THE ROLE OF $CD8^+$ REGULATORY T CELLS IN ANTI-TUMOUR IMMUNE RESPONSES IN HEPATOCELLULAR CARCINOMA

by

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ABSTRACT

Tumour specific effector T-cells can be detected in the blood and tumours of patients with hepatocellular carcinoma (HCC) but fail to mount effective immune responses. Attempts to amplify anti-tumour immune responses using immunotherapy show promise, but are hampered by the presence of suppressive regulatory T-cells (Treg) that inhibit anti-tumour immune responses. Many different subsets of Treg have since been identified including regulatory T-cells expressing the surface marker CD8 ($CD8^+$Treg). A set of experiments was designed in an attempt to increase our understanding on how $CD8^+$Treg may disrupt anti-tumour response and by what mechanisms they are induced.

$CD8^+$Treg was analysed by isolation of liver-derived T-cells from human HCC. Monocyte-derived dendritic cells (moDC) matured with tumour tissue conditioned medium were used to assess they potential to induce $CD8^+$Treg.

$CD8^+$Treg infiltrating HCC demonstrated a suppressive phenotype. The co-culture of naïve $CD8^+$ T-cells with tumour-conditioned moDC induces a population of $CD8^+$Treg through an IDO dependent mechanism. This population of induced T-cells was able to suppress via the CD39-adenosine pathway.

The findings of the mechanisms involved in the induction of $CD8^+$Treg by DC and the involvement of CD39 in the suppressive capacity of these novel T-cells, may guide the development of future immunotherapeutic in HCC.

DEDICATION

I dedicate this work to my wife Yee, my son Isaac and to my parents, who gave me their support to make this possible.

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1 INTRODUCTION

1.1 Introduction to hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common primary liver cancer in the world and is associated with one of the highest incidences of cancer related death globally. The prevalence of HCC varies widely in the world (Figure 1-1), largely due to differences in exposure to risk factors, which includes chronic viral infection, metabolic disease and exposure to aflatoxin [1].

Although HCC is relatively rare in the western hemisphere with a prevalence of 4 cases per 100,000 populations [2, 3]. It is the second most common cause of cancer-related death worldwide and in 2012, 782,000 new cases were reported worldwide. Resulting in an estimated 746,000 liver cancer deaths globally [4]. In developed countries its incidence is expected to rise both in the United States and Europe [5]. This is likely to be a reflection of the increased prevalence of cirrhosis as a result of chronic infection with hepatitis C virus (HCV), non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD). It is therefore likely to become a major health burden in the UK in the near future.

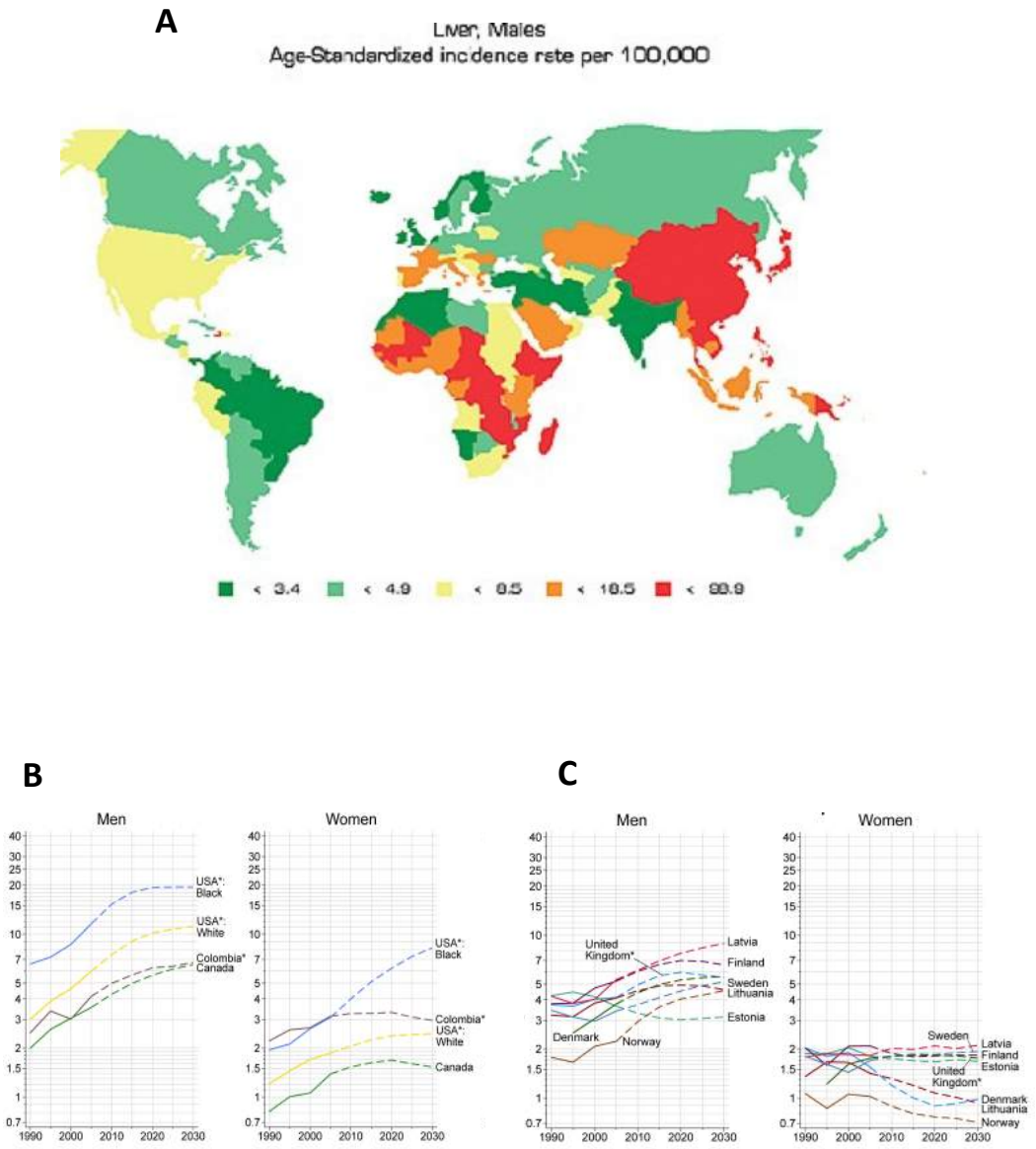

Figure 1-1 Global impact of hepatocelluar carcinoma.

(A) Age-Standardised incidence rates of HCC in males per 100,000 populations. (B) The predicted Age-Standardised Incidence Rates of HCC in America per 100,000 populations. (C) The predicted Age-Standardised Incidence Rates of HCC in Europe per 100,000 populations. Figure adapted from [4, 5].

Current treatments for HCC are limited and 5-year survival for all stages combined has been reported as low as 10% and with a medium survival of 10 months following diagnosis [6, 7]. In addition, the mortality rates of patients with HCC remained poor in comparison to patients with other leading causes of cancer [8]. The main reason for the poor survival seen in patients with HCC is partly due to the late presentation in the majority of patients. In addition, the presence of underlying liver cirrhosis in a large proportion of patients with HCC often limits their treatment options. Lastly, for HCC patients the availability of certain treatments, in particular liver transplantation, is restricted by the lack of donor organs and limitation in health resources, both of which are particular hurdles in developing nations that have the highest incidence of HCC.

1.2 Cancer surveillance

In view of the poor prognosis, regular radiological assessment of at risk individuals is advocated by international guidelines with the aim of detecting early cancers amenable to potentially curative therapy [9-11]. Thus, patients with liver cirrhosis are recommended to undergo six-monthly liver ultrasound scans (USS) as part of cancer surveillance. Until recently, the use of the tumour marker alpha feto-protein (AFP) in combination with USS had been the modalities of choice used for the surveillance of HCC. However, in recent guidelines published by the American Association for the Study of Liver Disease (AASLD) and European Association for the study of Liver Disease (EASL), the use of AFP is no longer recommended. This is due to the lack of evidence to demonstrate is effectiveness as a screening test [12, 13]. However, this

decision has remained controversial in the clinical world. Even more contentious, is the use of HCC surveillance in any form. Despite several studies suggesting possible improved tumour-related outcomes in patients receiving HCC surveillance, robust data supporting HCC surveillance remains limited. This is largely the result of difficulties in conducting large randomised controlled trials (RCTs) in the area of cancer surveillance. In HCC, only two RCTs have been conducted to evaluate the benefit of surveillance in patients with liver cirrhosis, both being subject to significant methodological flaws, poor compliance and high drop out rate [14, 15]. In addition, observational studies that had suggested the possible benefit of HCC surveillance are often heavily influenced by lead-time and length-time bias. Other indirect study methods such as cost-effectiveness analysis have supported the use of surveillance, but there remains much debate as to the optimal interval and method of HCC surveillance [16-21]. Despite the lack of clarity on the benefit of HCC surveillance in cirrhotic patients, it is likely we will continue with the current recommended practice until the development of more sensitive diagnostic techniques or well-conducted RCTs looking at the effectiveness of current screening methods.

It has long been recognised that viral hepatitis can induce hepatocarcinogenesis independent of liver cirrhosis, a risk particularly noticeably in patients of east-Asian and Black-African origin [22]. Selected guidelines have reflected on this and suggested the use of surveillance in non-cirrhotic patients with chronic viral infection. The evidence supporting HCC surveillance in this selected group of patients are limited, especially on the background of increasing evidence suggesting the risk of HCC in non-cirrhotic patients with chronic viral infection may be lower than

previously predicted. [23-25]. Hence, surveillance in this group is arguably below the threshold at which cost-effectiveness is likely met.

1.3 Treatment for Hepatocellular carcinoma

The treatment options for patients with HCC is dependent on the size and number of tumours present, the degree of underlying liver impairment as a result of chronic liver disease and the overall health status of the patient. Different staging systems incorporating all of the above have been used to guide treatment for individual patients. One of the most commonly used staging systems has been the Barcelona Cancer Liver Centre (BCLC) staging score [26] (Figure 1-2).

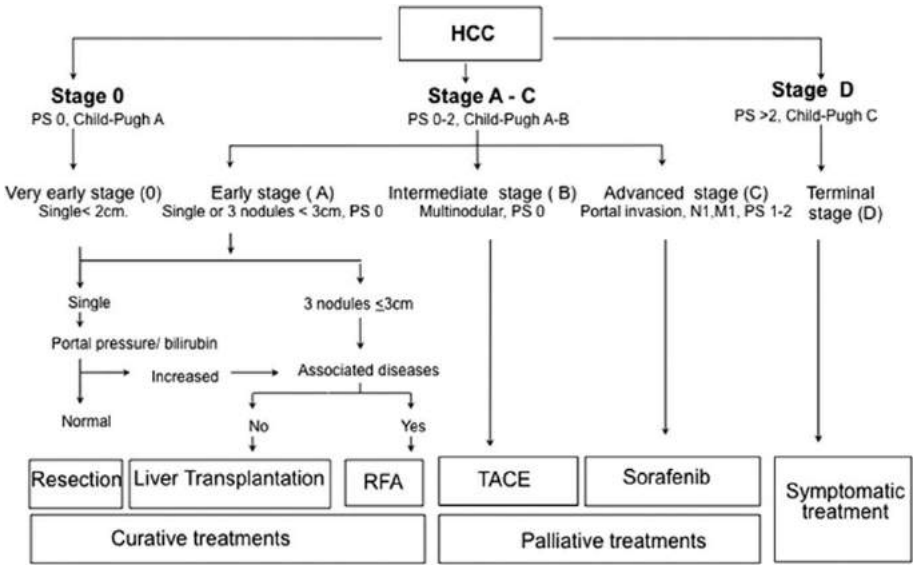

Figure 1-2 The BCLC staging system for HCC

(M, metastasis classification; N, node classification; PS, performance status; RFA, radiofrequency ablation; TACE, transarterial chemoembolization). Figure adapted from [13].

At present, surgery, either tumour resection or liver transplantation, is the main curative treatment for HCC. HCC differs from other solid organ cancers due to its occurrence on the background of chronic liver disease. Tumour resection is often contraindicated in patients with liver cirrhosis, due to the inability of the remnant non-tumour bearing liver to regenerate or maintain sufficient liver function following surgery. Hence, tumour resection is feasible in only a limited number of patients [27, 28].

In patients with small tumours and no evidence of vascular invasion but advanced chronic liver disease (poor hepatic function) liver transplantation is the only curable option [29]. Selection criteria for transplantation are designed to include those patients for whom transplant will give a survival benefit and in whom there is little or no risk of tumour recurrence. Most transplant centres base selection of patients with HCC for transplantation on the Milan criteria (one tumour <5cm, or three tumours each <3cm), which uses tumour size and number rather than tumour biology to classify patients. Use of the Milan criteria is associated with survival rates after liver transplantation similar to those seen in patients with non-malignant disease [30]. Some argue that these criteria are too restrictive and result in some patients being denied transplantation who would benefit from the procedure [31]. As such, extended criteria such as the University of California San Francisco (UCSF) criteria have been developed (one tumour ≤6.5 cm, or two or three tumours each ≤4.5 cm in diameter with a total tumour diameter ≤8 cm). Early studies comparing the UCSF criteria and the Milan criteria showed that these criteria have similar 5-year survival rates (74% compared with 72%) [32]. In a more recent validation study, based on radiological

assessment of the size of the tumours, the 5-year survival rates were 82% in the group selected using the UCSF criteria and 80% in the group selected using the Milan criteria [33]. However, the use of criteria other than the Milan criteria remains highly controversial; the main concern being that the use of extended criteria will jeopardize the outcome of patients being transplanted for non-HCC disease, due to the limited availability of donor organs.

