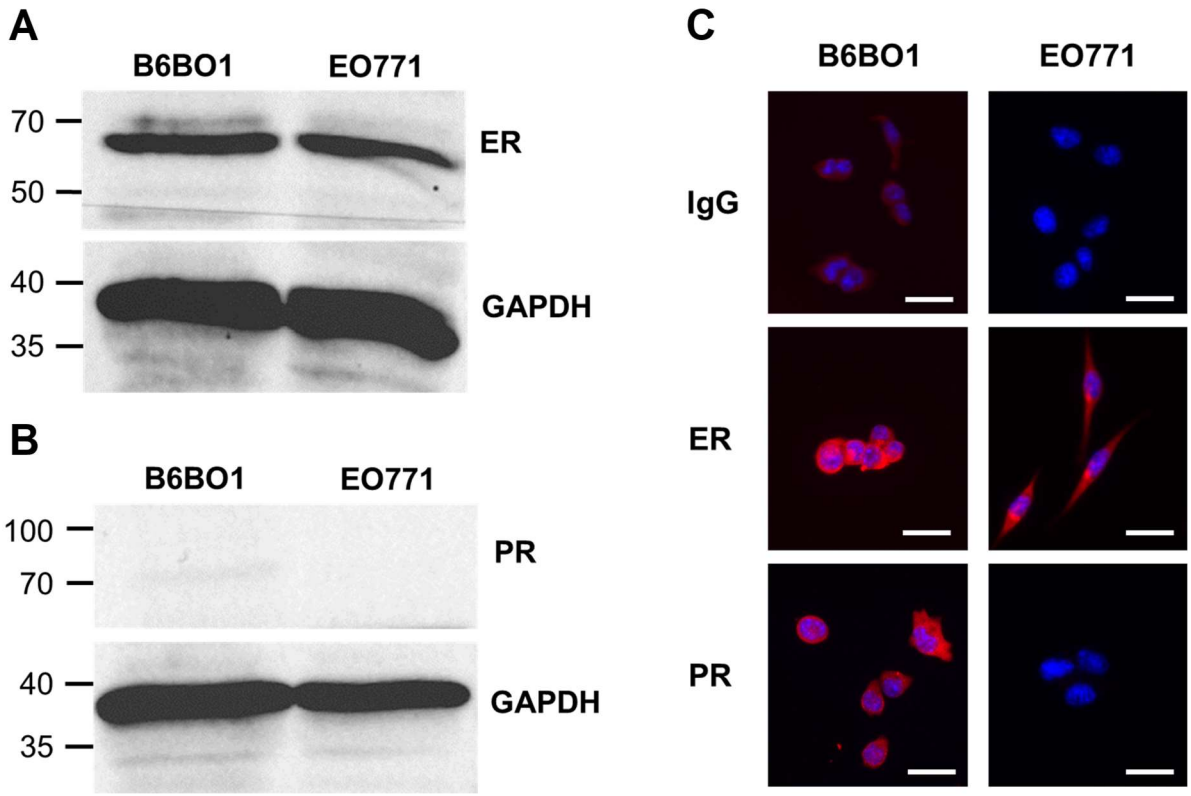


Figure 3.1 – Hormone receptor status of B6BO1 and EO771 murine BC cell lines: A+B) Whole tumour lysate was western blotted to determine the presence of either A) Estrogen receptor (ER) or B) Progesterone receptor (PR). GAPDH was used as a loading control and scale is in kDA. **C)** Representative images of immunocytochemistry to evaluate the presence and distribution of HRs in cultured cells. Staining was evaluated against an isotype IgG control. Scale bar = 100μm.

3.2. Treatment with broad spectrum antibiotics results in accelerated tumour growth across both tumour models and with different antibiotic regimens

There is significant evidence to suggest that administration of ABX results in significant and lasting impacts on microbial diversity in the gut. Knowing that the gut microbiota plays a key role in guiding immune responses, other groups have shown that ABX mediate disruption of the gut microbiota results in an impaired anti-cancer immune response. However as yet, no data exists to probe whether there is a relationship between the health of an individuals' microbiota and the extent of breast tumour growth. To evaluate this potential association, we tested the breast cancer cell lines detailed above in an orthotopic injection model. To determine the extent of the microbiota's involvement, animals were administered a harsh cocktail of antibiotics (VNMA) designed to severely disrupt the microbiome. Treatment with ABX was initiated 5 days before tumour cell injection to establish a microbial dysbiosis and maintained throughout the experiment (Figure 3.2A). In both tumour models, a significant increase in tumour volume was observed at the end point of the experiment by *ex vivo* calliper measurements (Figure 3.2, C&D). This acceleration of tumour growth was accompanied by an almost complete knockdown of the gut microbiome. Fecal DNA extractions from animals treated with the VNMA cocktail at day 5 and 22 yielded very low concentrations of DNA. When using this DNA to conduct 16S rRNA PCR, we were unable to amplify the 16S region (Figure 3.2E). This inability to detect bacterial signatures in a sensitive molecular assay suggests the extent of the microbial knockdown as a result of VNMA administration is extremely robust.

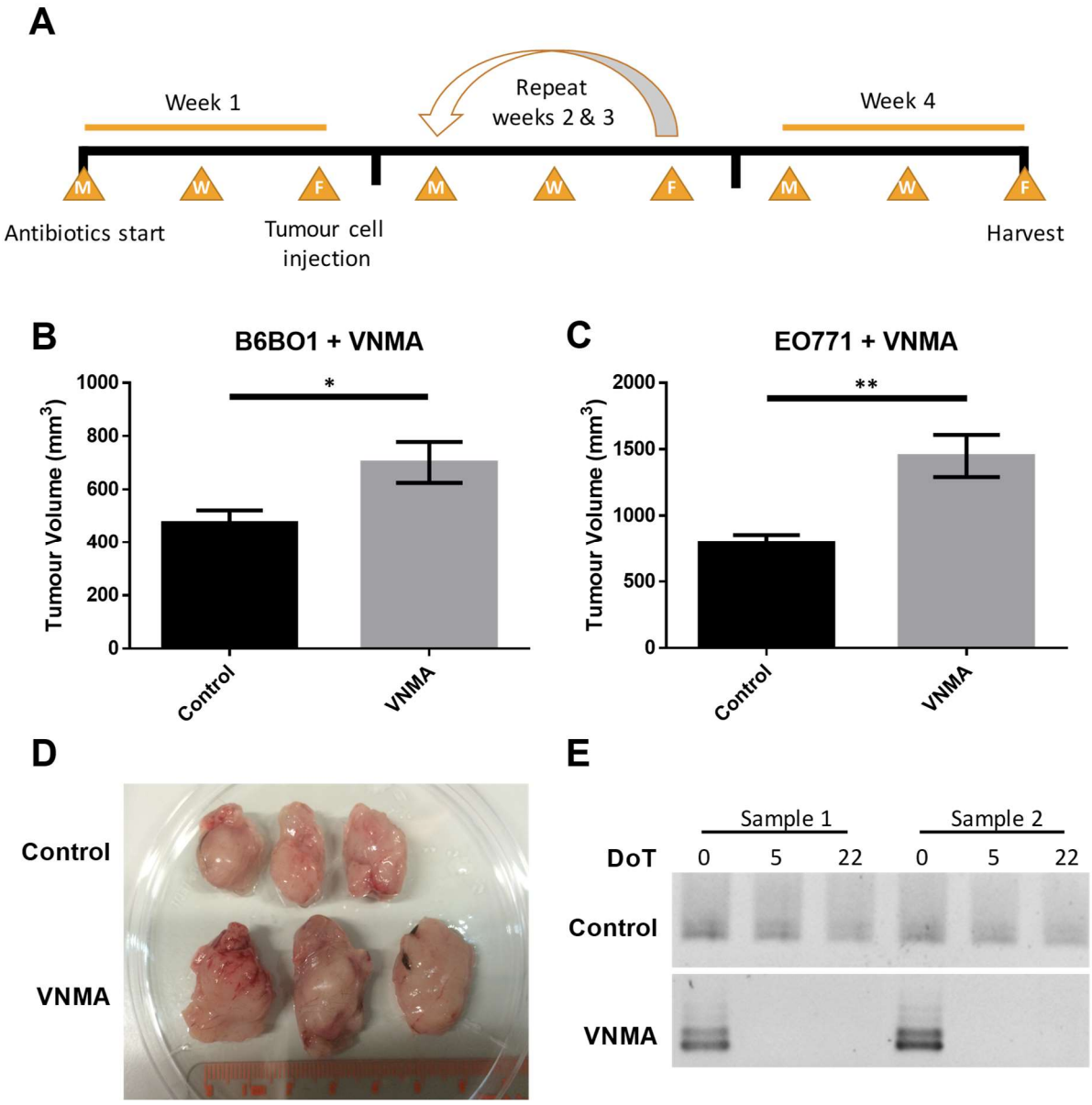


Figure 3.2 – Antibiotic administration results in accelerated tumour growth and comprehensive depletion of the gut microbiota: A) Schematic of VNMA ABX regimen detailed in 2.1.1, animals are dosed 5 days before tumour injection and three times weekly for the duration of the experiment. **B&C)** Animals subjected to VNMA were injected with either B6BO1 or EO771 cells in the inguinal mammary fat pad. Graphs show mean ± SEM of tumour volume at final time point, 14 and 26 days post injection respectively (n=15 for B6BO1 and ≥ 6 for EO771). Asterisks indicate significance: * $p<0.05$, ** $p<0.01$, calculated by unpaired, two-tailed *t* test. **D)** Photograph of representative B6BO1 tumours excised from water treated control and VNMA treated animals. **E)** Extracted fecal DNA underwent PCR to amplify the V1 and V2 regions of 16S rRNA. Samples were visualised on an agarose gel, image shows representative samples from two biological replicates from varying days of antibiotic treatment (DoT).

3.3. Low level profiling of intratumoural leukocytes reveals no differences in immune cell numbers after VNMA treatment

To begin to dissect the immune contributions to accelerated tumorigenesis after VNMA treatment, we undertook wide profiling of general immune populations in the tumour, spleen and mesenteric lymph nodes. The latter was used a proxy for changes in gut associated lymphoid tissues as attempts to profile gut mucosal tissues and Peyer's patches were unsuccessful. Our analysis was split into two staining panels, the first designed to evaluate changes in myeloid cell infiltration. The markers used in this panel identified neutrophils and macrophages by Ly6G and F4/80 expression respectively. Macrophages were further characterised according to their M1/M2 polarisation by use of the surface markers MHCII and CD206. This myeloid staining panel was only applied to the tumour due to the relatively low numbers of these cell types in peripheral lymphoid tissues.

The general characteristics of the tumour immune compartment reveal that B6BO1 tumours are typically between 20-30% leukocytes. Surprisingly though, the majority of these cells belong to the myeloid lineage. The leukocyte populations were typically comprised of between 80-90% CD11b+ myeloid cell populations. However, we were unable to detect any stastically significant changes in the proportion of tumour infiltrating myeloid cells at any level (Figure 3.3A). In addition to profiling the number of cells presenting M1/M2 markers, we also quantified the amount of MHCII or CD206 present at the cell surface in the F4/80+ subset by median fluorescence intensity (MFI). However, we found no difference in the ratio of M1 to M2 cells between the control and VNMA treated groups (Figure 3.3B). Additionally, we utilised a second panel designed to identify lymphoid populations including T helper cells, CTLs and NKs. The latter was unable to be evaluated due to non-specific staining of tumour cells resulting in an aberrantly inflated population size. Consistent with our observations of high myeloid cell numbers amongst the CD45+ population, the proportion of T cells was usually between 3-5% but was highly variable between individual samples. The distribution of CD4/CD8 cells was generally skewed towards CD4 cells with ratios of ~60:40. However, our analyses revealed no differences in the total number of CD3+, CD4+ or CD8+ T cells infiltrating the tumour between control and VNMA treated groups (Figure 3.3D). To extend our analyses to assess ABX mediated changes to systemic immunity, we also profiled the mLN and splenic lymphoid populations but also found no significant differences between groups (Figure 3.3C&E).

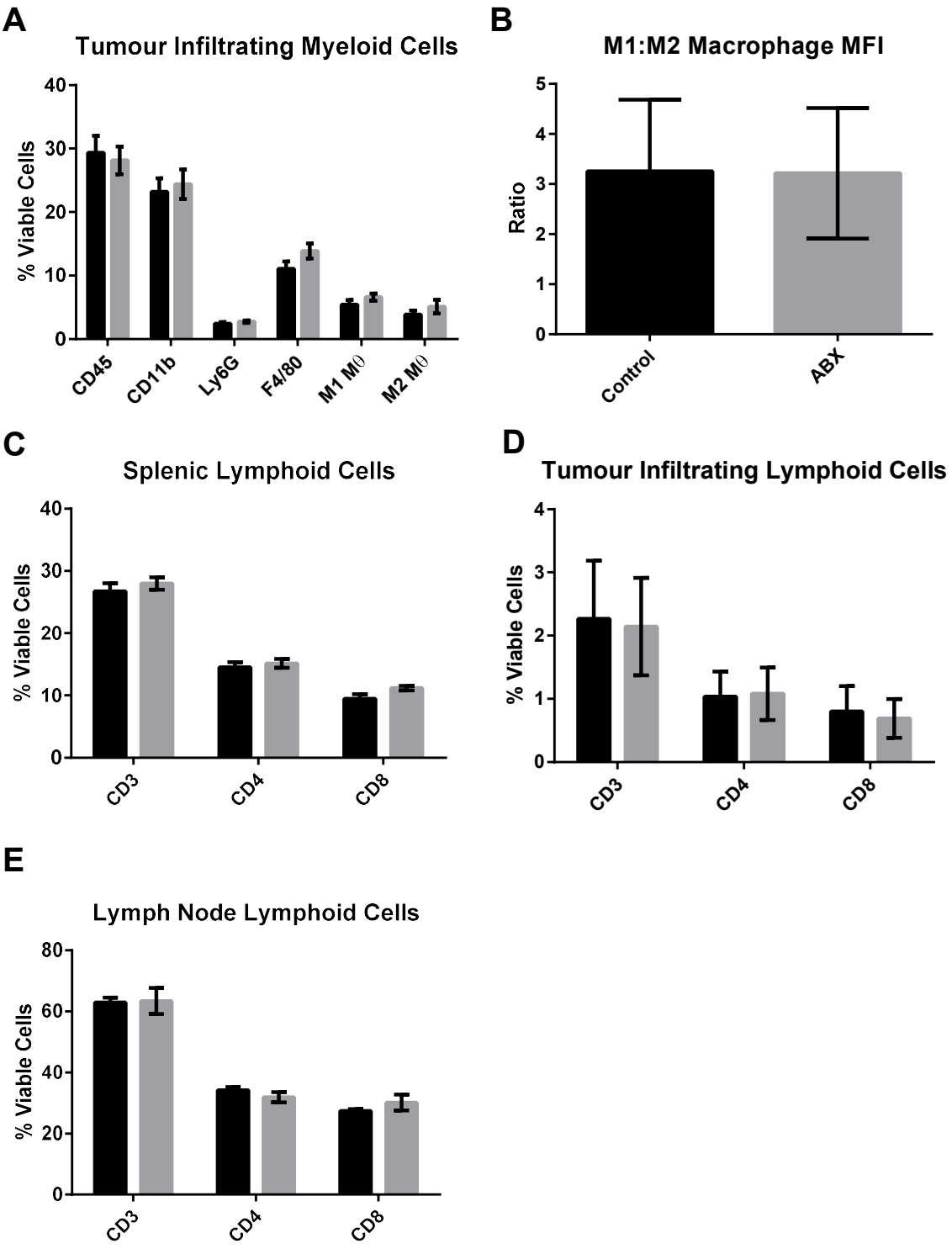


Figure 3.3 – Flow cytometric analysis of immune populations in the tumour, spleen and mLN of animals treated with VNMA ABX: Multi-colour flow cytometric data comparing immune populations in the indicated tissues. Bars represent the mean percentage of each cell type normalised to the total number of cells in each tissue (mean ±SEM from 3 independent experiments, n ≥ 11 for tumour, n=7 for spleen and n ≥ 5 for mLN) All means show non-significant differences by unpaired, two-tailed *t* test.

3.4. Unbiased intratumoural cytokine analysis by membrane-based array reveals several differentially produced proteins after VNMA treatment

After discouraging flow cytometric analyses, we sought to understand how the cytokine environment of the tumours may be differing between treatments. Unfortunately, bona fide proteomic approaches such as mass spectrometry were not possible due to cost. Therefore, we instead chose cytokine profiling arrays, allowing us to quantify production of over 100 cytokines. Whole tumour lysates from control or VNMA treated samples were used in each array. Of the 111 analytes, 73 were detected in at least 2 samples of one experimental condition. Of those that were consistently detectable, 15 were significantly differentially expressed, all of which were downregulated in tumours harboured by VNMA treated animals. Antibiotic treated tumours showed a general reduction in pro-inflammatory cytokine production across several branches of immunity (Figure 3.4A&B). A significant reduction is seen in secreted cytokines involved in macrophage and neutrophil recruitment such as CCL6 and CXCL16. Additionally, reduced levels of ICAM-1 are observed, a key mediator of leukocyte adhesion and recruitment from the vasculature. Reductions are also seen in cytokines associated with immune cell activation. Levels of IL-33 are significantly reduced, as is CD40, a key activating receptor found on antigen presenting cells. Interestingly, proteins involved in anti-microbial activities were also reduced, including the opsonising protein Reg3γ and the complement component 1q receptor (C1q R1), complement factor D (CFD) and C reactive protein (CRP).

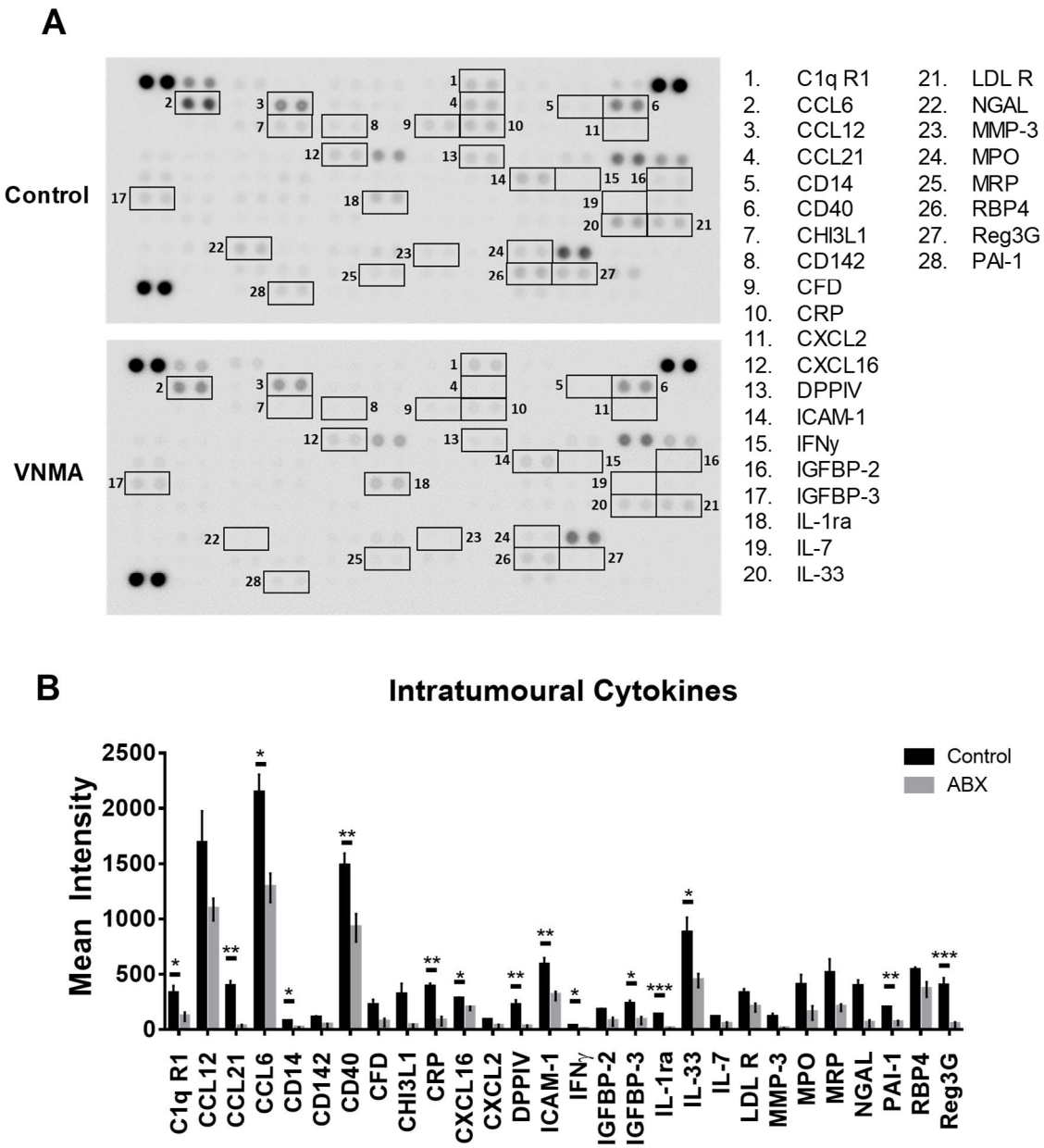


Figure 3.4 – Unbiased cytokine arrays reveal differentially produced intratumoural cytokines with VNMA treatment: Whole tumour protein lysates from animals harbouring B6BO1 tumours undergoing VNMA treatment were applied to cytokine profiling arrays. **A)** Representative images of arrays comparing control and VNMA treated tumours with identification of differentially regulated cytokines. **B)** Quantification of protein array images showing differentially regulated cytokines. Bars represent means ± SEM of pixel intensity measurements from two 'spots' per cytokine. Data taken from two independent experiments, n≥6 in both conditions. Asterisks represent statistical significance, *** $p<0.001$, ** $p<0.01$, * $p < 0.05$. Bars without asterisk represent $p < 0.1$. Calculated by unpaired, two-tailed *t* test.

3.5. Expanded profiling of intratumoural and intestinal cytokine production demonstrates microbial dysbiosis has a profound impact on cytokine regulation in the gut

The dysregulation of intratumoural cytokines we observed by protein array was encouraging, however the arrays suffer from two major limitations: 1) They are only semi-quantitative; 2) They are not particularly sensitive. We considered that we may be missing key pieces of information if certain, critical cytokines were not detectable in the arrays. Additionally, given we were unable to assess mucosal immune cell populations, we felt it was essential to profile intestinal cytokine production. We achieved this by using MSD V-PLEX arrays. This technique allows for screening of a relatively small number of cytokines but has the advantage of being incredibly sensitive, detecting concentrations less than 1pg/mL for most analytes. Using whole tissue protein extracts, we profiled cytokine production in both the tumour and large intestine. Intratumoural cytokines were not significantly changed after VNMA treatment (Figure 3.5A), however the large intestine showed significant downregulation of the pro-inflammatory cytokines CXCL1, IL-1β, IL-2 and TNFα (Figure 3.5B), indicating that the immune processes of gut associated lymphoid tissues are compromised by VNMA induced microbial dysbiosis.

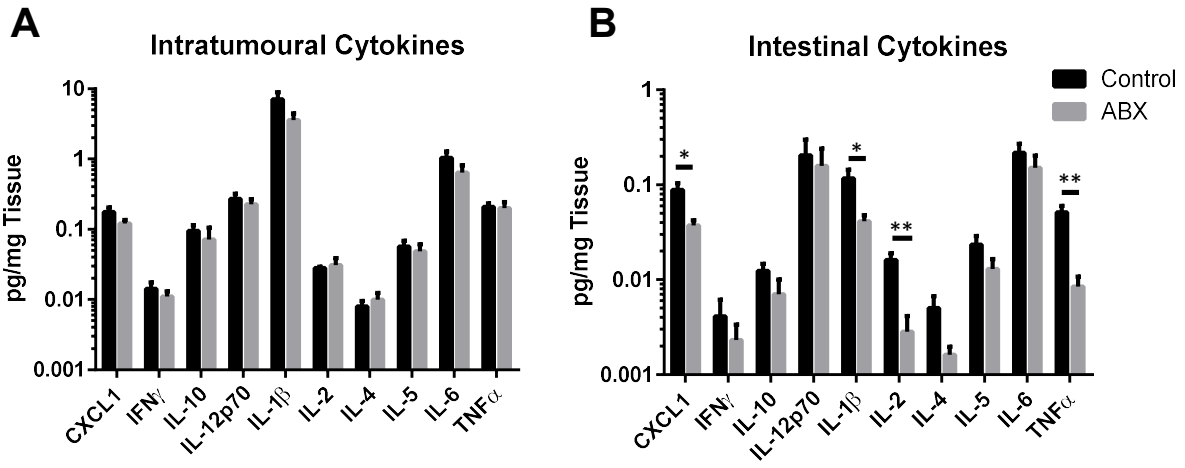


Figure 3.5 – Wider analysis of cytokine production reveals significantly downregulated pro-inflammatory cytokine production in the large intestine, but no changes intratumorally: Whole tissue protein extracts from either tumour (A) or large intestine (B) were analysed for cytokine production by MSD V-PLEX assay. Detected cytokine quantities were normalised against tissue weight in both cases. Bars show mean ± SEM, n ≥ 6. Asterisks indicate statistical significance, * $p<0.05$, ** $p<0.01$, determined by unpaired, two-tailed *t* test.

3.6. Microbial dysbiosis results in upregulation of IL-17 production in breast tumours

Despite some leads generated by the cytokine data from our arrays and MSD assays, we were still unclear as to the potential driving mechanisms of our antibiotic phenotype. Therefore we focused our attention on production of IL-17A as its overproduction in breast tumours has been demonstrated to negatively correlate with prognosis and has also been shown to drive tumorigenesis[337], [571]. Additionally, production of IL-17A is strongly associated with dysbiosis of the gut microbiome [572], [573]. To assess IL-17A levels in our tumours we undertook ELISAs on whole tumour protein lysates and found production to be significantly upregulated in B6BO1 tumours from VNMA treated animals. Interestingly, we were unable to detect IL-17A in any of our EO771 tumours (Figure 3.6A, EO771 data not shown). The IL-17 family consists of 6 isoforms and several have differential functions. We were curious to assess whether any other isoforms were being affected by VNMA treatment in B6BO1 tumours. Unfortunately, established protein-based assays for all isoforms do not exist, therefore we instead undertook TaqMan® qPCR using whole tumour RNA to evaluate their expression levels. Surprisingly, we were unable to detect mRNA transcripts of the *IL17A* gene. The only isoforms which were consistently detectable were *IL17C* and D, the former being significantly elevated in VNMA treated B6BO1 tumours (Figure 3.6B&C).

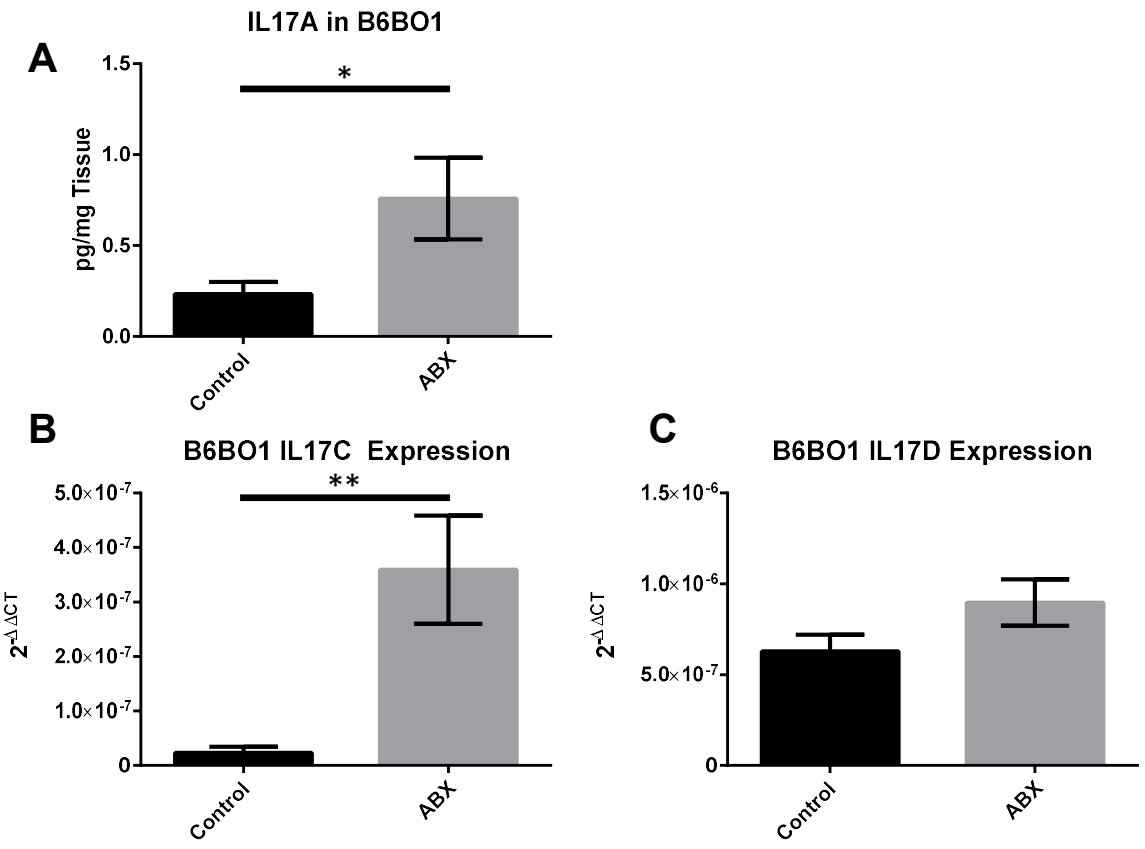
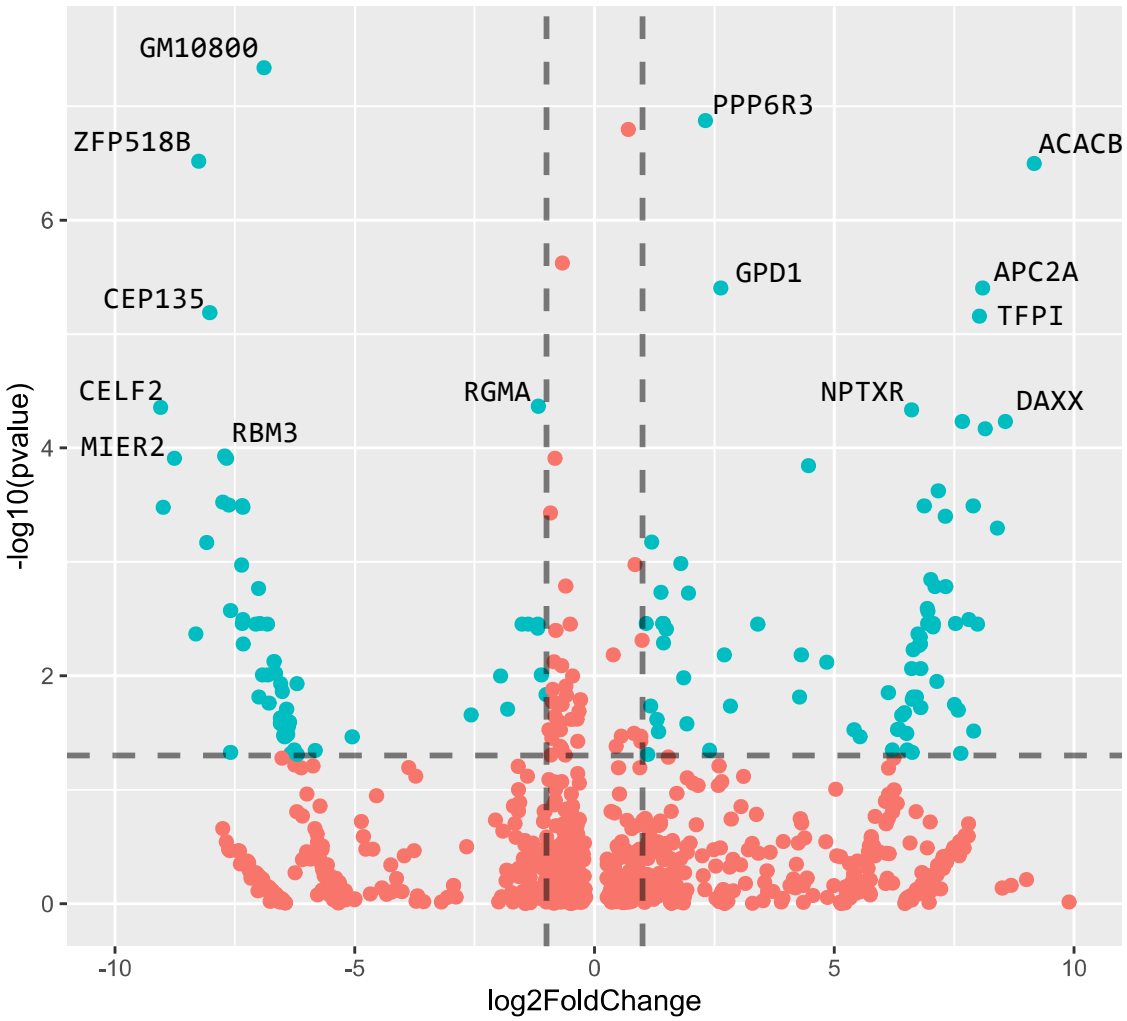