The majority of patients with HCC are deemed incurable at the time of diagnosis. For patients with incurable but limited disease, radiofrequency ablation (RFA) is the treatment of choice [34, 35]. RFA involves the percutaneous insertion of a probe into the tumour that emits radio waves, which get converted to heat resulting in killing of local tumour cells. In patients with more extensive disease, transarterial chemoembolization (TACE) is beneficial for selected patients [36, 37]. TACE involves the intra-arterial infusion of a chemotherapeutic agent such as doxorubicin, followed by embolization of the blood vessels supplying the tumour with gelatine sponge or other embolic agents. This leads to a combination of cytotoxic and ischaemic induced tumour cell death.

Pharmacological therapy with tyrosine kinase receptor inhibitors has demonstrated efficacy in patients with advanced disease who are beyond both surgical and ablative treatment criteria. Sorafenib is an oral multi-kinase inhibitor that blocks cell surface tyrosine kinase receptors (e.g. vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) receptors) and downstream intracellular

serine/threonine kinase signalling (e.g. Rapidly Accelerated Fibrosarcoma-1 (Raf-1)), resulting in inhibition of tumour cell proliferation and angiogenesis [38].

The fore-mentioned curative and non-curative interventions have become the mainstay of treatments for patients with different stages of HCC, but the prognosis for the majority of patients remains poor despite the availability of multiple treatment options. Even in patients who have undergone curative surgical treatment, recurrent disease is not uncommon, in particular in those who undergo surgical resection with reported recurrence rates as high as 60% after five years [39]. Hence there is an urgent need to develop more effective treatments. One potential novel treatment involves the manipulation of the immune system to target cancer cells, so called 'immunotherapy'. However, to aid in the development of effective immunotherapeutic agents we must improve our understanding of tumour immunology.

1.4 Immune response

The immune system has evolved primarily to provide protection against invading pathogens. It is a complex interacting network of different cell types and cytokines that under normal circumstances is able to recognise and destroy harmful foreign substances without causing damage to surrounding healthy cells.

The immune system is commonly separated into two parts: innate and adaptive. The innate immune system is the first line of defence and deals non-specifically with potential harmful pathogens by a combination of physical barrier (i.e. skin epithelia),

secretory molecules (i.e. nasal secretion, tears, lactoferrin and lysozyme in saliva) and cellular components [40]. The cellular components of the innate immune system consist mainly of phagocytic immune cells including; macrophages, neutrophils, eosinophil and natural killer (NK) cells. These cells possess a number of pattern recognition receptors (PPR) for the detection of harmful pathogens by identifying structures called pathogen associated molecular patterns (PAMP) such as lipopolysaccharide (LPS), which are found on many pathogens.

By contrast, the adaptive immune system acts specifically towards pathogens by recognising antigens via a various number of specialised immune cells [41]. Unlike the innate immune system, cellular components of the adaptive immune system require prior activation and differentiation towards a specific antigen. As a result, compared to the instant action of the innate immune system, the adaptive response will often take a few days prior to its effective function. However, following the first challenge by any particular pathogen, the adaptive system is able to generate memory against it, to ensure rapid response in subsequent engagement with the same pathogen. The adaptive immune response is mediated mainly by lymphocytes, which comprise primarily $CD4^+$T-cells, $CD8^+$T-cells and B-cells. Their activation and differentiation requires the aid of the innate immune system by providing addition stimulation signal and in particular though the process of antigen presentation [42]. The task of antigen processing and presentation is preformed mainly by the cellular components of the innate immune system and these cells are often termed antigen-presenting cell (APC). The most important of the APC are dendritic cells (DC) and are often described as professional APC. However, immune cells from the adaptive

immune system and even non-immune cells such as liver stromal cells can also present antigens [43].

1.5 Dendritic cells

DCs are constantly monitoring the periphery for antigens while in an immature state and on engagement with antigens is able to capture, process and present antigens via the major histocompatibility complex (MHC) molecules to other immune cells, in particular T-cells [44]. DC are produced from the bone marrow, but methods have been developed to produce DC in vitro from immature myeloid cells by using combinations of growth factors, such as granulocyte macrophage-colony stimulating factor (GM-CSF) and Flt3 ligand [45]. DC are powerful antigen presenting cell, but our consisted of many subsets, but the two predominate subsets are myeloid DCs, and plasmacytoid DC [46]. Each DC subsets has unique markers and are often confined to certain tissue compartment with distinct migration pathways. Importantly, each subset is able to generate specific phenotype under certain environment. Leading to a different immune response.

Following antigen capture, DC will undergo maturation in the presence of a danger signal, such as PAMP. DC maturation results in the increase in surface expression of MHC molecules, production of the pro-inflammatory cytokine interleukin-12 (IL-12) and the up-regulation in the expression of co-stimulatory molecules such as CD80 and CD86, which are all essential components required for the activation of T-cells. In addition, matured DC up-regulate the expression of the chemokine receptor CCR7

leading to the migration towards the local lymph nodes in response to CCL19 and CCL21 [47], where they are able to interact with naïve T-cells and present antigen in association with MHC molecules on DC to the T-cell receptor (TCR) on T-cells (Figure 1-3) [45]. The combination of high expression of MHC complex, co-stimulatory molecules and pro-inflammatory cytokines results in the induction of naïve T-cells into functional antigen specific Th 1, Th 2, Th17 or CTL [48].

In the absent of maturation, DC are ineffective in the activation of T-cells and can result in the generation of T-cell tolerance [49]. The ability of DC to induce tolerance against a wide range of harmless/self-antigens is essential and this is of special importance in the liver due to its need to process a wide array of harmless antigens derived from the gut.

The importance of DCs can further be demonstrated by their ability to present a wide range of different antigens to different immune cell subsets. DCs are able to process and present endogenous antigens to $CD8^+$T-cells via MHC class-I molecules and exogenous antigens via MHC class-II molecules to $CD4^+$T-cells. This results in the activation of $CD8^+$T-cells that are able to eliminate virally infected or damaged self-cells and $CD4^+$T-cells that respond to pathogens such as bacteria, fungi and parasites. In addition, DCs are able to cross present antigens by processing exogenous antigens into MHC-I molecules for presentation to $CD8^+$T-cells [50].

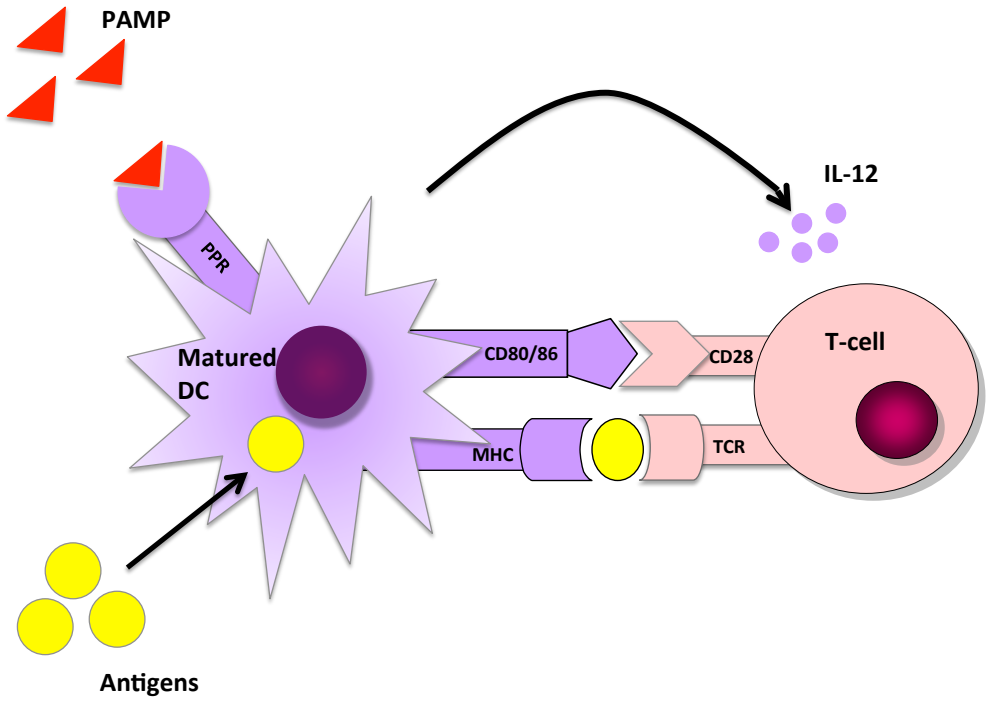

Figure 1-3: The activation of T-cells by antigen presentation DC

Following antigen capture, DC are matured in the presence of PAMP resulting in an increase in the expression of the co-stimulatory molecules CD80/86 and MHC. Matured DCs present antigens to T-cells via the MHC molecules, which are recognized by the TCR on T-cells. In addition to antigen presentation, the activation of T-cells is dependent on the production of IL-12 and signaling via CD28 by CD80/86. (PPR, Pattern recognition receptors; PAMP, pathogen associated molecular patterns; MHC, major histocompatibility complexes; TCR, T-cell receptor.)

1.6 Major histocompatibility complexes

The major histocompatibility complexes (MHC), also known as human leukocyte antigen (HLA) in humans, are essential in antigen presentation. Antigens are processed prior to their presentation by MHC molecules. Due to the diversity of pathogens the MHC system has also evolved. Human possess multiple MHC class-I genes (HLA-A, B and C) and MHC class-II genes (HLA-DR, DP and DQ). Such assortment of MHC genes allows peptides to be presented in multiple combinations. In addition, in human there are many different alleles of each MHC gene. This provides further diversity in antigen presentation via MHC molecules [51].

For antigen presentation to occur, antigens are presented via the MHC molecules. MHC class-I molecules are present on all nucleated cell. Antigens in the form of protein are transported into the cytoplasm of cells. The proteins are degraded into peptides by the proteasome and moved into the endoplasmic reticulum (ER), by transporter associated with antigen processing (TAP) proteins. MHC class-I molecules are made in the ER. The final step involves the packaging of peptides into MHC-I molecules before there are transported to the cells surface. MHC class-I molecules present peptides from intracellular pathogens such as viruses to CD8 T-cells. On their recognition of foreign peptides, $CD8^+$T-cells are able to kill such infected cells [52].

In contrast to MHC class-I molecules, MHC class-II molecules present longer peptides and are recognized by $CD4^+$T-cells. They are present mainly on immune cells, but under certain situation other non-immune cells such as fibroblast and

stromal cells can also present antigen via the MHC class-II molecules [53, 54]. Peptides from extracellular proteins are taken up into cells via endocytosis, into vesicles called endosomes. Endosomes containing these peptides then combine with lysosomes. Following enzyme degradation, the end product peptides are packaged into the MHC class-II molecules. This is followed by their release from the ER and is transported to the endocytic vesicles [52].

1.7 Immune tolerance

The immune system is essential in providing protection against harmful pathogens and this can be clearly demonstrated in patients with genetic associated immunodeficiency (i.e. severe combined immunodeficiency (SCID)) or acquired immunodeficiency (i.e. human immunodeficiency virus (HIV)). Here, the compromised immune response results in recurrent infection from both common and opportunistic pathogens and can result in death. Inversely, inappropriate activation of the immune system can lead to destruction of the host's healthy cells and even organs, resulting in autoimmune diseases which can be debilitating and even life threatening. Hence, multiple mechanisms are employed to maintain the fine balance between immunity towards harmful pathogens and tolerance towards self-antigens. The avoidance of inappropriate or excessive immune responses involves multiple layers of control, commonly referred to as central and peripheral tolerance. In particular, the liver has been identified to play an important role in the maintenance of peripheral tolerance.

Central tolerance takes place during T-cell and B-cell development within the thymus and bone marrow respectively [55]. T-cells (not B-cells) first undergo positive selection in which cells that recognise self-MHC molecules are maintained. This is followed by negative selection in which cells that recognise self-antigens are removed. However, central tolerance is unable to remove all auto-reactive cells hence a backup system, referred to as peripheral tolerance, is required to protect from the remaining auto-reactive cells [56]. Peripheral tolerance employs multiple mechanisms to overcome auto-reactive immune cells. Such mechanisms includes; suppressive immune cells and inhibitory signals and will be discussed in detail in later this chapter.

1.8 The liver as an immune organ

The liver is a unique organ well known for its functions in detoxification and metabolism, but the role of the liver as an immune organ is often overlooked [57]. The distinctive vasculature of the liver contributes enormously to its important functions, by receiving a blood supply from both the hepatic artery (20%) and the portal vein (80%) [58], resulting in the constant delivery of numerous antigens and immune active molecules such as LPS from the gut [59, 60]. The low flow rate in the liver blood vessels and the fenestration of the hepatic sinusoid endothelial cells (HSEC) aid in the capture of antigens by resident APC in the liver. However, despite constant bombardment from a wide array of antigens, the liver is able to prevent the inappropriate activation of the immune system towards harmless antigens [61]. Hence, the liver plays an essential role in both tolerances against harmless antigens, while maintaining the ability to respond to harmful pathogens.

The immune tolerance function of the liver can be demonstrated by its resistance to organ rejection following allogeneic liver transplantation, when compared to transplantation of other organs such as the heart and kidney [62]. Liver tolerance can also be demonstrated by the principle of oral tolerance, in which hypo-responsiveness can be initiated towards an orally ingested antigen as a result of antigen processing by the liver [63]. In comparison, if the same antigen was directly injected into the systemic circulation, it will result in an immune response. Unfortunately, the liver bias towards immune tolerance can be targeted by harmful pathogens, evidenced by the inability of the liver to destroy certain hepato-tropic pathogens such as HBV and HCV, resulting in chronic infection of the liver [64, 65].

The importance of antigen processing has been recognised as the pivotal component in the ability of the liver to confer tolerance [66]. Resident and transient immune cells of the liver such as kupffer cells (liver resident macrophages) and DC contribute towards the major population of APC. However, the role of non-immune cells acting as APC within the liver has emerged over the recent years. This includes HSEC, hepatic stellate cells, cholangiocytes and hepatocytes [67].

As already discussed, DC play an important role in orchestrating the immune response, in particular liver derived DC have been showed to play a central r^{o}le in the generation of peripheral tolerance [68]. The tolerogenic nature of liver DC was first observed by their poor ability to stimulate allogeneic T-cells expansions when compared to DC isolated from the skin [69]. The ineffectiveness of liver DC to

stimulate T-cells has been suggested to be the result of their inability to produce pro-inflammatory cytokines and the increased production of suppressive cytokines [70-72]. In addition, the lower expression of MHC molecules and co-stimulatory molecules has also been suggested to be involved in liver DC tolerance [73].

1.9 Inflammation & tumour initiation

Classically the development of HCC follows a stepwise process over many decades. The initial insult (i.e. viral, metabolic, cholestasis) induces damage to the liver parenchyma through a number of mechanisms such as DNA damage, reactive oxygen species (ROS), endoplasmic reticulum (ER) stress and necrosis of injured hepatocytes. The damage to the liver parenchyma results in the activation of resident immune cells such as kupffer cells to release a wide array of cytokines/chemokines. This is followed by further recruitment and activation of immune cells to the site of injury [74-76]. If the on-going insult persists, chronic inflammation will follow, leading to the activation of pro-fibrogenic cells and accumulation of extracellular matrix resulting in progressive fibrosis and cirrhosis. The combination of continued damage by the original insult and persistent inflammation results in mutations, genomic instability, and epigenetic modifications leading to the development of liver tumours [76, 77] (Figure 1-4).

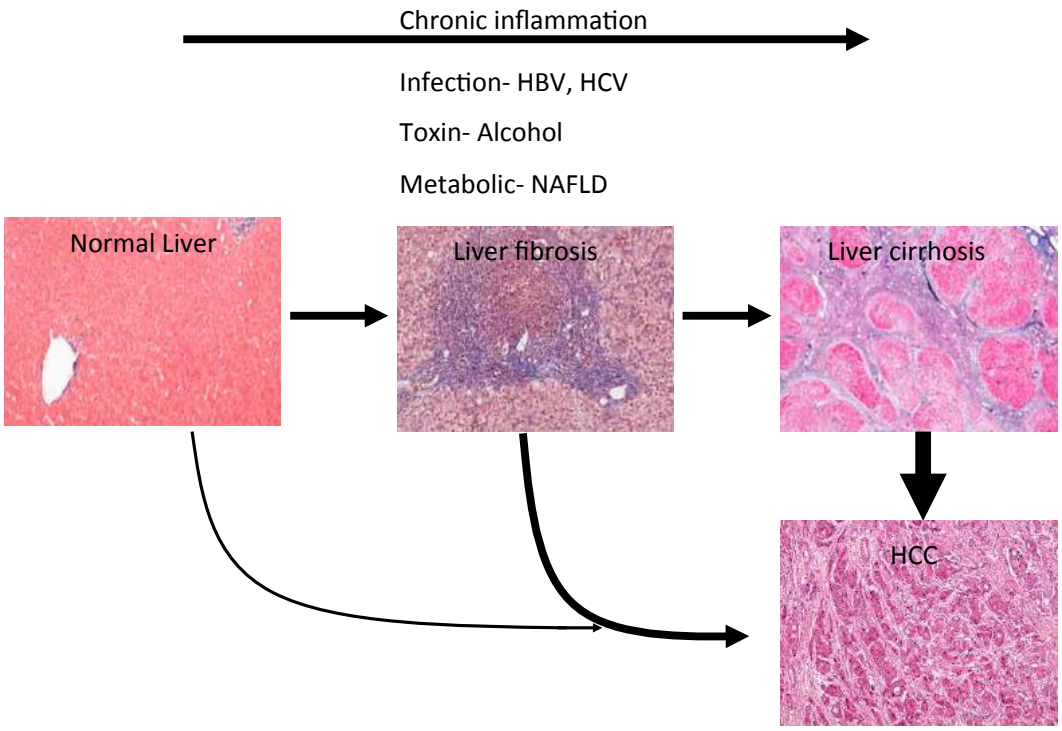

Figure 1-4: Natural history of hepatocellular carcinoma

Following the initial damage to the liver (i.e. infection), if the insult persists it will result in chronic inflammation. Chronic inflammation will lead to liver fibrosis, cirrhosis and ultimately the development of hepatocellular carcinoma. HCC; hepatocellular carcinoma, HBV; hepatitis B virus, HCV; hepatitis C virus, NAFLD; non-alcoholic fatty liver disease.

Following the initial formation of the tumour, chronic inflammation continues to drive tumour progression. The release of a wide range of cytokines and chemokines by tumour cells, tumour associated stromal cells and tumour infiltrating immune cells results in tumour growth. In particular, interleukin-6 (IL-6) has been identified to be a critical cytokine in HCC progression by promoting tumour cell growth [78-80]. Multiple roles have been described for IL-6, including its involvement in lymphocyte differentiation, cell proliferation, cell survival and inhibition of apoptosis [81]. It also plays an role in orchestrating metabolic and endocrine functions [82]. The importance of IL-6 in tumour development was first demonstrated in a murine model of HCC, in which animals deficient in IL-6 developed less HCC in response to the chemical carcinogen diethylnitrosamine (DEN) [83]. In addition, the male-bias for the development of HCC had often been contributed to the increased production of IL-6 in males [83]. Further evidence supporting the important role of IL-6 derives from the association of high levels of circulating IL-6 with tumour progression and HCC risk factors, such as ALD, viral liver disease and obesity [84-87]. Other cytokines have also been linked with tumour progression, including IL-11 [88], Tumour Necrosis Factor alpha (TNF-α) [89], IL-1β [90] and IL-23 [91]. Similar to IL-6, these cytokines results in the activation of a number of transcription factors, such as signal transducer and activator of transcription-3 (STAT-3), nuclear factor kappa-light-chain-enhancer of activated B cells **(**NF-kB) and activator protein-1 (AP-1), resulting in tumour cell survival. In addition, chronic inflammation also plays an important role in tumour angiogenesis and metastatic disease.

Angiogenesis is the growth of new blood vessels and is an essential component in tumour growth, aided by chronic inflammation [77]. Angiogenesis is initiated by tumour hypoxia, resulting in the recruitment of pro-angiogenic immune cells such as tumour-associated macrophages (TAM), leading to the release of a wide array of pro-angiogenic factors including vascular endothelial growth factor (VEGF), IL-8 and hypoxia-inducible factor 1-alpha (HIF-1α) [92]. Angiogenesis further aids tumour progression by allowing further infiltration of immune cells [93].

The importance of inflammation in the development of metastatic disease can be demonstrated in experiments where, following depletion of tumour associated immune cells, the metastatic potential of tumours was dramatically decreased [94]. Each of the critical steps of tumour metastasis requires the support of immune factors, beginning with the release of tumour associated transforming growth factor beta (TGF-β), resulting in the modulation of cancer cells to invade though the epithelial linings/basal membranes, so called epithelial-mesenchymal transition (EMT) [95]. Inflammation also aids in tumour cell metastases though the breakdown of extra-cellular matrix (ECM) and an increase in vascular permeability by the release of matrix metalloproteinases (MMP) [96] and cytokines [97] respectively. Inflammatory cytokines also provide survival signals to tumour cells during their transit within blood vessels and chemokines provide the signals needed to direct tumour cells to metastatic sites [98].

1.10 Cancer immune surveillance

As previously described, the immune system is well adapted to recognise and eliminate harmful pathogens, but it is increasingly acknowledged that it is also able to distinguish a wide range of tumour cells, with the potential to eliminate them, so called 'cancer immune surveillance'.

The concept of cancer immune surveillance was first supported by the observation that immune mediated elimination of tumours occurred following transplantation of tumour cells into immune competent mice. The existence of such a concept was further demonstrated in mice deficient in the recombination-activating gene (RAG) [99]. RAG is essential in the rearrangement of lymphocyte antigen receptors, its deficiency results in a complete lack of NK-cells, B-cells and T-cells. Furthermore, RAG-deficient mice develop spontaneous tumour when compared to wild type.

In addition to the studies carried out in RAG deficient mice demonstrating the importance of lymphocytes in immune surveillance, further support for the role of lymphocytes arises from studies in mice lacking α/β T-cells or γ/δ T-cells. It was observed animals lacking these T-cells were more susceptible to chemically induced tumour and tumour implantation, when compared to wild type mice, indicating the importance of T-cell subsets in cancer immune surveillance [100].

Further evidence to support the importance of tumour surveillance includes the detection of T-cells specific for tumour-associated antigens (TAA) [101]. The

association of increased TAA-specific T-cells with a better outcome in patients has been demonstrated in a wide range of malignancies [102, 103], whist an increased risk of malignancy associated with immune deficiency is seen in HIV infected patients and patients taking long term immune suppressants (i.e. solid organ transplantation and autoimmune conditions) [104]. Additional but indirect evidence for cancer immune surveillance has been demonstrated by reports of spontaneous tumour destruction accompanied by increased infiltration of activated immune cells that recognize TAA [104].

Since its conception, multiple immune mechanisms have been suggested to be involved in cancer immune surveillance. NK and NK-T cells, components of the innate immune system, were initially recognized for their important role in direct tumour cell killing in-vivo [105]. Unlike other subset of lymphocytes such as T-cells and B-cells, NK cells function mainly by non-specific targeting of cells that lack MHC molecules, so called 'missing self' recognition. The importance of NK cells in immune surveillance was confirmed by demonstrating their ability to protect the host from tumour initiation and metastasis. In these experiments, mice that were depleted of both NK and NK-T cells were shown to be more susceptible to tumour development and metastases, when compared to control mice [106, 107]. Similarly, animals that are genetically modified to lack selected NK cell functions were more susceptible to tumour formation when compared to wild type animals [108]. Further support for the important role of NK cells stems from the observation that the activation of NK cell function thought the administration of inflammatory cytokines resulted in the elimination of implanted tumours in animal models and defective

cytolytic activity in NK cells increases the risk of tumours in humans [109]. In addition, NK cell infiltration correlates positively with disease prognosis in cancer patients [110, 111].

The interferon (IFN) family are important cytokines of the immune system and are well known for their role in anti-viral responses [112]. Recent evidence has also demonstrated a role for IFNs in cancer immune surveillance. Three classes of IFN exists in humans, but it is mainly type-I IFNs (IFN-α and IFN-β) and type-II IFN (IFN-γ) that have been proposed to been involved in cancer immune surveillance, through their activation of immune cells (i.e. DC, NK cells and T-cells), anti-tumour proliferative effects and their inhibition of suppressive immune cells [113]. The importance of IFN-γ in tumour immunology was first recognized though the observation that endogenously secreted IFN-γ protected the host against chemical induced and transplanted tumours [114]. Subsequently, experiments demonstrated mice developed rapid growth of transplanted tumours following neutralization of IFN-γ [115]. Similarly, in a model of mice lacking sensitivity to either IFN-γ (IFN-γ growth receptor-deficient mice) or all classes of IFN, chemically induced tumours progressed more rapidly and with greater frequency [114]. Less evidence exists for the role of type-I IFN in cancer immune surveillance, however rapid tumour growth occurs following the neutralization of type-I IFN or in mice lacking the IFN-α receptor-1 subunit [116, 117].

In addition to IFN, the pore forming protein perforin has been implicated in cancer immune surveillance. Cytotoxic T-cells and NK cells can induce targeted cell death

either thought the granule exocytosis pathway or the death receptor pathway [118, 119]. The granule exocytosis pathway involves the secretion of perforin, which disrupts the cell membrane of the targeted cell, allowing the transportation of serine proteinase (i.e. granzymes A and B) into cells resulting in apoptosis of targeted cells. The importance of perforin in cancer immune surveillance can be demonstrated in knockout animals lacking perforin, resulting in an increased susceptibility to chemically induced tumours [120].