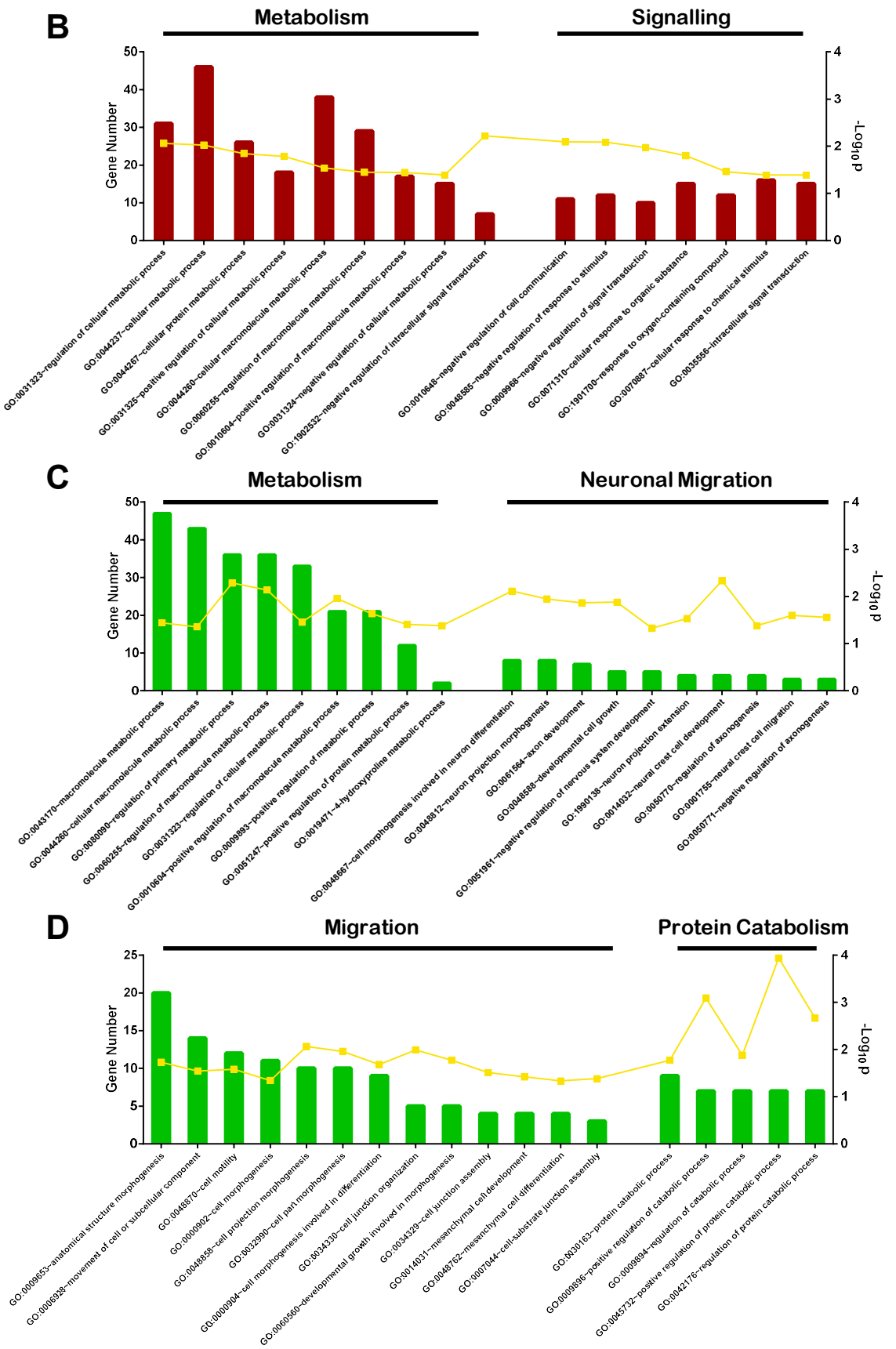
Figure 3.6 – Members of the IL17 family are upregulated in B6BO1 tumours from VNMA treated animals: (A) Levels of IL-17A were assessed at the protein level by ELISA of whole tumour protein lysates. Bars show mean concentration ± SEM of two independent experiments (n ≥ 20 per group). Amount of detected IL-17A was normalised to tissue weight. **B & C)** Expression levels of IL-17 family members were assessed by qPCR of whole tumour RNA extracts. *IL17A*, B, E and F were not consistently detectable and the data is therefore not shown. Bars show mean ± SEM of two independent experiments (n ≥ 9 in both groups). Asterisks indicate statistical significance, * $p<0.05$, ** $p<0.01$. Significance calculated by unpaired, two-tailed *t* test.

3.7. Transcriptomic analysis of tumours from VNMA treated animals reveals differential regulation of several biological processes

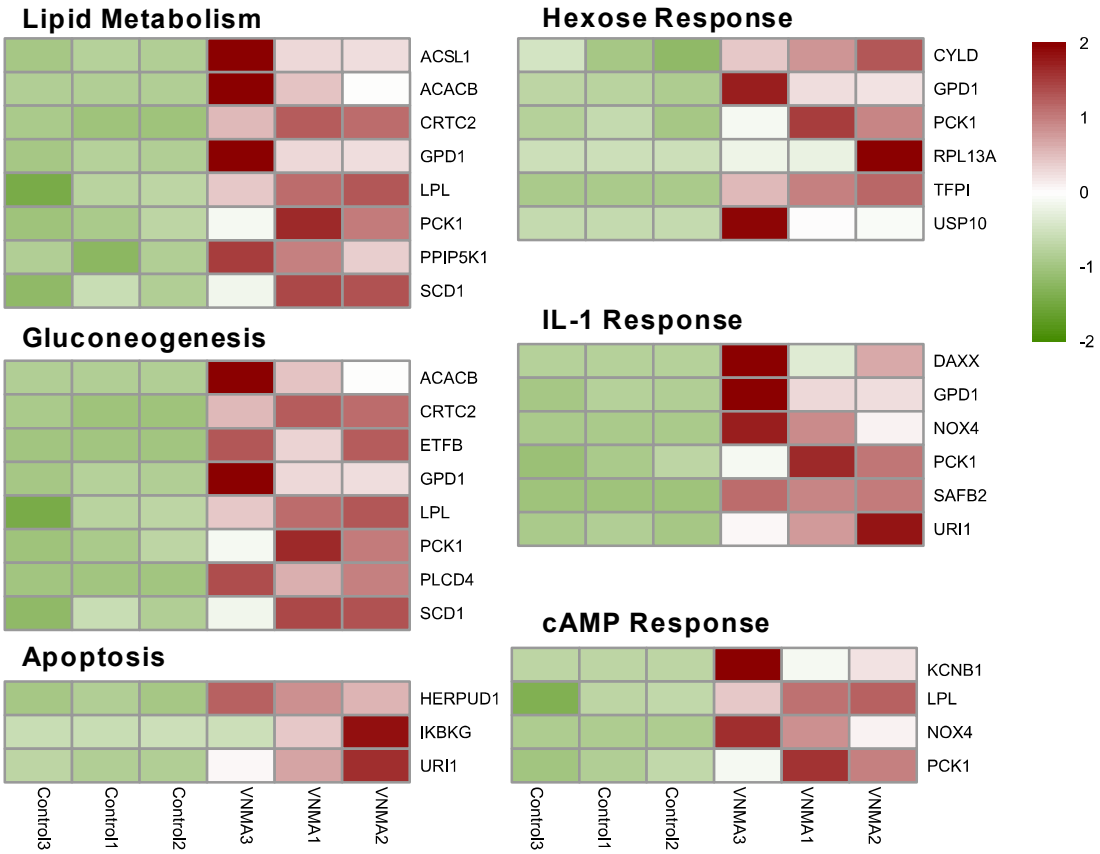
To probe the impact of antibiotic treatment on intratumoural processes, whole tumour RNA extracts were sequenced by the Wellcome Trust Sanger Institute using the Illumina HiSeq v4 platform. For details of bioinformatic analysis see 2.8.2.2. Transcript IDs were annotated according to mouse reference genome GRCm38.p6 and graphed using R. Volcano plot analysis using cutoff values of Log2FC > 1 and p_{adj} <0.05; this yielded 176 differentially expressed genes, 87 downregulated and 89 upregulated in the antibiotic treated samples relative to the controls (
Figure 3.7A). A full list of differentially regulated genes is available in Appendix Figure 7.2. To assess the biological impact of this differential regulation, up- and down-regulated gene sets were used to probe biological process enrichment using DAVID. Enrichment was determined using an EASE score of <0.05 and having >2 genes associated with the process in the dataset. This analysis yielded 85 significantly upregulated processes and 44 significantly downregulated processes. A full list of biological process enrichment can be found in Appendix Table 7.1 & Table 7.2. Parent/child analysis of biological process trees was used to cluster processes according to their overarching biological function and those functions with >5 enriched processes were deemed to have biological relevance (
Figure 3.7B,C&D). Surprisingly, the most significantly enriched biological processes were those with a metabolic function accounting for 26 of the 85 upregulated processes and 21 of the 44 downregulated processes. Additionally, processes associated with cell signalling were significantly upregulated and migratory processes were significantly downregulated in the antibiotic treated animals. The individual genes contributing to the highest order biological processes within each function are presented by heatmapping in
Figure 3.7E&F. This analysis revealed regulation of multiple metabolic processes including upregulation of lipid metabolism and gluconeogenesis in antibiotic treated animals (
Figure 3.7E). Additionally, signalling responses to several molecules such as hexose, IL-1 and cAMP were upregulated, as were genes which negatively regulate apoptosis. Protein metabolic processes were downregulated in VNMA treated tumours in addition to pathways controlling cell migration (
Figure 3.7F).

A


GM10800
ZFP518B
CEP135
CELF2
MIER2
RBM3
RGMA
PPP6R3
GPD1
ACACB
APC2A
TFPI
NPTXR
DAXX
-log10(pvalue)
log2FoldChange
0
2
4
6
-10
-5
0
5
10


B
Metabolism
Signalling
Gene Number
-Log10 p
GO:0031323~regulation of cellular metabolic process
GO:0044237~cellular metabolic process
GO:0044267~cellular protein metabolic process
GO:0031325~positive regulation of cellular metabolic process
GO:0044260~cellular macromolecule metabolic process
GO:0060255~regulation of macromolecule metabolic process
GO:0010604~positive regulation of macromolecule metabolic process
GO:0031324~negative regulation of cellular metabolic process
GO:1902532~negative regulation of intracellular signal transduction
GO:0010648~negative regulation of cell communication
GO:0048585~negative regulation of response to stimulus
GO:0009968~negative regulation of signal transduction
GO:0071310~cellular response to organic substance
GO:1901700~response to oxygen-containing compound
GO:0070887~cellular response to chemical stimulus
GO:0035556~intracellular signal transduction
C
Metabolism
Neuronal Migration
Gene Number
-Log10 p
GO:0043170~macromolecule metabolic process
GO:0044260~cellular macromolecule metabolic process
GO:0080090~regulation of primary metabolic process
GO:0060255~regulation of macromolecule metabolic process
GO:0031323~regulation of cellular metabolic process
GO:0010604~positive regulation of macromolecule metabolic process
GO:0009893~positive regulation of metabolic process
GO:0051247~positive regulation of protein metabolic process
GO:0019471~4-hydroxyproline metabolic process
GO:0048667~cell morphogenesis involved in neuron differentiation
GO:0048812~neuron projection morphogenesis
GO:0061564~axon development
GO:0048588~developmental cell growth
GO:0051961~negative regulation of nervous system development
GO:1990138~neuron projection extension
GO:0014032~neural crest cell development
GO:0050770~regulation of axonogenesis
GO:0001755~neural crest cell migration
GO:0050771~negative regulation of axonogenesis
D
Migration
Protein Catabolism
Gene Number
-Log10 p
GO:0009653~anatomical structure morphogenesis
GO:0006928~movement of cell or subcellular component
GO:0048870~cell motility
GO:0000902~cell morphogenesis
GO:0048858~cell projection morphogenesis
GO:0032990~cell part morphogenesis
GO:0000904~cell morphogenesis involved in differentiation
GO:0060560~developmental growth involved in morphogenesis
GO:0034330~cell junction organization
GO:0034329~cell junction assembly
GO:0014031~mesenchymal cell development
GO:0048762~mesenchymal cell differentiation
GO:0007044~cell-substrate junction assembly
GO:0030163~protein catabolic process
GO:0009896~positive regulation of catabolic process
GO:0009894~regulation of catabolic process
GO:0045732~positive regulation of protein catabolic process
GO:0042176~regulation of protein catabolic process

E


Lipid Metabolism
ACSL1
ACACB
CRTC2
GPD1
LPL
PCK1
PPIP5K1
SCD1
Hexose Response
CYLD
GPD1
PCK1
RPL13A
TFPI
USP10
Gluconeogenesis
ACACB
CRTC2
ETFB
GPD1
LPL
PCK1
PLCD4
SCD1
IL-1 Response
DAXX
GPD1
NOX4
PCK1
SAFB2
URI1
Apoptosis
HERPUD1
IKBKG
URI1
cAMP Response
KCNB1
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Control3
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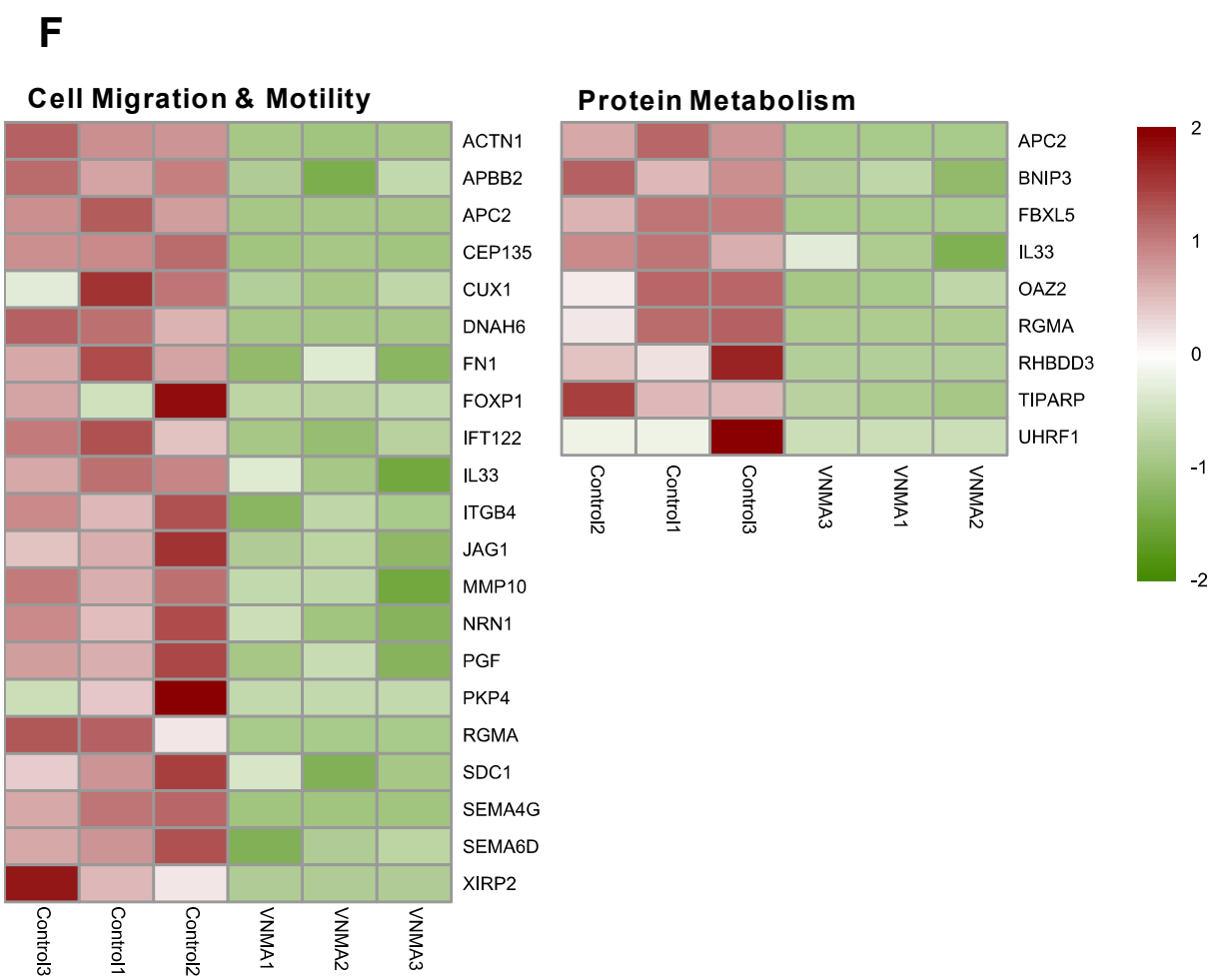


Figure 3.7 – Sequencing of whole tumour RNA reveals differential gene regulation across several biological processes: A) Volcano plot showing significantly up and down regulated genes, determined by a fold change cutoff of >1 and p_{adj} value of <0.05. **B, C, D)** Significantly up (B) and down regulated genes (C&D) were used in biological process enrichment analysis revealing regulation of multiple biological processes. Biological processes are presented according to their Gene Ontology reference and are clustered by their physiological function. Only functions which demonstrated enrichment in >5 GO designations are presented. Full biological process enrichment analysis is presented in Appendix Table 7.1 & Table 7.2. **E&F)** The biological functions by which processes are presented in panels B, C&D were broken down into their highest order GO biological process and the individual genes contributing to each process are presented. E shows processes and genes which are upregulated in the antibiotic treated samples whilst F shows those which are downregulated.

3.8. Administration of VNMA antibiotics results in severe disruption in bacterial metabolite production

As we were unable to amplify 16S rRNA from fecal samples, sequencing of bacterial populations from VNMA treated animals was not possible. Therefore, to gain some insight into how antibiotic treatment may be regulating bacterial function, we undertook analysis of fecal bacterial metabolites and substrates by ^{1}H NMR. Analysis of quantified metabolites was performed using MetaboAnalyst 3.0 [574]. Analysis of sample clustering by PCA revealed distinct grouping of control and VNMA treated samples (Figure 3.8A), however one replicate in the control group was highlighted as an outlier. This was further confirmed in heatmapping of all metabolites across biological replicates (Appendix Figure 7.3). The outlying replicate was found to be aberrantly elevated in 17 of the 51 measured compounds. As outliers can severely impact downstream analysis, this replicate was excluded from our analysis. A volcano plot of the remaining replicates revealed 22 metabolites were significantly differentially regulated (Figure 3.8B). Of these 22 compounds, 12 were enriched in VNMA treated fecal samples. These compounds were largely composed of amino acids (Alanine, Histidine, Aspartate) and substrates for bacterial metabolism, such as Raffinose and Sialic acid. The reverse was true of the depleted compounds which contained a number of bacterial metabolic products such as the SCFAs butyrate and acetate in addition to other fermentation products such as succinate (Figure 3.8C).

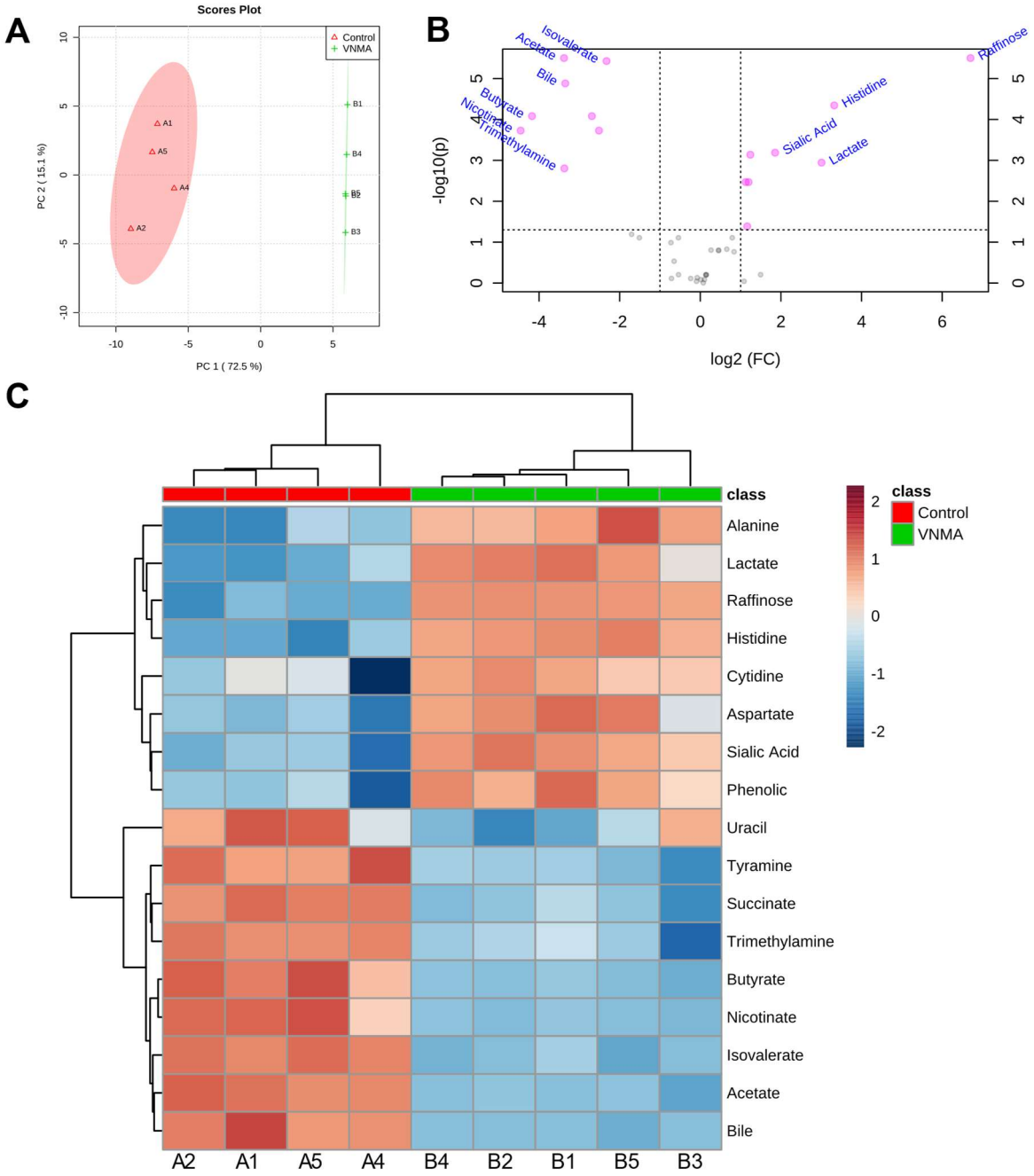


Figure 3.8 – Fecal metabolomic analysis of VNMA treated animals: Fecal metabolites from control and VNMA treated animals harbouring B6BO1 tumours were compared by ^{1}H NMR. One biological replicate had significant outliers in multiple metabolites and was excluded from analysis. Full results of all metabolites are shown in Appendix Figure 7.3. **A)** Two component PCA of included biological replicates. **B)** Volcano plot of remaining replicates; x-axis specifies the Log_2 fold change of VNMA relative to control animals and the y-axis specifies the negative logarithm to the base 10 of the t-test p-values. Dashed lines represent cut off values for differential regulation (FC ± 1, p_{adj} < 0.05). The 10 most significantly regulated metabolites are named. **C)** Filtered heatmap clustered by average Euclidean distance showing only significantly regulated analytes ($p<0.05$), calculated by unpaired, two-tailed *t* test. All graphs produced using MetaboAnalyst 3.0 software.

3.9. Reconstituting VNMA treated animals with a healthy microbiome reverses the pro-tumorigenic effects of antibiotic treatment

In the absence of amplifiable 16S from fecal DNA samples, we are unable to determine which bacterial populations are being impacted by VNMA treatment. As a result, we were unable to evaluate the contribution of the microbiome to accelerated tumorigenesis. Whilst some groups have shown that some bacterial species can improve anti-tumour immune responses and therefore inhibit tumour growth, others have shown some bacteria can act as pathobionts. These species drive tumour growth through promotion of inflammatory responses, particularly through induction of IL-17 production [575]. To address the contribution of the microbiome in our model, we used a co-housing experiment to homogenise the microbiome between experimental groups. Co-housing of mice has been shown to resolve microbiome drifts due to both separate caging and experimental treatments resulting in significant clustering of co-housed animals' when evaluating their microbial diversity [576], [577]. Animals were not directly co-housed due to delivery of antibiotics via drinking water, but co-housing was simulated by transfer of bedding and pellets between cages. The antibiotic treatment regimen was followed as before, beginning five days preceding tumour injection, during which time animals from each experimental condition were housed separately. At tumour injection, water and VNMA treated animals were "co-housed". This was maintained throughout the experiment with bedding swaps every two days and antibiotic treatments were also continued throughout the experiment. Regular re-supplementation with feces from animals with a healthy microbiome led to a significant reduction in VNMA treated tumour volume with respect to a non-cohoused, VNMA treated control (Figure 3.9A). Importantly, exposure to VNMA treated feces did not increase tumour volumes in the water treated co-housed animals, suggesting a pathobiont is not responsible for our observed tumour effects. To investigate whether *IL17C* overexpression is driving these effects, we conducted qRT-PCR on tumour RNA extracts to assess intratumoural expression. Whilst the trend of expression is similar to that of the tumour volumes, none of the comparisons between groups were statistically significant (Figure 3.9B). To assess the contribution of immune cells to the differences in tumour volume, we conducted low level profiling of intratumoural leukocytes, this time extending our analyses to include dendritic cells and T regulatory cells (Figure 3.9C&D, DCs in Figure 3.9E). However, again, no significant differences were seen in the number of immune cells infiltrating the tumours.

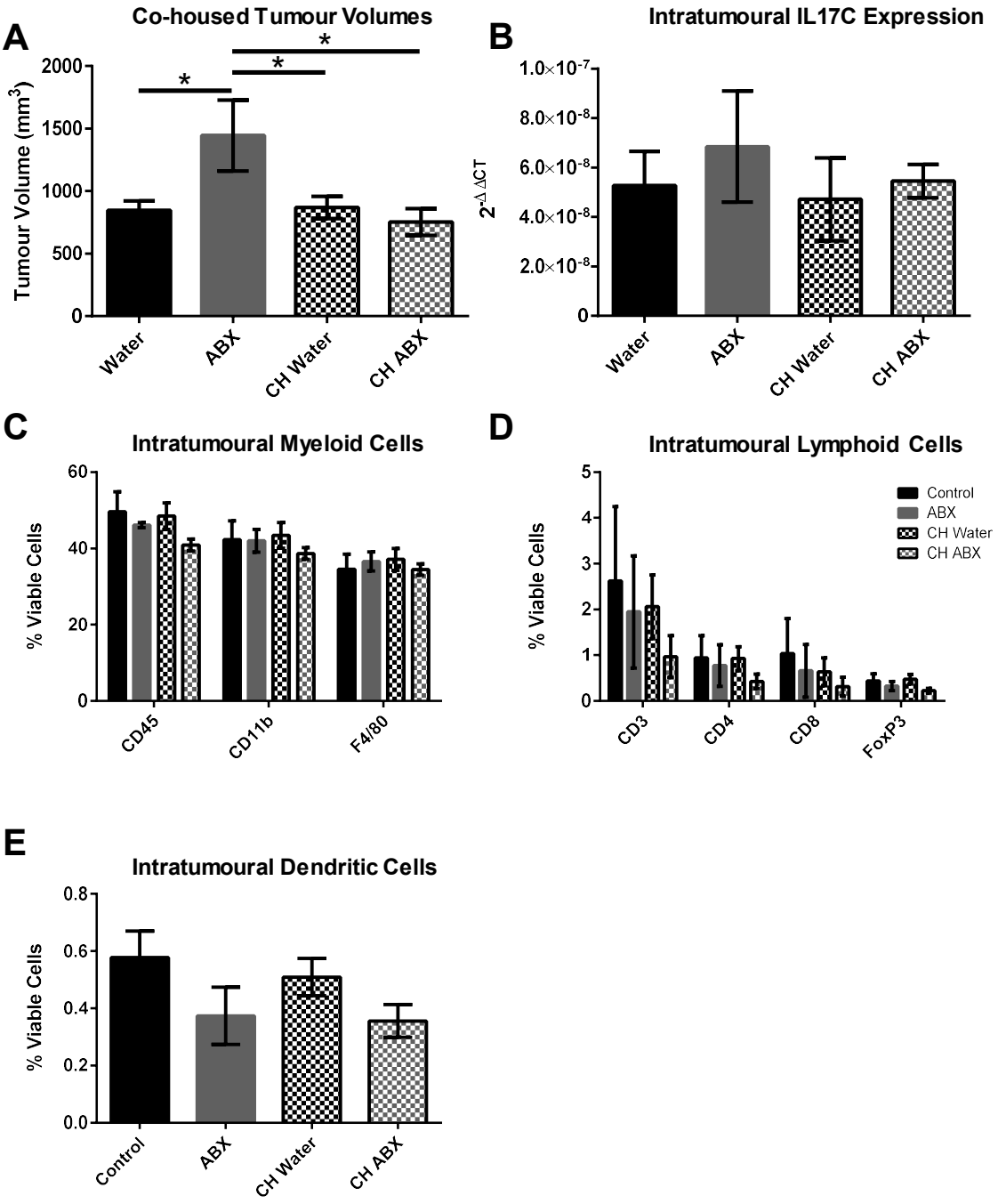


Figure 3.9 – Co-housing water and VNMA treated animals reverses accelerated tumour growth caused by antibiotic treatment: A) *Ex vivo* tumour volumes of animals treated with water or VNMA and housed separately or those which have been housed with the feces from oppositely treated animals (CH water given VNMA feces, CH ABX given water feces). **B)** Intratumoural IL17C expression measured by qRT-PCR of whole tumour total RNA extracts. **C)** Flow cytometric analyses of intratumoural myeloid cell infiltration as a percentage of total intratumoural cells. **D)** As in C) but for lymphoid cell infiltrates. **E)** As in C), but plot only showing dendritic cell infiltrates. All bars represent means of ≥5 biological replicates ±SEM. Asterisks indicate statistical significance, * $p<0.05$, evaluated by unpaired, two-tailed *t* test.

3.10. Use of a single, clinically relevant antibiotic Cephalexin, also results in accelerated tumour growth

Whilst the increased tumour volumes observed after VNMA treatment suggest the microbiota plays a significant role in guiding anti-cancer immune responses, these antibiotics are not conventionally prescribed to BC patients. We sought to alter our antibiotic regimen to include a single antibiotic agent that is routinely prescribed to patients. Our aims were two-fold. Firstly, to improve the clinical relevance of our studies with a milder, patient relevant dose of antibiotics. Secondly, to profile changes in the microbiome via 16S and shotgun whole genome sequencing. We have demonstrated that the VNMA cocktail is not suitable for these analyses due to comprehensive microbial knockdown. Therefore, we hoped administration of a milder antibiotic may preserve 16S fecal DNA and allow for genomic analysis of bacterial populations.

After discussion with a breast oncologist, we were recommended the broad-spectrum cephalosporin, Cephalexin (Keflex®). Keflex is a first generation cephalosporin that is commonly prescribed to patients following mastectomy in the US (where our collaborator is based), particularly in patients who have undergone breast reconstruction surgery or who require post-operative closed suction drains [578], [579]. We administered Keflex at patient relevant concentrations (14mg/kg) to animals harbouring B6BO1 breast tumours. This resulted in significant increases in tumour volume throughout the course of the experiment (Figure 3.10A). To determine if Keflex treatment is suitable for genomic analysis of the microbiota, we isolated fecal DNA from Keflex treated animals and sought to amplify the 16S V1 & V2 regions. In contrast to fecal DNA samples from the VNMA regimen, in which we were unable to detect any 16S, Keflex treated animals had detectable 16S in their feces up to 22 days after antibiotic treatment began (Figure 3.10B&C). These samples were used in all downstream analysis of gut microbial composition.