Collectively, evidence suggests that the innate and adaptive immune systems combine to detect and destroy cells with malignant potential as part of cancer immune surveillance. However, despite evidence demonstrating the existence of the ability of the immune system to detect and remove cancer cells, tumours still develop in immune competent individuals. The reason for tumour development despite cancer immune surveillance has been increasingly recognised to be the result of 'immune editing' [121, 122]. The concept of immune editing can be divided into three steps; elimination, equilibrium and escape.

Immune elimination encompasses the concept of cancer immune surveillance in which the immune system is constantly seeking cells with malignant potential and actively removing them from the host. However, the constant elimination of mutated cells can sometimes lead to the selection of cells with reduced immunogenicity. This can result in the persistence of mutated cells, which can survive attacks by the host's immune response. This mutated cell selection is commonly termed as 'immune equilibrium'. This step can take many years and even decades, but with time, cells

that are resistant to immune destruction acquire multiple mechanisms to evade from the host's immune system, resulting in uncontrolled tumour growth. This final step is termed 'immune escape'.

1.11 The suppressive tumour environment

A number of different mechanisms have been discovered to allow for immune escape to take place, despite the present of a functioning immune system [123]. These include the down regulation of MHC molecules and TAA on tumour cells the disruption of antigen presentation by down regulation of co-stimulatory molecules and defects in TAA presentation [124-129], However, it has been increasingly recognised, that creation of a suppressive tumour microenvironment is achieved by the recruitment and induction of regulatory immune cells within the tumour environment, such as regulatory T-cells (Treg), myeloid derived suppressor cells (MDSC) and TAM, leading to immune escape [130-132]. In addition, a number of recently discovered inhibitory molecules such as programmed death receptor-1 ligand (PD-L1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) produced by both tumours associated immune cells and stromal cells also contribute to the maintenance of a suppressive tumour environment [133, 134] **(**Figure 1-5**)**.

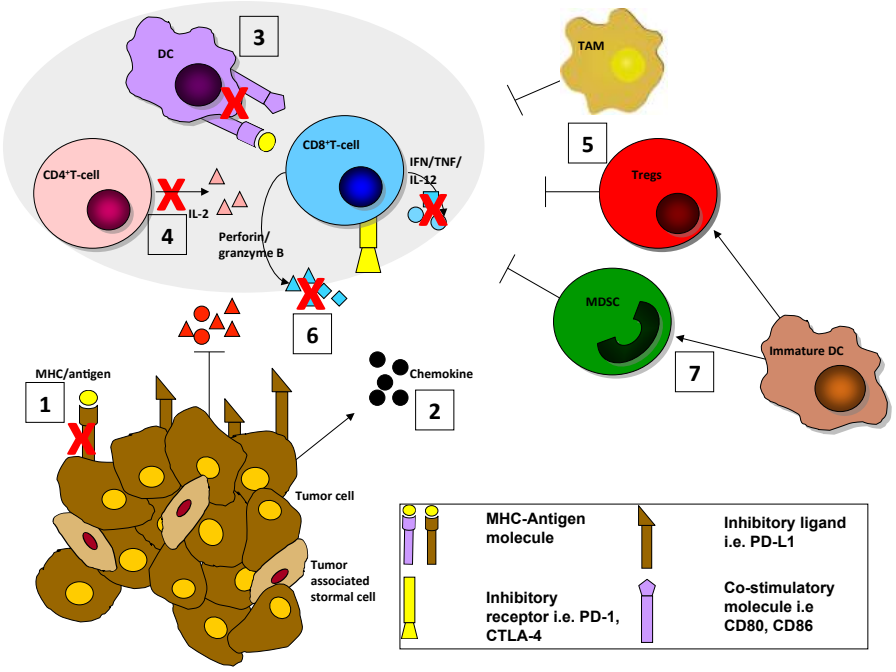

Figure 1-5 Overview of immune escape

Multiple means have been recognised in which tumours use to evade immune response, these include; (1) Reduced expression of MHC molecule and TAA on tumour cells. (2) The production of chemokines by tumour and tumour associated stormal cells to recruit suppressive cells. (3) Reduced co-stimulatory molecule expression and interference of antigen processing. (4) Suppression of helper $CD4^+$T-cells. (5) Suppression of effector cells. (6) Activation of inhibitory receptors by its ligands such as PD-L1 on tumour cells, resulting in suppression of effector $CD8^+$T-cells. (7) The induction of Tregs and MDSC. MHC, major histocompatibility complex; TAA, tumour associated antigen; Tregs, regulatory T-cells; DC, dendritic cells; TAM, tumour associated macrophages; MDSC, myeloid derived suppressor cell; transforming growth factor beta; PD-L1, programme death-ligand-1; CTLA-4, cytotoxic T-lymphocyte antigen-4; IL-2, interleukin-2; TNF, tumour necrosis factors; IFN-γ, interferon-γ. Adopted from Li et al. [135].

1.12 Regulatory immune cells & cancer

Regulatory immune cells are crucial in the maintenance of immune tolerance and in the prevention of auto-reactive immune response [136], but in the context of cancer they can be hijacked to suppress beneficial anti-tumour immunity. A number of different subsets of regulatory immune cells had since been implicated in this process.

$CD4^+$ Regulatory T-cells

Treg are critical in suppressing immune responses following their activation to eliminate pathogens, thereby allowing the restoration of immune homeostasis without autoimmunity [136]. The importance of Treg in peripheral tolerance can be seen in animals, in which mice depleted of Treg result in a wide variety of autoimmune disease [137, 138]. The majority of research conducted on Treg had so far has focused on regulatory T-cells expressing the surface marker CD4 ($CD4^+$Treg). They have been observed to be present in a wide range of human tumours and their presence correlates negatively with survival [139]. Increased numbers of $CD4^+$Treg have also been demonstrated in patients with HCC and have been associated with a poorer prognosis and recurrence following treatment [140-142]. $CD4^+$Treg express high levels of the interleukin-2 receptor α subunit (CD25) and low levels of the IL-7 receptor (CD127). They are characterised by expression of the transcription factor forkhead box P3 (FOXP3) that is critical for their differentiation and functional survival [143, 144]. The importance of FOXP3 in $CD4^+$Treg development can be emphasised in FOXP3 mutant scurfy mice lacking $CD4^+$Treg, resulting in lethal autoimmune syndrome [145]. Similarly, a rare X-linked syndrome in humans linked

to the mutation of the FOXP3 gene; IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) results in $CD4^+$Treg dysfunction, resulting in a wide array of autoimmune diseases [146].

The study of Treg has often been limited by the lack of specific marker. The surface expression of CD25 on T-cells had been used as a marker for Treg but its expression can also be found on newly activated effector T-cells [147]. Similarly, the reduced expression of CD127 can also be seen on effector T-cells [148, 149]. Currently, the expression of FOXP3 is being used as the gold standard marker for the identification of Treg. Once again, unlike in mice, FOXP3 expression in humans is not restricted to Treg alone and can be detected on effector T-cells [147, 148, 150]. Therefore, to be confident that T-cells with markers of Treg are bona fide suppressive T-cells, confirmation of their suppressive function is often required.

The origin of $CD4^+$Treg has been intensely debated over the past decade and increasingly evidence has suggested that they can occur both naturally or thought induction from non-Treg cells. Naturally occurring $CD4^+$Treg are generated from the thymus and recruited into a wide range of organs though the action of multiple receptor molecules and ligands present on both immune cells and tissues. In particular chemokine receptors and their ligands have been recognised to play a pivotal role in Treg recruitment in both health and disease. Chemokine receptors are a family of G-protein coupled receptors expressed on the surface of immune cells [151]. On binding its ligand, chemokine receptor activation leads to a range of cell activities including cell migration, differentiation and effector functions.

Studies involving $CD4^+$Treg confirmed the importance of the chemokine receptors CCR4, CXCR3 and CCR10 in their migration [152-156]. In addition, the importance of chemokines in recruitment of $CD4^+$Treg into tumours has been demonstrated by a number of studies, including CCR4 expressing $CD4^+$Treg in ovarian, colorectal, gastric and breast cancer [157-159], CCR5 expressing $CD4^+$Treg in pancreatic cancer [160] and more recently, CXCR3 expressing $CD4^+$Treg in ovarian cancer [161].

In addition to the recruitment of naturally occurring $CD4^+$Treg, the induction of $CD4^+$Treg from non-regulatory cells also plays a major role in their accumulation. A number of mechanisms in which $CD4^+$Treg can be induced have been identified including the generation of $CD4^+$Treg from non-Treg by suppressive soluble factors such as TGF-β and IL-10 and the generation of $CD4^+$Treg by tolerogenic APC [162, 163].

Intensive research has been focused on unravelling the mechanisms by which $CD4^+$Treg induce immune suppression. Both in-vivo and in-vitro experiments in humans and animals has suggested the involvement of multiple mechanisms which include; IL-2 consumption, release of inhibitory cytokines such as IL-10 and TGF-β, direct cytotoxic killing via the release of granzyme and perforin, the inhibition of effector immune cells via inhibitory molecules expressed on the surface of $CD4^+$Treg and metabolic disruption of responder cells [136, 164]. However, controversies exist on many of the proposed mechanisms, due to failure in translating findings seen during in-vivo experiments with in-vitro studies[165-169].

In addition to the 'classical $CD4^+$Treg' defined by they expression of CD25 and FOXP3, other $CD4^+$ regulatory T-cells subsets had been recognised. They differ from the classical $CD4^+$Treg mainly by the absence of stable expression of FOXP3 and being solely derived from extra-thymic generation from non-Treg cells in the presence of cytokines such as IL-10 and IL-27. These $CD4^+$ regulatory T-cells include IL-10 and TGF-β expressing T regulatory cell type-1 (Tr1) and Th3 regulatory $CD4^+$T-cells [170-173].

$CD8^+$ Regulatory T-cells

In recent years there has been increasing interest in another subset of Treg, beyond $CD4^+$Treg, characterised by the expression of the surface marker CD8. Historically, regulatory $CD8^+$T-cells were the first T-cell population identified to exhibit suppressive capacity [174-177]. The importance of regulatory $CD8^+$T-cells was further emphasised in animal model of autoimmune myocarditis, in which mice lacking in $CD8^+$T-cells developed more severe disease compared to wild type mice and in a similar study, mice with $CD8^+$T-cell deficiency developed increased susceptibility to autoimmune arthritis following inoculation with an auto-reactive antigen [178-180]. However, due to the lack of reliable markers available at the time, research into $CD8^+$Treg had taken a back seat until recently. Since the resurgence of research in regulatory T-cells, a number of different subsets of $CD8^+$T-cells with regulatory functions have since been identified in both animal models and man.

Qa-1-specific $CD8^+$T-cells are the best-defined subset of regulatory $CD8^+$T-cells so far [181], first described in murine studies of experimental allergic encephalomyelitis

(EAE), a model of human multiple sclerosis (MS). Mice inoculated with the antigen myelin basic protein, were noted to become resistant to EAE, compared to mice inoculated in the presence of defective $CD8^+$T-cells [182-185]. Additional studies confirmed the protection from EAE was related to a subset of regulatory $CD8^+$T-cells, which recognize the non-classical MHC class 1b molecule; Qa-1. Qa-1-specific $CD8^+$T-cells induce immune regulation by the direct cytotoxic killing of auto-reactive $CD4^+$T-cells expressing Qa-1 [186, 187]. Human HLA-E restricted $CD8^+$T-cells; a homolog of Qa-1 $CD8^+$T-cell have since been discovered in humans. A defect in this subset is associated with targeting against auto-reactive myelin specific $CD4^+$T-cell and have been linked in patients with active MS [185, 188-190].

In humans, $CD8^+CD28^-$T-cells have been identified as another subset of regulatory $CD8^+$T-cells. $CD8^+CD28^-$T-cells are often further classified into $FOXP3^+$ and $FOXP3^-$ subsets. The mechanisms in which $CD8^+CD28^-FOXP3^+$ T-cells induce their suppressive capacity is believed to be though the inhibition of APC such as DC, either via the up regulation of the inhibitory receptors immunoglobulin-like transcript-3 (ILT3) and immunoglobulin-like transcript-4 (ILT4) or the down regulation of the co-stimulatory molecules CD80 and CD86 on APC [191, 192]. Unlike $CD8^+CD28^-$ $FOXP3^+$ T-cells, $CD8^+CD28^-$ lacking FOXP3 expressions appear to mediate immune suppression though the production of IL-10 [193].

Since the discovery of $CD8^+$ regulatory T-cells, their involvement in animal and human diseases had been described, including, Grave's disease [194], inflammatory bowel disease (IBD) [195], systemic lupus erythematosus (SLE) [196-198] and

rheumatoid arthritis [180, 199], as well as a wide range of human cancers, which includes gastro-intestinal, prostate, melanoma, lung and lymphoma. Importantly, the suppressive capacity of $CD8^+$ Treg correlated with the clinical stage of the disease and patients overall survival, further supporting its role in the disruption of the cancer immune response [200-205]. Similarly, $CD8^+$ Treg has also been detected in patients with chronic infections such as HIV and HCV [206-212]. The presence of $CD8^+$ Treg was associated with poorer response to anti-viral treatment [213-216].