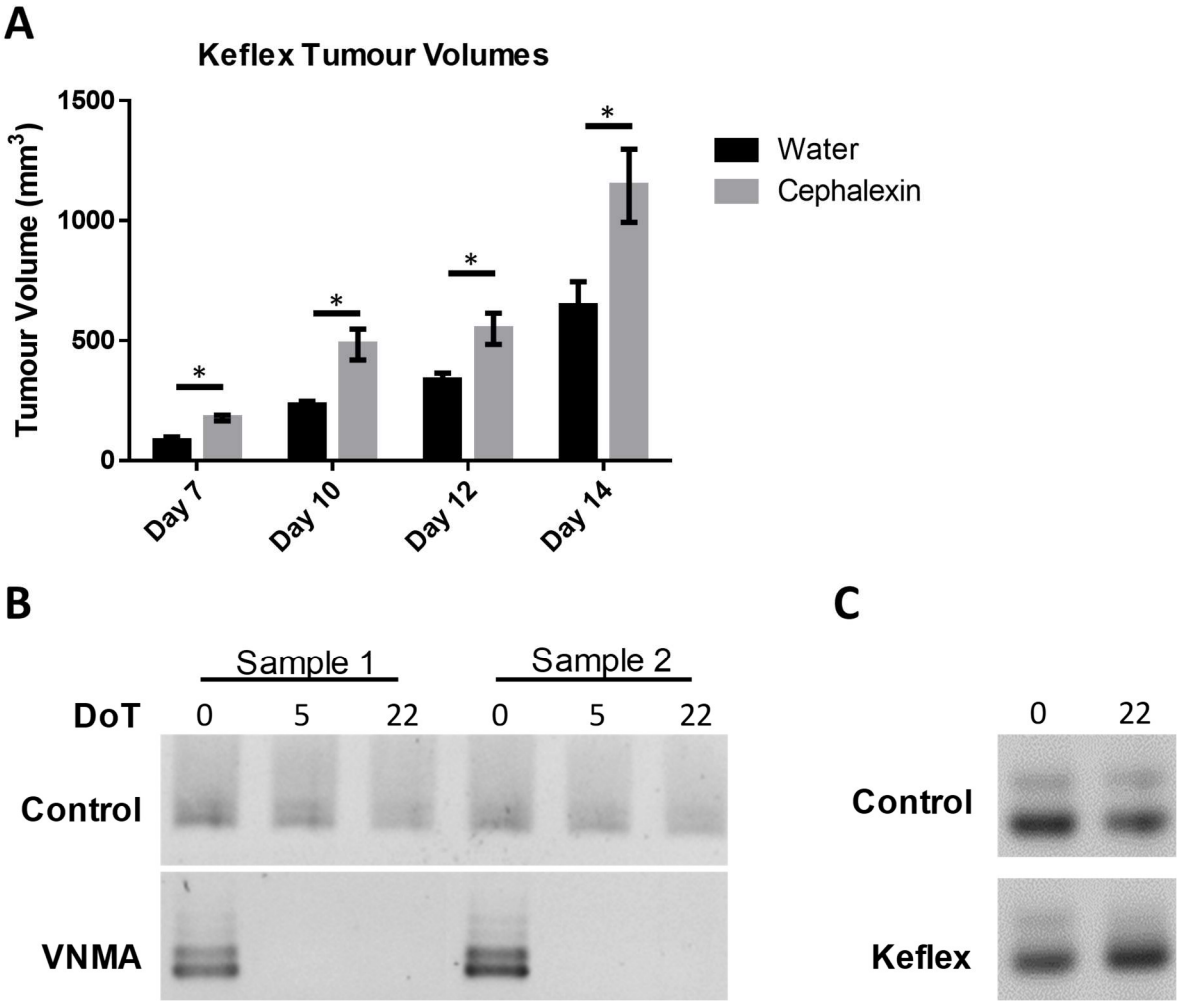


Figure 3.10 – Treatment with the clinically relevant, broad-spectrum antibiotic, Cephalexin, results in signficantly accelerated breast tumour growth whilst preserving the microbiome for genomic anlaysis: A) Volumes of B6BO1 tumours in animals treated with Keflex or water control. Days represent post tumour injection. Days 7, 10 & 12 are skin measurements. Day 14 is an *ex vivo* measurement. Asterisks represent statistical significance, * $p<0.05$, evaluated by unpaired, two-tailed *t* test. **B)** Representative agarose gel images showing amplification of the V1&V2 regions of the 16S rRNA gene in fecal DNA samples. Top image is samples taken from control animals, bottom is samples from VNMA treated animals. DoT represents Days of antibiotic Treatment. **C)** As in B), but representative fecal samples from control or Keflex treated animals.

3.11. Treatment with Cephalexin results in significant alterations in colonic microbial diversity at a genus level

To understand the impact that Keflex treatment has on microbial composition in the gut, we undertook 16S sequencing of isolated fecal DNA. Samples were taken before commencement of treatment to profile the baseline microbiome of both groups and again at the final timepoint. Extracted DNA was used in 16S amplification using the primers detailed in Table 2.4 and sequenced by the Wellcome Trust Sanger Institute using the Illumina MiSeq platform. Reads were quality assessed using FASTX-Toolkit and then aligned against the SILVA database. Data was subsequently normalised, visualised and analysed using Megan6. Initial analysis was undertaken using principle component analysis to identify any outliers and generalise the composition of each treatment group. The starting microbiomes of each group display considerable overlap in genus diversity, likely reflecting the prior randomisation and co-housing of each group (Figure 3.11A). The microbiome at this timepoint is predominantly composed of *Lactobacillus* and *Faecalibaculum* spp. with a slight, but non-significantly, elevated presence of the latter in the animals allocated to the control treatment. End-point samples from both treatment groups show significant shifts in composition relative to their starting microbiome. For the control group, this results in a reduction in the number of lactobacillus and the predominance of *Faecalibaculum* (Figure 3.11B). On the other hand, the Keflex treated samples show reductions in both lactobacillus and *Faecalibaculum* relative to their starting point in addition to significant reductions in other genera such as *Alistipes*, *Staphylococcus* and *Odoribacter*. Many genera also display increases after antibiotic treatment, the most dramatic of which are the increases seen in *Bacteroides*, *Roseburia* and *Lachnoclostridium* (Figure 3.11C&D).

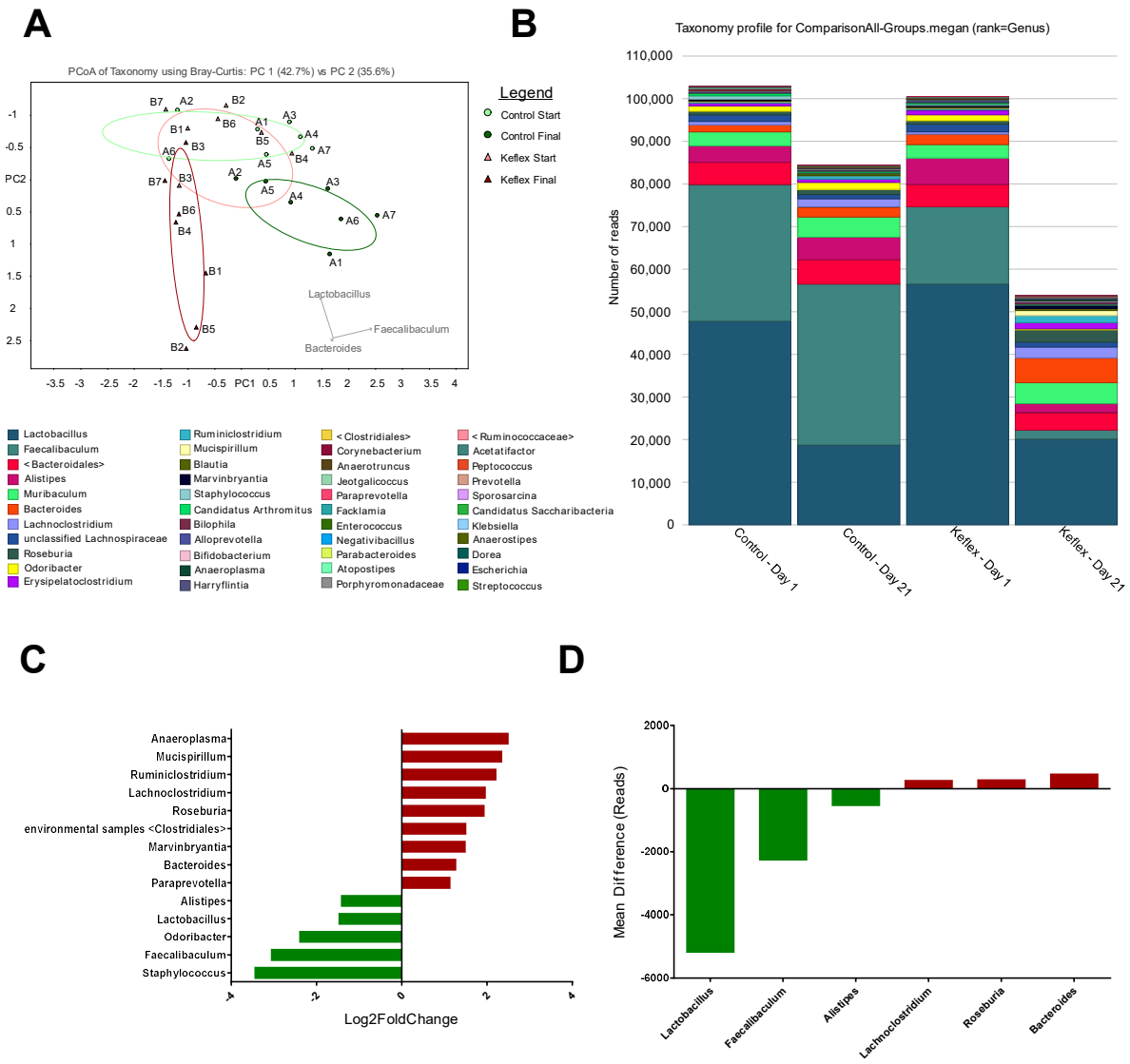


Figure 3.11 – Analysis of the gut microbiome via 16S sequencing reveals significant alterations in composition at a genus level after treatment with Keflex: A) Principle component analysis of all replicates using Bray Curtis distances shows that both control and Keflex treated samples experience microbiome shifts over time. Greens depict control replicates and reds Keflex treated, the pastel colours are the starting microbiomes and the darker colours represent samples taken from the experimental end-point. **B)** Full microbiome composition by genera in control vs Keflex treated animals at experimental start and end-points. Bars represent percentage of total reads for each genus and are sorted by increasing number from bottom to top. Legend is ordered by greatest abundance. **C)** Mean fold change of significantly altered genera, green indicates genera which are significantly depleted whilst red are enriched in Keflex treated animals at the final sampling point relative to their experimental start-point. **D)** Top three most depleted and enriched genera reported by difference in mean read number relative to the day 1 samples. Significance determined by paired, two-tailed *t* test, n=7 per group, per time point.

3.12. Administration of VNMA or Cephalexin *in vitro* does not affect B6BO1 survival or migration in scratch wound assays

Whilst the oral bioavailability of all the antibiotics in the VNMA cocktail (with the exception of metronidazole) are generally poor, low concentrations of each are found in sera of treated individuals, particularly during colitis [580]–[583]. Furthermore, low concentrations of antibiotics have been shown to cause oxidative stress in some mammalian cell lines, resulting in dramatic changes in gene expression [584], [585]. Therefore, we sought to understand whether direct administration of VNMA antibiotics to B6BO1 cells results in any changes to their proliferative or migratory potential, *in vitro*. We assessed this using scratch wound assays of cultured B6BO1 cells exposed to serial dilutions of the VNMA antibiotic cocktail or Keflex alone. The initial concentration was equal to that administered to the animals and was then diluted 10-fold to a minimal concentration of 10^{-4}. No significant differences were seen in either VNMA (Figure 3.12A) or Keflex (Figure 3.12B) treated samples across all dilutions, with the exception that significant cell death was observed at the highest concentrations of antibiotic.

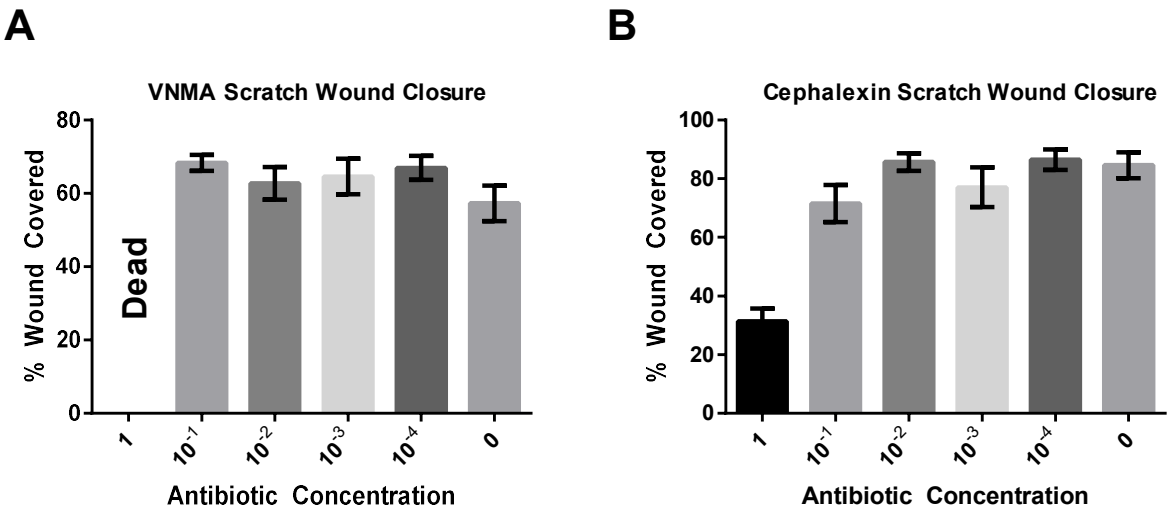


Figure 3.12 – Scratch wound assays show antibiotics have no effect on B6BO1 cell growth or migration *in vitro*: (A&B) Cultured B6BO1 cells were grown to confluence and a physical wound was created. Cells were dosed with serially diluted VNMA (A) or Cephalexin (B) antibiotics and wound closure was measured after 2 days in culture. Bars show mean of percentage wound closure across 3 biological replicates. Significance was determined by unpaired, two-tailed *t* test.

3.13. Discussion

The use of antibiotics is widespread amongst cancer patients to prevent opportunistic infection during periods of immunocompromisation. However, now more than ever is the time to re-evaluate the way antibiotics are used in the clinic. The threat of antibiotic resistant pathogens is imminent and serious. According to the 2016 review by the UK Department of Health, antimicrobial resistance is already killing 700,000 people per year worldwide and this figure is expected to grow exponentially over the next 30 years [586] . Additionally, some evidence suggests that antibiotic use may not be beneficial to all patients. Recent studies have demonstrated an unequivocal role of the patient microbiome in orchestrating anti-tumour responses and many have found that the use of antibiotics compromises treatment efficacy in several cancers. It is therefore paramount that clinicians begin to carefully consider whether using antibiotics will be effective for their patients. To do so, we must fully understand how the microbiome impacts different cancer pathologies. Research groups led by Thomas Gajewski and Laurence Zitvogel have made great progress in understanding how antibiotics affect cancer, particularly in melanoma. However, currently no studies have been conducted to assess the relevance of the microbiome-immune-tumour axis in BC. One aim of this thesis was to begin to understand how the gut microbiome influences the anti-cancer immune response during BC using pre-clinical murine models.

Our first goal was to understand whether the use of antibiotics has any impact on primary tumour growth. To address this, we undertook tumour studies using orthotopically implanted PyMT derived luminal (B6BO1) or spontaneously derived basal (EO771) tumours in animals which had been administered a robust, VNMA antibiotic cocktail. This revealed that disruption of the gut microbiota results in accelerated tumour growth across both models, with volumes reaching 1.5-1.6x the size of their control, water treated counterparts. This suggests that VNMA treatment is disruptive to anti-cancer responses regardless of the molecular subtype, however we are yet to test this in TNBC. This is largely in agreement with the findings of other groups, albeit in different cancers. Use of ABX has been shown to drive tumour growth in both pre-clinical and human studies across multiple cancers, however these studies mainly focus on the influence of the microbiome on anti-tumour therapies. For example, Vetizou *et al.* and Routy *et al.* probe the impact of antibiotics on anti-CTLA4 and anti-PD-1 therapies respectively, finding that these treatments are rendered ineffective when the microbiome is depleted [554], [555]. However, when comparing control and ABX treated animals without administration of anti-tumour agents, these groups found no difference in tumour volume. This begs the question, why is our model different? There

are several potential explanations. The first is an issue that is likely to confound many microbiological studies; reproducibility of results. The microbiome of experimental animals will differ across animal facilities and sites and it is very likely that initial microbiome of our animals differs significantly from those used by other groups. This is evident from looking at the results of our 16S sequencing, the microbiome of our mice is dominated by Lactobacillus and Faecalibacterium, whilst the animals used by Vetizou *et al* is almost entirely composed of Bacteroides and the animals in Iida *et al.* is split evenly between Bacteroides and Clostridia. Therefore, the beneficial species or species that exist in our animals, may not be present in the cited studies. This may limit the beneficial properties of their microbial profiles so that they are only influential during treatments and not solely as a result of antibiotic induced dysbiosis, as is seen in our studies. Furthermore, these contributions will be context dependent and differ wildly from cancer to cancer. For example, administration of ABX has actually been shown to be protective in pancreatic cancer, therefore wildly different responses can be expected in other diseases [587]. Most studies in the literature focus on melanoma, the pathology of which will undoubtedly be significantly different to BC. Therefore, whilst the intrinsic anti-cancer response is not susceptible to microbiological perturbations in melanoma, the nature of BC pathophysiology may contribute to its susceptibility to antibiotic induced dysbiosis. This is partially supported by the findings of Rossini *et al.*, who in 2006 demonstrated, using HER2/*neu* transgenic mice, that antibiotics increase the incidence of spontaneous BC. In these studies, however, no mechanism was deteremined. This was likely due to the difficulties of studying spontaneous BC models; animals present with numerous foci at different stages of tumour development making it difficult to make specific observations.

We hoped to build on this work using our orthotopic implantable models. Based on the known roles of the gut microbiota in guiding anti-cancer immune responses, we hypothesised that the mechanism driving our phenotype was likely to be immunological. Our first step in gaining mechanistic insight was to profile the immunological landscape of both the tumour and peripheral immune organs. To do so, we undertook wide profiling of immune cell populations in the tumour, spleen and mesenteric lymph nodes by flow cytometry. The latter was used as a proxy for immune cell populations in the gut as we were unable to profile Peyer's patches or the lamina propria due to technical difficulties. Surprisingly, we were unable to find significant differences in any immune cell populations at any site. Based on our RNAseq data, it is unlikely that intratumoural immune population or activation changes are occurring. However, for completeness, it may be interesting to use an unbiased approach such as CyTOF to rule out any other changes. Additionally, whilst we have comprehensive data describing the immune landscape at the later stages of

tumour growth, we are yet to look at any other time points. It would be interesting to examine earlier time points as, according to data from our collaborators using the B6BO1 model, the immune cell distribution differs significantly earlier in disease progression. For example, the percentage of infiltrating T cells is around 10-15% rather than the 4-5% seen at later stages (data not shown). Therefore, the nature of their response may be sensitive to antibiotic treatment at this stage. However, based on current findings, we do not believe antibiotic administration is disrupting the overall number of infiltrating immune cells.

We therefore instead turned our attentions to profiling the contributions of the immune cells present rather than their identity. Doing so by flow cytometry would have been time consuming and potentially fruitless. Therefore, we undertook unbiased analysis of cytokine production initially by membrane-based array. The array revealed 15 differentially regulated cytokines, all of which were significantly downregulated in the VNMA treated tumours. Literature analysis of these cytokines found that only 5 had a known role in breast tumorigenesis. CC21 and CXCL16 have been shown to modulate the tumour microenvironment in BC by promoting the recruitment of immune cells [588], [589]. However, given we did not observe any population differences in our flow cytometric analysis, they are unlikely to be playing a role in our model. Both IGFBP-3 and IL-1RA have been shown to promote BC tumour growth, the former through modulation of epidermal growth factor receptor (EGFR) signalling. Upregulation of IGFBP-3 has been shown to drive expression of sphingosine kinase (Spk1) which through phosphorylation of sphingosine-1-phosphate can transactivate EGFR leading to upregulated growth signalling in BC [590]. Downregulation of IL-1RA may lead to increased IL-1 signalling. IL-1RA antagonises IL-1 signalling via binding of secreted IL-1, preventing its interaction with IL-1R. In BC, potentiation of IL-1 signalling has been shown to potently drive tumour growth and metastasis by suppression of apoptosis and increased proliferation [591]. Confusingly, IL-33 levels were also downregulated at the protein level, despite its known role in promoting breast cancer tumorigenicity [592]. This will be discussed in more detail later. To gain a more focused insight, we turned to using MSD V-PLEX assays to quantitatively assess intratumoural and intestinal cytokine production. Whilst none of the profiled cytokines were significantly regulated intratumourally, several were significantly decreased in intestinal tissue. The biological impact of these decreases on tumour growth are not clear, however it suggests a general dysregulation of the GALT during VNMA treatment. Both IL-1β and TNFα play key roles in orchestration of gut-immune responses through chemoattraction and modulation of inflammation. Both have also been shown to drive CXCL1 expression and their decreased production may explain our observed significant reduction of CXCL1 in intestinal tissues following VNMA treatment [593], [594]. The role of CXCL1 in intestinal

homeostasis is unclear, however it is known to be a critical regulator of intestinal inflammation in response to septic injury via neutrophil recruitment. It would be prudent to assess the degree of neutrophil infiltration and phenotypes by flow cytometry/FACS to determine what impact the reduction of CXCL1 has at the gut/immune interface. Neutrophils have been shown to directly influence immune cell populations at the GALT, particularly through recruitment and expansion of Th17 cell populations [595]. Therefore, alongside our MSD analysis we also used ELISAs to assess IL-17A production in the gut and tumour. Whilst there was no difference in the intestinal tissues, IL-17A production was significantly upregulated in the tumour. This is consistent with the known role of IL-17A in BC tumorigenesis; increased IL-17A production has been shown to drive BC cell proliferation through upregulation of Erk1/2 phosphorylation. Additionally, IL-17A has been shown to mediate immunosuppressive functions by modulation of the suppressive actions of MDSCs. However, when probing this further by looking at IL17 isoform expression by qPCR, we were unable to detect IL17A transcripts. Instead, a significant increase in *IL17C* expression was observed in the VNMA treated tumours. The presence of *IL17C* in tumours is relatively understudied. Unlike IL-17A, it is not secreted by immune cells and is instead produced by epithelial cells in response to pathogenic infection. Its role in BC tumorigenesis is unknown, but its expression has been observed in human BC samples [596]. Furthermore, it has been shown to drive tumour growth in lung cancer via neutrophil accumulation, however given we do not observe any increases in neutrophil number by flow cytometry, this is unlikely to be its mechanism of action in our model. Alternatively, Song *et al.* suggest that *IL17C* expression is upregulated in colon cancer by microbial dysbiosis resulting in increased pro-survival signalling and accelerated tumorigenesis [597]. Whilst the microbial environment that colorectal cancers are exposed to is far more diverse than that of the mammary gland, there is evidence to suggest the mammary has its own unique microbiome that plays a role in tumorigenesis [598]. We are currently unclear as to how VNMA treatment impacts the mammary microbiome as we were unable to sample bacterial DNA from breast tumour tissue. However, the ampicillin, metronidazole and amphoteracin B used in our VNMA cocktail are orally bioavailable. Additionally, the cytokine array highlighted intratumoural downregulation of multiple bactericidal complement associated proteins including Reg3G, CRP and CFD which may suggest a decreased number of bacterial species in the breast. Therefore, it is sensible to presume that the tumour modulating effects of VNMA treatment may be a result of mammary microbiota dysfunction rather than the gut. However, without isolation of bacterial DNA from breast tissue and subsequent sequencing, we are unable to make a definitive hypothesis.

To gain a better understanding of the tumoural processes that antibiotic administration regulates and identify any potential target mechanisms, we employed RNA sequencing of whole tumour RNA extracts. The cytokine data we had already generated led us to hypothesise we would observe differential regulation of immune processes. However, to our surprise, enrichment is predominantly seen in metabolic processes, particularly in lipid metabolism and gluconeogenesis. Metabolic reprogramming is a well-established hallmark of cancer and upregulation of lipid metabolism is strongly associated with tumorigenesis, particularly in BC. Expression of fatty acid synthase (FAS) is upregulated in BC lesions from the earliest stages of carcinogenesis and persist through to metastasis [599]. Additionally, high levels of FAS in BC is known to be a negative prognostic marker and its inhibition has been shown to promote apoptosis [600], [601]. However, in our model FAS is not transcriptionally upregulated, instead several other members of the cellular fatty acid metabolic pathway are elevated. Tumours require lipids to fuel their dysregulated growth and the majority of lipid uptake during malignancy is destined for use in membrane biosynthesis [602]. Lipid sources vary depending on the type of cancer, however BC is equipped to utilise circulating, dietary fats and for *de novo* generation of fatty acids [603]. The significant upregulation of lipoprotein lipase (LPL) in our model suggests in this case that the tumours are attempting to utilise circulating fats. Secreted LPL adheres to the luminal surfaces of endothelial cells and catalyses the degradation of circulating triglycerides into fatty acids (FAs) for import into the nearby cells via CD36. Elevated expression of both CD36 and LPL has been shown in BC. Furthermore, high LPL expression correlates with negative prognosis [604]. Interestingly, genes involved in promotion of β oxidation are also upregulated in our tumours. Acyl-CoA Synthetase 1 (ACSL1) activates long chain fatty acids and uses them to synthesise acyl-CoA and is the first committed step in fatty acid metabolism. The acyl-CoA is then imported into the mitochondria via Carnitine palmitoyltransferase I (CPT1) and used in β-oxidation after conversion to acetyl-CoA. Upregulation of ACSL1 has been demonstrated in malignant breast tissue when compared to local, non-malignant control tissue [605]. Furthermore, patients presenting with elevated ACSL1 in their tumours have significantly worsened survival rates than patients with low expression [606]. Additionally, administration of triascin C, a broad spectrum ACSL isoform inhibitor, suppressed BC cell growth *in vitro*; as yet, no studies have been performed *in vivo* [607]. Interestingly, upregulation of ACSL1 expression has been associated with microbial exposure. In studies using bone marrow derived macrophages, Rubinow *et al.* showed that the gram-negative bacteria *E. coli* and *S. typhimuirum* induce ACSL1 expression through LPS induced activation of TLR4 signalling [608]. Given the B6BO1 tumours used in our model consist of ~10-15% macrophages, it is possible that the increased *ACSL1* expression is a result of changes in macrophage

expression. Furthermore, if modulation of the breast microbiome is occurring as a result of VNMA treatment, these bacterial changes may increase LPS production in the breast microbiome. However, without bacterial population analysis of the breast microbiome, it is difficult to speculate.

The product of fatty acid β-oxidation, acetyl-CoA, is utilised by cells in the generation of cellular components. Acetyl-CoA carboxylase 2 (ACC2/ACACB) plays a key role in this by conversion of acetyl-CoA into malonyl-CoA which inhibits the transport protein CPT1 found on the mitochondrial membrane [609]. The functional significance of ACC2 activity in tumour metabolism is unknown. Some work has been conducted in glioblastoma cell lines using pharmacological inhibition and has shown that ACC inhibition blocks proliferation and promotes apoptosis [610]. However, the results are confounded by non-specific inhibition of both ACC (ACC1/2) isoforms therefore the contributions of each cannot be determined. Both work co-operatively to promote FA synthesis and inhibition of ACC1 in BC has been shown to induce apoptosis, however, the role of ACC2 is currently unknown [611]. Malonyl-CoA accumulation precedes fatty acid generation by FAS, which are then free for cellular utilisation or storage. To keep pace with the cell's proliferative demands, phospholipids must be readily available for membrane generation. A key enzyme in this process is Stearoyl-CoA desaturase (SCD1), which is also significantly upregulated in our antibiotic tumours. Its primary function is catalysing the production of monounsaturated fatty acids (MUFAs) from their saturated counterparts. Production of MUFAs is noted to be elevated in several cancers, including breast, and is required for generation of cellular lipids, particularly membrane phospholipids [612]. Additionally, elevated SCD1 levels prevent the accumulation of saturated fatty acids which can induce apoptosis. This is demonstrated by SCD1 inhibition in BC cells which demonstrate decreased proliferative ability and increased rates of cell death [613]. Furthermore, high levels of SCD1 have been observed in BC and are associated with poor prognosis [614].

Together, this network is suggestive of a tumour with significantly dysregulated lipid metabolic pathways, a hypothetical model of which is summarised in Figure 3.13. However, we are currently unable to reconcile the upregulation of both lipogenic and β oxidative programmes. It is possible that one is a compensatory consequence of the other. Metabolomic analysis is required to determine if the significant upregulation of *ACSL1* is sufficient to increase β oxidation rates. Additionally, we are unable to determine whether the tumour cells themselves, or other cells within the microenvironment, are upregulating their expression of these genes. As discussed, macrophages have also been shown to upregulate *ACSL1* after immunological challenge. Therefore, the significantly increased

ACSL1 expression that we have observed may be a result of metabolic alterations in stromal elements. Ideally, we would use single cell sequencing to probe the heterogeneity of the tumour and identify specific changes in the microenvironment, however this is prohibited by cost. Instead, it would be advantageous to use isolation platforms such as FACS and/or magnetic beads to probe the transcriptomic and proteomic state of different populations within the tumour microenvironment, including, for example, macrophages.

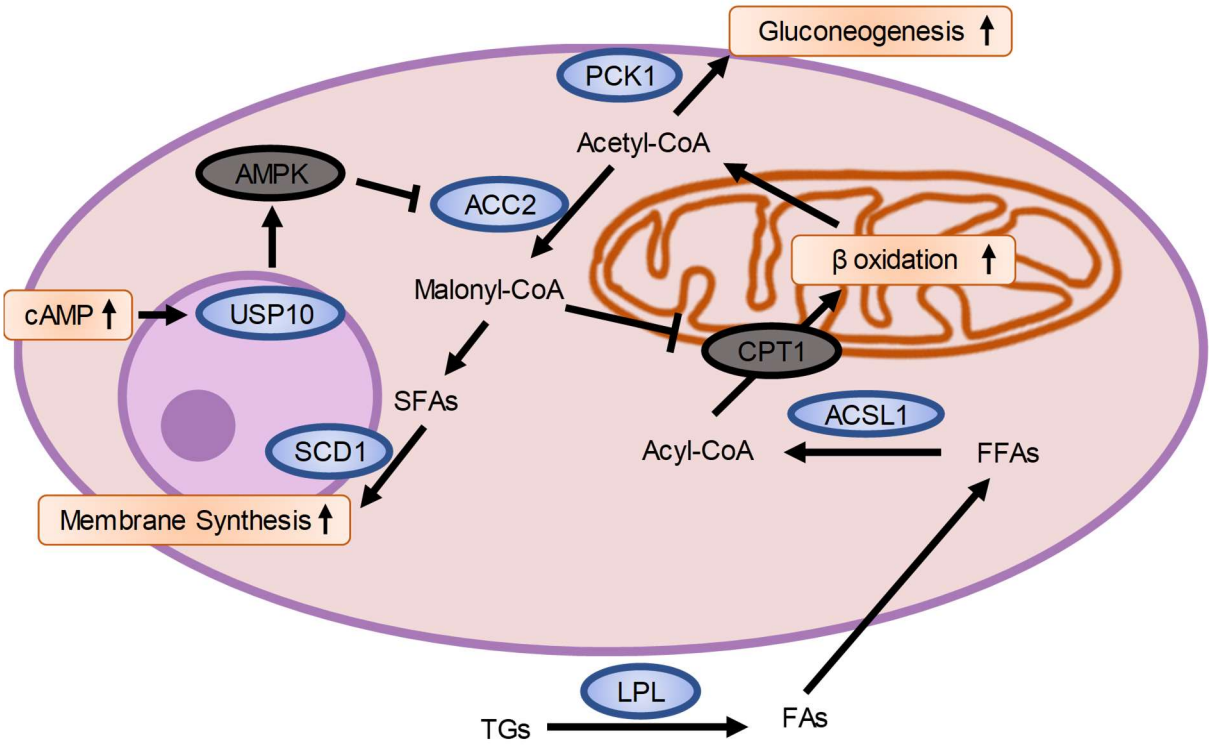


Figure 3.13 – Suggested model of metabolic dysregulation based on transcriptomic analysis: Analysis of our RNAseq dataset suggests that multiple metabolic pathways are disrupted by antibiotic administration, particularly in lipid metabolism. Increased LPL expression suggests increased amounts of FAs are available for cellular import. This, coupled with increased ACSL1 expression indicates higher rates of β-oxidation. Additionally, genes which facilitate the use of acetyl-CoA in biosynthetic processes are also upregulated. Higher expression of ACC2 leads to production of malonyl-CoA from acetyl-CoA, a committed step in cellular lipid biogenesis and a process that requires SCD1 to generate MuFAs for membrane lipid production. However, USP10 is also significantly increased and suppresses ACC2 mediated production of malonyl-CoA which may act to filter acetyl-CoA into gluconeogenic programmes. An upregulation of PCK1 supports this theory and initiates glucose production from acetyl-CoA derived metabolites, likely to support cell growth by providing intermediates for biosynthesis. Light blue proteins, expression is upregulated in our transcriptomic data set, grey are known interacting proteins.

TGs – Triglycerides, **LPL** – Lipoprotein lipase, **FAs** – Fatty acids, **FFAs** – Free fatty acids, **ACSL1** - Acyl-CoA Synthetase Long Chain Family Member 1, **CPT1** - Carnitine palmitoyltransferase I, **PCK1** - Phosphoenolpyruvate Carboxykinase 1, **ACC2** - Acetyl-CoA carboxylase 2 (ACACB), **SFAs** – Saturated fatty acids, **SCD1** - Stearoyl-CoA desaturase-1, **USP10** - Ubiquitin Specific Peptidase 10, **AMPK** - AMP-activated protein kinase.

However, based on the known phenotypic effects of lipid biosynthesis, it is likely that the upregulation of genes associated with fatty acid synthesis and/or metabolism are playing some part in the antibiotic phenotype. Lipid biosynthesis is required for rapid cell proliferation and has been shown to suppress stress induced apoptosis. This is supported by our observed upregulation of multiple genes which are known to negatively regulate apoptotic processes. Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein (HERPUD1*)* and Unconventional prefoldin RPB5 interactor (URI1*)* have been shown to suppress stress induced apoptosis that normally occurs as result of dysregulated metabolic processes. Whilst neither have a demonstrated role in BC, both have been implicated in other cancers. Expression of HERPUD1 has been shown to protect HELA cells from oxidative stress by control of mitochondrial calcium flux [615]. Additionally, URI1 is elevated in aggressive ovarian cancer cells and suppresses metabolic stress induced apoptosis by inhibition of phosphatase 1 gamma (PP1γ), a key regulator of ribosomal S6 kinase 1 (S6K1) induced pro-survival signalling, leading to uncontrolled S6K1 mediated phosphorylation and inactivation of Bcl-2-associated death promoter (BAD) [616]. Interestingly, inhibition of S6K1 signalling has been shown to promote apoptosis in BC cells and therefore metabolic upregulation of URI1 expression may represent a possible mechanism of accelerated tumour growth in our model [617]. These findings are also consistent with work performed by Iida *et al.* who found that antibiotic depletion of the gut microbiome prevented response to CpG oligonucleotide therapies in MC38 colon adenocarcinoma. This was in part due to suppression of intratumoural oxidative stress responses by downregulation of p53 response genes. However, the transduction mechanism from gut to tumour in these studies was not elucidated [552].

In addition to metabolic processes, several nutrient response processes were also significantly upregulated in the antibiotic treated tumours. The enrichment in genes involved in responding to hexose and cAMP are likely interconnected and related to low energy levels. The whole tumour RNA used in our sequencing was extracted at a late stage of disease. Given that tumours primarily utilise increased glycolysis to provide their energy requirements, we are likely seeing a tumour that has outstripped its glucose supply, resulting in low ATP, increased AMP, and a reliance on lipid oxidation to meet its energy demands. This is highlighted by significantly increased expression of the genes Ubiquitin specific peptidase 10 (*USP10)* and Phosphoenolpyruvate Carboxykinase 1 (*PCK1)* which are involved in hexose and cAMP responses, respectively. The former is a deubiquitinase and has been shown to promote activation of AMP-activated protein kinase (AMPK), a key regulatory enzyme of lipid metabolism. AMPK phosphorylates several enzymes involved in lipid metabolism, one of which is ACC2 [618]. Under normal energy conditions AMPK is

inactivated by ubiquitination and is unable to inactivate ACC2 by phosphorylation. Therefore acetyl-CoA is converted into malonyl-CoA and fed into lipogenesis programmes rather than the Krebs cycle. However, during periods of stress, increased cellular cAMP concentrations activate USP10 which in turn deubiquitinates AMPK resulting in ACC2 phosphorylation and acetyl-CoA is now available for use in energy generation [619]. Therefore, upregulation of *USP10* expression is suggestive of a cell that is struggling to meet its energy demands and has therefore switched to β-oxidation to provide its ATP. This is further supported by the upregulation of *PCK1* which encodes the enzyme Phosphoenolpyruvate Carboxykinase 1 (PEPCK1) and catalyses the rate limiting step in gluconeogenesis [620]. Increased PEPCK1 has been observed in numerous cancers and likely represents a tumour that is attempting to A) replenish glucose supplies which are notoriously low and B) provide glycolytic intermediates which are essential for biosynthesis of cellular components.

Of the significantly downregulated processes, cell migration and protein metabolism were the most prominent. The former is suggestive of a tumour with decreased metastatic potential and is potentially reflected in our metastatic findings. This will be discussed in Chapter 5. However, the latter is more difficult to reconcile with increased tumour growth rates. Generally, a rapidly proliferating tumour will be synthesising proteins at increased rates to supply cellular components. Therefore, one would expect a tumour to present with increased protein metabolic processes. However, several of the genes highlighted by our analysis have tumour suppressor functions. Expression of BNIP3 is known to induce apoptosis in response to oxidative stress by mitophagy and is seen at reduced levels in more aggressive BC subtypes [621], [622]. Additionally, Ornithine Decarboxylase Antizyme 2 (OAZ2) has been shown to inhibit the enzyme ornithine decarboxylase (ODC) which synthesises polyamines from ornithine and promotes proliferation in numerous cancers [623], [624].

Taken together, the transcriptional profile of our antibiotic tumours suggests that the microbial changes induced by VNMA treatment results in severe metabolic dysfunction. The extent of this dysfunction and its relevance to tumorigenesis requires further investigation with metabolomics and more detailed proteomic analyses. However, our findings currently allude to a tumour that has shifted its metabolic profile to: 1) support its energy requirements with β-oxidation of fatty acids; 2) increase its utilisation of acetyl-CoA to synthesise cellular components; and, 3) suppress stress responses induced by ATP shortage and oxidative stress to avoid apoptosis.

The question remains, however, how does perturbation of the microbiome induce these effects? Due to the comprehensive bacterial knockdown induced by VNMA treatment, we were unable to gain any insights by studying population changes. Therefore, we opted to use fecal metabolomic analysis as a proxy for bacterial changes in the gut. We observed severe disruption of metabolite production in the gut leading to significant reductions in bacterial fermentation products and accumulation of metabolic substrates. Of particular interest is perturbations in production of the SCFAs acetate and butyrate. Acetate is produced by numerous commensals, including Bifidobacteria, and has been shown to play some role in regulation of metabolic diseases and obesity [625]. However, links to tumorigenesis are scant. Therefore, its reduction in our system is likely significant because of its role in bacterial cross feeding. Acetate most commonly feeds into butyrate producing bacterial species in the gut; this SCFA, too, is significantly downregulated in our antibiotic treated samples [626]. Gut derived butyrate is readily absorbed and has been suggested to play a role in multiple biological processes, including tumour inhibition. The primary methods by which butyrate achieves this is through alterations in host gene expression and modulation of immune cell populations. The latter is a product of increased peripheral T_{reg} differentiation and expansion in response to bacterially produced butyrate both *in vitro* and *in vivo* [627]. However, our flow cytometric analysis suggests that there are no changes in T_{reg} numbers at either the mesenteric lymph node, spleen or tumour. Therefore it is unlikely that decreased microbially produced butyrate is having an immunomodulatory effect in our tumours. Instead, if butyrate depletion is playing a role, it is more likely to be related to its ability to inhibit histone deacetylases (HDACs). Inhibition of HDACs by butyrate has profound impacts on gene expression and has been shown to sensitise cancer cells to DNA damage and ROS induced apoptosis [628]. *In vitro* studies using BC cells have shown that administration of exogenous butyrate (in the form of sodium butyrate) suppresses proliferation through cell cycle senescence and induces apoptosis by modulation of key apoptotic genes [629]. Whilst our transcriptomic data did not reveal any differences in transcription of cell cycle regulatory genes, we do observe decreased expression of pro-apoptotic genes such as *BNIP3* and increased pro-survival genes such as *HERPUD1* and *URI*; this is consistent with butyrate's bioactivity. Therefore, it is possible that decreased butyrate bioavailability is playing some role in our system. However, further verification is required to follow this hypothesis up. For example, full metabolomic analysis of tumour and serum is required to determine if butyrate concentrations are changing. Additionally, analysis of proliferation and apoptosis by ki67 and TUNEL staining respectively would be advantageous to understand whether these processes are being affected by antibiotic administration.

Several metabolic compounds were also upregulated in our antibiotic treated animals. The majority were amino acids such as histidine, aspartate, and alanine, in addition to the nucleoside cytidine. Whilst these molecules are readily bioavailable after absorption by the gut and have been shown to play significant roles in metabolic reprogramming during tumorigenesis [630], there was no evidence of this occurring in our transcriptomic data. Therefore, it is unlikely they are playing a role in driving tumorigenesis in our model. However, increased availability of lactate, raffinose and sialic acid may play some part in explaining the metabolic and immunological dysregulation observed in our tumours. Both alanine and lactate have been associated with increased gluconeogenesis in multiple cancers. However, the former has not been demonstrated to be sufficient to contribute to the increased energy demands of tumours [631]. Whereas lactate is capable of feeding into the Krebs cycle via its conversion into pyruvate, a process that has been demonstrated to occur in many cancers including BC. Therefore, lactate has been shown to be a viable metabolic substrate to support increased rates of cell growth [632]. Additionally, lactate has HDAC inhibitor activity and may modulate gene expression in a similar fashion to that of butyrate, though the implications of this on tumorigenesis are unclear [633]. However, we are unable to determine whether increased lactate availability is responsible for accelerating primary tumour growth after antibiotic administration. Increased pyruvate synthesis from lactate would likely result in upregulation of LDH and this is not present in our transcriptomic data. However, lactate metabolism yields alanine as a metabolic product which is known to feed into gluconeogenic programmes. Therefore, our observed upregulation of gluconeogenic genes is consistent with increased tumoural lactate metabolism. It would be interesting to test this using monocarboxylate transporter 1 (MCT1) inhibitors as MCT1 is the primary cellular importer of lactate. Administration of MCT1 inhibitors has been shown to induce apoptosis in lactate dependent cancer cell lines [634]. Therefore, if our tumours are primarily using lactate to meet their energy requirements, this should abrogate the negative effects of antibiotic administration.

Sialic acid (SA) accumulation has also been implicated in promoting tumorigenesis, but not through metabolic pathways. Gut derived SA is readily absorbed and has been implicated in supporting several physiological functions, particularly brain development [635]. It is also utilised by pathogenic bacteria to circumvent immune destruction and has been shown to facilitate opportunistic GI infection as levels of SA are significantly elevated in the gut after antibiotic treatment [636]. Through incorporation of SA into their membranes, bacteria can avoid activation of the complement pathway and prevent immune mediated destruction [637]. These principles have also been extended to tumour cells and increased SA has been shown to promote immunosuppression in some carcinoma cell lines [638]. Whilst

these effects have not been demonstrated *in vivo* or in BC, suppression of complement mediated immunity is consistent with our cytokine panel data. Key complement proteins were significantly downregulated and are known to play a significant role in anti-tumour immunity, therefore their potential involvement warrants further investigation.

Due to the inability to extract meaningful quantities of DNA from fecal samples or amplify 16S DNA from our samples, we were unable to determine the nature of the microbial changes induced by VNMA treatment. To evaluate whether antibiotic treatment depletes beneficial species or allows amplification of a pathobiont we used a co-housing study to attempt homogenisation of the microbiome across treatment groups. As bona fide cohousing with VNMA animals was not possible due to ampicillin being administered in the drinking water, animals were cross housed with bedding from the contrasting treatment group. Due to coprophagy, the microbiome of cohoused animals has been shown to converge rapidly, usually within a matter of days [639]. Therefore, we hoped the transfer of any bacterial species from water to VNMA treated animals or vice versa may inform us as to the biological relevance of the bacteria species which remain in the gut during VNMA administration. We found that cohousing VNMA treated animals with feces from eubiotic animals results in abrogation of accelerated tumour growth whilst the reverse has no effect. This suggests that VNMA treatment is removing beneficial bacterial species from the gut, however it does not completely rule out the possibility of pathobiont amplification. It is possible that depletion of other species by VNMA treatment provides the niche that is necessary for the pathobiont to flourish and therefore lateral transfer of species from VNMA to eubiotic animals may not be feasible. As before, we conducted profiling of intratumoural immune infiltrate and saw no significant differences between treatment groups. We also profiled IL17C expression levels to determine if it is a causal factor in the VNMA tumour phenotype and whilst trending in the same way as the tumour volumes; the results were not significant. Additionally, we intend to conduct fecal metabolomic analysis on the cohoused treatment groups to determine if the metabolite profiles are transmissible, however this data is not yet available.

To strengthen the clinical relevance of our data and attempt to gain some insight into microbial population changes, we turned to using a milder antibiotic regimen. We intended to follow a regimen that is representative of that given to BC patients in the clinic and our US oncologist collaborator recommended using Cephalexin (Keflex). Treatment at a patient relevant dose (14mg/kg) of animals harbouring implanted B6BO1 tumours resulted in significantly accelerated tumour growth at approximately the same scale as that of VNMA treatment. This suggests that cancer relevant doses of antibiotics may also accelerate

breast tumour growth. Given our cohousing data suggests these effects are due to loss of beneficial populations, we hypothesised that Keflex is sufficient to deplete the same, integral populations as VNMA administration. However, use of Keflex had a less profound impact on the microbiome and bacterial DNA was detectable in fecal samples. This DNA was used in 16S sequencing analysis to probe the composition of the microbiome after Keflex treatment. Two fecal time points were analysed for each group: the starting point before administration of any treatments and a sample taken on the final day of the experiment. Both groups have similar starting microbiomes but drifted significantly over the course of the study. This was expected in the Keflex treated group due to antibiotic administration, but less so in the control group. This is likely explained by cage effect. Animals housed in different cages show high degrees of microbial divergence within a matter of weeks [640]. There is some suggestion in the literature that this is an effect driven by horizontal transfer of *Helicobacter* spp. However, our SPF facility is *Helicobacter* free and it does not appear in our 16S analysis. There is also some possibility that the control animals are contaminated by the administered water or the gavage procedure itself. However, the differences between the control start and end point microbiomes are a result of expansion or contraction of already present species, therefore this is unlikely.

In the Keflex treated samples, the biggest change occurs in *Lactobacillus* spp. However, because of the decreases also seen in the control animals, it is difficult to determine if this is a result of antibiotic administration or cage drifts. Therefore, it is more likely that any phenotypic effects are a result of the losses in other bacterial genera. Many of the genera that are diminished after Keflex treatment are known producers of butyrate (*Faecalibacterium*, *Odoribacter* and *Alistipes*). Loss of microbially produced butyrate has been associated with increasing malignant proliferation rates due to their HDACi activities and may be contributing to our increased BC tumour growth after Keflex administration. Furthermore, loss of members of the *Faecalibacterium* genus has been associated with impaired anti-tumour immune responses during anti-PD-1 therapy [641]. This was shown to be a result of reduced intratumoural CD8+ T cell infiltration when *Faecalibacterium* spp. were reduced. Additionally, peripheral numbers of T regulatory cells were also decreased in the *Faecalibacterium* enriched samples. This is surprising given that *Faecalibacterium* spp. are known butyrate producers and butyrate production by microbial populations has been shown to strongly induce T_{reg} accumulations [642]. We unfortunately do not have any flow cytometric analysis of Keflex treated animals, therefore it is difficult to comment on the potential immunological effects of the induced microbial dysbiosis. However, depletion of *Facecalibacterium* spp. may be partially consistent with the metabolomic data generated in our VNMA treated samples. *Faecalibacterium* spp. have been shown to metabolise mucin

derived sialic acid which was significantly elevated in the VNMA treated fecal samples [643]. Moreover, *Faecalibactrium* are utilisers of raffinose and loss of their population may explain the accumulations of raffinose we observed [644]. This, alongside the reduction in complement associated proteins we observed intratumorally may be a potential mechanism of action but needs considerable amounts of follow up work to confirm.

Of the accumulated genera, most are understudied with respect to their contribution to human health. Several are producers of either acetate or butyrate (*Lachnoclostridium* [645], *Paraprevotella* [646], *Roseburia* [647]) and therefore may be contributing to increased tumorigenesis by increased Treg differentiation, however without flow cytometric analysis it is difficult to speculate. Of particular interest is the significant increases in Bacteroides spp. which is known to be resistant to several antibiotics including β-lactams [648]. Their role in human health and particularly in their contributions to tumorigenesis is mixed and heavily species dependent. An abundance of *Bacteroides fragilis (B. fragilis)* has been shown to potentiate immunotherapy in mouse sarcoma [555]. However, their presence in the microbiome has recently been associated with increased incidence of colon carcinogenesis [649]. Additionally, different species have also been shown to drive distant cancers. *Bacteroides thetaiotaomicron (B. thetaiotaomicron)* is associated with non-response to PD-1 therapies in melanoma through reduced infiltration of cytotoxic T cells [641]. Therefore, to determine the potential importance of Bacteroides in our system, it is essential we obtain data from metagenomic analyses to determine the species changes in the microbiome of Keflex treated animals. This data is forthcoming and should be available in the near future.

In conclusion, we have demonstrated that the gut microbiome plays an important role in controlling breast tumorigenesis. Whilst some changes occur in intratumoural cytokine production, there are no changes in immune cell populations at either the tumour, mLN or spleen. Therefore, the mechanism of action appears to be through facilitation of metabolic reprogramming with increased expression of genes associated with lipid metabolism in VNMA treated animals. The microbial determinants of these effects are yet to be elucidated due to the inability to extract bacterial DNA from fecal samples. Furthermore, many of the population changes observed during Keflex administration are not consistent with the metabolomic or transcriptomic data generated after VNMA treatment. Therefore, the mechanism of action may be different depending on the type and strength of the administered antibiotic.

4. Administration of probiotic Bifidobacteria impairs primary breast tumour growth by modulation of the gut microbiome and altered intratumoural immune processes

4.1. Introduction to the chapter

Consumption of health promoting bacterial species has likely occurred for millennia but was only explored scientifically in the early 1900s by the pioneering immunologist Élie Metchnikoff. His observations of increased longevity in Bulgarian populations consuming soured milk led him to produce the first known commercial probiotic. Today, probiotics are big business; the worldwide market is currently worth ~$40bn and is expected to exceed $60bn in the next five years [650]. However, despite hundreds of probiotic products available commercially as dietary supplements, none have been approved for clinical use. Primarily, this is due to concerns over safety. Whilst many probiotic species are generally regarded as safe, there is a lack of trials evaluating their safety in immunocompromised individuals. Furthermore, the purity of probiotic substances is currently technically difficult to assure [651]. Therefore, contaminant bacterial species may lead to unwanted side effects. Additionally, the efficacy of many probiotics is yet to be demonstrated at a clinical level. Whilst there are some associations between consumption of probiotics and alleviation of certain conditions, these are largely inconsistent [652]. This is representative of the gaps in the field's understanding regarding the ecological complexity of the human gut microbiota. However, the advent of high throughput sequencing technologies over the last decade has led to significant advancement via metagenomic analyses of the microbiome. Furthermore, whole genome sequencing of individual species has led to a greater molecular understanding of the processes by which they exert their beneficial effects. A primary example of this is members of the *Bifidobacterium* genus. The NCBI database currently holds 64 whole genome sequences of bifidobacterial strains and is still growing, along with a number of studies associating bifidobacteria (*Bif*) with improved health outcomes [653], [654]. This chapter will summarise the key characteristics of *Bif* to highlight why they represent such a promising probiotic species, specifically discussing their modulation of immune processes.

4.1.1. A general introduction to Bifidobacteria

Bifidobacterium (*Bif*) are a genus of gram-positive, branched anaerobic bacteria that are known to inhabit the microbiome of several animal species. Isolated from neonatal feces by Henri Tissier, *Bif* are now one of the best studied probiotic bacteria. As discussed earlier, *Bif* are a significant component of the gut microbiota, particularly in breast-fed infants. *Bif* are thought to be one of the earliest colonisers of the human microbiome; acquired vertically from mother to infant [655]. Interruption of this colonisation has been associated with long-term negative health outcomes, including susceptibilities to GI infection, asthma and development of allergic diseases [654]. The primary determinants of *Bif* diversity in the gut microbiome are vaginal birth and breast feeding; both have been strongly associated with high *Bif* numbers and positive health outcomes. Reduced numbers of *Bif* in infants delivered by caesarean are likely a result of limited acquisition due to not transiting the vaginal canal. However, in vaginally delivered, bottle fed infants, this is likely due to the absence of bifidogenic oligosaccharides found in formula milk. These human breast milk oligosaccharides (HMOs) are preferentially utilised by certain *Bifidobacterium* spp such as *bifidum* (*B. bifidum)* and *longum* ssp. *infantis* (*B. infantis)* resulting in their expansion in the microbiome [656]. However, post weaning these spp. diminish in number and are instead replaced by spp. which preferentially utilise plant derived starchy polysaccharides, such as *Bifidobacterium breve* (*B. breve)* and *Bifidobacterium longum* ssp. *longum (B. longum)* [657]. This transition highlights one of the selective advantages of *Bif*, the diversity of their carbohydrate metabolic processes.

Digestion of complex carbohydrates is primarily performed by GHs, of which the human genome encodes only eight that are known to participate in digestion [658]. This means that most ingested carbohydrates remain undigested by host factors in the small intestine and are instead digested by GHs derived from the microbial contents of the colon. *Bif* are well adapted to this function and 12% of their annotated open reading frames are predicted to encode carbohydrate metabolising enzymes [659]. Many of these are GHs. The *Bif* genome has been shown to encode up to 126 GHs across 57 families [654]. Furthermore, *Bif* possess enzymes designed to digest HMOs and plant celluloses into their constituent sugars, making them well suited to digestion of a variety of carbohydrate sources [660]. These subunits are then fed into the primary metabolic process of *Bif*, known as the bifid shunt. Centred around the enzyme fructose-6-phosphoketolase, this process drives a form of glycolysis that is unique to *Bif* and allows for utilisation of numerous plant and animal derived factors in their metabolic processes [661]. The end result is production of varying

amounts of ATP depending on the initial carbon source and production of the fermentation products acetate and lactate [660].

These SCFA products are thought to mediate a number of the physiologically beneficial effects of *Bif* in the microbiota. For example, production of acetate by *Bif* has been shown to reduce intestinal permeability and protect the host against infection by enteropathogens [662]. Furthermore, both acetate and lactate can feed into metabolic processes of other microbial constituents. This cross-feeding has been shown to mediate the expansion of other beneficial microbial species and support development of a robust microbiota in infants [663]. Additionally, other bacterial species have been shown to utilise *Bif* produced acetate and lactate to produce other SCFAs. For example, administration of *Bif* preferential prebiotics leads to generation of butyrate, despite *Bif* containing no butyrate production pathways in their genomes [664]. Furthermore, administration of *B. longum* to GF animals lead to increased acetate production and a complete absence of butyrate. However, when administered to GF animals after human microbiota transplantation, butyrate production was significantly elevated [665]. *Bif* cross-feeding is advantageous to the host as increased butyrate production has been demonstrated to be beneficial in numerous physiological processes. Butyrate has been suggested to inhibit colon carcinogenesis by preventing aberrant crypt formation [666]. Furthermore, butyrate production has been shown to modulate intestinal permeability by promoting tight junction assembly and driving mucin production [667]. Importantly, along with acetate, butyrate has been shown to modulate the immune system. Therefore, by indirect and direct SCFA production, *Bif* can have a profound impact on immunological processes. For this reason, *Bif* have been heavily implicated in shaping immune development in infants and maintaining tolerance in adults. These properties of bifidobacteria and their potential mechanisms will be discussed in detail in the next section.

4.1.2. Modulation of immunity by Bifidobacteria

It has long been accepted that the host gut microbiota plays a key role in the development and maintenance of immune function, but the key microbial drivers were unknown. However, with the advent of high throughput sequencing platforms it has been possible to begin unravelling the contributions of individual species. *Bif* have been heavily implicated in promotion of proper immunological function. This is highlighted by observational studies in both children and adults which show increased rates of allergic and inflammatory diseases in populations with low numbers of *Bif* in their microbiome [668]–[671]. These effects are largely considered to be a result of *Bif*'s tolerogenic properties particularly with respect to intestinal immunity. These effects are summarised in Figure 4.1. This has led to widespread consideration as to their therapeutic applications, and several pre-clinical studies have demonstrated benefit, particularly in inflammatory bowel disorders. Supplementation with *B. bifidum* in chemically induced colitis models has led to significantly improved histological disease scores. This effect was mediated by suppression of pro-inflammatory T_H1 responses in the colon, leading to downregulation of several pro-inflammatory cytokines associated with colitis pathogenesis [672]. Additionally, *Bif* administration has been shown to drive T_{reg} accumulation, a key tolerogenic cell type. By inhibition of T cell activation and induction of IL-10 production, T_{regs} have potent immunoregulatory functions. When *B. bifidum* is administered as part of a probiotic cocktail, colonic T_{reg} expansion is significantly increased. This is accompanied by T helper cell hyporesponsiveness and suppression of IBD symptoms [527]. These preclinical studies have translated into promising intervention trials, but consistent clinical efficacy is yet to be demonstrated in patients suffering with IBD. Efforts to improve the efficacy of *Bif* probiotics are underway but are bottlenecked by a lack of mechanistic understanding. Whilst immunological associations are frequently observed following *Bif* administration, the molecular orchestrators of these effects are unknown. This is partially due to *Bif* not being amendable to genetic manipulation. The *Bif* genome has a high GC content, typically ≥50% depending on the species and strain. This presents significant challenges to molecular approaches aiming to dissect the role of specific components. However, using isolation techniques, some mechanistic insights have been achieved, particularly with respect to the role of exopolysaccharides (EPSs). EPSs are carbohydrate polymers that form an extracellular layer on many prokaryotes. The primary function of EPS layers is to protect against gastrointestinal challenge by digestive and immunological factors. Their extracellular locality means they are regularly sensed by immune populations inhabiting the GALT. For this reason, they have been strongly implicated in modulation of host immune processes and *Bif* produced EPSs are no exception. Isolates from *B. longum* ssp. BCRC14634 have been shown to elicit IL-10

production by macrophages in culture. Furthermore, they limit the production of TNFα in response to LPS, suggesting that EPSs found in *Bif* may be partially mediating their tolerogenic effects [673]. This hypothesis is supported by studies using EPSs isolated from *B. animals* ssp. *lactis*. In *ex vivo* colon culture models, administration of isolated EPSs resulted in elevated IL-10 and suppressed TNFα production [674]. In preclinical disease models, these anti-inflammatory properties have demonstrated potentially therapeutic benefits. In models of T cell induced colitis, administration of EPSs isolated from *B. longum* 35624 resulted in significantly improved disease scores. This was accompanied by reduced expansion of T_H17 cells, key mediators of the inflammatory response in colitis [674]. Additionally, administration of EPSs isolated from *B. breve* UCC2003 resulted in reduced epithelial cell shedding after injection with LPS, a process which mimics that of IBD induced shedding. This was shown to be TNFα and T_{reg} independent and was a result of suppressed TLR mediated apoptotic signalling programmes [675].

These studies all support the hypothesis that *Bif* derived EPSs, at least in part, drive the immunoregulatory properties of *Bif*. However, other mechanisms have also been proposed and a combinatorial process is the most likely explanation. For example, as alluded to earlier, *Bif* have been suggested to modulate immunity by driving butyrate production in other commensal microbes. As discussed earlier, *Bif* produced acetate feeds into metabolic processes in other bacterial species to generate butyrate. This is demonstrated by increased butyrate production after administration of bifidogenic prebiotics, despite the *Bif* genome not encoding any butyrate production pathways [664], [676]. Butyrate production by the gut microbiota has been shown to drive T_{reg} accumulation in colonic tissues and ameliorate T cell induced colitis [677]. Although these effects have not been directly attributed to *Bif*, their probiotic administration has been shown to increase the number of colonic T_{regs} *in vivo* by induction of regulatory DCs [678]; however how *Bif* drive this effect is still unclear. In other studies, *Bif* has been shown to upregulate production of IDO in DCs, a key marker of the regulatory DC phenotype [527]. Additionally, butyrate has also been shown to promote a regulatory DC phenotype by induction of IDO production [679]. Whilst it has not been demonstrated experimentally, it is possible that *Bif* induces T_{reg} expansion by polarising DCs to a regulatory phenotype via indirect butyrate production as a result of nutrient cross-feeding.

Bif mediated DC alterations have also been linked to modulation of systemic immunity. High levels of *Bif* in the host gut microbiome have been associated with a reduced risk of developing allergic disease and asthma. Furthermore, an increased number of Bif in the microbiome is also associated with higher T_{reg} numbers suggesting the loss of *Bif* results in

impaired immune tolerance [680]. Therefore, *Bif* have emerged as a prime candidate for therapeutic use in allergic conditions. Intervention trials where *Bif* are administered as a probiotic lead to significant quality of life improvements in pollen and grass allergies in addition to allergic asthma [671], [681]. The mechanisms that drive these responses are unclear, but the prevailing theory is linked to *Bif* mediated expansion of regulatory DCs. In pre-clinical models of shellfish allergy, supplementation with *B. infantis* reduces allergic response by supporting the accumulation of CD103+ tolerogenic DCs which in turn led to accumulation of T_{regs} and suppression of the allergic response [682]. This principle has also been demonstrated in human subjects. Volunteers were fed *B. infantis* and their peripheral blood cytokines and immune cell populations were analysed. Patients fed with *B. infantis* presented with higher serum levels of the immunoregulatory cytokine IL-10 and increased numbers of FoxP3+ T_{regs} compared to the control [683]. These immunoregulatory mechanisms are yet to be demonstrated in clinical data, however, they are a likely mechanism by which *Bif* alleviates allergic disease.

Interestingly, the immunomodulatory effects of *Bif* are highly strain specific. For example, in profiling studies of *Bif* mediated cytokine production, whilst *B. bifidum* LMG11041 drives potent IL-10 production by DCs, *B. bifidum* A8 or IF10/10 induce almost no IL-10 production. Remarkably, the profile of cytokine production elected by different spp. is also species specific, whilst many spp. adhere to the classical view of *Bif* mediated cytokine production (i.e. an IL-10 predominated, tolerogenic response), some species actually favour a TNFα/IL-17 immunostimulatory response [684]. This suggests that in addition to suppressing aberrant immune responses in allergic and autoimmune diseases, *Bif* may also be able to augment immunity. This has been exploited in the cancer field to try and bolster anti-cancer immunity. In a landmark study by Sivan *et al.,* probiotic administration of *Bif* was shown to promote anti-cancer immunity in pre-clinical models of melanoma. This was shown to be a result of *Bif* mediated potentiation of DC activation. DCs taken from *Bif* supplemented animals had upregulated gene expression in T cell activation pathways which led to increased IFNγ production in activated, intratumoural CD8+ T cells [556]. Furthermore, supplementation with *Bif* improved the efficacy of anti-PD-L1 immunotherapy and high levels of *Bif* in the host microbiome has been shown to promote treatment efficacy in clinical studies [557]. In light of these findings, we believe it is pertinent to examine the effects of *Bif* supplementation on the anti-cancer immune response in pre-clinical models of breast cancer. This chapter describes our findings to date, discusses their relevance to the field and speculates as to their clinical potential.