More recently, $CD8^+$ T-cells expressing the E-cadherin receptor (CD103) have been identified. This novel subset of cells was first identified as a subset of effector T-cells in the setting of renal transplant rejection and graft vs. host disease (GVHD). However, further work has since confirmed their immune regulatory property [217-219].

A fourth subset of regulatory $CD8^+$T-cells share many similarities with classical $CD4^+$Treg and are commonly termed $CD8^+CD25^{high}$ Treg. Expression of FOXP3 can be demonstrated on $CD8^+CD25^{high}$ Treg and other molecules such as high surface expression of CD25, CD28, CD122, CD103, glucocorticoid-induced TNFR-related (GITR) protein, CTLA-4 and low surface expression of CD127 can also be detected [143, 220-225]. However, $CD8^+CD25^{high}$ Treg have been suggested to differ from $CD4^+$Treg by possessing greater suppressive capacity and more importantly they may have the potential to be redirected to effector cells by specific co-stimulatory signals [226-228]. However, similar to $CD4^+$Treg there are currently no specific markers for the identification of $CD8^+CD25^{high}$ Treg. Therefore, to be confident that T-cells

expressing $CD8^{+}CD25^{high}$ with other molecules expressing Treg markers are bona fide suppressive T-cells, confirmation of their suppressive function is often required.

In man, $CD8^{+}CD25^{high}$ Treg have been described to occur naturally within the thymus and share many common features with naturally occurring classical $CD4^{+}$Treg [220]. In addition to naturally occurring $CD8^{+}CD25^{high}$ Treg, a number of studies have demonstrated the ability to generate $CD8^{+}CD25^{high}$ Treg in-vitro from $CD8^{+}CD25^{low}$ T-cells, through the modulation by suppressive cytokines such as IL-10 and TGF-β [229, 230]. Also $CD8^{+}CD25^{high}$ Treg can be generated by the chronic stimulation of TCR with artificial CD3 antibodies or though the direct stimulation by antigen derived from pathogens such as mycobacterium and salmonella [231, 232].

The detection of $CD8^{+}CD25^{high}$ Treg has been associated with a wide range of animal and human diseases. Primates infected with the simian immunodeficiency virus (SIV), a retrovirus that resembles the HIV [233, 234], the level of $CD8^{+}CD25^{high}$ appeared to correlate with high viral load. This suggests the possible role of $CD8^{+}CD25^{high}$ Treg in supporting virus persistence, possibly through the inhibition of anti-viral immune response. In man, $CD8^{+}CD25^{high}$ Treg have been recognised to be responsible for a suppressed immune response against a number of different micro-organism pathogens including HIV, HCV and herpes viruses, resulting in chronic infection [210, 235-237]. At the other end of the spectrum, dysfunctional or reduced numbers of $CD8^{+}CD25^{high}$ Treg, had been shown to play a pivotal role in the development of autoimmune diseases such as ankylosing spondylitis, SLE and MS [198, 222, 223, 238]. In cancer, $CD8^{+}CD25^{high}$ Treg have been shown to infiltrate

human tumours, including colorectal, prostate cancers and more recently in HCC [203, 228, 239]. Similar to $CD4^+$Treg, a number of mechanisms have been proposed to be involved in the suppressive function of $CD8^+CD25^{high}$ Treg, which includes the production of suppressive cytokines, direct cell killing via cytolysis, metabolic disruption of effector immune cells, disruption of APC functions and inhibitory molecule directed suppression [240]. However, unlike $CD4^+$Treg the understanding of $CD8^+CD25^{high}$ Treg remains scarce.

Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSC) have been identified as an important population of suppressive immune cells. Importantly, is has been implicated in tumour induced immune suppression [241]. Similar to regulatory T-cell, MDSC appear to be consisted of a heterogeneous group of immature myeloid derived cells [242]. In healthy individual, these immature cells are rapidly differentiated into DC, macrophages and granulocytes. However, during time of illness such as infection and cancer, these immature cells are prevented to undergo maturation, resulting in the increase in MDSC. The transcription factor cytosine-cytosine-adenosine-adenosine-thymidine (CCAAT)-enhancer protein b (C/EBPb) has been suggested as a marker of MDSC [243]. However, similar to the use of FOXP3 for Treg, C/EBPb can also be found in non-MDSC [244, 245]. Increased frequencies of MDSC have been reported in tumours and peripheral blood of cancer patients, including HCC, and have been associated with advanced clinical stage and disease progression [246-248]. Human MDSC are commonly classified into granulocytic MDSC ($CD15^+$ LIN^-), which share many phenotypic features to neutrophil granulocytes or monocytic MDSC

($CD14^{+}HLA\text{-}DR^{low/-}$), which closely related to monocytes. Both granulocytic and monocytic human MDSC expresses CD33, CD11b, and CD124 [130]. It was initially believed, MDSC were derived from immature monocytes, but emerging evidence suggests MDSC can be induced from mature monocytes although the mechanism remains unclear [249, 250]. Similarly, the mechanisms in which MDSC employ to mediate immune suppression remains uncertain but may include the induction of Treg, direct suppression of tumour specific innate and adaptive responses via intracellular production of arginase, nitric oxide and ROS [251]. ROS and in particular peroxynitrite a byproduct of ROS reaction, results in the disruption of antigen presentation via acting thought the modulation of TCR [252]. Nitric oxide on the other hand act via the inhibition of intracellular signaling such as Signal transducer and activator of transcription-5 (STAT-5), Janus kinase-3 (JAK-3) and MHC molecules, resulting in T-cell suppression [253]. Arginase results in the depletion of the non-essential amino acid L-arginine. This result in reduced expression of the CD3 ζ-chain, leading to a decrease in T-cell proliferation [254].

Tumour associated macrophages

TAMs have been detected in human solid organ tumours, including HCC and their presence is associated with a poor prognosis [255, 256]. TAMs are known to be derived from circulating monocytes, in which following they recruitment into the tumour by the chemokine CCL2 and differentiate into TAM, as a response to tumour derived factors such as colony-stimulating factor-1 (CSF-1)[257]. Macrophages are commonly separated into two distinct populations; M1 and M2. M1 macrophages are often referred to as the killer macrophages responsible for tissue damage and

inhibition of cell proliferation. In comparison, M2 macrophages are often referred to as tissue repair and growth promoting cells. [258, 259]. In this context, TAM possesses features of M2 macrophages. Many factors have been implicated in driving the differentiation of TAM towards a M2 phenotype [260]. These include a wide range of chemokines and cytokines such as CCL2, IL-10, IL-4 and IL-13. Tumour cells produce such factors and a wide array of tumour associated cells such as Treg, tumour stromal cells and TAM them self. In addition, certain tumour environmental factors such as hypoxia can also skew TAM differentiation towards a M2 phenotype. TAM support tumour genesis in many ways. Firstly, it supports the progression of tumour growth by promoting angiogenesis and tumour invasion via the release of factors such as MMP and VEGF [261, 262]. Importantly, TAM can also suppress anti-tumour responses by the activation and recruitment of Treg hence promoting a suppressive environment that favours tumour growth [263, 264]. In addition, TAM are also important in aiding initiation of tumour genesis via the maintenance of chronic inflammation by the production of cytokines such as IL-6, IL-17 and IL-23 [265]. Similar to MDSC, TAM can suppress immune response by the up-regulation of NO, arginase and ROS production [266]. The process of metastasis of tumour cells is also aided by TAM through the production of cytokines required in EMT (discussed previously) [267].

Tumour associated dendritic cells

First discovered in 1868 as Langerhans cells of the skin, dendritic cells (DC) are specialists in antigen presentation and play an integral role in the mediation of host immunity. As already discussed, DCs play an important role in immunity and

tolerance, in which DC in the presence of danger signals such as PAMP become matured, resulting in effective presentation of antigens and activation of immune cells. Inversely, DC lacking maturation are poor activators of immune cells and can lead to the generation of regulatory cells. The tolerogenic property of DC can be 'hijacked' by tumour cells to aid in its progression.

DCs play a major role in the anti-tumour immune response. This has been highlighted by studies demonstrating the presence of DC within the tumour or tumour margin and the possible association with better prognosis [268, 269]. Contradictorily, the infiltration of DCs within the tumour environment has also been implicated in immune escape. DCs isolated from either within the tumour or blood of cancer patients, including HCC, often express an immature/tolerogenic phenotype, with lower expression of co-stimulatory and MHC molecules [270-272]. In addition, tumour associated DC are often defective in their ability to produce pro-inflammatory cytokines [271] [273] and activate functional T-cells [271], resulting in an ineffective anti-tumour immune response. Further support for the detrimental effect of the tumour environment on DC differentiation and functions can be observed in experiments in which DC primed in conditioned medium from tumour cells resulted in an immature and dysfunctional phenotype [274, 275].

In addition to the inability of tumour associated DC to induce an effective anti-tumour immune response, increasingly, studies have also identified the active role DC may have in the generation of a suppressive tumour environment, through the induction of suppressive immune cells including Treg and via the production of suppressive cytokines (i.e. IL-10, TGF-β) [276] and inhibitory molecules [277, 278].

A number of mechanisms have been proposed to be involved in the generation of tumour-associated tolerogenic DC, immune modulating factors, such as IL-10 [279], IL-6 [257], TGF [280] and VEGF [281] have all been implicated in tumour associated DC's differentiation, maturation or function.

Suppressive stromal cells

Tumour associated stromal cells make up a large proportion of the tumour bulk and have been shown to play an important part in the initiation and progression of tumours though the production of a wide range of cytokines, chemokines and growth factors [282]. Increasing evidence has also suggested an immune suppressive role for stromal cells in orchestrating immune escape.

Fibroblasts are activated cells, which in normal circumstances contribute to wound healing though the production of ECM and collagen. Unlike fibroblasts involved in wound healing, cancer associated fibroblast (CAF) are maintained in a activated state and aid in tumour progression by the constant production of growth factors [283, 284], chemokine [285] and ECM [283] resulting in tumour proliferation, angiogenesis and further recruitment of tumour promoting immune cells. In addition CAF have been implicated in the induction of suppressive immune cells though their antigen presentation properties [53, 283, 286, 287].

Hepatic stellate cells (HSC) play an important pathological role in the development of fibrosis and cirrhosis, increasing evidence has suggested their immune suppressive

potential. Similarly to CAF, HSC have been shown to directly suppress effector cell responses, probably through the expression of inhibitory receptor ligands such as PD-L1 [288]. HSC can also induce the differentiation of MDSC and Treg through the production of suppressive cytokines and retinoid [289, 290].

Mesenchymal stem cells (MSC) are multiple potent stromal cells, which have been defined as cells with the potential to differentiate into a wide array of cell types, including osteoblasts, chondrocytes and adipocytes, under specific conditions. MSC have been implicated in tumour initiation, but their immunological suppressive properties may also be involved in immune escape. MSC have been shown to suppress T-cell, B-cell and NK-cell differentiation and proliferation through the production of IL-10, TGF-β [291] and metabolic disruption by IDO [292]. The expression of PD-L1 on MSC has been implicated as another possible mechanism of lymphocyte suppression [293]. MSC have also been shown to affect the differentiation, maturation and activation of DC, resulting in inhibition of effector T-cell stimulation [294, 295]. In addition, MSC have been demonstrated to induce the development of Treg directly or via the interaction of immature DC [296-298].

1.13 Inhibitory receptors

CTLA-4 is a glycoprotein expressed on T-cells and is homologous to the receptor CD28, sharing the same ligands (CD80 & CD86) that are essential for effector T-cell activation [299]. In health, CTLA-4 plays an important role in immune tolerance and this was emphasised by animal experiments in which CTLA-4 deficient mice exhibit

lethal lymphocyte proliferation and multi-organ lymphocyte infiltration [300, 301]. The precise mechanisms in which CTLA-4 exerts its immune inhibitory function remains unclear, but includes the inhibition of DC maturation and function, induction of tolerogenic DC and the restriction of CD28-CD80/CD86 signalling by either out completing CD28 in the binding of its ligands or through internalisation of the ligand by CTLA-4 expressing cells [302-307]. CTLA-4 has been detected in a wide array of suppressive immune cells [308] and tumours [309], hence it has been suggested to contribute enormously to tumour escape.

The inhibitory receptor, PD-1, is expressed on a wide range of immune cells but predominately on T-cells. In response to its activation by its ligand PD-L1, T-cells undergo apoptosis and anergy, resulting in limitation of effector response [310]. In health, PD-1 provides an additional mechanism for the maintenance of immune tolerance. However, in the setting of tumour escape, PD-1 can act to terminate potential anti-tumour responses. In particular, the ligand for PD-1 is highly expressed on human HCC and importantly the level of expression has been associated with poorer prognosis [311-313]. Other inhibitory receptors implicated in T-cell dysfunction include T-cell immunoglobulin and mucin-domain-containing-molecule-3 (TIM-3) and lymphocyte-activation gene-3 (LAG-3)[314].

1.14 Current Immunotherapy

With the ability of cancer cells to evade the anti-tumour immune response, the manipulation of the host's immune system to overcome some of these mechanisms (immunotherapy) had been an attractive area of research. HCC is an excellent target

for immunotherapy, due to the important relationship of the immune system with HCC initiation and progression [315]. In addition, observation of spontaneous or artificially stimulated anti-tumour immune responses with better prognosis has offered further support to the potential of immunotherapy.

Cytokine Therapy

The aim of immunotherapy is to either overcome processes involved in immune escape or to enhance the host's tumour immune response. The first generation of immunotherapy consisted of cytokine-based therapy. In particular, IFNγ has been tested in a number of studies in HBV and HCV infected patients with HCC as an adjuvant to other treatments. The precise anti-tumour mechanisms of IFN are unclear, but it is likely to be related to its immune modulating properties as previously discussed. Early studies of IFN demonstrated promising results with improvement in recurrence-free survival in cancer patients [316-318]. However, more recent studies failed to confirm the efficacy of IFN based adjuvant therapy [319]. A number of studies have also incorporated standard chemotherapy such as 5-fluorouracil and doxorubicin with IFN [320, 321]. However, most of these studies were limited by size and with the majority of these studies failing to demonstrate significant efficacy. In addition the tolerability of IFN has been suggested as a major obstacle for its use, especially in the setting of HCC treated by organ transplantation.

Interleukin-2 (IL-2) plays an important role in differentiation of T-cells into effector cells and has been shown to have the potential to overcome the suppressive effect of

the tumour microenvironment. The few studies conducted in HCC patients treated with IL-2 have reported objective responses, but the interpretation of these results is difficult due to their small size and importantly the use of IL-2 has been associated with severe side effects [322, 323].

Monoclonal antibodies

The ineffectiveness of the effector response seen in cancer has prompted the development of a number of potential immunotherapeutic strategies based on monoclonal antibodies (mAb) to stimulate T-cells, including mAb against CD137 (also known as 4-1BB) and CD28, with the aim of providing additional co-stimulatory signals required for T-cell activation [324-326]. Despite their potential to stimulate an anti-tumour response, severe toxicity has so far limited their development. More recently, CD134 (also known as OX40) a member of the TNF receptor superfamily has demonstrated early promise as a potential immunotherapeutic target. CD134 is expressed on both $CD4^+$ and $CD8^+$ T-cells and its activation induces T-cell proliferation [327]. Pre-clinical studies have demonstrated increases in tumour immunity following CD134 mAb treatment [328]. Early phase-1 clinical trials, using a mouse antibody targeted against human OX40 in patients with advanced cancer has been well tolerated, but further studies are needed to confirm its use as an future immunotherapeutic agent [328, 329].

The discovery of the importance of a number of inhibitory molecules in the suppression of anti-tumour immune responses has offered an attractive target for

immunotherapy. Inhibition of such pathways by antagonistic mAb, commonly being referred to as checkpoint inhibitors, has shown early promise. In particular, the importance of CTLA-4 has translated into approved therapy for melanoma and the licensing of a PD-1 inhibitor is expected in the near future. [330-334]. Importantly, a recent trial of CTLA-4 blockade with the mAb tremelimumab in patients with HCC demonstrated its safety with objective anti-tumour response [335]. Similarly, the administration of anti-PD-1 mAb has demonstrated antitumor effects in a murine model of HCC and currently a phase-1 dose escalation trial of PD-1 blockade with the mAb Nivolumab is underway in human HCC [336]. In addition, increasing evidence has also suggested the blocking of both receptors may have an additive anti-tumour effect in patients with melanoma [337].

Adoptive cell therapy – T cells

Despite the presence of effector immune cells within the tumour environment, it is often the case they are rendered dysfunctional by the many immune evasive mechanisms already mentioned. In an attempt to overcome these issues, adoptive T-cell transfer was developed which involves the transfer of ex-vivo generated and stimulated T-cells into cancer patients [338]. Although a number of different methods have been employed for the generation of adoptive T-cells, in general, T-cells are isolated from the blood or tumour of patients, followed by their expansion, priming to recognise TAA and activation, prior to re-infusion back into the patient. The majority of clinical trials using adoptive T-cell transfer in HCC patients have been small, but these trials have confirmed the feasibility and safety of this approach [339-341]. Despite the potential of such methods, immunotherapy based on adoptive transfer has

been limited, largely due to the inability to expand a sufficiently large number of ex-vivo generated T-cells and the identification of suitable TAA. For example, a number of TAA have been identified to be present in varying degrees in human HCC, including AFP, melanoma-associated antigen (MAGE), synovial sarcoma X breakpoint (SSX), testis-specific protein on Y chromosome (TSPY), New York-esophageal squamous cell carcinoma-1 (NY-ESO-1) and Glypican-3. [342-347]. However no single antigen has been proven to be present in all cases of HCC and, with the exception of AFP, the knowledge of T-cell reactivity against HCC tumour antigens is limited.

Adoptive therapy- Dendritic cells

DC are powerful APC which are important in the generation of immune responses against a wide range of antigens including those derived from tumours [348]. However, tumours often disrupt the functions of DC though multiple mechanisms and can even modulate DC to contribute to the generation of a suppressive tumour environment, through the induction of suppressive immune cells. Attempts have been made to overcome some of these problems via the ex-vivo generation of functional autologous DC that can recognise and generate a TAA specific effector response, so called 'DC vaccines'.

For more than a decade, numerous clinical trials have been conducted using DC vaccines against a wide range of cancers, with over 77 trials conducted between the years 2008 to 2012, with excellent safety profile [349]. The number of studies

involving HCC had also increased over the years **(**Table 1.1), but the total number of patients recruited has remained small.

Table 1-1 Recent dendritic cell vaccine trials in hepatocellular carcinoma.

Study	**Number of Patients**	**Vaccine**	**Antigen**	**Adverse reaction**
Tada 2012[350]	5	GM-CSF–IL-4 DCs	Peptides	No grade 3-4
El Ansary 2013[351]	30	GM-CSF–IL-4 DCs	Tumour lysate	No grade 3-4
Palmer 2009[352]	17	GM-CSF–IL-4 DCs	Tumour lysate	No grade 3-4
Ladhams 2002[353]	2	GM-CSF–IL-4 DCs	Tumour lysate	Non reported
Iwashita 2003[354]	10	GM-CSF–IL-4 DCs	Tumour lysate	Non reported
Stift 2003[355]	2 (out of 20 solid organ cancers)	GM-CSF–IL-4 DCs	Tumour lysate	No grade 3-4
Mazzolini 2008[356]	9 (out of 17 solid organ cancers)	GM-CSF–IL-4 DCs	Transfected with an adenovirus encoding interleukin-12 genes	4 grade 3 lymphopenia
Lee 2005 [357]	31	GM-CSF–IL-4 DCs	Tumour lysate	No grade 3-4
Chi 2005[358]	14	GM-CSF–IL-4 DCs	N/A	No grade 3-4

GM-CSF, Granulocyte-macrophage colony-stimulating factor; IL-4, Interleukin-4; DC, dendritic cells.

The rapid development of DC vaccines has been put down to the ability to generate large quantities of DC, either through the differentiation of peripherally derived monocytes or $CD34^+$ stem cells [359]. The production of DC vaccines involves loading DC with TAA, employing a variety of techniques. These include pulsing with recombinant proteins [360], peptides and tumour lysates [361], RNA transfection [362] and transfection with plasmid vectors encoding tumour associated antigens [363]. Furthermore, the antigens used in DC vaccine trials often differ between studies and at present there is no strong evidence to support the use of a particular type of antigen or loading method, but the targeting of multiple TAA such as by the use of tumour lysates may overcome some of the issues with tumour heterogeneity [352].

For DC to effectively stimulate T-cells, their appropriate maturation is essential. In early DC vaccine trials, common DC maturation cocktails consisted of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and IL-6 [364]. However, research into improving the effectiveness of DC vaccines has demonstrated the activation of DC through the physiological pathway of PPR activation, such as toll-like receptors (TLR), may improve the efficacy of the vaccine [365, 366].

Despite the intense research in DC vaccines, most studies have not demonstrated correlation between immune response and clinical impact [352, 367]. The reason for the failure of these studies to demonstrate a clinical response despite an adequate immune response is poorly understood, but may reflect our current lack of

understanding on what is needed to produce the most effective DC vaccine. This is illustrated by the enormous variation in DC generation methods between vaccine trials. In addition, the failure of recent DC trials emphasises the multiple mechanisms tumour cells employ to evade the immune system.

1.15 Targeting the suppressive tumour environment

The majority of previous trials on immunotherapy have focused on enhancing tumour immunity. However, as described previously, the tumour environment contributes significantly to the inhibition of anti-tumour immune responses. Hence, it is likely the suppressive tumour environment can counteract immunity enhancing treatment. The targeting of tumour-induced suppression either alone or in combination with immunotherapy enhancing tumour immunity may reverse the inhibition of anti-tumour immune response. With our increasing understanding of the importance of the suppressive tumour microenvironment in the progression of cancer, a number of promising novel treatments targeting suppressive immune cells had emerged. The therapeutic potential of Treg depletion has been demonstrated in animal models. In which the depletion of Treg alone or combined with immunotherapy has been shown to enhance anti-tumour immunity [368-370]. In particular, early animal studies have demonstrated the enhancement of anti-tumour responses following Treg depletion through the targeting of CD25 with mAb [371, 372]. In humans, the use of Denileukin diftitox, a fusion protein of IL-2 and diphtheria toxin that targets CD25 expressing cells, leading to their killing, has been shown to reduce the level of circulating Treg in patients with melanoma, ovarian cancer and renal cell carcinoma [373-375].

However, the persistence of Treg depletion with Denileukin diftitox was often transient due to its short half-life. More recently a humanized anti-CD25 mAb; Daclizumab has demonstrated promising results as a treatment for the depletion of Treg in breast cancer patients [376]. Currently, we are still awaiting further results on the effectiveness of CD25 targeted therapy, but a major obstacle in the targeting of this molecule exists due to the depletion of CD25 expressing effector T-cells.

Another promising agent that has been investigated for the depletion of Treg is the alkylating agent cyclophosphamide. Cyclophosphamide is commonly used in a wide range of human malignancies and autoimmune diseases. In addition to its cytotoxic effect, cyclophosphamide may have potential immunomodulating effects. This was first recognised by its ability to enhance vaccination responses in humans [377]. Evidence to support the Treg modulation properties of cyclophosphamide came from the selective depletion of $CD4^+$Treg in mice following treatment [368, 378], where importantly, the administration of low dose cyclophosphamide delayed tumour growth and enhanced the efficacy of anti-tumour vaccination.

In humans with metastatic solid tumours, a metronomic low dose cyclophosphamide regime reduced the numbers of Treg and was associated with reversal of suppression of both NK cell function and T-cell proliferation [379]. Importantly, a small pilot study in human HCC showed that low-dose cyclophosphamide reduced circulating Treg numbers [380]. More recently, the use of low dose cyclophosphamide was confirmed to specifically prevent the generation of $CD8^+CD25^+$Treg [381].

Tumour infiltrating Treg can be induced from non-Treg cells or recruited into the tumour environment through the action of chemokines produced by tumour cells, tumour associated stromal cells or immune cells. The prevention of Treg migration into the tumour has provided an attractive therapeutic target and exploitation of an increased expression of the chemokine receptor CCR4 by Treg when compared to effector T-cells may provide such a target. The chemokines CCL17 and CCL22 are ligands for CCR4 and have been shown to be expressed at a high level in the tumour microenvironment, including human HCC. Early studies in the targeting of Treg migration in a model of ovarian cancer with the use of a mAb against the chemokine CCL22 was encouraging, with the demonstration of a decrease in $CD4^{+}$Treg infiltration following treatment [126]. Similarly, the use of an anti-CCR4 mAb in tumour bearing mice resulted in reduction in Treg infiltration and tumour size [382]. Importantly, recent clinical trials using a humanized version of anti-CCR4 mAb in patients with haematological cancer resulted in anti-tumour activity [383, 384]. However, due to the redundancy of the chemokine system, the blockade of just one ligand is likely to result in the trafficking of Treg via an alternative pathway.

1.16 Immune Responses in Conventional Treatment

Treatment options for incurable liver cancer depend on the tumour stage. For patients with incurable but limited disease, RFA is the treatment of choice whilst in patients with more extensive disease TACE is beneficial for selected patients [34-37]. The effectiveness of these treatments is down to their direct killing of tumour cells but evidence suggests that control of liver cancer can also be mediated indirectly by activation of anti-tumour immune responses during therapy.

Evidence for the possible role of local ablative treatment in the activation of anti-tumour immune response comes from studies showing changes in immune cell kinetics following treatment. The observation includes; enhanced NK cell activation, cytoxicity activity and IFN production [385], increased frequency of TAA specific T-cells [386, 387] and increased DC maturation and function [387]. Importantly, in some of these studies the changes in immune response following ablative treatment was associated with better recurrence free survival, when compared with patients with a limited immune response.