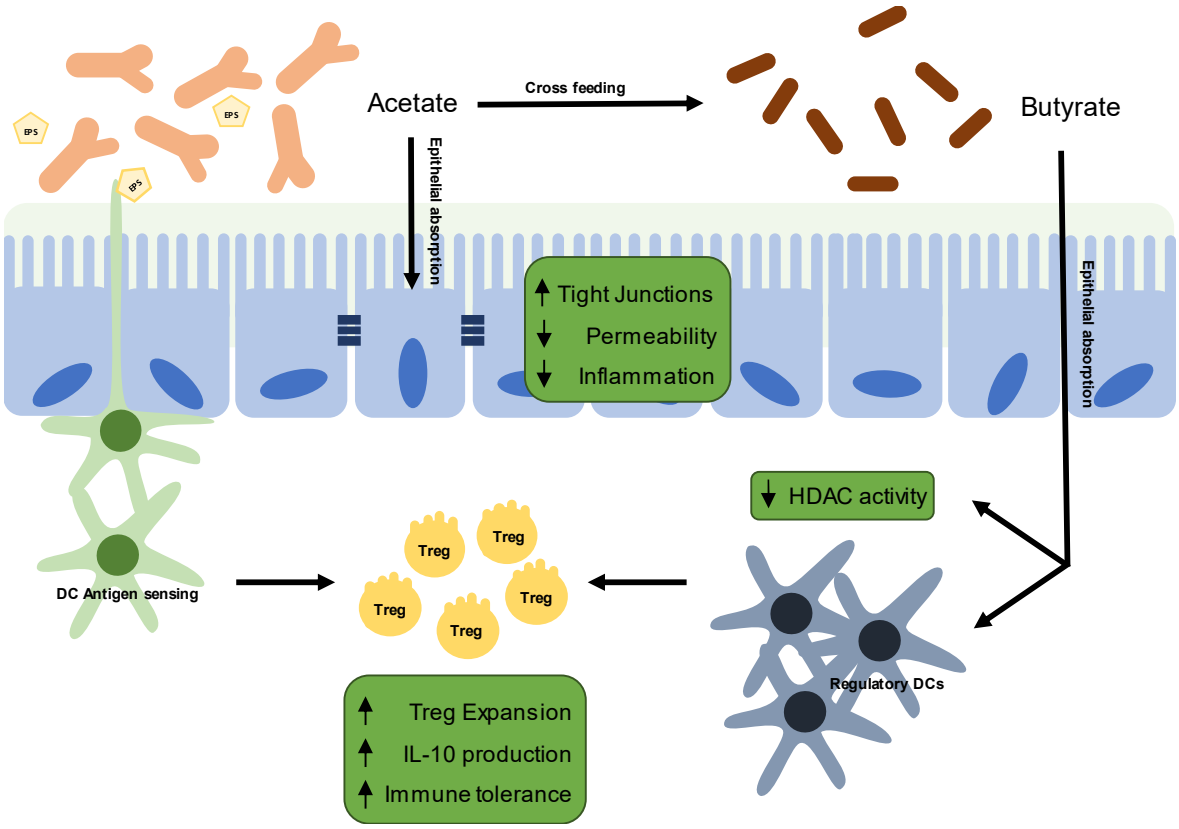


Figure 4.1 – Schematic of *Bif* interactions with the intestinal immune system: The presence of *Bif* in the microbiota has been shown to promote tolerogenic immune responses. *Bif* have been shown to achieve this through promotion of Treg accumulation both in the colon and the periphery. The factors that contribute to *Bif's* tolerogenic actions are summarised. *Bif* ferment starchy carbohydrates to provide energy, the main by-product of which are SCFAs, particularly acetate. Acetate has been demonstrated to cross-feed other bacterial species in the microbiome and allows *Bif* to drive butyrate production. Butyrate is absorbed by the gut epithelium and drives Treg accumulation by conversion of DCs to a regulatory phenotype, or by acting directly on CD4+ T cells and directing them to a regulatory phenotype by altering their gene expression through HDAC inhibition. Additionally, *Bif* drives Treg expansion directly via DC mediated antigen sensing. *Bif* produced EPSs along with other factors are sampled by DCs resulting in adoption of a regulatory phenotype and subsequent expansion of Tregs resulting in immune tolerance.

4.2. Supplementation with Bifidobacteria results in impaired primary tumour growth

Given the demonstrable positive effects of *Bif* on host immunological processes and promising results in the literature when their administration is combined with immune checkpoint therapies, we sought to understand if *Bif* play any role in breast cancer growth. To assess this, we modelled a probiotic in a preventative setting by dosing female animals with *Bif* (for composition and full dosing regimen, see section Probiotic Administration 2.1.2) two days before B6BO1 tumour cell injection (Figure 4.2A). This treatment was maintained throughout tumour growth. Using *in vivo* calliper measurements, primary tumour growth rate was shown to be significantly reduced compared to the PBS treated controls at the earlier time points. However, upon sacrifice, *ex vivo* measurements were only slightly reduced, and the effect was not significant (Figure 4.2B&C, p=0.31).

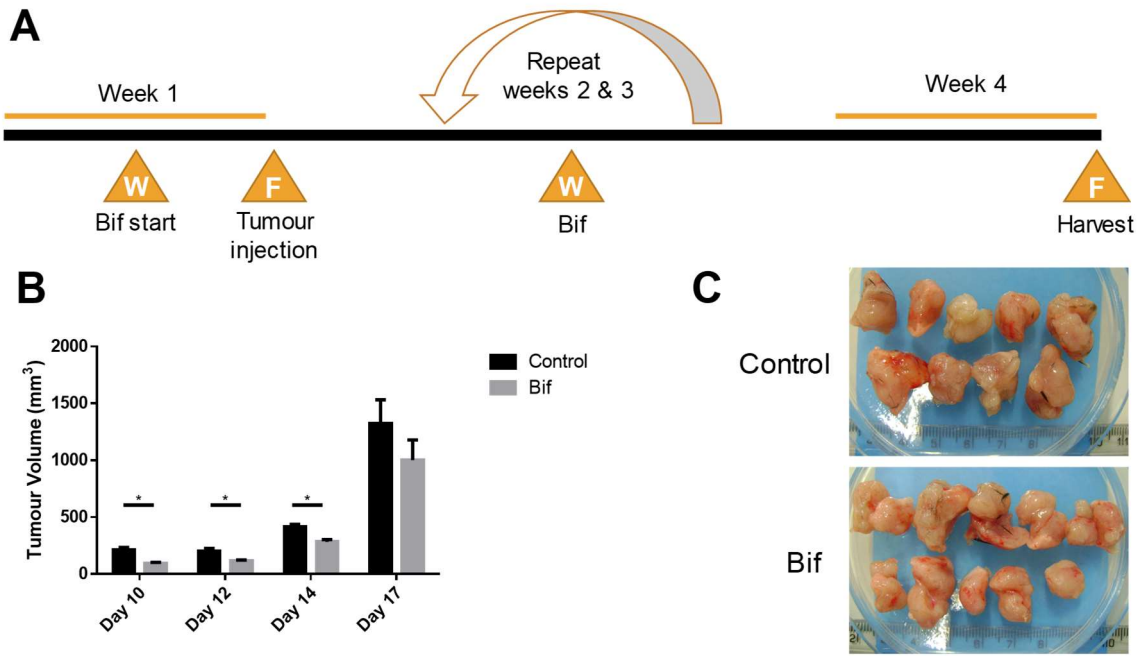


Figure 4.2 – *Bif* administration significantly impairs early tumour growth: A) Schematic of initial *Bif* treatment regimen used to model probiotic, preventative administration. Animals were randomised and co-housed before treatment. Animals received oral gavage with either PBS control or a *Bif* cocktail once per week two days before tumour injection and throughout the experiment. **B)** Primary tumour growth was assessed by *in vivo* calliper measurements throughout and showed significant reductions during early tumour growth. Day 17 represents ex vivo measurements. Bars represent mean tumour volume ±SEM. Asterisks indicate statistical significance, * $p<0.05$, calculated by unpaired, two-tailed t test. $n \geq 9$. **C)** Photograph of excised tumours, water treated control tumours are shown in the top image, whilst *Bif* treated are in the bottom.

4.3. Suppression of primary tumour growth is associated with altered intratumoural cytokine production

In order to determine the mechanisms by which *Bif* supplementation may inhibit tumour growth, we performed wide cytokine profiling using the MSD V-PLEX mouse pro-inflammatory panel 1 array. To gain insight as to how *Bif* may be modulating both intratumoural and gut associated immunity, we performed the assay using whole protein extracts from both tumour and large intestinal tissue. Intratumoural IL-10 production was the only significantly altered cytokine and was elevated in the *Bif* treated tumours (Figure 4.3A). The intestine did not show any differentially regulated cytokines (Figure 4.3B).

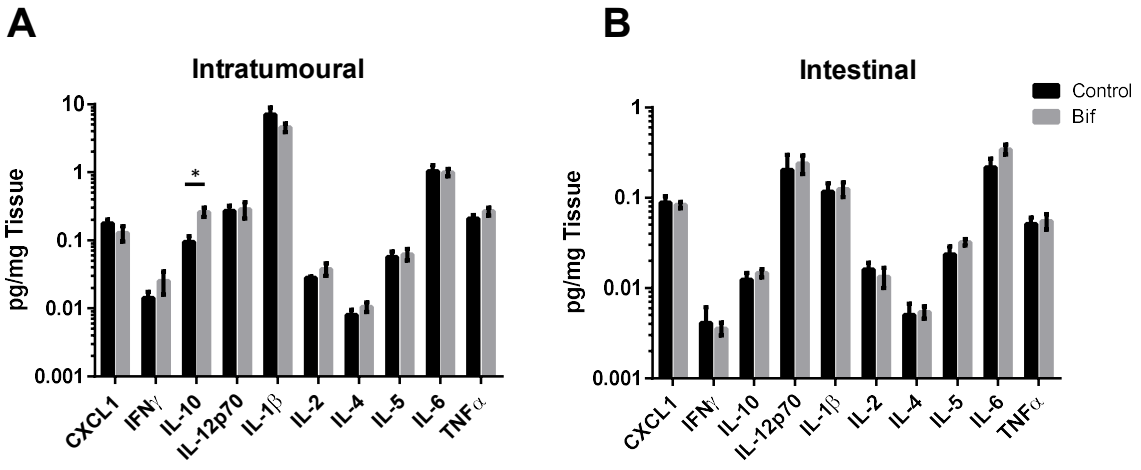


Figure 4.3 – Cytokine profiling reveals upregulated IL-10 production in *Bif* tumours: Whole tissue protein extracts from tumour (A) and large intestine (B) were analysed via MSD V-PLEX assay. Detected cytokine quantities were normalised against lysed tissue weight. Bars show mean ± SEM, n ≥ 6 in both groups. Asterisks indicate statistical significance, * $p<0.05$, determined by unpaired, two-tailed *t* test.

4.4. Modification of the Bifidobacteria cocktail results in improved efficacy with respect to primary tumour growth inhibition

Despite encouraging results during early tumour growth, supplementation with *Bif* was unable to exert a durable response. We speculated that this may be due to overcolonisation of particular species within our cocktail, therefore diluting the effects of other, more efficacious species. To test this, we eliminated *B. breve* UCC2003 from our cocktail. As a model *Bif* strain, UCC2003 has been shown to readily colonise the murine gut microbiota [685]. Therefore, we speculated that it was the most likely to be overcolonising and impairing the efficacy of other members of our probiotic cocktail and was omitted in an attempt to improve the anti-tumour response. To further improve the efficacy of our cocktail, we extended our dosing regimen to three times weekly to maximise the potential for intestinal colonisation and began treatment five days prior to tumour cell injection (Figure 4.4A). Under this regimen, the original *Bif* cocktail including UCC2003 (*Bif* v1.0) did not result in significantly reduced tumour growth at day 17 ($p=0.24$). However, when UCC2003 was omitted from the probiotic cocktail (*Bif* v2.0), significantly reduced final day tumour volume was observed (Figure 4.4B). In order to evaluate whether IL-10 was playing a part in the inhibition of BC tumour growth, we performed ELISAs on whole tumour protein extracts. Production of IL-10 was upregulated in *Bif* v1.0 treated tumours compared to the PBS controls, but this effect was not significant ($p=0.14$). However, in *Bif* v2.0 treated tumours, IL-10 was significantly upregulated when compared to the PBS treated control tumours (Figure 4.4C). This suggests that the anti-tumour effects of probiotic *Bif* supplementation is at least in part driven by elevated intratumoural IL-10 production.

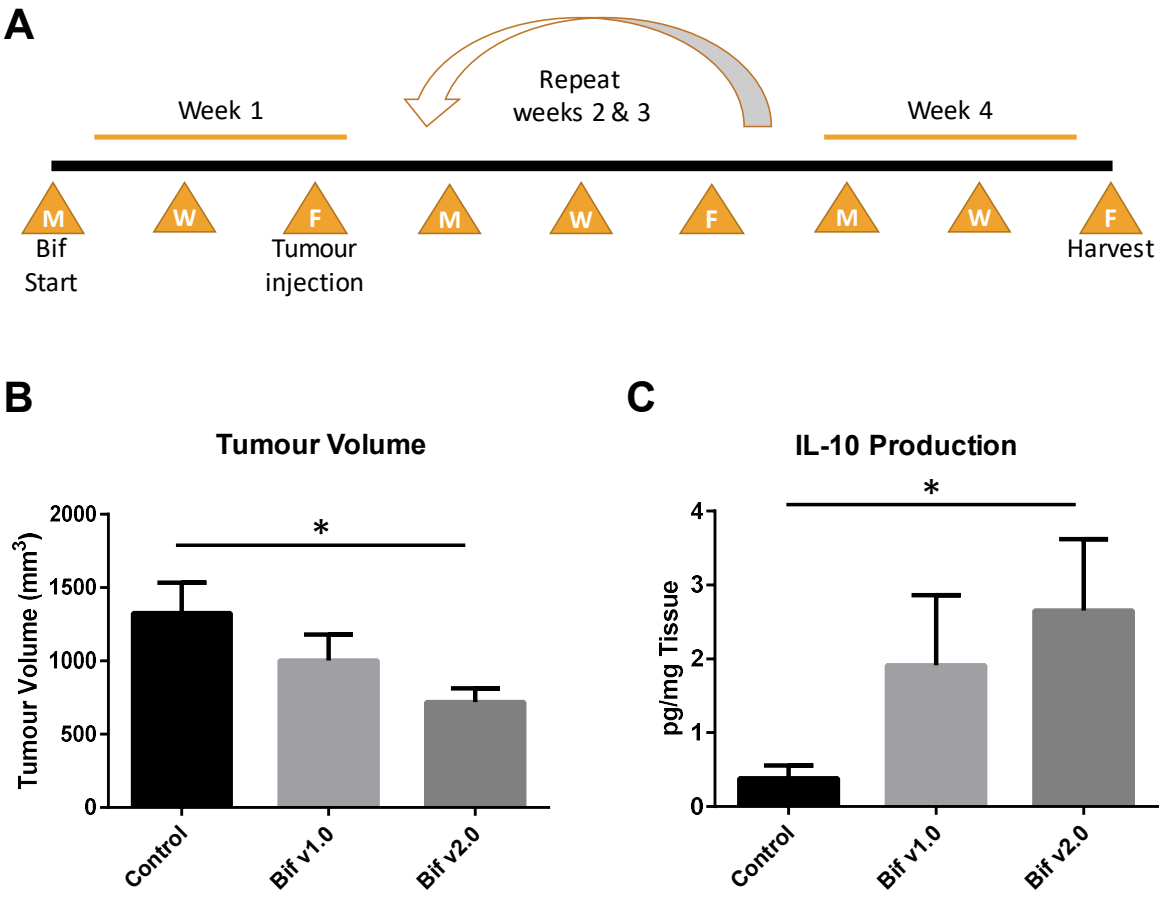


Figure 4.4 – A modified probiotic cocktail improves the anti-tumour effects of *Bif* supplementation: A) Schematic detailing the extended *Bif* supplementation regimen. Weekly doses are increased from once per week to three times and supplementation is initiated five days prior to tumour cell injection as opposed to two in previous experiments. **B)** The *Bif* cocktail used in Figure 4.2 (*Bif* v1.0) and a refined cocktail lacking *B. breve* UCC2003 (*Bif* v2.0) was administered according to the regimen described in A. After 17 days, tumours were excised and measured using digital callipers. Bars represent mean tumour volume ± SEM. **C)** Whole tumour protein extracts were taken from excised tumours and the levels of IL-10 were analysed by ELISA. The quantity of IL-10 in each sample was normalised to processed tissue weight. Bars represent mean IL-10 concentration in tumour tissue ±SEM. Asterisks indicate statistical significance, * $p<0.05$, determined by unpaired, two-tailed *t* test. $n\geq9$ in all cases.

4.5. Reduced primary tumour volume is not associated with changes in intratumoural immune infiltration

To begin understanding which immune cells may be contributing to increased intratumoural IL-10 production, we undertook wide profiling of tumour infiltrating immune cells by flow cytometric analysis. We intended to use established population markers with intracellular cytokine staining of IL-10 to determine which cells are producing the cytokine. However, due to technical difficulties, we were unable to perform such analyses. We undertook profiling of tumours from PBS treated control animals and those treated with the *Bif* v2.0 cocktail under the three times per week regimen. Disappointingly, no significant differences were seen between the two groups at this level. Like the VNMA treated tumours, *Bif* treated tumours are predominated by myeloid cells (CD45+, CD11b+), with most of the myeloid cells being macrophages (F4/80+, Ly6G-). Neutrophils and DCs (CD11b+, Ly6G+ and CD11b-, CD11c+, MHCII+ respectively) are also present in the tumours, albeit at very low levels (~2% and <1% respectively) (Figure 4.5A). Again, like the VNMA treated tumours, *Bif* treated tumours have very low numbers of T cells at this late stage timepoint (2-3%). The ratios of CD4/CD8 cells are slightly skewed towards T helper cells, however there is no significant bias. T regulatory (CD4+, FoxP3+) cells are also present, but at very low levels (<0.5%) (Figure 4.5B). To determine if *Bif* administration may be having any impact on peripheral immune cell expansion, we also undertook profiling of the T cell populations in the mLN and spleen. As expected, much larger numbers of T cells were observed (~30% and ~40% respectively), however again, there was no significant differences between treatment groups (Figure 4.5C&D). To assess the impact of bif supplementation on MHCII and FoxP3 expression in myeloid cells and T_{regs}, respectively, median fluorescence intensity (MFI) analysis was conducted. Populations of interest were gated based on their marker expression, and MFI for each marker was calculated by FlowJo. No differences are observed in MHCII expression in intratumoural DCs or Mθs, neither are there any differences in T_{reg} FoxP3 expression in any of the tissues profiled (Figure 4.5E).

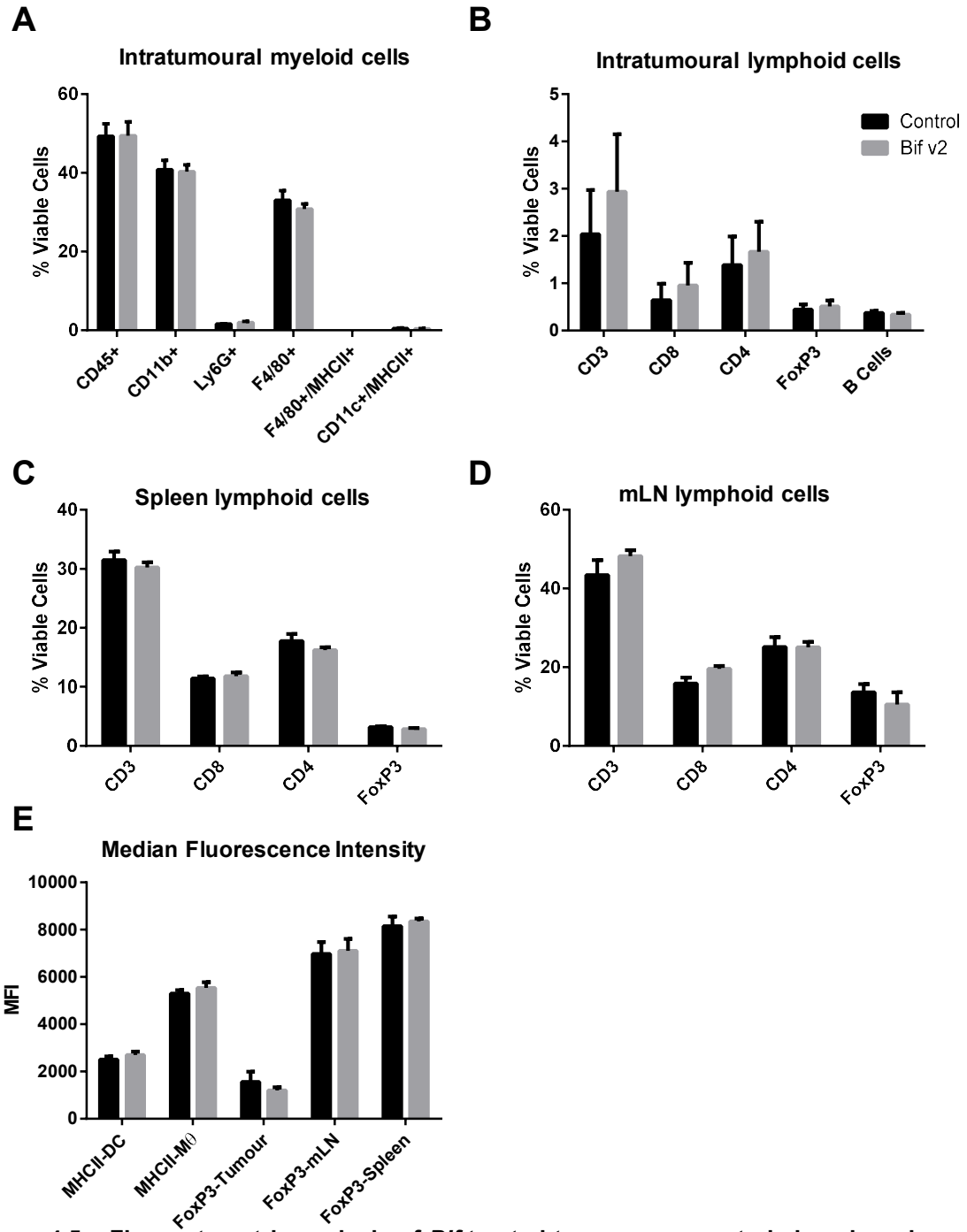


Figure 4.5 – Flow cytometric analysis of *Bif* treated tumours, mesenteric lymph nodes and splenic immune cell populations: All tissues were homogenised, turned into a single cell suspension, stained and analysed by flow cytometry according to section 2.6. **A&B)** Intratumoural myeloid (A) and lymphoid (B) cell populations were identified by using the markers detailed in Figure 7.1. C&D) Peripheral T cell populations were assessed in the mesenteric lymph node (mLN) **(C)** and the spleen **(D)**. Bars in A-D represent mean number of cells normalised against total cell number ±SEM. **E)** Median fluorescence intensity was calculated for DCs and MΘs to assess cell surface MHCII levels. The same analysis was also conducted to assess FoxP3 expression in Tregs. Bars represent mean MFI of indicated cell population as calculated by FlowJo. n≥8 for tumour and spleen, n=3 for mLN for all graphs.

4.6. Discussion

In the past half a decade a clear connection has emerged between the microbiota and anti-cancer immunity. The presence of several bacterial species has been associated with improved prognosis in multiple cancers. Furthermore, administration of probiotic bacteria has been shown to modulate immune checkpoint therapies resulting in higher rates of response and greater efficacy. Despite this encouraging data, the impact of the microbiota on BC is yet to be evaluated. For the first time, we have probed these relationships and have shown that probiotic administration of a bifidobacteria cocktail results in impaired tumour growth in an orthotopically implanted model of BC. Our findings are generally consistent with the sparse information available about the role of *Bif* in tumorigenesis from the literature. *Bif* supplementation has been shown to limit the growth of colorectal cancers in a variety of preclinical models [686], [687]. Furthermore, Sivan *et al.* have demonstrated that supplementation with *Bif* alone is sufficient to impair tumour growth in subcutaneous implant models of melanoma [556]. The mechanisms of *Bif* mediated impairment of carcinogenesis in CRC are unclear and appear to be multifaceted. However, the action of *Bif* supplementation in melanoma is better understood and is primarily driven by modulation of immunological processes. For this reason, we undertook profiling of cytokine production in both the large intestine and tumour.

Surprisingly, there were no significant changes in intestinal cytokine production despite *Bif* being widely associated with modulation of intestinal immunity. This may allude to the fact that the mechanism driving *Bif* mediated tumour inhibition are driven by alterations in metabolite production, however this will be discussed later. Intratumorally, IL-10 production was significantly upregulated and is an effect that is consistent with the known actions of *Bif* supplementation. Supplementation with *Bif* has been shown to induce IL-10 production by T_{regs} in the colon, an effect which has been demonstrated to temper the inflammation caused by IBDs [678]. Additionally, *Bif* supplementation has also been shown to promote IL-10 production systemically via T_{regs}. Probiotic administration of *B. breve* ssp. M-16 V in an ovalbumin induced model of allergic asthma resulted in significantly increased IL-10 production in the lung and improved symptoms [688]. Therefore, it is plausible that supplementation of the gut microbiome with *Bif* induces distant changes in IL-10 production in mammary tumours.

Unfortunately, at this point the optimisation of our tumour flow cytometry was not completed, so we instead turned out attentions to improving the efficacy of our cocktail. We decided to increase the dosing frequency from one to three times per week. Additionally, we removed

UCC2003 from the probiotic cocktail; this was primarily to improve the colonisation of other *Bif* spp. but was also advantageous for commercialisation reasons. These changes to our cocktail administration led to improvements in efficacy; significant reductions in tumour volume were observed up to day 17 with the updated cocktail. Additionally, this was accompanied by a strong negative correlation with IL-10 production, whilst the original cocktail still resulted in IL-10 upregulation, the adjusted cocktail resulted in an improved and significant increase over the control samples. This strengthened our belief that intratumoural IL-10 upregulation is a consequence of oral *Bif* supplementation and may, at least in part, be mediating the phenotypic effects on tumour growth. However, the role of IL-10 in tumorigenesis is controversial. The traditional view is that the immunosuppression elicited by IL-10 results in evasion of immunity, improved proliferation and a greater ability to metastasise. This is reflected by association studies that show high intratumoural IL-10 production is associated with poor prognosis in breast, lung and skin cancers, to name a few [689]–[691]. Mechanistically, the presence of IL-10 in breast tumours has been shown to inhibit CD8+ T cell cytotoxicity in preclinical models of BC [692]. However, recently a more nuanced role for IL-10 in tumorigenesis has been uncovered. It was already known that IL-10 production was capable of suppressing angiogenesis and inhibiting tumour growth in some contexts [693]. However, it has now also been associated with promoting anti-cancer immune responses by modulation of T cell responses. In preclinical studies, expression of IL-10 in BC has been shown to elicit anti-tumour T cell responses by induction of IFN-γ production [694], [695]. Whilst we do not have any data alluding to the cytotoxic capabilities of tumour resident CD8+ T cells, we have probed IFN-γ production and found no differences in *Bif* treated tumours. Therefore, it is unlikely that IL-10 is performing this function in our tumours. Alternatively, IL-10 has been shown to regulate IL-6 levels in subcutaneous models of HPV induced cervical cancer. The loss of IL-10 in these tumours results in increased IL-6 production and induction of an inflammatory tumour environment. This in turn results in MDSC recruitment to the tumour and suppression of anti-cancer immunity [696]. However, we do not observe any differences in IL-6 production in our *Bif* treated tumours.

To gain further insight into the potential sources and role of IL-10, we conducted flow cytometric analysis of tumour immune infiltrate, mLN and spleen. Administration of *Bif* has been shown to strongly induce peripheral T_{reg} expansion and T_{regs} are known to produce high levels of IL-10. We therefore hypothesised that we would observe some regulation of either Treg numbers or FoxP3 expression in CD4+ T cells. However, this was not the case and no population changes were seen in any of the cell types we profiled. Whilst surprising, we hypothesised that whilst population numbers may not be changing, there may be

qualitative changes in the immune cells that are driving *Bif* mediated tumour inhibition. As suggested earlier, IL-10 production has been shown to upregulate MHCII production in APCs [326], however we did not observe any changes in MHCII expression in either macrophages or DCs in our tumours. Furthermore, *Bif* administration has been shown to induce higher levels of FoxP3 expression irrespective of changes in population numbers which correlates positively with IL-10 production by T_{regs} [688]; however we did not observe any changes in the FoxP3 MFI in T_{regs}. We found this surprising, given that *Bif* is known to play a key role in T_{reg} expansion. It is possible that changes in T_{reg} numbers are seen in the lamina propria or peyer's patches, however we were unable to profile the immune populations at these sites due to technical issues. Furthermore, gut derived, antigen loaded DCs migrate from the LP and PPs to the draining mLN in order to present antigens to resident T cells [697]. Therefore, any T cell population changes are likely to occur at the mLN and it is likely we missed any changes by not sampling the LP or PPs. It is also possible that whilst there are no changes in T_{reg} numbers or their FoxP3 expression, they could still be producing more IL-10. A study by Wei *et al.* found that FoxP3 expression was dispensable for T_{reg} mediated IL-10 production [698]. Instead, IRF4 mediated activation of the transcription factor Blimp-1 was shown to be essential in initiation of T_{reg} production of IL-10 [699]. Therefore, it would be advantageous to assess IL-10 production in T_{regs} by intracellular cytokine staining and flow cytometric analysis. Despite attempts to achieve this, technical issues meant this analysis was not possible. Alternatively, we could assess Blimp1 positivity in T_{regs}, either by transcription factor staining or by biochemical analyses such as Western blot after T_{reg} isolation by FACS/MACS.

Alternatively, we hypothesised that the IL-10 production in our tumours may be produced by non-T_{reg} sources. Both B cells and CD8+ T cells have also been shown to produce IL-10 under certain conditions [700], [701]. Additionally, macrophages are potent producers of IL-10 and have been shown to be its primary source in the MMTV-PyMT spontaneous mammary tumour model [692]. Furthermore, macrophage mediated production of IL-10 has been shown to be sensitive to microbial perturbation and interestingly appears to be driven by *Bif*. Exposure of cultured macrophages to either heat killed *Bif* spp. or EPS isolates strongly induces IL-10 production, however this has not yet been explored *in vivo* [702], [703]. Further confounding our findings of increased IL-10 production in mammary tumours is the potential for the mammary cells themselves to be producing it. Established BC cell lines were profiled for IL-10 expression and 5/8 were positive for *IL10* transcripts [704]. Therefore, to further understand the implications of IL-10 in our system, it is essential that we understand which cells are contributing to intratumoural IL-10 production. This could be

accomplished by intracellular cytokine staining and analysis by flow cytometry, however, as discussed earlier, this was not possible in our project due to technical difficulties.

It is also possible that rather than directly mediating the inhibition of tumour growth, upregulation of IL-10 is a consequence of an alternative mechanism. For example, increased TGF-β production has also been shown to drive IL-10 expression [705]. Furthermore, supplementation with *Bif* has also been shown to regulate TGF-β production *in vivo*; Yang *et al.* demonstrated that probiotic *Bif* administration increases TGF-β1 expression in the colon [706]. Importantly, this effect was also observed in the serum of treated animals, therefore these effects may be transmissible to distant tumours if replicated in our model. The tolerogenic function of *Bif* in this setting was mediated by expansion of regulatory CD103 expression DCs in PPs. Whilst we have generated data as to DC numbers in our model, we do not have any phenotypic data about their functional properties, therefore it is possible that *Bif* are driving the same effects in our model. The authors also observed a consequent increase in FoxP3+ T_{reg} numbers in PPs, the mLN and spleen, an effect which we have not observed and therefore may not be consistent with our data. However, in a separate study, Izumi *et al.* made similar findings in DSS induced colitis models and observed significant increases in TGF-β1 production in the colon after *Bif* supplementation [707]. Interestingly, the source of the TGF-β1 was revealed to be T_{regs}, but there was no accompanying increase in their overall population size. Therefore, it is possible that *Bif* may modulate T_{reg} responses without affecting their numbers. It would therefore be interesting for us to profile TGF-β production in the gut and tumour after supplementation with our *Bif* cocktail in order to gain further mechanistic insights.

We also considered whether *Bif* may not be the primary bacterial genera mediating the inhibition of tumour growth. As already discussed, *Bif* exhibit social behaviour in the gut microbiota by cross-feeding metabolites into other bacterial species. This has been shown to support the growth and maintenance of other beneficial species in the microbiome. The best studied interactions are those between *Bif* and butyrogenic bacterial species as production of butyrate is known to modulate a range of host interactions. Acetate and lactate produced by *Bif* have been shown to provide nutrition for other species such as *Eubacterium rectale* and *Anaerostipes caccae* leading to increased population numbers and a concomitant increased in butyrate production [708], [709]. Additionally, digestion of oligofructose by *Bif* allows *Roseburia intestinalis* to utilise the released fructose and has been shown to increase their growth rate [710]. Interestingly, *R. intestinalis* has also been shown to promote IL-10 production but does so by suppressing IL-17 production [711]. Therefore, in order to understand how the microbiome may be influencing BC growth, we

must analyse how *Bif* impacts the wider bacterial community. We plan to achieve this by performing shotgun metagenomic analysis of the microbiome after *Bif* supplementation, however this data is, unfortunately, not yet available. Additionally, it would be advantageous to understand how *Bif* supplementation modulates metabolite production in the gut microbiome. Therefore, we intend to perform fecal metabolomic analysis, particularly focused on SCFA production, however this analysis is unlikely to be performed in the near future due to funding restrictions.