In addition to the possible effect of local ablative treatment on effector cell anti-tumour responses, this form of therapy has also been suggested to contribute to the disruption of the suppressive tumour environment. A number of recent studies have observed changes in suppressive immune cells such as MDSC [388] and $CD4^{+}$Treg [389, 390] following local ablative treatment and were associated with a positive outcome in patients with a reduction of suppressive immune cells.

Further evidence supporting the enhancement of anti-tumour immune responses in patients undergoing local ablative treatment has been proposed to be related to the release of immune-activating molecules, such as heat shock protein and pro-inflammatory cytokines such as IL-17 and VEGF, primarily as a result of tumour cell death, leading to an enhanced immune response directed against the remaining viable tumour cells [391-394].

Despite evidence to suggest the possible involvement of ablative treatment in the enhancement of anti-tumour immune responses, the precise mechanisms of how anti-tumour immune responses are enhanced following local ablative treatment remains unclear. Importantly, induced immune response seen post treatment is not adequate to prevent tumour progression. However, combining the tumour killing and immune related effect of ablative treatment with other forms of immunotherapy may offer a potential strategy to further boost this anti-tumour immune response. It is therefore critical that we further our understanding of the immune events that take place following ablative treatment to guide research into identifying the best target and timing for any potential adjuvant immunotherapeutic strategy.

1.17 Hypotheses

Chapter 3 deals with the hypothesis that a novel subset of suppressive T-cells expressing the surface marker CD8 is presence within human HCC. Chapter 4 focus on the hypothesis that the tumour environment is able to induce suppressive $CD8^+$T-cells though the interaction with dendritic cells. Chapter 5 concerns the hypothesis that tumour infiltrating suppressive CD8 T-cells can modulates the activation of tumour effector T cells, leading to the suppression of anti-tumour immune responses and promoting tumour progression. Finally, chapter 6 looks at weather the standard ablative treatment against human HCC may have an immune-modulating role.

1.18 Aim

In recent years, enormous interest had been focused on understanding the different components involved in the generation of a suppressive tumour microenvironment, with the goal of identifying possible therapeutic targets. The aim of this project was to determine the role regulatory immune cells; in particular the novel regulatory T-cells $CD8^+CD25^+$Treg, had on human hepatocellular carcinoma. The objectives of the project are:

What is the phenotype of tumour-infiltrating $CD8^+CD25^+$Treg?

Phenotypic analysis of T-cells isolated from primary liver tumours, matched peripheral blood, and matched non-tumour tissue without in-vitro expansion to confirm the phenotype and frequency of $CD8^+CD25^+$Treg.

Where do $CD8^+CD25^+$Treg come from?

Early studies suggest that $CD8^+CD25^+$Treg differentiation depend on specific co-stimulatory signals provided by dendritic cells or specific cytokines. Here I investigate the signals required for $CD8^+CD25^+$Treg generation in human hepatocellular carcinoma.

What is the function of tumour infiltrating $CD8^+CD25^+$Treg?

The suppressive ability of $CD8^+CD25^+$Treg will be studied using assays optimised as part of this study. I will further define $CD8^+CD25^+$Treg function though they cytokine secretion profile.

Do ablative treatment effect Treg?

Efficacy of current immunotherapeutic strategies has been disappointing, but combining it with other treatment modalities may offer potential benefit. I will define the immune response in patients undergoing ablative treatment, in particular the effect on Treg numbers, phenotype and function.

2 Materials & Methods

2.1 Human samples

Matched tumour and tumour free distal liver tissue were obtained from patients undergoing either liver transplantation or resection at the University Hospital Birmingham. Tumour tissue was identified by its macroscopic appears and matched non-tumour tissues were obtained as distally as possible from the tumour. The weight of tissues was recorded prior to processing. Blood samples were taken from healthy donors, patients with hereditary haemachromatosis or HCC undergoing treatment. Blood samples were collected and placed into ethylenediaminetetraacetic acid (EDTA) bags or bottles. For protein isolation, $1cm^2$ cubes of liver tissue were snap frozen with liquid nitrogen and stored at -80^0C until processing. For immunohist^{oc}hemistry and immunofluorescence staining, tissue sections were obtained from paraffin embedded and frozen tissue blocks. Written informed patient consent and ethical approval were obtained prior to collection of all samples.

2.2 Mononuclear cells extraction from liver tissue

Liver and tumour tissues were processed immediately on arrival to the lab. The tissues were first sliced into small cubes ($<0.2cm^2$) and transferred to a gentleMACS C tube **(**Figure 2-1**;** mitenyi Biotec). This was followed by mechanical dissociation with the gentleMACS dissociator (Figure 2-1; Mitenyi Biotec) using the pre-set gentleMACS program (h_spleen_01). The dissociated materials were then passed

though a sterile fine mesh (John Staniar & Co Ltd UK) and washed twice with RPMI-1640 1% GPS and pelleted in a centrifuge (800 x *g*, 5mins). The sediment was re-suspended with RPMI-1640 1% GPS and separated over a Lympholyte®-H (Cedarlane) gradient at a 1:1 ratio and centrifuged for 30 minutes at 800 x *g*. After centrifugation, a well-defined mononuclear cell (MNC) layer interface appeared and was removed using a Pasteur pipette. The collected cells were washed twice with phosphate buffer saline (PBS) (Oxoid, UK) and pelleted in a centrifuge (800 x *g*, 5mins). The MNC were re-suspended with MACS buffer (PBS, 0.5% FCS (Foetal Calf Serum), 2mM EDTA) and used for downstream experiments. The number and viability of cells were confirmed using trypan blue staining and manual haemocytometer counting.

A

B

Figure 2-1 Liver mononuclear cells extraction kits

Human liver tissue dissociation was performed using the (A) gentleMACS dissociator and (B) gentleMACS C tubes.

2.3 Peripheral blood mononuclear cells extraction from whole blood

Peripheral blood mononuclear cells (PBMC) were extracted from whole blood, by first centrifuging the blood for 30mins at 800 x *g* to create a buffy coat. The buffy coat interface was recovered using a Pasteur pipette and diluted 1:4 with autologous plasma. PBMC were then separated from the buffy coat by layering over a Lympholyte®-H (Cedarlane) gradient at a 1:1 ratio and centrifuged for 30mins at 800 x *g*. After centrifugation, the well-defined PBMC layer interface was removed using a Pasteur pipette. The collected cells were washed with PBS and centrifuged (800 x *g*, 5mins) twice and re-suspended in MACS buffer for downstream experiments. The number and viability of cells was confirmed using trypan blue staining and manual haemocytmeter counting.

2.4 Specific T-cell subsets isolation

T-cells isolation by cell sorting

To isolate specific T-cell subsets for functional assays, MNC isolated from tumour, matched tumour free tissues or peripheral blood samples were labelled with fluorescently conjugated antibodies raised against CD3, CD4, CD8, CD25 and CD127 (detailed protocol in flow cytometry section). $CD8^{+}$Treg ($CD3^{+}CD8^{+}CD25^{high}CD127^{low}$), $CD4^{+}$Treg ($CD3^{+}CD4^{+}CD25^{high}CD127^{low}$) and responder T-cells ($CD3^{+}CD25^{-}$) were obtained by using a Mo-Flow XDP cell sorter (Beckman Coulter*)*. Cells were sorted into a 1.5mL sterile eppendorf tubes containing

ice-cold RPMI-1640, 1% GPS and purity of cells isolated was confirmed to be >95% by flow cytometry. The sorted cells were centrifuged (350 x *g*, 10mins) and resuspended in RPMI-1640, 1% GPS for further downstream experiments. The number and viability of cells was confirmed using trypan blue staining and manual haemocytometer counting.

Magnetic isolation of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells

$CD4^+$Treg and responder T-cells ($CD4^+CD25^-$) were isolated using a Dynal regulatory $CD4^+CD25^+$T-cells kit (Invitrogen). Prior to use, the desired volume of each type of beads was transferred to a polystyrene tube (BD falcon) and mixed with 1mL of MACS buffer. The beads were placed in a magnet for 1min and the supernatant was removed. The beads were removed from the magnet and resuspended with the same volume of MACS buffer as the initial volume of beads taken. The isolation of $CD4^+$Treg was performed in three main steps.

Step 1. Negative isolation of $CD4^+$ T cells:

Negative isolation of $CD4^+$T-cells was performed by first depleting B cells, NK cells, monocytes, $CD8^+$T-cells and red blood cells. PBMC obtained from patients or healthy donors were firstly extracted as previously described and re-suspended at a concentration of $2x10^8$ per mL of MACS buffer. PBMC were indirectly labelled with an antibody mix against CD8, CD14, CD16, CD19, CD56, CD36, CD123, and Glycophorin A and then Depletion Dynabeads were added to capture the antibody bound cells. These labelled cells were then removed with a magnet.

Step 2. Positive isolation of $CD4^+CD25^+$ Treg cells:

After negative isolation of the $CD4^+$ cells, Dynabeads CD25 were added to positively isolate the $CD4^+CD25^+$T-cells. The bead-labelled $CD4^+CD25^+$T-cells were captured in a magnet. The supernatant containing the un-labelled $CD4^+CD25^-$ cells (responder T-cells) were removed for later use.

Step 3. Detachment of the isolated $CD4^+CD25^+$ Treg cells:

After positive isolation of the $CD4^+CD25^+$ Treg, the cells were detached from the beads by adding DETACHaBEAD, which cleaves the magnetic particle from the $CD25^+$ cells. The beads are removed with a magnet, leaving a pure population of $CD4^+CD25^+$ cells. The purity of cells was improved by repeating each magnet selection step twice.

All washing steps were carried out using MACS buffer and cells were centrifuged at 350 x *g* for 5mins. The volume of antibody and beads used was according to manufacturer instruction.

Naïve $CD8^+$ T-cells isolation

Naïve $CD8^+$ T-cells ($CD8^+CD45RA^+$) were extracted from PBMC by negative magnetic isolation using an EasySep™ Human Naïve $CD8^+$ T-cell enrichment kit (StemCell technologies).

This was performed by depleting B-cells, NK cells, monocytes, $CD4^+$ T-cells, memory T-cells and red blood cells. PBMC from healthy donors was firstly extracted as previously described and re-suspended at a concentration of $5x10^7$ cells per mL of MACS buffer. PBMC were indirectly labelled with an antibody Mix against CD4, CD14, CD16, CD19, CD20, CD36, CD45RO, CD56, CD57, CD94, CD123, CD244, TCRγ/δ and glycophorin A and then paramagnetic beads were added to capture the antibody bound cells. These labelled cells were then removed with a magnet. To improve the purity of the naïve $CD8^+$T-cells, the magnetic separation step was repeated a total of 5 times.

For autologous $CD14^+$ derived DC: naïve CD8+T-cells co-culture experiments, $CD14^+$ monocytes were first isolated from PBMC (see section 0), followed by the isolation of naïve $CD8^+$ T-cells from the non-$CD14^+$ cells fraction. The naïve $CD8^+$ T-cells were kept in medium containing RPMI-1640 supplemented with 10% FCS, 1% GPS and 50IU/mL IL2 at a concentration of $5x10^6$ cells per mL in a 48-well plate. The naïve $CD8^+$ T-cells were used in co-culture experiments after the $CD14^+$ cells had been differentiated into monocyte derived dendritic cells (moDC). The purity of naive $CD8^+$T-cells was confirmed by flow cytometry immediately after isolation and following culture in IL-2.

All washing steps were carried out using MACS buffer and cells were centrifuged at 350 x *g* for 5mins. The volume of antibody and beads used was according to manufacturer's instructions.

$CD14^+$ monocytes isolation

$CD14^+$ monocytes were isolated from PBMC using a positive magnetic isolation kit. PBMC were re-suspeneded with 80µL of MACS buffer per 10^7 cells. $CD14^+$ micro beads (Miltenyi Biotec) were added per 10^7 cells. The cells were applied over a primed LS column (Figure 2-2) (ferromagnetic spheres) that had been attached to a MACS separator (Figure 2-2) (magnetic field) and washed though with MACS buffer three times to obtain the unlabelled cells. The $CD14^+$ micro beads labelled monocytes are retained in the column. To remove the $CD14^+$ monocytes, the LS column was removed from the MACS separator and MACS buffer was added to the column and the cells were flushed out, releasing the previously attached $CD14^+$ cells. To improve the purity of the $CD14^+$ monocytes, the cells are passed though a new MACS column/separator and the separation procedure described above was repeated. The unlabelled cell fraction ($CD14^-$ PBMC) was used to isolate naïve CD8 T-cells for autologous co-culture experiments. The volume of antibody and beads used was according to manufacturer's instructions.

Figure 2-2 $CD14^+$ cells isolation kit

(A) LS column (B) MACS separator

2.5 Tissue conditioned medium

Approximately 1g of matched tumour and tumour free distal tissue was weighed and washed in RPMI-1640. The tissues were finely sliced into sections by scalpel. The sections were transferred to a T45 tissue culture flask (Corning) and incubated in RPMI-1640 supplemented with 1% GPS at 2mL per 0.1g of tissue for 24 hours at 35^{o}C, 5% CO_2 (Figure 2-3). The tissue-conditioned medium was collected and centrifuged twice at 800 x *g* for 5mins to remove any tissue debris. The medium was passed though a 0.22μm sterile filter (Millipore) and aliquoted into 1.5mL cryovials (Corning) for storage at -80^{o}C until use.