In conclusion, we have shown that probiotic supplementation with bifidobacteria is effective at suppressing primary tumour growth in murine models of BC. This appears to be through modulation of anti-tumour immunity. However, no immune cell populations show any significant changes either in the tumour, mLN or spleen. Despite this, some intratumoural immune modulation appears to be occurring as IL-10 production is significantly elevated. This appears to be driven by *Bif* supplementation as refinement of our cocktail resulted in improved anti-tumour efficacy that was accompanied by further elevated IL-10 production. However, we are not clear as to the mechanisms that link probiotic *Bif* supplementation and IL-10 upregulation. Neither do we understand why increased intratumoural IL-10 production results in inhibition of primary tumour growth. As discussed, further qualitative profiling is required of intratumoural immune populations to determine how *Bif* supplementation alters their functional properties. Specifically, to address which immune cells are responsible for producing IL-10 in our model allowing us to focus our mechanistic investigations. Additionally, profiling of gut associated immune populations is also required, particularly in Peyer's patches. The proportion of DCs is high in PPs, and the known host interactions of *Bif* suggest that they promote DCs to adopt a regulatory phenotype and may explain how supplementation with *Bif* drives distant IL-10 production. Whilst there are many questions that remain to be answered, we believe our findings represent a promising avenue for therapy in BC. Probiotic administration is generally considered to be safe, inexpensive, and has very few side effects. It is our hope that *Bif* supplementation will synergise with current chemo and immuno therapies and make its way into the clinic, though this likely represents the next chapter in a very long road.

5. The microbiota plays a role in establishment of the pre-metastatic niche in breast cancer by modulation of immune cell infiltration

Metastasis describes the spread of cancer cells from a primary tumour to distant organs of the body. Once a tumour has metastasised, it is generally considered to be incurable and treatment modalities switch from cure to control [441]. Many metastatic cancers will eventually become terminal and it is estimated that 90% of all cancer deaths are the result of metastatic spread [712]. Worryingly, this statistic has remained unchanged for over 50 years [713]. This is particularly relevant to BC. If BC is diagnosed before metastatic spread, or even if spread is limited to local lymph nodes, prognosis is good. Over 80% of all women diagnosed with such disease will live for over five years. However, once metastatic cells have reached distant organs, the prognosis worsens significantly. Only 15-20% of women will survive for five years after their diagnosis and survival rates have barely improved over the last 10 years, despite significant advances in primary tumour treatments [714]. New ways of thinking are required with respect to treatment of metastatic breast cancer. To address this, we have applied our preliminary findings regarding the contribution of the gut microbiota to anti-cancer immune responses in BC to determine if there is also any effect on metastatic dissemination. This chapter first introduces the theories behind metastatic dissemination and establishment of the pre-metastatic niche, before presenting our data so far.

5.1. Introduction to the chapter

5.1.1. The process of metastasis

Metastasis is the result of malignantly transformed cells escaping the tumour via extravasation and being transported to distant sites, usually through the vasculature or lymph vessels. Cells will then lodge at metastatic sites, intravasate and establish a new metastatic colony at the distant organ [715] (Figure 5.1). However, it's estimated that less than 0.01% of tumour cells can form metastases [716]. This is likely due to the considerable challenges involved in reaching metastatic sites. This section will highlight these challenges and describe the mechanisms by which metastatic cells can avoid them and flourish.

The first step in developing metastatic potential is acquisition of a motile phenotype. This is frequently attributed to cells undergoing an epithelial-to-mesenchymal transition (EMT).

Cells undergoing this transition downregulate cell-cell and basement membrane adhesion factors, upregulate migratory genes and increase their resistance to apoptosis. The canonical markers of EMT in cancer are the cell-cell adhesion molecule E-cadherin and the migratory protein Vimentin [717], [718]. The expression of the former has been shown to significantly reduce during EMT, resulting in loss of cellular adhesion, upregulation of β-catenin mediated gene expression and induction of a motile phenotype. This loss of E-cadherin in malignant cells has been strongly associated with a metastatic phenotype *in vivo* [719]. Once malignant cells have developed a migratory phenotype, they still need to cross the endothelial barrier to reach the vasculature. It has been suggested that tumour cells achieve this by increasing vascular permeability via several secreted factors. Hepatocyte growth factor (HGF) can induce vascular permeability by reducing occludin expression, a key cell-cell adhesion molecule [719]. Also, the highly metastatic B16 cell line can release collagenases which directly impair endothelial barrier function by digestion of endothelial collagens [720]. Both modalities have been shown to contribute to metastatic cell extravasation. Whilst the movement of cells via the circulatory system has a considerable contribution to metastasis, the primary method of cell spreading is via lymphatics. The lymphatic system spans the entirety of the body and plays a key role in movement of interstitial fluid and distribution of immune cells. Crucially however, in contrast to the circulatory system, lymphatics are not a closed system. The basement membrane of lymphatic vessels is not continuous, therefore their permeability is significantly increased compared to circulatory vessels [721], [722]. However, the role of lymphatics in metastasis is not limited to passive movement of tumour cells into vessels. Instead, peritumoural lymphatics have been shown to actively respond to tumour secreted factors, particularly VEGF-C. Signalling by VEGF-C has been shown to increase flow rate in lymphatic vessels, increasing metastatic dissemination [723], [724]. Additionally, tumour associated lymphatic endothelial cells show altered cytokine expression profiles which can result in increased production of chemokines such as CCL21 and CXCL12. These chemokines can directly interact with tumour cells to present a homing signal, therefore facilitating lymphatic metastasis [725], [726].

Gaining access to the vasculature is only one half of the battle for a tumour cell attempting to spread to distant organs. Once transmigration has occurred, tumour cells come under attack from two angles: fluid shear stress and interrogation by immune cells. Flow rates in the circulatory system can be anything up to 40cm/s depending on the vascular location [727]. How circulating tumour cells (CTCs) manage to survive these extreme conditions is poorly understood. There is some suggestion that migratory tumour cells may change their mechanical properties to cope with circulatory forces. Migratory cells have been shown to

remodel their cytoskeletal actin structures, resulting in a softening of the cell cortex that facilitates migration through small spaces [728]. Whilst this is primarily required for migration through extracellular matrices, it also appears to confer a survival advantage during circulation. Modelling of fluid shear stresses *in silico* shows that a softer cell cortex promotes cell survival during exposure to high flow rates [729]. Additionally, tumour cells have been suggested to be capable of imitating leukocyte adhesion and rolling. This behaviour partly contributes to survival under high shear stress, but has also been implicated in facilitating extravasation of CTCs at metastatic sites [730]. In addition to surviving the forces of circulation, CTCs will frequently collide with, and therefore be profiled, by circulating immune cells. Interactions with cells such as NKs, macrophages and neutrophils play a considerable role in controlling CTC numbers. In BC patients with compromised NK function, CTCs are present at much greater proportions [731]. However, relatively speaking these events are still extremely rare. In this study, patients with elevated CTC numbers were described as having more than five detectable cells. Furthermore, CTCs are detected at a rate of 1 per billion relative to normal blood cells [732]. Therefore, significant technical challenges have had to be overcome in order to biologically profile CTCs. This has led to a gross lack of understanding with respect to how CTCs subvert the immune response. However, recent advances in single cell sequencing have begun to shed some light on the differential expression profiles of individual cells that confer a selection advantage in metastatic dissemination. In colorectal cancer, CTCs have been shown to overexpress the immune regulatory receptor CD47. Interactions between CTC mediated CD47 and its receptor SIRPa on T cells and macrophages has been shown to potently disrupt cytotoxic responses [733], [734]. This work has been extended to BC, where presence of CD47 CTCs has been shown to correlate with relapse [731].

Therefore, in the context of preventing tumour cell circulation, the immune response appears to be protective. However, in the context of facilitating metastatic dissemination, the immune response has a more sinister role. There are strong associations between immune produced cytokines and attraction of tumour cells to metastatic sites. What's more, a high proportion of immune cells in tissues such as the lung and bone have been shown to not only support, but promote growth of metastatic lesions [735]. These interactions and the proposed conditioning of the metastatic 'soil' by immune cells will be discussed in detail in the following section.

5.1.2. Establishing the early-metastatic niche

The earliest predictions as to how metastatic dissemination is determined was thought to be through emboli of circulating tumour cells in the microvasculature. According to this model, the distribution of metastatic growth should be inherently random. However, in 1889, Stephen Paget examined post-mortem records of hundreds of women with BC. He deduced that metastatic spread favoured certain organs over others, coining the 'seed and soil' hypothesis [736]. This states that certain organs provide a suitable environment for growth of metastatic lesions, whilst others will reject any disseminated tumour cells. These principles were largely ignored by the medical community and not explored further for almost 100 years. That is, until Isaiah Fidler's seminal findings during the 1970s and 80s. Along with Ian Hart, they published key findings that suggested metastatic colonisation is unlikely in certain organs, but will occur freely in others such as the lung [737]. These findings were extended in 2009 by Psaila *et al.*, who demonstrated that primary tumours could manipulate the microenvironment in distant organs to promote growth of disseminated cells [738]. This gave birth to the prevailing theory of the moment, the metastatic niche model.

Whilst still controversial, the metastatic niche has gained considerable attention. Its basic ideas suggest that tumour secreted factors drive recruitment and activation of essential effector cells in metastatic organs. These effectors have several key functions, such as permitting CTC extravasation, facilitating their growth and preventing destruction by immune cells [739]–[741]. These processes are in part driven by activation of resident stromal cells such as fibroblasts and endothelial cells, however recruited bone marrow derived cells are essential for proper formation of the pre-metastatic niche [742]. A diverse array of immune cells has been shown to congregate at sites of metastatic seeding, particularly those of the myeloid lineage. Both macrophages and neutrophils have been shown to accumulate in large numbers in distant organs during tumorigenesis, particularly in the lung in the context of BC [743], [744]. These cells often precede the infiltration of metastatic cells and inhibition of this immune accumulation has been shown to strongly suppress formation of metastases.

The mechanisms by which tumours drive immune cell accumulation at metastatic sites are only now beginning to be understood and appear to be heavily influenced by tumour secreted factors. The chemokine CCL2 is central to BMDC recruitment at the PMN. Several tumours, including BC, have been shown to secrete CCL2 leading to high serum levels. Interaction of CCL2 with its cognate receptor, CCR2, increases the survival and proliferation

of tumour cells, but also drives recruitment and differential activation of stromal elements [745]. Qian *et al*. have shown that tumour derived CCL2 drives accumulation of CCR2+ monocytes and macrophages at metastatic sites in the lung. These have been shown to significantly contribute to formation of early metastatic lesions, so much so that inhibition of CCL2 signalling alone inhibits formation of lung metastases [746]. Interestingly, tumours have also been shown to modulate the metastatic environment by release of exosomes. Tumour exosomes in BC home to the lung in an integrin dependent manner. Here they release their contents and induce Src phosphorylation in stromal cells. This results in increased secretion of the pro-inflammatory S100 cytokines, particularly S100A4 [747]. Upregulation of S100A4 in the PMN drives T cell infiltration and promotes metastatic seeding [748].

Once collected in the metastatic organ, immune cells have a diverse set of unique functions, from improving vascular permeability to promoting remodelling of the extracellular matrix. The former is primarily driven by CCR2+ myeloid cells. In the study by Qian *et al*. they observed high expression and secretion of the angiogenic factor VEGF-A in infiltrating monocytes and macrophages. This increased angiogenic signalling promoted local vascular remodelling at the PMN and increased vascular permeability. These effects appear to facilitate extravasation of CTCs into the PMN and in studies using VEGF-A KO animals, metastatic seeding was severely impaired [749]. In addition to activation of classical angiogenic signalling machinery, the BMDCs in the PMN have been shown to alter vascular permeability by non-canonical pathways. Specifically, through activation of innate immune signalling in endothelial cells. Using an orthotopic implant model with the murine EO771 BC cell line, CCR2+ immune cells at the PMN were shown to upregulate another S100 family protein, S100A8. In turn, this induced expression and secretion of Serum Amyloid A3 (SAA3). Interaction of SAA3 with its functional receptor TLR4/MD2 induced vascular permeability through activation of focal adhesion kinase (FAK). Inhibition of MD2 signalling prevented these effects and minimised metastatic seeding to the lung [750]. Therefore, modulation of the local vasculature in the metastatic site by BMDCs is essential for correct function of the metastatic niche.

In addition to their role in facilitating CTC extravasation, BMDCs have also been implicated in promotion of ECM remodelling during establishment of metastases. Many immune cells initiate secretion of proteases, particularly the Matrix Metalloproteases (MMPs), after infiltration of the PMN. For example, mac-1+ macrophages and neutrophils express high levels of MMP9 in the lung during establishment of the PMN [751], [752]. Through degradation of collagens, MMP9 has numerous effects at the niche. The primary component

of endothelial basement membrane is collagen IV, which MMP9 readily enzymatically digests. This has been shown to contribute to endothelial barrier dysfunction and promote CTC invasion into the lung [753]. Furthermore, this digestion of collagen IV can release biologically active fragments that have been shown to promote tumour cell adhesion and therefore may drive metastatic seeding. These processes have been shown to promote adhesion and infiltration of immune cells at the PMN, resulting in a positive feedback loop of BMDC recruitment [754]. Fragments of type IV collagen have also been shown to serve as adhesion factors for BMDCs. Additionally, the lysyl oxidase (LOX) enzymes are significantly upregulated in the PMN. Their crosslinking of type I and type IV collagen is required for infiltration of CD11b+ immune cells such as MDSCs [755]. These cells are required to facilitate the final crucial ingredient for a functional metastatic niche, evasion of cytotoxic immune cells.

Whilst inflammatory responses play a key role in generating a PMN that supports invasion and growth of metastatic cells, it is inevitable that during this process metastatic cells will interact with cytotoxic leukocytes. It is therefore necessary to prevent these immunological terminators from successfully eradicating the foreign metastatic invader. To this end, several immunosuppressive immune cells are also recruited to the niche. For example, breast tumours have been shown to secrete high levels of G-CSF. At the bone marrow, this drives proliferation of several neutrophil subsets, including inhibitory CD11b+ Ly6G+ cells. These are recruited in large numbers to the lung PMN and produce high levels of ROS. This was shown to effectively inhibit T cell cytotoxicity and therefore promote survival of disseminated metastatic cells [756]. A closely related cell type, the MDSC is also recruited into the PMN and is the cell type that is best characterised with respect to promotion of metastatic growth through local immunosuppression. Whilst the role of MDSCs in the PMN is relatively well studied, very little is known about the phenotype or characteristics of the MDSC itself. They are identified in mice by expression of the surface markers CD11b and Gr-1. However, these markers are also expressed by several other cell types, therefore the populations described as MDSCs in the literature are still extremely heterogeneous. They can broadly be subdivided into monocytic and granular MDSCs, which share morphological similarity with monocytes and neutrophils, respectively [757]. The differentiation cascades responsible for MDSC production and accumulation are also very poorly understood. The kinetics of MDSC appearance during immune responses are gradual, with few cells observed during the initiation of immunity. However, as the inflammatory environment matures MDSC numbers gradually increase. It has been suggested that collaborative signalling mediated by several cytokines may drive MDSC differentiation from other myeloid precursors [757]. For example, administration of GM-CSF alongside IFN-γ has been shown

to promote monocytes in culture to take on an immunosuppressive phenotype that resemble that of MDSCs [758].

The immunosuppressive functions of MDSCs are primarily centred around inhibiting T cell function. This is most effectively achieved by production of ROS and reactive nitrogen species. These suppress T cell activities by preventing TCR chain expression and interference with IL-2 signalling mechanisms resulting in T cell anergy [759]. Additionally, MDSCs can prevent clonal expansion of T cells through arginase expression. Proliferation of T cells is L-arginine dependent and its metabolism by MDSCs significantly impairs T cell growth [760]. Through direct cell-cell contact, MDSCs have been shown to promote T_{reg} expansion, however the mechanisms of this interaction are not fully understood [761]. In addition to inhibition of T cell mediated immune responses, MDSCs can also inhibit the phagocytic activity of macrophages. Production of IL-10 by MDSCs suppresses IL-12 production in macrophages and skews them towards an M2 phenotype. In addition to suppressing local immune responses, this effect has been shown to promote tumorigenesis by increasing M2 macrophage polarisation [762].

These potent immunoinhibitory functions make MDSCs well adapted to contributing to PMN formation. The presence of MDSCs at the PMN is strongly implicated in driving metastatic seeding. Their accumulation is thought to be a result of tumour derived factors, but the factors that are specifically required for MDSC recruitment are yet to be identified. Recently, Wang *et al.* have shown that the cytokine CXCL1 is required for tracking of MDSCs to the live PMN in models of colorectal cancer [763]. However, it is yet to be seen whether this will translate to other cancers. In BC, MDSCs at the PMN have been shown to promote metastatic seeding through several mechanisms. In murine models, conditioned media taken from hypoxic tumour cells induced MDSC accumulation in the lung and this is accompanied by increased metastatic seeding. This was shown to be a result of MDSC mediated inhibition of NK cytotoxicity, suggesting MDSCs can protect metastatic cells from immune destruction [764]. In addition to impairing the anti-tumour immune response at the PMN, MDSCs have also been demonstrated to modulate the properties of CTCs. By secreting IL-1β in the PMN, MDSCs increase E-selectin expression in local endothelial cells. This drives CTC arrest in the lung and increases metastatic seeding to the lung [765].

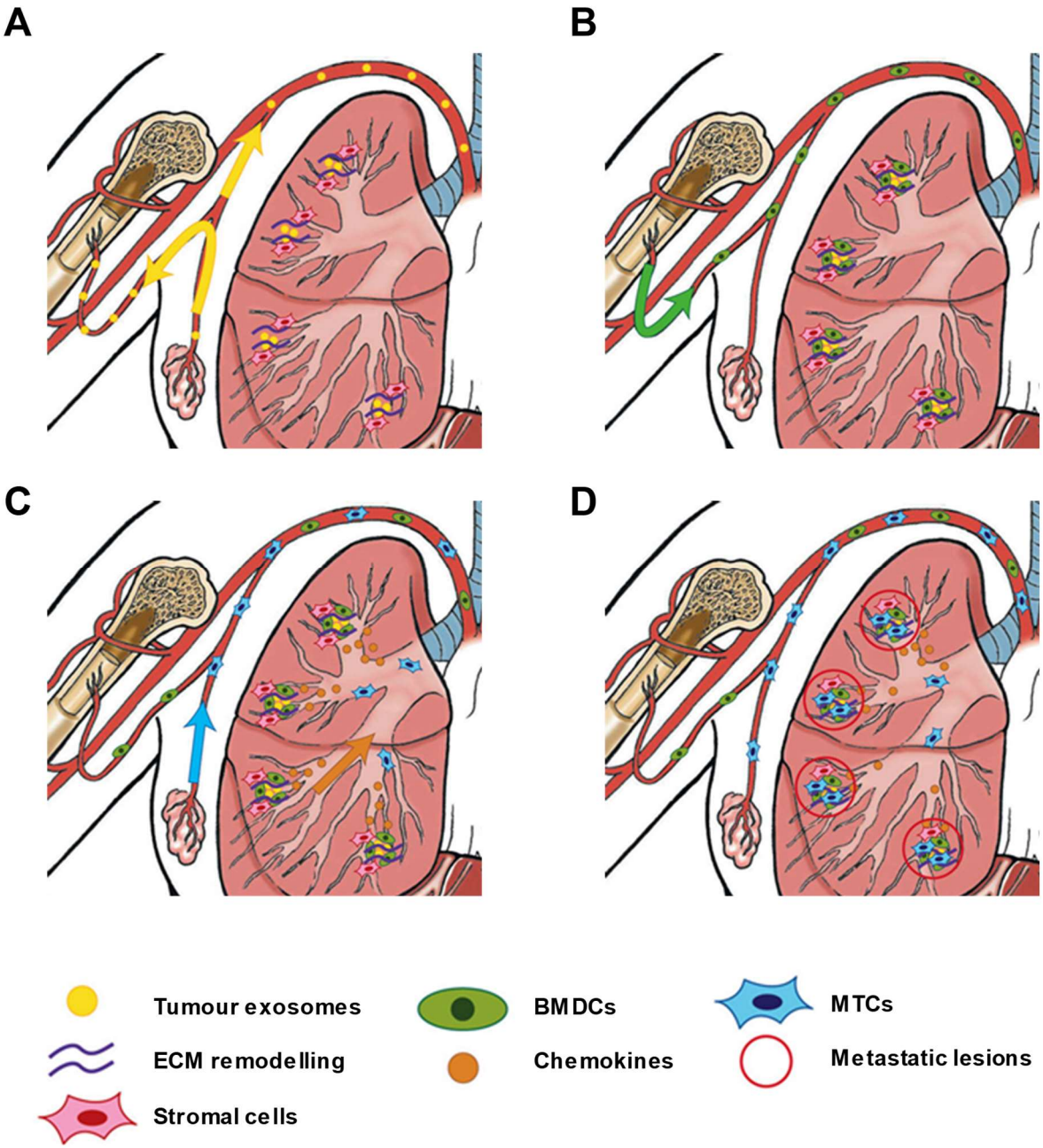


Figure 5.1 – Establishing the pre-metastatic niche: Schematic representation of the processes involved in setting up the lung metastatic niche during BC. A) Tumour exosomes home to metastatic organs. Their payload induces changes in expression of stromal cells, particularly with respect to secreted cytokines and chemokines. **B)** Stromal cell chemokines recruit BMDCs to the metastatic niche. Remodelling of the local ECM is initiated in addition to production of angiogenic cytokines to facilitate CTC extravasation. **C)** BMDCs and stromal cells secrete chemotactic factors which promote CTC recruitment to the niche. **D)** CTCs extravasate and occupy the niche, beginning the development of metastatic lesions in the lung. Adapted from [790].

5.1.3. Growth of metastatic lesions

The early view of the metastatic cascade was that of rapid dissemination, metastatic seeding, lesion growth and mortality. This model of continuous growth was the prevailing theory of metastatic outgrowth, until computational modelling using tumour growth kinetics suggested that continuous growth does not satisfactorily explain clinically observed rates of metastatic incidence [766]. Despite evidence to suggest that initial metastatic dissemination occurs early in tumorigenesis, many patients do not relapse for many years after their initial diagnosis and treatment [767]. This is particularly apparent in BC and, interestingly, the time to metastatic relapse is dependent on molecular subtype. Patients who present with TNBC or HER2+ tumours that go on to develop distant metastases do so rapidly within 5 years. However, HR+ luminal tumours have a constant rate of metastatic development that occurs over a period of up to 15 years [768]. Therefore, disseminated metastatic cells likely undergo a period of dormancy after invasion at the metastatic site. The mechanisms that govern this refractory period are largely unknown, however recent theories suggest that disseminated cells struggle to adapt to the foreign microenvironment. This is despite the formation of a PMN that encourages and permits CTC extravasation, suggesting that the metastatic niche must enter a period of remodelling after metastatic seeding to support growth. Several studies have concluded that metastatic cells enter a period of senescence after entering the metastatic organ [769]. This is highlighted by upregulation of cell survival signalling in disseminated cells in response to stromal factors. For example, disseminated BC cells in the bone increase Akt phosphorylation through activation of Src by stromal cell-derived factor (SDF)-1/CXCR4 signalling [770]. Additionally, metastatic cells in the lung also induce Akt activation, but macrophages contribute significantly. Interaction of tumour cell derived VCAM-1 with α4β1-integrin on infiltrating macrophages at the metastatic niche increases Akt phosphorylation via the Ezrin/PI3K signalling cascade [771]. Inhibition of these survival pathways results in reduced metastatic seeding, suggesting these early survival signals are essential for metastatic outgrowth. The necessity of this signal upregulation has been suggested to permit disseminated cells some time to form the necessary matrix associations to facilitate proliferation. Metastatic BC cells have been observed to do this in several ways. Firstly, BC cells can directly produce the matrix components required for proliferative signalling. For example, during pulmonary metastasis, disseminated BC cells themselves begin producing tenascin C (TNC). In later stages of outgrowth, recruited S100A4+ fibroblasts also begin producing TNC. Interactions between TNC and tumour cells result in increased Notch and Wnt signalling, therefore improving disseminated cell survival and permitting proliferation [772], [773]. Importantly, in TNC KO animals, growth of BC pulmonary metastatic lesions is significantly impaired. However,

primary tumour growth and other stem cell niches are unaffected [774]. Therefore, inhibition of TNC/MDC interactions may prove to be a promising therapeutic strategy to prevent metastatic growth. Finally, to establish macrometastatic lesions, disseminated cells must subvert other stromal elements to permit growth. Infiltrating metastatic cells often display mesenchymal cell characteristics. Whilst invasive, this cellular state is not permissive of rapid proliferation. The reverse process, the mesenchymal to epithelial transition (MET) requires significant contribution from stromal BMDCs. Monocytes in the lung have been shown to produce versican, which, through inhibition of TGFβ signalling, promotes MET and drives metastatic cell proliferation [775]. The last piece of the puzzle to develop a fully-fledged metastatic lesion is an ample blood supply. Using the MMTV-PyMT model of BC, Mazzieri *et al.* have shown that prevention of tumour cell derived Angiopoeitin-2 from interacting with its cognate endothelial receptor, Tie2, prevents metastatic growth. This is primarily by prevention of a robust angiogenic response and hypovascularisation of metastatic lesions [776]. Interestingly, use of Ang-2 inhibitors also caused regression of established metastatic lesions and therefore may prove to be a useful therapeutic strategy to minimise metastatic outgrowth.

5.2. Treatment with VNMA antibiotics reduces the number of animals presenting with disseminated metastatic cells in the lung

To assess the contributions of the microbiome to early metastatic processes, we undertook profiling of early tumour cell dissemination. The B6BO1 cell line we typically use in our orthotopic model metastasises to lung and bone, but the rate of metastatic spread to the latter is very rare (typically less than 5%), so we chose to focus on the lung. Animals harbouring luciferase tagged B6BO1 tumours were treated with the previously documented regimen of VNMA antibiotics or the v1.0 regimen of Bifidobacteria probiotics. In a typical metastasis experiment, tumour resection occurs at 1000mm^3 and the animals are left for a further 3-4 weeks to allow metastatic lesions to develop (Figure 5.2A). However, in these experiments, animals were sacrificed at the point where tumours would normally be resected and the lungs were collected, homogenised, and lysed. Lung lysates were used in plate based luciferase assays to detect luciferase tagged, disseminated tumour cells (Figure 5.2B). Luciferase positive disseminated metastatic cells were detected in 50% of individuals in the control arm. Individuals treated with the Bifidobacteria cocktail did not significantly deviate from this rate of metastatic seeding. However, animals undergoing treatment with VNMA antibiotics displayed a significantly decreased number of individuals presenting with disseminated metastatic cells in their lungs (Figure 5.2C). This suggests that antibiotic induced dysbiosis may in fact be protective in the context of metastatic seeding.

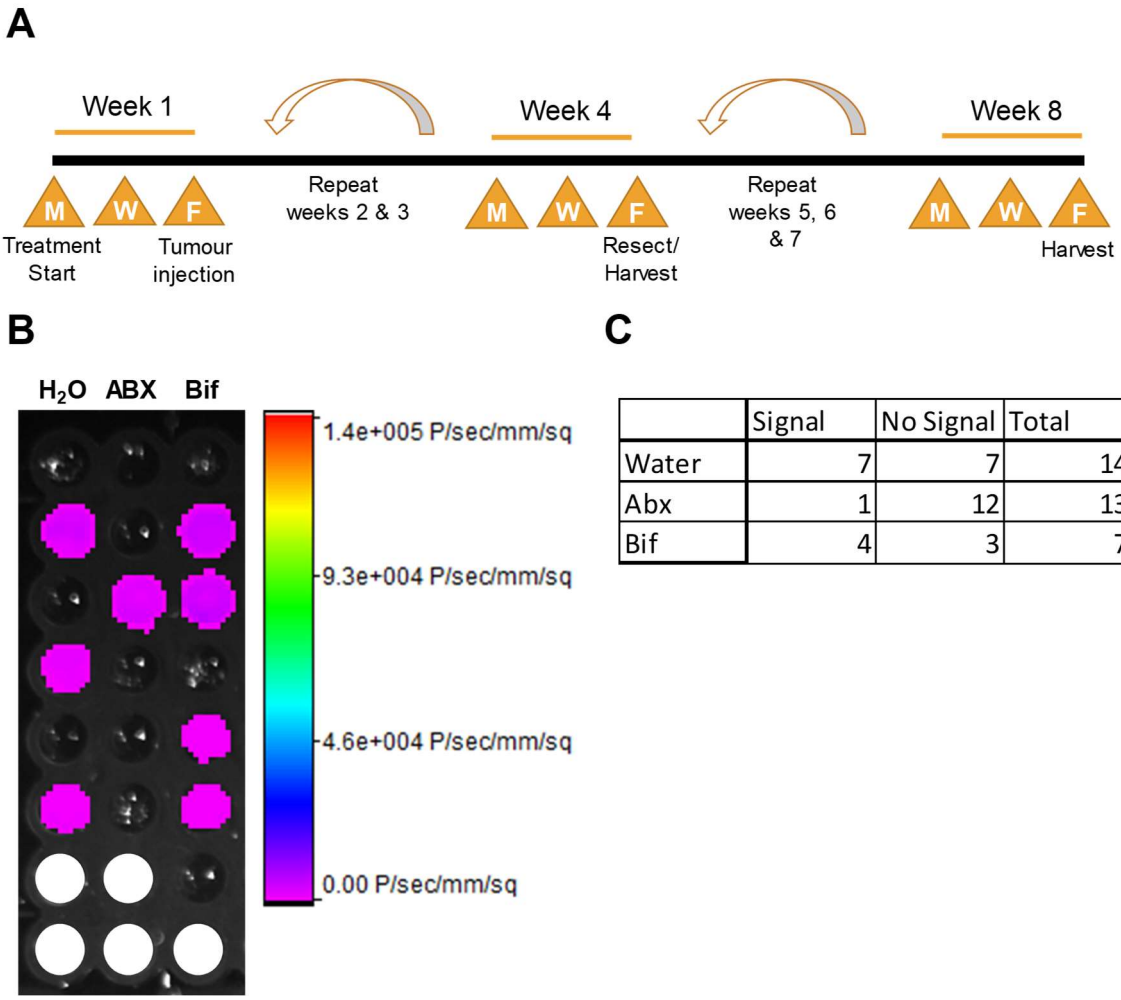


Figure 5.2 – Treatment with antibiotics results in impaired metastatic seeding to the lung: A) Treatment regimen for metastatic experiments. As in previous experiments, treatment begins 5 days prior to tumour injection and is administered for the duration of the experiment (M, W, F, as indicated). In full term metastatic experiments, as described in Figure 5.4, tumours are resected once they reach 1000mm^3 in volume and allowed to continue for 3-4 weeks to allow larger metastatic lesions to develop. In early-metastatic seeding experiments, animals were sacrificed at 1000mm^3 volume and the presence of metastatic cells profiled using luciferase assays. **B)** Representative image of luciferase assay. Lung lysates from animals treated with the indicated regimen were profiled for luciferase activity using the Promega luciferase assay system. Rows are individual samples from each group. White circles indicate wells with no sample. Scale shows photons/second/mm^2. **C)** Collated results of luciferase assays from two biological replicates. Samples are split into individuals with detectable luciferase or negative samples. Asterisks indicate significance, * $p < 0.05$, determined by Fisher's exact test.

5.3. Reduced metastatic dissemination to the lung is associated with changes in immune cell infiltrate

During development of the pre- and early metastatic niche, the immune response plays a key role in recruiting CTCs and in promoting metastatic seeding. Therefore, to assess how perturbations of the microbiome may impact this process, we undertook high level flow cytometric analysis of immune populations in the lungs. To assess myeloid cell infiltration, we used a panel consisting of CD45, CD11b, Gr-1 and F4/80; unfortunately, the F4/80 stain failed in this instance. In all groups, the percentage of CD45+ cells were approximately 45-50% and there were no significant differences in total leukocyte infiltration into the lung (Figure 5.3A). The proportion of total myeloid cells (CD11b+) in the lung was comparable in control and VNMA treated animals at 22% and 27%, respectively. However, animals treated with Bifidobacteria exhibited an almost significant increase in the number of CD11b+ cells (μ = 39%, p=0.05). Relative to total cell number, there were no significant changes in Gr-1+ cells in any group. However, when normalised to the total number of myeloid cells, significant differences were observed between groups. Animals treated with VNMA antibiotics exhibited a significant decrease in CD11b+, Gr-1+ cell numbers when compared to the control arm. Additionally, Bifidobacteria treated samples displayed an almost significantly increased number of these cells (p=0.05) (Figure 5.3B). Cells exhibiting these markers are often described as MDSCs and can be further characterised into monocytic (M-MDSC) or granular (G-MDSC) by their side scatter (SSC) profile. This analysis revealed that control and Bifidobacteria treated samples have similar proportions of M/G MDSCs, whilst the VNMA treated samples exhibit a significant shift towards G-MDSCs (Figure 5.3C&D). To investigate changes in lymphoid cell populations, a panel of CD45, CD3, CD8 and CD4 was used; unfortunately, the CD4 stain was undetectable in these samples. When normalised to total cell number, no significant differences are seen in either marker between all groups (Figure 5.3E). However, when normalised to the total leukocyte number, significant differences are observed in antibiotic treated animals. The total number of CD3+ T cells are significantly elevated, whilst the number of cytotoxic CD8+ T cells is significantly reduced (Figure 5.3F). These data suggest that perturbation of the microbiota by antibiotics disrupts the immunological development of the early-metastatic niche.

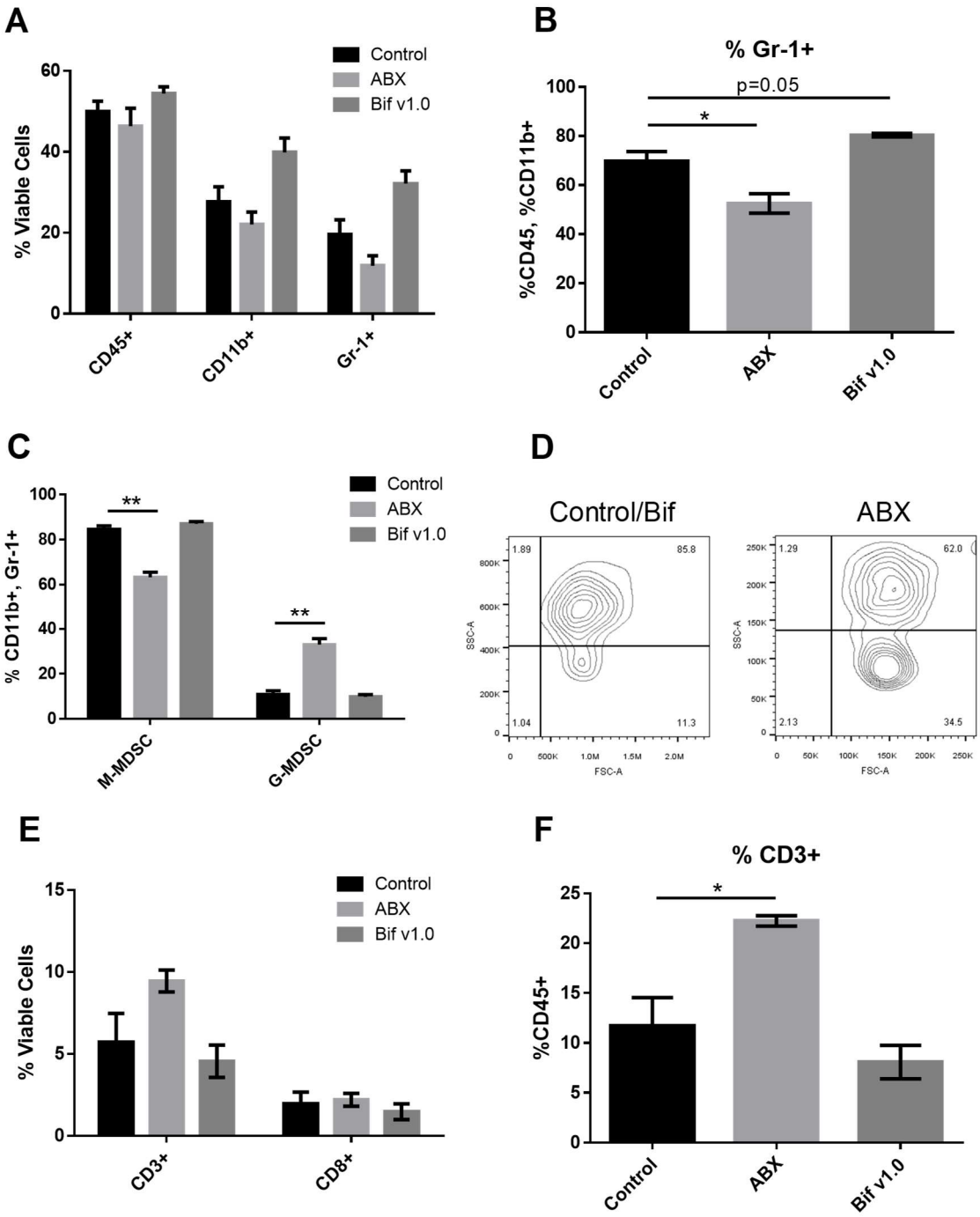


Figure 5.3 – Modulation of the gut microbiome results in immunological changes at the early-metastatic niche: Flow cytometric analysis of lungs taken from animals harbouring B6BO1 tumours at a resectable tumour volume. **A)** Analysis of myeloid populations normalised against total viable cell number **B)** Proportions of Gr-1+ positive events normalised to total myeloid infiltrate (CD11b+ events). **C&D)** Analysis of the Gr-1+ population, monocytic and granular MDSCs were separated by their granularity using the SSC channel. Representative plot shown in D. **E)** Lymphoid population analysis, normalised against total viable cell number. **F)** Percentage of lung infiltrating CD3+ T cells, normalised to total immune infiltrate (CD45+ events). Asterisks indicate significance, * $p < 0.05$, ** $p < 0.01$, determined by unpaired, two-tailed *t* test, n=3.

5.4. Long-term metastatic experiments show accelerated growth of lung metastatic lesions in VNMA treated animals

After the surprising finding that VNMA induced microbial dysbiosis inhibited metastatic cell seeding, we wanted to understand how it might impact growth of metastatic lesions. To address this, animals harbouring B6BO1 tumours were administered the VNMA antibiotic regimen. At 1000mm^3, tumours were resected by mastectomy and the experiment continued. Animals were intended to be maintained on the VNMA regimen for 4 weeks after resection, however rapid primary tumour regrowth in some individuals meant animals had to be sacrificed after 3 weeks instead. Despite this, macrometastases (defined as metastatic lesions visible to the naked eye) were observed in both groups. Lungs were histologically processed and sectioned. To detect metastases, sections were stained with anti-luciferase to highlight luciferase expressing tumour cells in the lung. The number, size, and relative area of these lesions were determined using ImageJ analysis software. There was no significant difference in the number of metastatic lesions per area in the lung between the two groups (Figure 5.4A. However, metastatic lesions in VNMA treated animals were significantly larger than the water treated controls. Many of the lesions present in the control group were still micrometastases, consisting of small collections of disseminated cells (Figure 5.4B&C). However, several had formed larger metastatic areas and occupied a significant proportion of the lung's total surface area. This suggests that whilst dysbiosis induced by antibiotics may slow the initial stages of metastatic seeding, its later impacts on formation of metastatic lesions mirrors the effects on primary tumour growth.

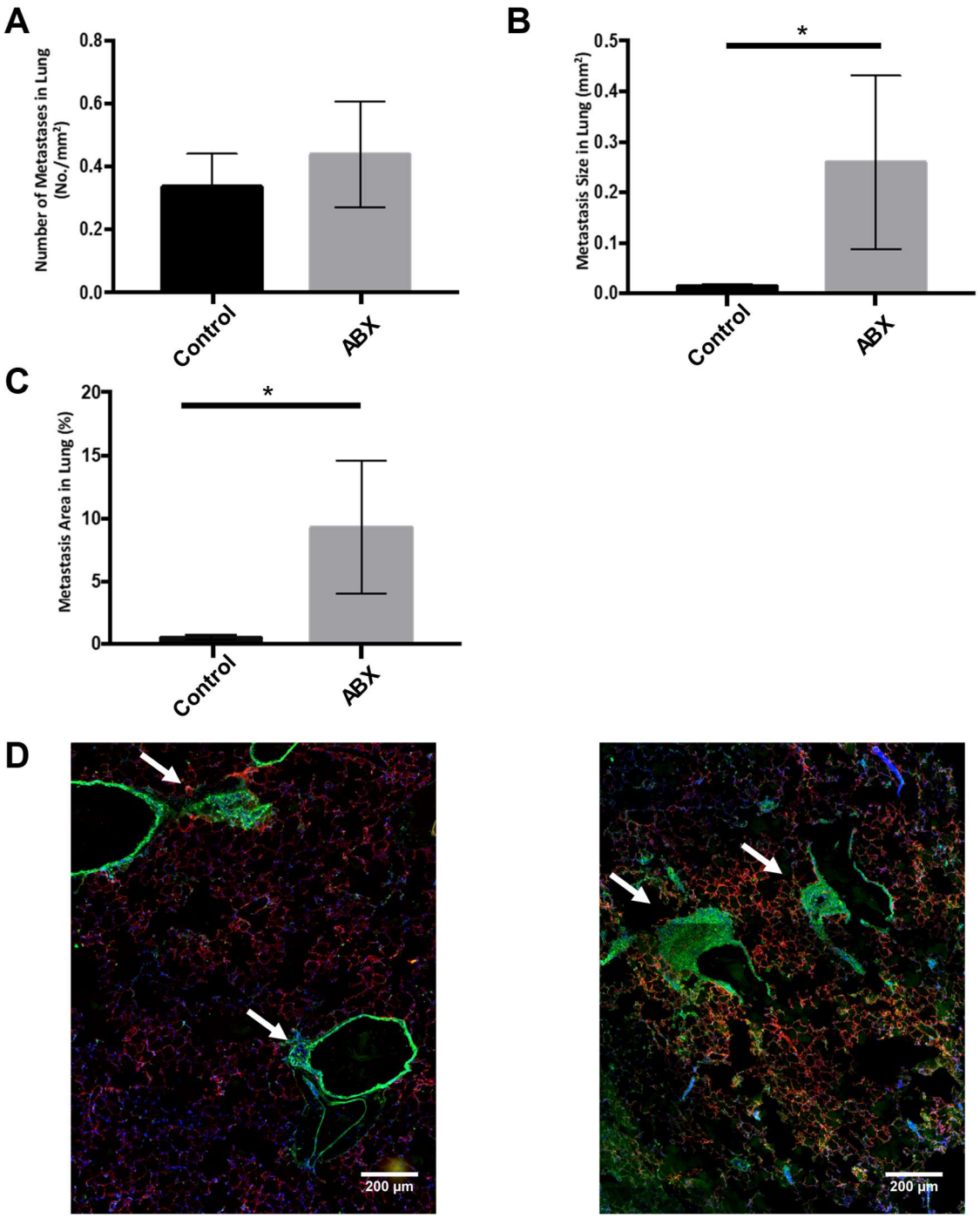


Figure 5.4 – Microbial dysbiosis results in accelerated growth of metastatic lesions in the lung: Quantification of histological analysis to determine properties of metastatic lesions in lung. **A)** Number of metastatic clusters were counted and normalised to the total area of the lung. Bars represent mean number of lesions per lung in each group **B)** Quantification of lesion size, bars represent mean of each group. **C)** Lesion size was normalised against total area of the lung, bars represent mean coverage of metastatic lesions as a percentage of total lung area. n=8 in each group in all cases. Asterisks indicate significance, * $p < 0.05$, determined by unpaired, two=tailed *t* test. **D)** Representative images of metastatic lesions in each group. White arrows highlight lesions. Green = luciferase, Red = endomucin, Blue = DAPI.

5.5. Antibiotic treatment promotes a more mesenchymal phenotype in breast tumour cells

Whilst the impact of microbial dysbiosis on the metastatic potential of the distant organ has been assessed, we sought to understand if this dysbiosis may result in any changes at the primary tumour that may impact their metastatic potential. Epithelial to mesenchymal transition is a key factor in the ability of tumour cells to metastasise. The advancement of this process can be determined by looking at expression of the epithelial marker E-Cadherin and the mesenchymal marker vimentin. We undertook western blots of whole tumour lysates to investigate the expression level of these proteins in our tumours. Whilst levels of vimentin remain unchanged, E-cadherin expression is significantly reduced in tumours from VNMA treated animals (Figure 5.5A&B). This suggests that microbial dysbiosis may have direct impacts on the phenotype of tumour cells that could increase their proclivity to tissue invasion.

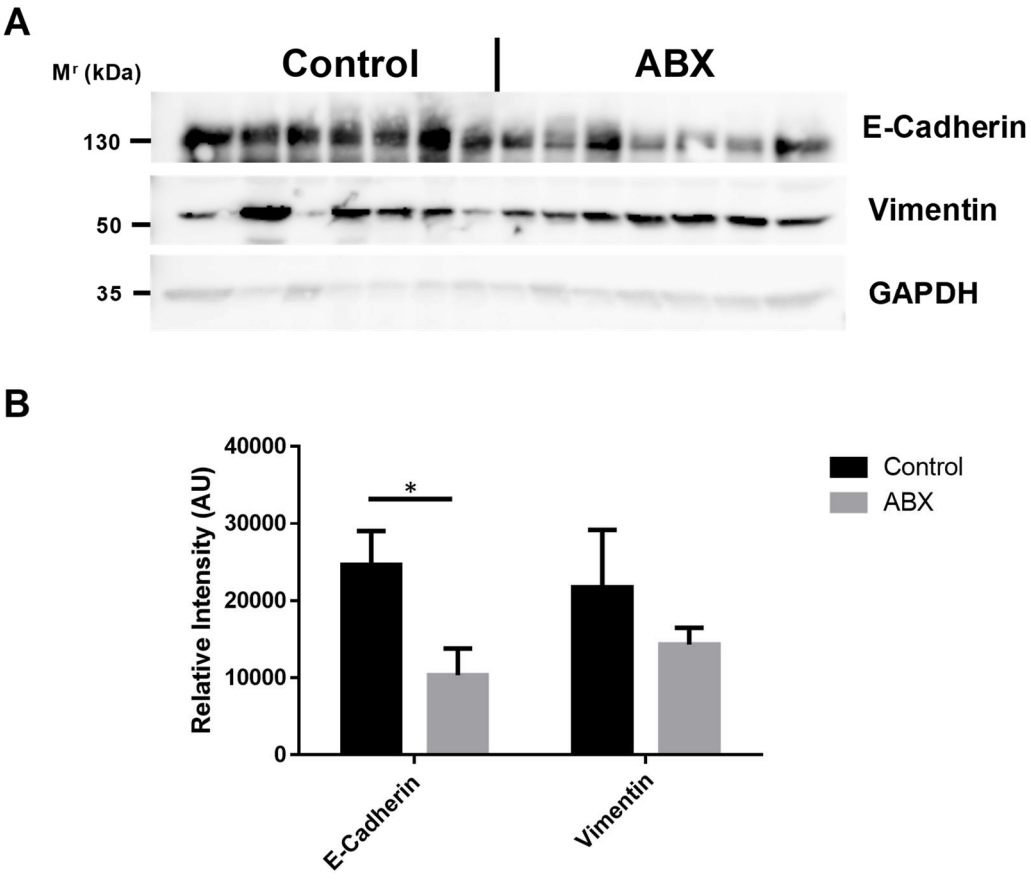


Figure 5.5 – Antibiotic induced dysbiosis drives EMT in breast tumours: Western blot analysis for the markers of EMT, E-cadherin and vimentin. **A)** Blot image showing 7 biological replicates being analysed for expression of these two proteins. **B)** Quantification of the blot shown in A, protein levels are normalised to GAPDH expression. Bars represent mean levels from replicates shown in A. Asterisk represents statistical significance, $p < 0.05$, determined by unpaired, two-tailed *t* test.

5.6. Discussion

The significance of the gut/tumour axis has only recently been realised. As a result, the field is in an infant stage, but is receiving a growing amount of attention from researchers worldwide. Despite this, investigations delving into the role of the gut microbiome in the metastatic cascade are almost completely absent. This is surprising given the importance of the gut microbiome in shaping immune responses and the indispensable role that BMDCs play in establishing both the early and mature metastatic niches. To address this, our study focused on how manipulations of the gut microbiome impact metastatic development in murine models of BC, particularly with respect to its modulation of pro-metastatic immune responses. We have previously shown that the disruption of the gut microbiota through administration of antibiotics accelerates primary tumour growth through dysregulation of anti-tumour responses. Therefore, we hypothesised that the same dysbiosis would also increase early metastatic seeding and accelerate the growth of pulmonary lesions. Unexpectedly, antibiotic administration impaired early metastatic seeding, suggesting the gut microbiota is a negative regulator of pulmonary BC metastasis. Whilst there are published associations between certain bacterial species and the rate of metastasis in colon cancer, there are yet to be any such studies with respect to non-GI cancers [777]. Furthermore, even in colon cancer, the mechanistic basis for these associations are yet to be described. Therefore, to probe the mechanism that may be driving our metastatic phenotype, we turned to the establishment of the pre- and early metastatic niche. We hypothesised that a dysbiotic gut microbiome may inhibit the immunological processes that contribute to proper development of the PMN. High level profiling of lung infiltrating immune cells at the early stages of metastatic colonisation revealed significantly decreased numbers of CD11b+, Gr-1+ cells and significantly increased numbers of CD3+ T cells in antibiotic treated lungs. These data agree with other literature that suggests these cells types are pro and anti-metastatic respectively.

The CD11b+, Gr-1+ cell population observed in our experiments is broadly described to be that of MDSCs. However, anti-Gr-1 has specificity for both Ly6G and Ly6C. Therefore, this population remains incredibly heterogeneous and will include inhibitory monocytes and neutrophils. Furthermore, due to the loss of F4/80 staining as a result of technical difficulties, this population will also include some macrophages. It would have been advantageous in this experiment to include separate stains for both Ly6G and Ly6C, however antibody availability prevented this. In light of these limitations, the work will be discussed on the assumption that these are predominantly MDSCs but must be heavily caveated until the populations can be assessed in more detail.

The presence of MDSCs in the early metastatic niche is generally considered to be pro-metastatic. Yan *et al*. show that in models of murine BC, pulmonary MDSC accumulation precedes metastatic seeding. The authors go on to show that MDSCs exert their pro-metastatic effects by secretion of MMP9 and therefore induction of vascular permeability. Further to this, they demonstrate that inhibition of MMP9 reduces metastatic seeding to the lung [778]. Whilst we were unable to explain the role of MDSCs in our model, it is sensible to presume that we may see similar mechanistic effects. Unfortunately, because metastatic seeding does not occur homogenously across the organ of interest, the luciferase assay requires whole lung homogenisation to avoid spatial bias. Therefore, we were unable to return to these samples and perform histological analysis of the vasculature. However, these data provide a focal point for any future experimental analysis. To determine how the microbiota may influence MDSC infiltration to the lung, it is pertinent to perform measurements of serum cytokine levels. We have previously shown that intestinal production of CXCL1 is significantly reduced by antibiotic administration. This is important, given that other works have found CXCL1 to be a key driver of CD11b+, Gr-1+ cell recruitment. A study by Acharyya *et al*. found that tumour produced CXCL1 promoted pulmonary infiltration of CD11b+, Gr-1+ cells which support metastatic seeding in murine models of BC [779]. It is therefore reasonable that a loss of microbiota driven CXCL1 may impair seeding of metastatic cells in our model. It is interesting that treatment with Bifidobacteria resulted in an almost significant ($p=0.05$) decrease in CD11b+, Gr-1+ cell number in the lung. If our hypothesis is correct regarding the importance of this cell population in promotion of metastatic seeding, we would expect to see increased dissemination in bifidobacteria treated individuals. However, contrary to this, we see no changes in the number of individuals presenting with metastatic cells in the lung. The reasons for this are unclear but may be related to the proportion of M- and G-MDSCs present in the metastatic niche. We observed a significant shift from M-MDSCs to G-MDSCs in antibiotic treated lungs, whilst animals treated with Bifidobacteria resembled the control. This may begin to explain two facets of our metastatic phenotype. Both subsets of MDSC are immunosuppressive, however M-MDSCs are proposed to be more immunosuppressive than G-MDSCs due to their potent production of NO via NOS2 [780], [781]. Therefore, reduced proportions of M-MDSCs may hinder the pulmonary immunosuppression required for effective formation of the early-metastatic niche. On the other hand, increased infiltration of G-MDSCs has been shown to support metastatic growth [782]. This may partly explain why, despite reduced tumour cell seeding, antibiotic treated animals present with significantly larger pulmonary metastatic lesions. As yet, we have not analysed immune populations in mice with overt pulmonary metastases. It would be interesting to see if the

increased G-MDSC proportions we have observed are maintained through metastatic growth.

In contrast to the reduction of pro-metastatic cell types in the lung, we also observed significant increases in CD3+ T cells. Unfortunately, we were unable to determine the proportion of CD4/CD8 cells due to a technical issue with the CD4 staining, however we did not observe any significant differences in the proportion of CD8+ cells. Without any changes in CD8+ T cells, it is interesting to speculate which cytotoxic cell types may be responsible for the increased pulmonary CD3+ cell infiltration. One such cell type could be NKT cells, which express an αβ TCR, NK associated receptors, and CD3. Importantly NKTs do not always express either CD4 or CD8 and therefore may be responsible for the increased numbers of CD3+, CD8- T cells. Accumulation of NKTs at metastatic sites has been shown to mediate anti-metastatic immunity. Gebremeskel *et al*. showed that activated NKT cells can potently clear pulmonary metastatic cells in the 4T1 BC model [778]. Furthermore, the microbiota has been shown to negatively regulate NKT expansion, particularly in the lung. Germ free animals show significantly elevated levels of NKT cells in both the lamina propria and lung. Co-housing of GF animals with SPF mice returned NKT numbers to baseline, suggesting this effect is mediated by exposure to microbial antigens [783]. These findings are elaborated on by Ma *et al*. who used experimental models of metastasis to investigate the role of the microbiota in liver metastasis. Using an antibiotic regimen of vancomycin, neomycin and primaxin, the authors found that depletion of the microbiome significantly impaired the development of micro and macro metastatic lesions. This effect was accompanied by expansion of NKT cells in the metastatic organ [784]. Therefore, the severe diminishment of the microbiome in our model by VNMA antibiotic administration could impair metastatic seeding through dysregulated NKT expansion in the lung.

Whilst we have generated compelling evidence that the microbiome plays a key role in establishing the PMN, we sought to understand if it has any significance in the progression of metastatic lesions. To address this, we allowed animals with resected breast tumours to continue experimentally for up to 4 weeks after surgery in order to model clinical metastatic growth after treatment. At this time point, there was no difference in the number of pulmonary lesions. However, in antibiotic treated animals the lesions were significantly larger and occupied a larger proportion of the lung when compared to the control treated animals. These findings mirror our previous findings with respect to primary tumour growth, in that antibiotic administration accelerates tumour growth. It appears that similar effects are seen with respect to growth of metastatic lesions. However, we do not have data to suggest why this may be. Our primary method of analysis in this experiment was histological

in order to detect larger metastatic lesions. Our intention was also to histologically analyse immune cell infiltrate, but due to technical difficulties we were unable to do so. It would be interesting to examine if the protumorigenic mechanisms that we observe in primary tumours can be extended to metastatic lesions. However, these findings suggest a dual role for the microbiota in the metastatic cascade. Depletion of the gut microbiome impairs early metastatic seeding which suggests a protective role for antibiotics. However, this does not translate into significantly improved outcomes. In late timepoints, metastatic growth is significantly accelerated. Whilst we were unable to examine the impact of this on mortality, based on the extent of metastatic growth, the antibiotic treated animals would almost certainly have died sooner than the control animals. To understand this duality, we aimed to determine if the microbiota has any impact on the metastatic potential of primary tumour cells. To do so, we evaluated the common EMT markers E-cadherin and vimentin by western blot of whole tumour protein lysates. We observed significant decreases in E-cadherin expression in VNMA treated tumours, but no difference in vimentin expression suggesting a more metastatic cellular phenotype with antibiotic treatment. This may, in part, explain the dual role we have observed for the microbiota in development of metastasis. Whilst early seeding is impaired through a dysregulated PMN, the tumour cells have a more metastatic phenotype. Studies by other groups have shown that metastatic dissemination and seeding of metastatic organs occurs asynchronously [785]. Therefore, whilst the PMN is initially not permissive, a higher metastatic potential in antibiotic treated animals may outweigh this over time. To test this, it would be interesting to evaluate the number of CTCs in antibiotic treated animals. Furthermore, it would be pertinent to monitor the rate of metastatic seeding over time to determine if the negative regulation of the PMN by microbial dysfunction is maintained. However, it is also necessary to validate these results using further controls. It is possible that the quantity of E-cadherin per cell is not actually increasing, but perhaps the relative number of cancer cells in the tumour is. This would artificially increase E-cadherin levels in whole tumour analysis, simply because the tumour consists of a greater number of E-cadherin expressing tumour cells relative to the other stromal elements. To test this, an epithelial cell marker such as cytokeratin should also be run on the western blot to ensure the proportion of tumour cells remains constant across treatment conditions.

There are still several questions that persist despite our encouraging data. However, we have performed pioneering work that suggests the gut microbiome plays a key role in metastatic seeding of distant organs. The translational impact of this work is high and may lead to restructuring of antibiotic treatments in clinical practice for BC patients. However, there are still several areas in which this work needs to be strengthened. The flow cytometry

needs to be expanded to clarify the identity of the CD11b+, Gr-1+ cell population. The CD3+ cell fraction needs a far more extensive characterisation, including addition of NK markers to determine if the CD3+ expansion is mediated by NKTs. Cytokine analysis of serum and the lung needs to be performed to determine the mechanism by which the primary tumour may drive immune cell recruitment to metastatic organs and how the microbiota may modulate these systems. Finally, it would be pertinent to investigate the impact of milder antibiotics that are likely to be administered clinically to BC patients. This would mirror our earlier findings in primary tumour growth and crucially allow for correlation of metastatic determinants with changes in the gut microbiome.

6. Final Discussion

We have demonstrated for the first time that the growth of breast tumours is sensitive to perturbations of the gut microbiota. Using antibiotics, we have shown that a dysregulated microbiome can accelerate BC tumorigenesis. Unexpectedly, this appears to be due in part to metabolic changes occurring at the tumour. Furthermore, we have shown that antibiotic administration has a profound impact on bacterial metabolite production, which may be the driving factor behind the altered metabolic expression we observe. However, we are not yet certain that the metabolic changes we observe by transcriptomics have any impact on tumour metabolism. Additionally, we do not know if the changes in bacterial metabolite production we observed in feces can translate to the tumour. Therefore, it is essential that we undertake a more robust metabolomic profiling after antibiotic administration. Work is already underway to analyse metabolites in the tumour, serum and feces to try, but intervention studies are required to test our hypotheses. In addition to this metabolic work, it is essential that we build on our Keflex findings. Due to the milder dysbiosis when compared to VNMA administration, we are able to profile changes in the microbial populations. This allows us to correlate intratumoural changes with the loss or gain of particular microbial constituents of the gut microbiome. Whilst we have already generated some data with respect to these bacterial changes, we do not have any tumoural mechanistic data. Therefore, we should target our analysis towards gene expression analysis of Keflex treated tumours to determine if our RNAseq data can be replicated. Alongside this, we should generate a more detailed picture of the dysbiosis caused by Keflex administration. The use of shotgun metagenomic analysis will allow us to profile the microbial changes in greater resolution and allow us to identify species level changes that may be driving our phenotype.

Conversely, we have shown that by administration of probiotic *Bif,* BC tumour growth can be inhibited. Whilst this is not associated with any changes in immune cell populations, increased levels of intratumoural IL-10 are observed. However, we do not know whether this is causal or coincidental as we are yet to undertake any inhibitor studies. It would be interesting to conduct experiments using pharmacological IL-10 inhibitors to determine if the tumour suppressing effects of *Bif* are ameliorated. Additionally, it is essential that we begin to understand which cell populations are producing IL-10 in the tumour. This is achievable by intracellular cytokine staining during flow cytometry, however this was not technically possible in our current studies. Our work would also benefit heavily from shotgun metagenomic analysis as we do not yet know how *Bif* is affecting the microbiome. To

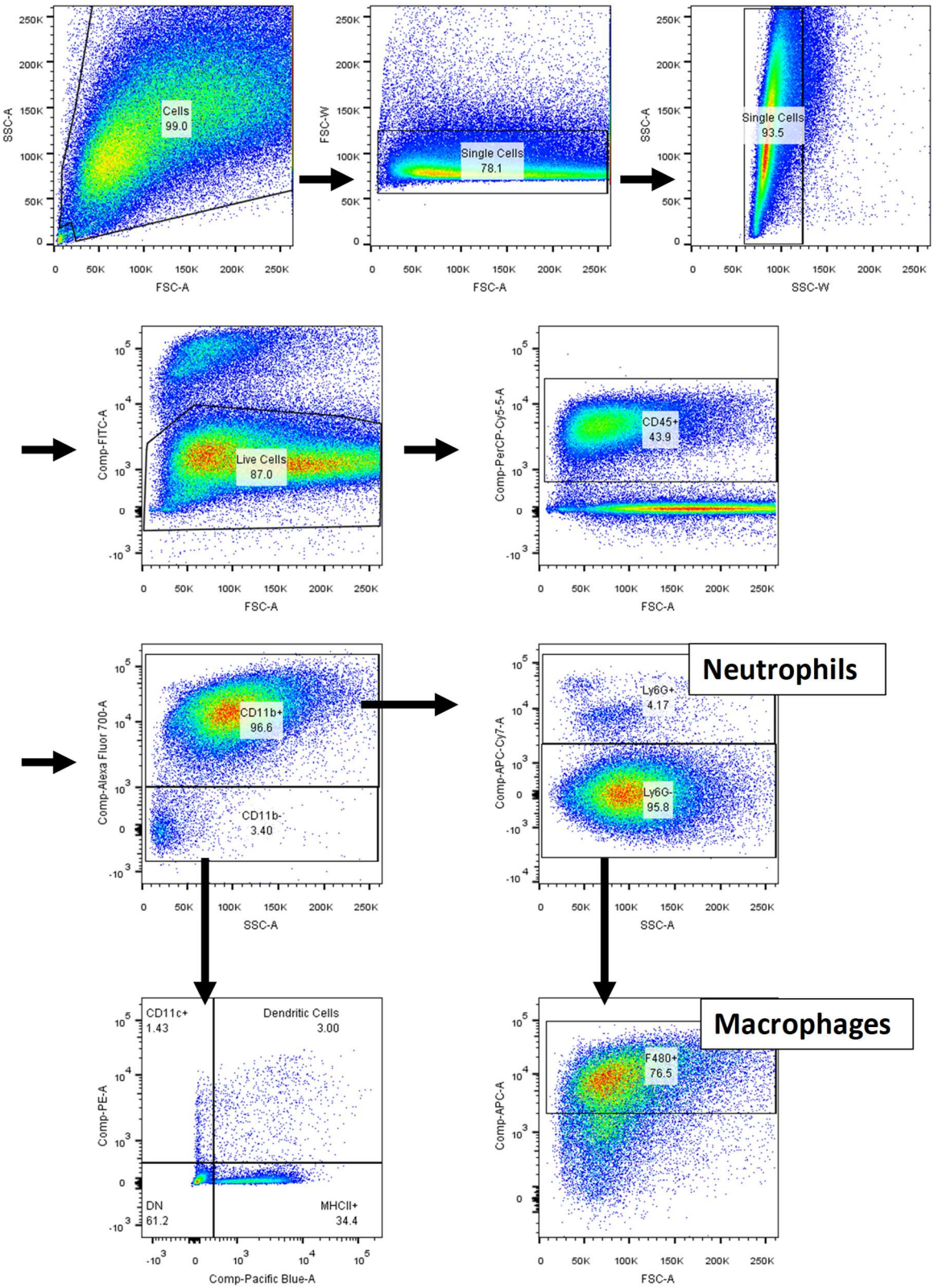
understand whether the microbial changes are solely in *Bif* colonisation, or if *Bif* colonisation merely provides an ecological niche for other beneficial species will help us start to identify a mechanism for these effects.

Finally, we have shown that whilst administration of *Bif* has no impact on metastatic dissemination, antibiotic administration appears to impair early metastatic seeding of the lung in BC. We believe this is driven mechanistically by impaired pre- or early-metastatic niche formation, primarily the immunological components of this process. Antibiotic administration results in significantly decreased numbers of MDSCs and increased numbers of T cells at the early metastatic niche. This is suggestive of a niche that is hostile to disseminating cancer cells thereby preventing their infiltration into the lung. However, when we extend our treatment regimen in order to allow overt metastatic lesions to form, antibiotics appear to accelerate metastatic growth. We do not yet understand the reasons for this but it may be related to antibiotics promoting a mesenchymal phenotype at the primary tumour, however, this is currently inconclusive.

In summary, we have shown that the microbiota represents a promising new avenue of research in the BC field. We hope that our work will allow clinicians to reconsider their approach to antibiotic use in cancers patients to minimise their deleterious effects. We also hope that our *Bif* cocktail will one day see clinical use alongside conventional therapies, however there is still a very long road until this can become reality.

7. Appendix Figures

A



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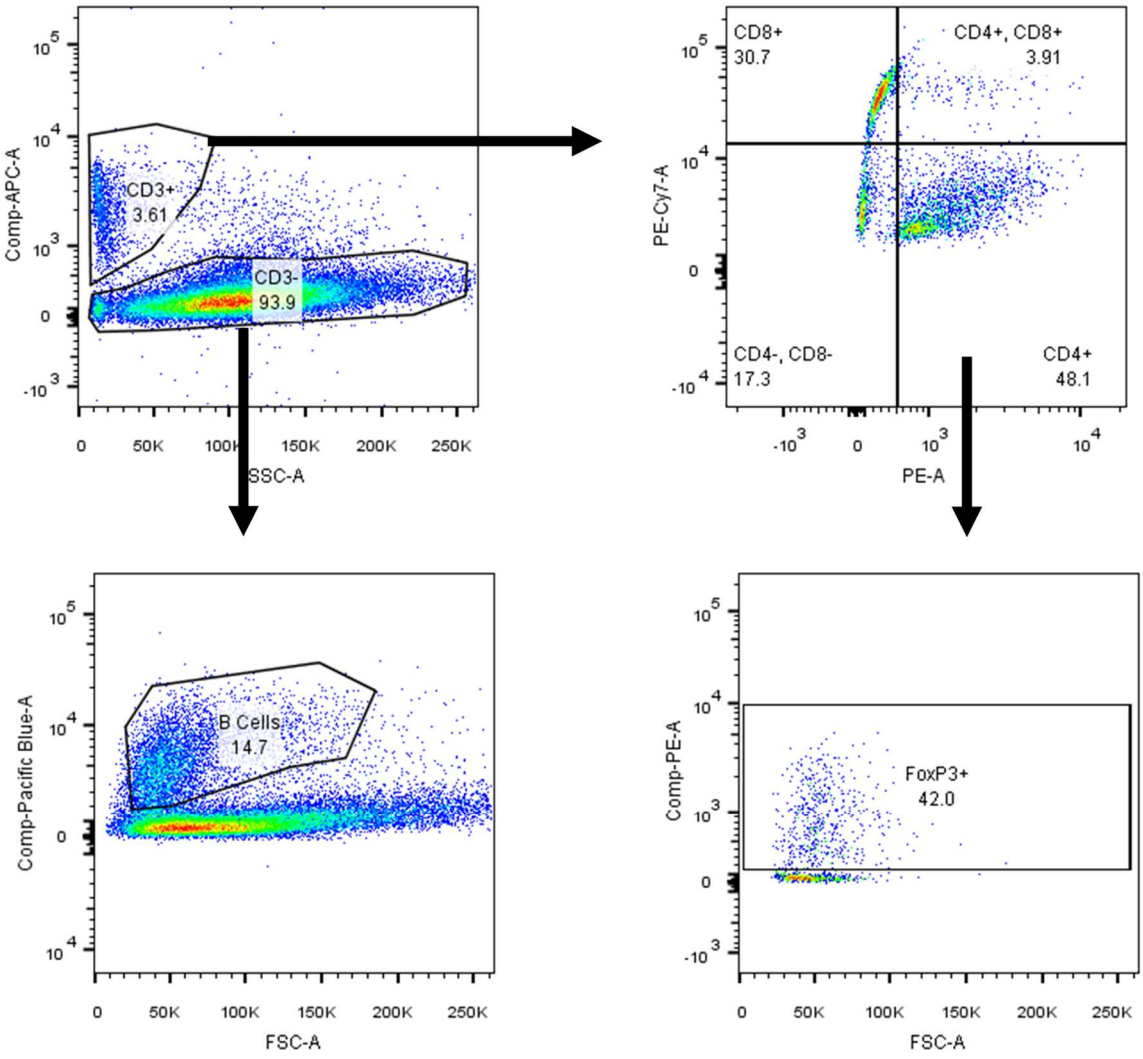
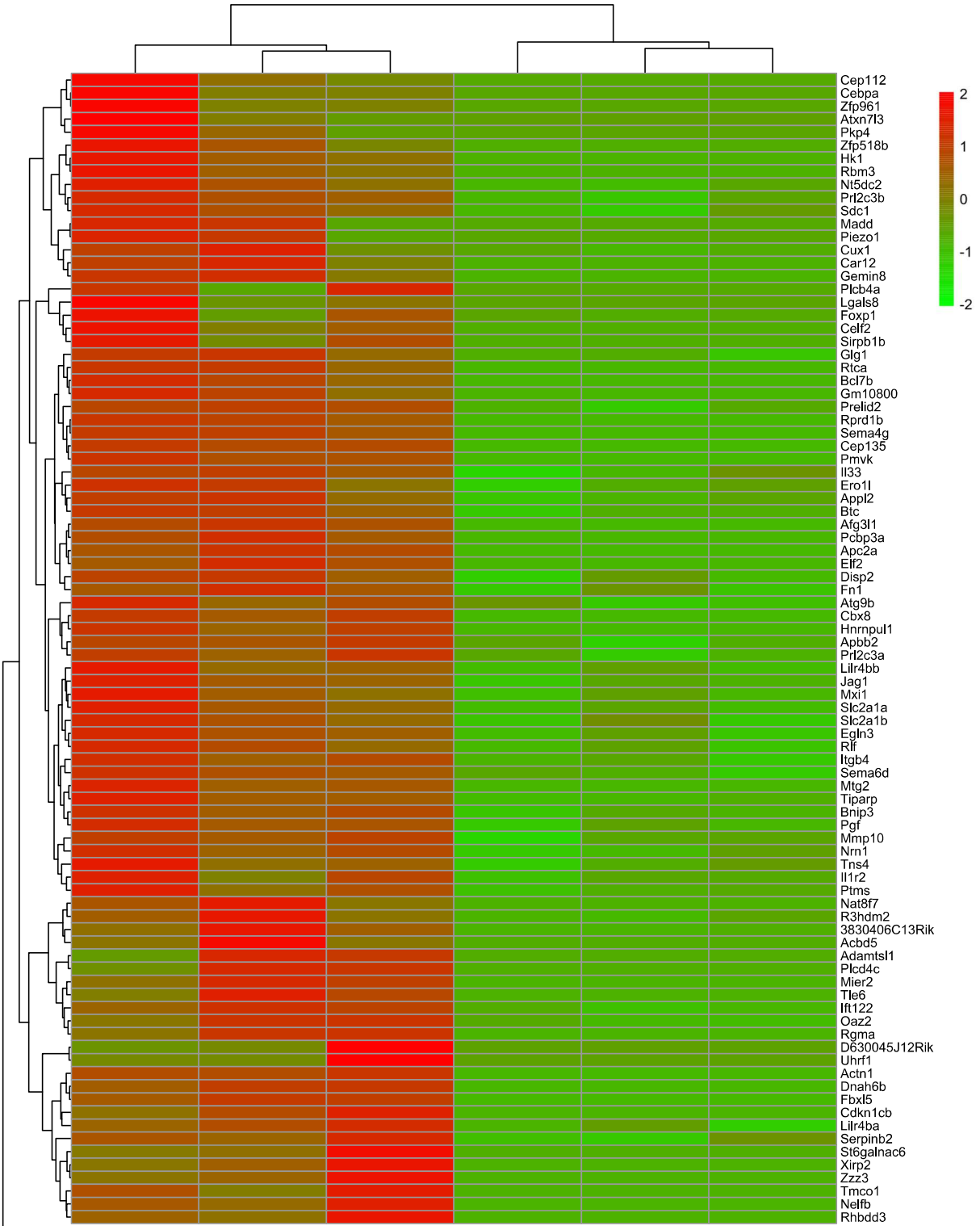


Figure 7.1 – Flow cytometry gating strategies: Figure detailing the flow cytometry gating profiles used for figures in sections 3.3, 4.5 and 5.3. **A)** Gates used to separate myeloid cells, events are first gated to remove debris, then single cells and finally dead cells using a fixable fluorescent live/dead stain. Live cells are gated by their CD45 status and are then described as myeloid by CD11b+. Myeloid cells are separated into neutrophils (Ly6G+) and Ly6G- cells are then gated into macrophages (F4/80+). CD11b- cells are separated by their MHCII and CD11c signals, events which are double positive are termed dendritic cells. **B)** Lymphoid cells go through the same gating as in A), but CD45+ events are gated by their CD3 status. CD3+ cells are gated according to their CD4/8 signal to identify Thelper and Tcytotoxic cells respectively. CD4+ cells are then used for FoxP3 analysis to quantify Treg numbers. CD3- cells are used to identify B cells via B220 staining.

A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Lgals8
Foxp1
Celf2
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Mmp10
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Lilr4ba
Serpinb2
St6galnac6
Xirp2
Zzz3
Tmco1
Nelfb
Rhbdd3
2
1
0
-1
-2

B

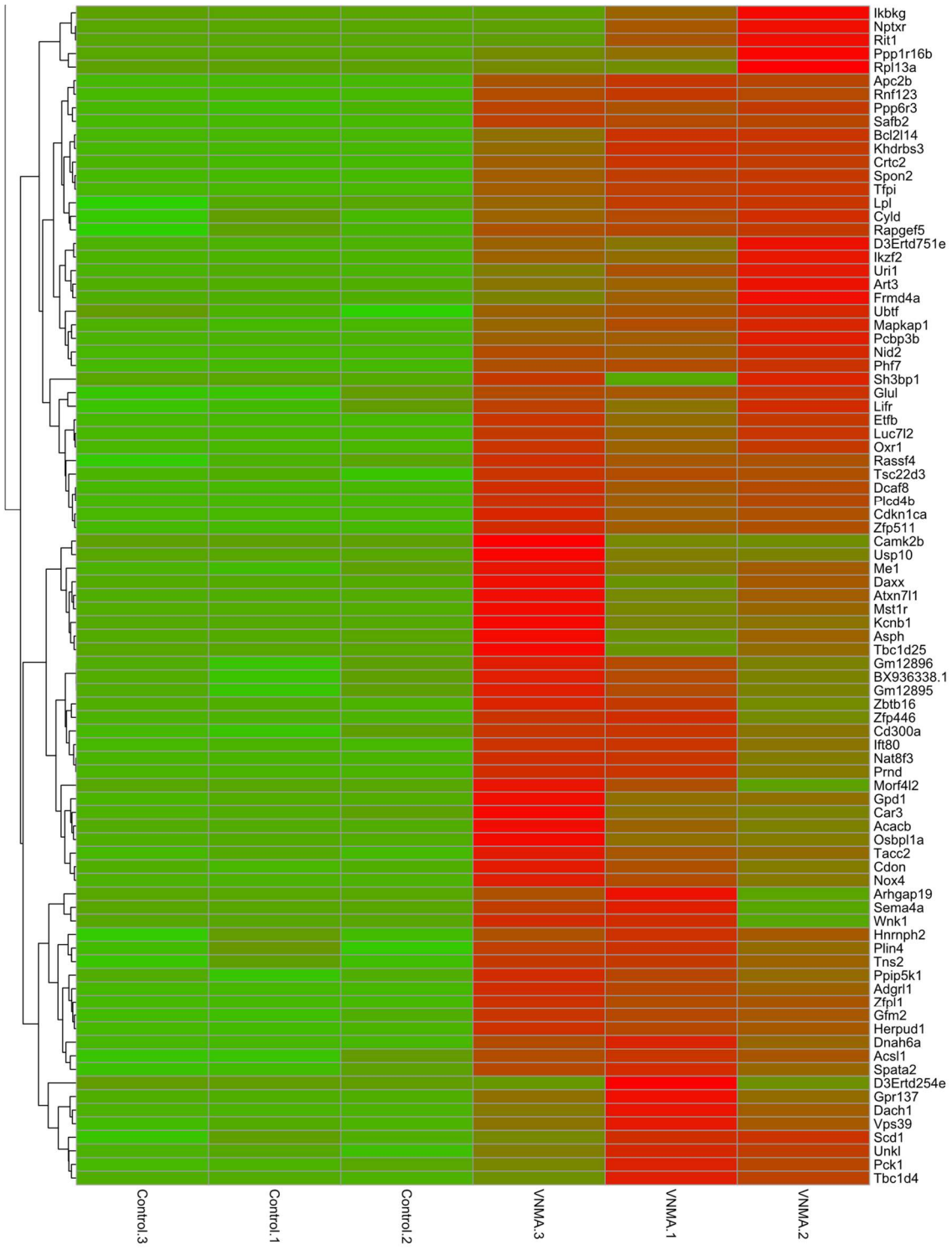

Figure 7.2 – Full RNAseq data set: Figure showing all differentially regulated genes in our transcriptomic dataset. **A)** Genes downregulated in antibiotic treated animals. **B)** Genes upregulated in antibiotic treated animals. Samples and genes are clustered according to Bray Curtis distance.

Table 7.1 – Full list of upregulated biological processes

Gene Ontology Term	Number of genes	pValue
GO:0009987~cellular process	64	0.019552
GO:0050789~regulation of biological process	49	0.041826
GO:0050794~regulation of cellular process	48	0.02781
GO:0044237~cellular metabolic process	46	0.009464
GO:0044238~primary metabolic process	46	0.010877
GO:0071704~organic substance metabolic process	46	0.038532
GO:0044260~cellular macromolecule metabolic process	38	0.028967
GO:0019222~regulation of metabolic process	32	0.013494
GO:0080090~regulation of primary metabolic process	31	0.007727
GO:0031323~regulation of cellular metabolic process	31	0.008713
GO:0016043~cellular component organization	30	0.026979
GO:0071840~cellular component organization or biogenesis	30	0.038692
GO:0048519~negative regulation of biological process	29	0.002468
GO:0048518~positive regulation of biological process	29	0.012779
GO:0060255~regulation of macromolecule metabolic process	29	0.035642
GO:0048523~negative regulation of cellular process	28	0.001742
GO:0019538~protein metabolic process	27	0.032549
GO:0044267~cellular protein metabolic process	26	0.014316
GO:0048522~positive regulation of cellular process	26	0.024093
GO:0006464~cellular protein modification process	22	0.008285
GO:0036211~protein modification process	22	0.008285
GO:0043412~macromolecule modification	22	0.017708
GO:0042221~response to chemical	22	0.047206
GO:0048583~regulation of response to stimulus	20	0.022282
GO:0010033~response to organic substance	19	0.015156
GO:0009893~positive regulation of metabolic process	19	0.016813
GO:0065009~regulation of molecular function	18	0.001582
GO:0031325~positive regulation of cellular metabolic process	18	0.016341
GO:0010646~regulation of cell communication	18	0.017235
GO:0023051~regulation of signaling	18	0.018588

GO:0009966~regulation of signal transduction	17	0.012349
GO:0010604~positive regulation of macromolecule metabolic process	17	0.036006
GO:0051128~regulation of cellular component organization	16	0.021114
GO:0070887~cellular response to chemical stimulus	16	0.040883
GO:0050790~regulation of catalytic activity	15	0.002011
GO:0071310~cellular response to organic substance	15	0.015857
GO:0031324~negative regulation of cellular metabolic process	15	0.041055
GO:0035556~intracellular signal transduction	15	0.041055
GO:0042592~homeostatic process	13	0.017453
GO:0006357~regulation of transcription from RNA polymerase II promoter	13	0.022938
GO:0044093~positive regulation of molecular function	12	0.005254
GO:0048585~negative regulation of response to stimulus	12	0.008224
GO:0019220~regulation of phosphate metabolic process	12	0.029996
GO:0051174~regulation of phosphorus metabolic process	12	0.030114
GO:0045935~positive regulation of nucleobase-containing compound metabolic process	12	0.030589
GO:1901700~response to oxygen-containing compound	12	0.034714
GO:0006366~transcription from RNA polymerase II promoter	12	0.038798
GO:0051173~positive regulation of nitrogen compound metabolic process	12	0.045075
GO:0010648~negative regulation of cell communication	11	0.008084
GO:0023057~negative regulation of signaling	11	0.008274
GO:1902531~regulation of intracellular signal transduction	11	0.049424
GO:0051336~regulation of hydrolase activity	10	0.004569
GO:0009968~negative regulation of signal transduction	10	0.010701
GO:0009967~positive regulation of signal transduction	10	0.04715
GO:0043085~positive regulation of catalytic activity	9	0.021822
GO:0048878~chemical homeostasis	9	0.042917
GO:0044283~small molecule biosynthetic process	7	0.005798
GO:1902532~negative regulation of intracellular signal transduction	7	0.006045
GO:0008285~negative regulation of cell proliferation	7	0.028835
GO:2001233~regulation of apoptotic signaling pathway	6	0.012017
GO:0051051~negative regulation of transport	6	0.036256
GO:0071407~cellular response to organic cyclic compound	6	0.038902
GO:0071345~cellular response to cytokine stimulus	6	0.045136

GO:0032787~monocarboxylic acid metabolic process	6	0.048466
GO:0035303~regulation of dephosphorylation	5	0.002455
GO:0046486~glycerolipid metabolic process	5	0.019972
GO:0016311~dephosphorylation	5	0.039141
GO:0010921~regulation of phosphatase activity	4	0.009234
GO:2001242~regulation of intrinsic apoptotic signaling pathway	4	0.016507
GO:0006475~internal protein amino acid acetylation	4	0.016802
GO:0006473~protein acetylation	4	0.026629
GO:0043543~protein acylation	4	0.040357
GO:0009746~response to hexose	4	0.040357
GO:0034284~response to monosaccharide	4	0.043263
GO:0071320~cellular response to cAMP	3	0.01395
GO:0006094~gluconeogenesis	3	0.026969
GO:0019319~hexose biosynthetic process	3	0.029113
GO:0046364~monosaccharide biosynthetic process	3	0.030579
GO:0035304~regulation of protein dephosphorylation	3	0.033597
GO:0035305~negative regulation of dephosphorylation	3	0.037526
GO:2001243~negative regulation of intrinsic apoptotic signaling pathway	3	0.039965
GO:0006641~triglyceride metabolic process	3	0.044158
GO:0071347~cellular response to interleukin-1	3	0.046749
GO:0090129~positive regulation of synapse maturation	2	0.03885
GO:0090128~regulation of synapse maturation	2	0.049184

Table 7.2 – Full list of downregulated biological processes

Gene Ontology Term	Number of genes	pValue
GO:0009987~cellular process	51	0.018049
GO:0050794~regulation of cellular process	38	0.04489
GO:0008152~metabolic process	38	0.045647
GO:0044237~cellular metabolic process	36	0.025271
GO:0044238~primary metabolic process	36	0.028135
GO:0043170~macromolecule metabolic process	33	0.035113
GO:0044260~cellular macromolecule metabolic process	31	0.027333
GO:0080090~regulation of primary metabolic process	26	0.005648
GO:0060255~regulation of macromolecule metabolic process	26	0.007428
GO:0019222~regulation of metabolic process	26	0.0169
GO:0010467~gene expression	25	0.004479
GO:0048518~positive regulation of biological process	25	0.005635
GO:0034641~cellular nitrogen compound metabolic process	25	0.039816
GO:0048522~positive regulation of cellular process	22	0.016569
GO:0010468~regulation of gene expression	21	0.009255
GO:0044271~cellular nitrogen compound biosynthetic process	21	0.027459
GO:0016070~RNA metabolic process	20	0.023098
GO:2000112~regulation of cellular macromolecule biosynthetic process	19	0.015911
GO:0010556~regulation of macromolecule biosynthetic process	19	0.020005
GO:0031326~regulation of cellular biosynthetic process	19	0.030131
GO:0009889~regulation of biosynthetic process	19	0.034194
GO:0051171~regulation of nitrogen compound metabolic process	19	0.036026
GO:0010604~positive regulation of macromolecule metabolic process	17	0.003285
GO:0009893~positive regulation of metabolic process	17	0.006793
GO:0006355~regulation of transcription, DNA-templated	16	0.049549
GO:1903506~regulation of nucleic acid-templated transcription	16	0.049878
GO:0006351~transcription, DNA-templated	15	0.047354
GO:0006357~regulation of transcription from RNA polymerase II promoter	11	0.024612
GO:0010557~positive regulation of macromolecule biosynthetic process	10	0.030706
GO:0010629~negative regulation of gene expression	10	0.036653

GO:0006366~transcription from RNA polymerase II promoter	10	0.046358
GO:0010628~positive regulation of gene expression	10	0.047535
GO:0051247~positive regulation of protein metabolic process	9	0.045224
GO:2000113~negative regulation of cellular macromolecule biosynthetic process	9	0.047615
GO:0000122~negative regulation of transcription from RNA polymerase II promoter	8	0.004851
GO:0051051~negative regulation of transport	6	0.014157
GO:0045732~positive regulation of protein catabolic process	5	0.001615
GO:0009896~positive regulation of catabolic process	5	0.006064
GO:0042176~regulation of protein catabolic process	5	0.011499
GO:0009894~regulation of catabolic process	5	0.040151
GO:0051224~negative regulation of protein transport	4	0.02585
GO:1904950~negative regulation of establishment of protein localization	4	0.029197
GO:0061387~regulation of extent of cell growth	3	0.043087
GO:0033622~integrin activation	2	0.041498

Table 7.3 – Upregulated KEGG pathways

KEGG Term	Number of genes	pValue	Gene List
mmu04922: Glucagon signalling pathway	4	0.002368	CRTC2, CAMK2B, ACACB, PCK1
mmu04931: Insulin resistance	4	0.003105	CRTC2, TBC1D4, ACACB, PCK1
mmu04920: Adipocytokine signalling pathway	3	0.016065	IKBKG, ACACB, PCK1
mmu03320: PPAR signalling pathway	3	0.019605	SCD1, LPL, PCK1
mmu04152: AMPK signalling pathway	3	0.045992	SCD1, CRTC2, PCK1

Table 7.4 – Downregulated KEGG pathways

KEGG Term	Number of genes	pValue	Gene List
mmu05146: Amoebiasis	3	0.021023	IL1R2, PLCB4, FN1
mmu05200: Pathways in cancer	4	0.03872	CEBPA, PLCB4, APC2, FN1

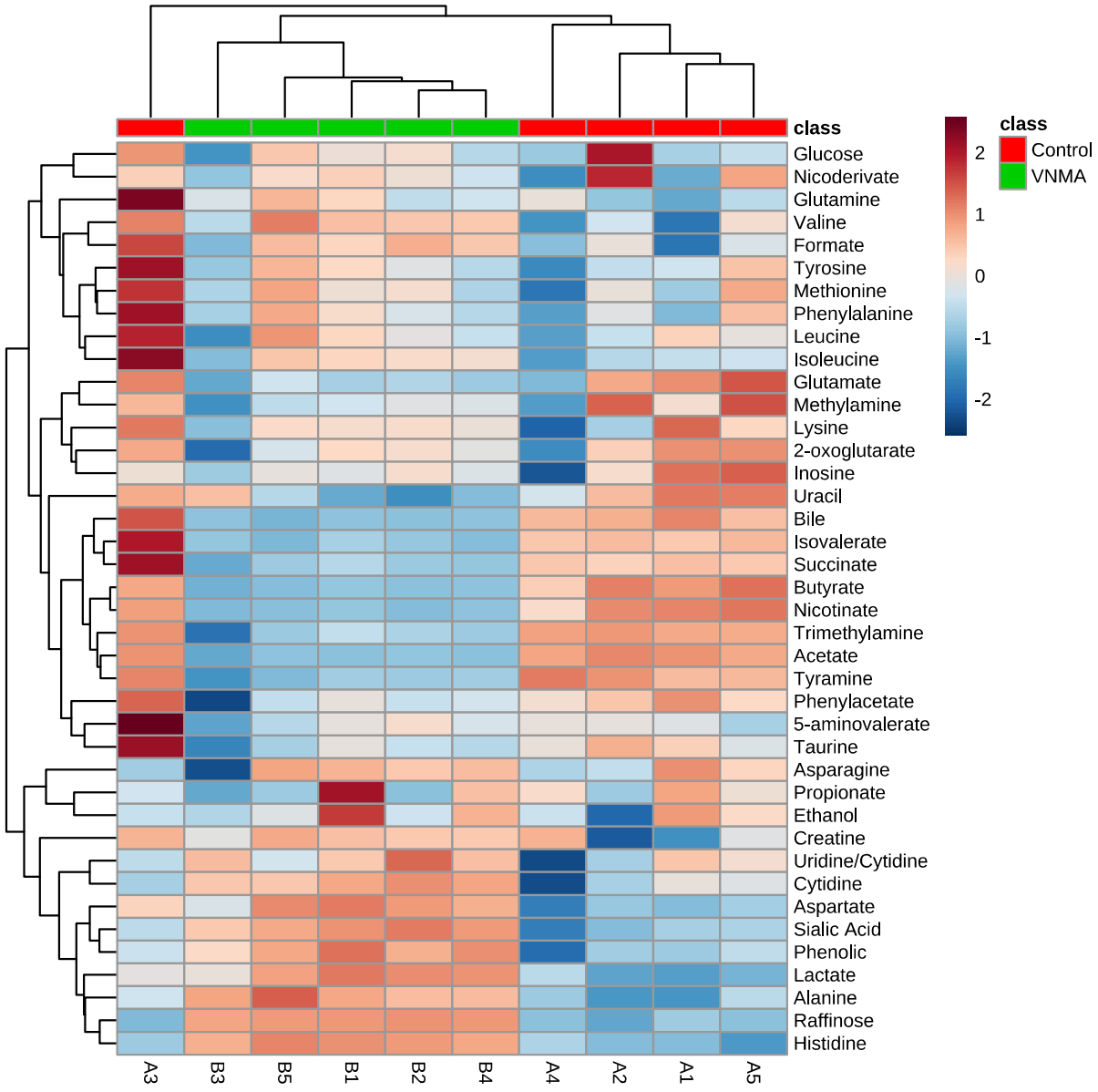

Figure 7.3 – Full metabolomic dataset: Figure showing all profiled metabolites in our fecal analysis. This figure also includes the outlier A3 which was excluded from our analyses due to erroneously elevated signal in several metabolites. Samples and metabolites are clustered according to Bray Curtis distance.

8. List of Abbreviations

ABX - Antibiotics

ACC - Acetyl-CoA Carboxylase

ACSL - Acyl-CoA Synthetase

Ag - Antigen

AID - Activation Induced Deaminase

AMPK - AMP-activated protein kinase

APC - Antigen Presenting Cell

B6BO1 - MMTV-PyMT Derived Breast Cancer Cell Line

BAD - Bcl-2-associated death promoter

BC - Breast Cancer

BCR - B Cell Receptor

Bif - Bifidobacteria

BLyS - B Lymphocyte Stimulator

BM - Bone Marrow

BMDM - Bone Marrow Derived Macrophage

BRCA - Breast Cancer Susceptibility Genes

C1q R - complement component 1q receptor

CAR-T - Chimeric Antigen Receptor T Cell

CCL - C-C motif Chemokine Ligand

CCR - C-C Chemokine Receptor

cDC - Classical Dendritic Cell

CDP - Common Dendritic Cell Progenitor

CFD - Complement Factor D

CFU - Colony Forming Units

CH - Co-housing

CLP - Common Lymphoid Progenitor

CMP - Common Myeloid Progenitor

CNA - Copy Number Aberrations

CpG - cytosine-phosphorothioate-guanine nucleotides

CPT - Carnitine-Palmitoyl Transferase

CRC - Colorectal Cancer

CRP - C Reactive Protein

CSF - Colony Stimulating Factor

CTC - Circulating Tumour Cell

CTL - Cytotoxic T Lymphocyte

CTLA - Cytotoxic T Lymphocyte Associated Protein

CTX - Cyclophosphamide

DAMP - Damage Associated Molecular Pattern

DAT - Desaminotyrosine

DC - Dendritic Cell

DCIS - Ductal Carcinoma in situ

DN - Double Negative (T Cell)

DNGR - C-type lectin domain family 9 Member A

DoT - Days of Treatment

DPI - Days Post Injection

EBF - Early B Cell Factor

EGFR - Epidermal Growth Factor Receptor

EMT - Epithelial to Mesenchymal Transition

EO771 - Spontaneously Derived Breast Cancer Cell Line

EPS - Exopolysaccharide

ER - Estrogen Receptor

ESAM - Endothelial Cell Selective Adhesion Molecule

ESL - E-Selectin Ligand

FA - Fatty Acid

FAE - Follicle Associated Epithelium

FAK - Focal Adhesion Kinase

FAS - Fatty Acid Synthase

FFA - Free Fatty Acid

FL - Fucosyllactose

Flt - FMS-like Tyrosine Kinase

FMT - Fecal Microbiota Transplant

FoxP3 - Forkhead Box P3

FSC - Forward Scatter

GALT - Gut Associated Lymphoid Tissue

GF - Germ Free

GH - Glycoside Hydrolase

GM-CSF - Granulocyte Macrophage Colony Stimulating Factor

G-MDSC - Granular Myeloid Derived Suppressor Cell

HDAC - Histone Deacetylase

HER - Human Epidermal Growth Factor Receptor

HERPUD - Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member

HGF - Hepatocyte Growth Factor

HMO - Human Milk Oligosaccharide

HPV - Human Papilloma Virus

HR - Hormone Receptor

HSC - Haematopoietic Stem Cell

ICAM - Intercellular Adhesion Molecule

ICI - Immune Checkpoint Inhibitor

IDC - Invasive Ductal Carcinoma

IDO - Indoleamine 2,3-dioxygenase

IFN - Interferon

IFNAR - Interferon Alpha and Beta Receptor

IFR - Interfollicular Region

IL - Interleukin

IL-1RA - Interleukin-1 Receptor Antagonist

ILC - Invasive Lobular Carcinoma

iNKT - Invariant Natural Killer T Cell

iNos - Inducible Nitric Oxide Synthase

IntClust - Integrative Cluster

JAK-STAT - Janus Kinase-Signal Transducer and Activator of Transcription

LAG - Lymphocyte Activation Gene

LDH - Lactate Dehydrogenase

LFA - Lymphocyte Function Associated antigen

LN - Lymph Node

LOX - Lysyl Oxidase

LPL - Lipoprotein Lipase

LPS - Lipopolysaccharide

M Cell - Microfold Cell

MCA - 3'-methylcholanthrene

M-CSF - Macrophage Colony Stimulating Factor

MCT - Monocarboxylate Transporter

MDP - Macrophage Dendritic Cell Progenitor

MDSC - Myeloid Derived Suppressor Cell

MFI - Median Fluorescence Intensity

MFP - Mammary Fat Pad

M-MDSC - Monocytic Myeloid Derived Suppressor Cell

MMP - Matrix Metalloproteinase

MPP - Multipotent Progenitor Cell

MSC - Mesenchymal Stem Cell

MUFA - Monounsaturated Fatty Acid

MWF - Monday Wednesday Friday

MZ - Marginal Zone

NICE - National Institute of Clinical Excellence

NK - Natural Killer Cell

NO - Nitric Oxide

NSCLC - Non-small Cell Lung Carcinoma

OAZ - Ornithine Decarboxylase Antizyme

ODC - Ornithine Decarboxylase

ORR - Objective Response Rate

PAMP - Pathogen Associated Molecular Pattern

PAP - Prostatic Acid Phosphatase

PCK - Phosphoenolpyruvate Carboxykinase

PECAM - Platelet Endothelial Adhesion Molecule

PEPCK - Phosphoenolpyruvate Carboxykinase

PI3K - Phosphatidylinositol-4,5-bisphosphate 3-kinase

PLC - Pregnancy Lactation Cycle

PMN - Pre-Metastatic Niche

PP - Peyer's Patch

PP1γ - phosphatase 1 gamma

PR - Progesterone Receptor

PSA - Polysaccharide A

PSGL - P-Selectin Glycoprotein Ligand

PTPN - phosphatase Tyrosine-protein phosphatase non-receptor type

Rae - Retinoic Acid Early Inducible

RAG - Recombination Activating Genes

S6K - ribosomal S6 kinase

SA - Sialic Acid

SAA3 - Serum Amyloid A3

SCD - Stearoyl-CoA desaturase

SCF - Stem Cell Factor

SCFA - Short Chain Fatty Acid

SDF - Stromal-cell Derived Factor

SED - Sub Epithelial Dome

SFB - Segmented Filamentous Bacteria

SLEC - Short Lived Effector Cell

SP - Single Positive (T Cell)

SPK - Spingosine-1-Phosphate of Sphingosine Kinase

SSC - Side Scatter

STING - Stimulator of interferon genes

TAMP - Tumour Associated Molecular Pattern

TCR - T Cell Receptor

TF - Transcription Factor

T_H - T helper cell

TIL - Tumour Infiltrating Leukocyte

TNC - Tenascin C

TNF - Tumour Necrosis Factor

T_{reg} - T regulatory Cell

URI - and Unconventional prefoldin RPB5 interactor

USP - Ubiquitin specific peptidase

VEGF - Vascular Endothelial Growth Factor

VNMA - Vancomycin Neomycin Metronidazole Amphoteracin Antibiotics

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