Figure 2-3 Generation of tissue conditioned medium

2.6 Immunohistochemistry & immunofluorescence

Immunohistochemistry staining was carried out using formalin fixed, paraffin embedded sections. 5µm thick sections were cut from tissue blocks using a microtome and mounted onto X-tra® Adhesive Snowcoat slides (Surgipath). Slides were incubated at 60^{o}C for 1 hour and stored at room temperature until use.

Immunofluorescence staining was carried out using fresh liver tissue (1cm^{2} cubes), snap frozen in liquid nitrogen and stored at – 80^{o}C until use. 5µm thick sections were cut using a cryostat (Bright OTF) and mounted on glass microscope slides (BDH UK) and fixed in acetone (Fisher Scientific), wrapped in foil and stored at -20^{o}C until use.

A full list of primary antibodies used for immunohistochemistry and immunofluorescence are summarised in (Table 2-1).

Table 2-1 Antibodies used for immunohistochemistry & immunofluorescence staining

Antibody	Clone	Source	Concentration
CD8a Ms IgG^{2a}	OKT-8	eBioscience	10 μg/mL
CD4 Ms IgG^{1}	RPA-T4	BD Bioscience	10 μg/mL
FOXP3 Ms IgG^{1}	236/E7	Abcam	5 μg/mL
FOXP3 Rb IgG	Polyclonal	Abcam	1 μg/mL
CD11c Ms IgG^{1}	F24	Abcam	10 μg/mL
Mouse IgG^{2a} isotype control	DAK-GO5	DAKO	Use at same concentration as primary antibody
Mouse IgG^{1} Isotype control	DAK-GO1	DAKO	Use at same concentration as primary antibody
Rabbit polyclonal IgG **Isotype control**		DAKO	Use at same concentration as primary antibody
Goat anti-mouse IgG^{2a} Alexa Fluor 488		Invitrogen	2 μg/mL
Goat anti-mouse IgG^{1} Alexa Fluor 488		Invitrogen	2 μg/mL
Goat anti-rabbit IgG (H+L) Alexa Fluor 534		Invitrogen	2 μg/mL
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)		Invitrogen	300nM

Immunohistochemical staining

Formalin fixed, paraffin embedded sections were deparaffinised with xylene (Surgipath) for 4 minutes, followed by dehydration with 100% ethanol (Fisher Scientific) for 4 minutes. Endogenous peroxidise activity was blocked with 0.3% hydrogen peroxide solution (30% H_2O_2 (Sigma-Aldrich) dissolved in ethanol (Fisher Scientific)) for 20 minutes followed by washing in PBS for 5 minutes. Antigen retrieval was carried out by pre-heating EDTA buffer (1mM EDTA (Abcam) in distilled H_2O, with 0.1% Tween20 (Promega), adjusted to pH8) in a microwave at 800 watts for 10 minutes, followed by heating the sections in EDTA buffer for a further 20 minutes. The sections were allowed to cool at room temperature and washed with PBS for a further 5 minutes. To block non-specific binding, the sections were blocked by incubation with normal horse serum (Vector Labs). To block endogenous biotin activity, sections were incubated in avidin solution for 20 minutes, followed by incubation in biotin solution for 20 minutes (Vector Labs). The sections were incubated with primary antibodies or isotype control diluted with normal horse serum, at room temperature for 60 minutes. The sections were washed 3 times in PBS, followed by incubation with biotinylated secondary antibody raised in an appropriate species (Vector Labs) for 30 minutes and washed 3 times with PBS. Staining was visualised using the immunoperoxidase technique with avidin-biotin peroxidase (VECTASTAIN®ABC) according to manufacturer's instructions and incubated with the enzyme substrate Vector® NovaRED™ (Vector Labs) or ImmPACT DAB™ (Vector Labs) for 5 minutes, followed by washing in distilled H_2O (dH_2O) for 5 minutes. The sections were counter stained with haematoxylin (Dako Ltd, UK) for 30 seconds and washed in (dH_2O) for 5 minutes. The sections were then dehydrated in

100% ethanol for 4 minutes and cleared in xylene for 4 minutes. The sections were mounted in DPX (Sigma-Aldrich) and allowed to dry over night. Images were analysed using a Zesis Axioscope microscope and Axiovision software (Carl Zeiss).

For dual immunohistochemistry staining, the sections were washed 3 times in PBS following incubation with the first primary antibody and enzyme substrate. The blocking of non-specific binding and endogenous biotin binding was repeated as described previously. The sections were incubated with the second primary antibody or isotype control diluted with normal horse serum at room temperature for 60 minutes and washed three times with PBS. The sections were once again incubated with biotinylated secondary antibody raised in the appropriate species (Vector Lab) for 30 minutes. The sections were incubated with VECTASTAIN®ABC followed by the second enzyme substrate (ImmPACT DAB with Nickel or Vector Blue, (Vector Labs)). The sections were counterstained, dehydrated, cleared and mounted as previously described.

Immunofluorescent staining

Acetones fixed frozen sections were left at room temperature for 30 minutes, followed by repeat fixation with acetone for 5 minutes. The sections were washed in PBS and incubated with normal horse serum for 20 minutes to block non-specific binding. The sections were incubated with primary antibodies or isotype control diluted with normal horse serum at room temperature for 60 minutes. The sections were washed 3 times in PBS, followed by incubation with fluorescent secondary antibody raised in the appropriate species (Table 2-1) for 30 minutes in the dark and washed 3 times

with PBS. The sections were counterstained with DAPI (Invitrogen) and washed with PBS. The sections were mounted in Immunomount (GeneTex) and allowed to dry over night whilst protected from light. The sections were analysed using a Zeiss LSM 510 UV confocal microscope (Carl Zeiss).

2.7 Flow cytometry

The phenotype of different subsets of immune cells was assessed by multi-colour flow cytometry using a Cyan ADP flow cytometer and data analysed using Summit 5.2 (Beckman Coulter).

For surface staining, $1x10^6$ cells were diluted in 100 µL MACS buffer and incubated with fluorescent antibodies or isotype controls (Table 2-2) for 30 minutes at 4^oC then washed in MACS buffer followed by centrifugation at 800 x *g* for 5 minutes. Cells were re-suspended in 200µL of MACS buffer for flow cytometry analysis.

For staining lymphocytes isolated from liver tissues, a live/dead cells marker (life technologies) was added into the antibody mix. $1x10^6$ cells were diluted in 1mL of MACS buffer and incubated with1µL of live/dead marker for 15 minutes at 4^oC then washed in MACS buffer followed by centrifugation at 800 x *g* for 5 minutes. Cells were re-suspended in 200µL of MACS buffer for flow cytometry analysis.

For experiments that required absolute cell counts, 20µL of AccuCheck counting beads (Invitrogen) were added to the final sample and the final cell counts obtained using the formula below.

Absolute Count (cells/µL) = number of cells counted/Total number of beads counted x Number of AccuCheck Counting Beads Beads per µL

For intracellular staining, the cells were first stained with surface antibodies or isotype controls followed by fixation and permabilsation using the BD cytofix/cytoperm kit (BD biosciences) according to manufacturer's instructions. Cells were then stained with intracellular antibodies or isotype controls for 30 minutes at 4^{o}C. For FOXP3 staining, 10µL of goat serum was added with the antibody to reduce non-specific staining.

For intracellular cytokine detection in T-cells, cells were stimulated prior to antibody staining. Cells were re-suspended in RPMI-1640 supplemented with 5% human serum (TCS Biosciences) at 10^7 cells/mL and stimulated with 20µL of CytoStim, a TCR MHC cross-linking antibody, per mL of cells suspension (Miltenyi Biotec) for 2 hours at 37^{o}C, 5% CO_2. To enhance the detection of intracellular cytokines 1µg per mL of brefeldin A (Sigma-Aldrich) was added into the stimulated cells to prevent protein trafficking before a further incubation for 4 hours. Surface and intracellular staining was then carried out as described previously. A full list of monoclonal antibodies used for flow cytometry is summarised in (Table 2-2)

Mononuclear cells (MNCs) were identified on forward scatter (FSC) and side scatter (SSC). The MNC were gated against CD3 and Live/Dead marker (L/D) to identify viable T-cells. Viable $CD3^+$T-cells were gated against CD8 and CD4 to identify $CD3^+CD8^+$ T-cells and $CD3^+CD4^+$ T-cells subsets. $CD3^+CD8^+$ T-cells are gated against CD25 and CD127 to identify $CD8^+$Tregs ($CD3^+CD8^+CD25^{high}CD127^{low}$cells). $CD3^+CD4^+$T-cells are gated against CD25 and CD127 to identify $CD4^+$Tregs ($CD3^+CD4^+CD25^{high}CD127l^{ow}$ cells).

To identify the expression of marker of interest, the histogram regions for negative/positive populations were set. The regions were set so that the negative population included 98.5% of events using a cocktail fluorochrome conjugated isotype matched control antibody.

Table 2-2 Antibodies used for flow cytometry

Antibody Isotype	Clone	Source	Volume per 10^6 cells
CD3 V450 Ms IgG[1]	UCHT1	BD bioscience	5 µl
CD3 PE Ms IgG[1]	UCHT1	BD bioscience	5 µl
CD4 V500 Ms IgG[1]	RPA-T4	BD bioscience	5 µl
CD8 PE Cy7 Ms IgG[1]	RPA-T8	BD bioscience	3 µl
CD8 PerCP-Cy5.5 Ms IgG[1]	SK1	BD bioscience	5 µl
CD25 APC Ms IgG[1]	M-A251	BD bioscience	5 µl

CD25 APC Cy7 **Ms IgG^{1}**	M-A251	BD bioscience	5 µl
CD127 FITC **Ms IgG^{1}**	HIL-7R-M21	BD bioscience	5 µl
CD14 V500 **Ms IgG^{2a}**	M5E2	BD bioscience	5 µl
HLA DR APC **Ms IgG^{2a}**	G46-5	BD bioscience	5 µl
HLA-ABC APC **Ms IgG^{2a}**	G46-2.6	BD bioscience	5 µl
CD80 PE **Ms IgG^{1}**	L307.4	BD bioscience	5 µl
CD86 FITC **Ms IgG^{1}**	2331 (FUN-1)	BD bioscience	5 µl
IL10 PE **Rat IgG^{1}**	JES3-9D7	Miltenyi Biotec	10 µl
IFN APC **Ms IgG^{1}**	45-15	Miltenyi Biotec	10 µl
IL12 APC **Ms IgG^{1}**	C11.5	BD bioscience	10 µl
LAP (TGF-β1) PE **Ms IgG^{1}**	27232	R&D Systems	10 µl
FOXP33 PE **Ms IgG^{1}**	236A/E7	eBioscience	10 µl
CTLA-4 PE **Gt IgG**	Sf 21	R&D Systems	5 µl
CD39 PE **Ms IgG^{1}**	A1	eBioscience	5 µl
GITR APC **Ms IgG^{1}**	109101	R&D Systems	10 µl
CD73 PE **Ms IgG^{1}**	AD2	BD bioscience	5 µl

CD103 PE Ms IgG^{1}	Ber-ACT8	BD bioscience	5 µl
CD28 PE Ms IgG^{1}	CD28.2	BD bioscience	5 µl
Perforin PE Ms IgG^{2b}	G9	BD bioscience	10 µl
Granzyme B PE Ms IgG^{1}	GB11	BD bioscience	10 µl

2.8 Proliferation & Suppression assay

Responder T-cells (CD25-) was isolated from the PBMC of healthy donors as previously described. The responder cells were labelled with CellTrace™ violet cell proliferation kit (Figure 2-4; Invitrogen). $1.5x10^6$ responder T-cells were re-suspended in 1mL of MACS buffer and incubated with 1μL of CellTrace violet at room temperature for 20 minutes. The cells were quenched with 5mL of ice-cold MACS buffer and incubated for a further 5 minutes on ice. Labelled cells were centrifuged at 350 x *g* 5 minutes and re-suspended in RPMI-1640 1% GPS. The number and viability of cells was confirmed using trypan blue staining and manual hemocytometer counting.

Stimulation of responder T-cells to induce proliferation was initially preformed with CD3/CD28 Dynal beads (Invitrogen) and was subsequently carried out with CD3/CD28 activating beads (Treg inspector; Miltenyi Biotec). Responder T-cells were cultured in a 96 well round bottom plate (BD) in the presence of CD3/CD28 activating beads (Treg inspector; Miltenyi Biotec) to induce T-cell proliferation at a 1:1 bead to T-cell ratio. Different ratios of $CD8^+$Treg or $CD4^+$Treg were added with the responder T-cells before culture at 37^oC, 5% CO_2 for 3 days (Table 2-3).

For allogeneic T-cell proliferation assays, moDC were co-cultured with $5x10^4$ CellTrace violet labelled responder T-cells at a 1:10 ratio and cultured in RPMI-1640, 10% FCS, 1% GPS for 5 days.

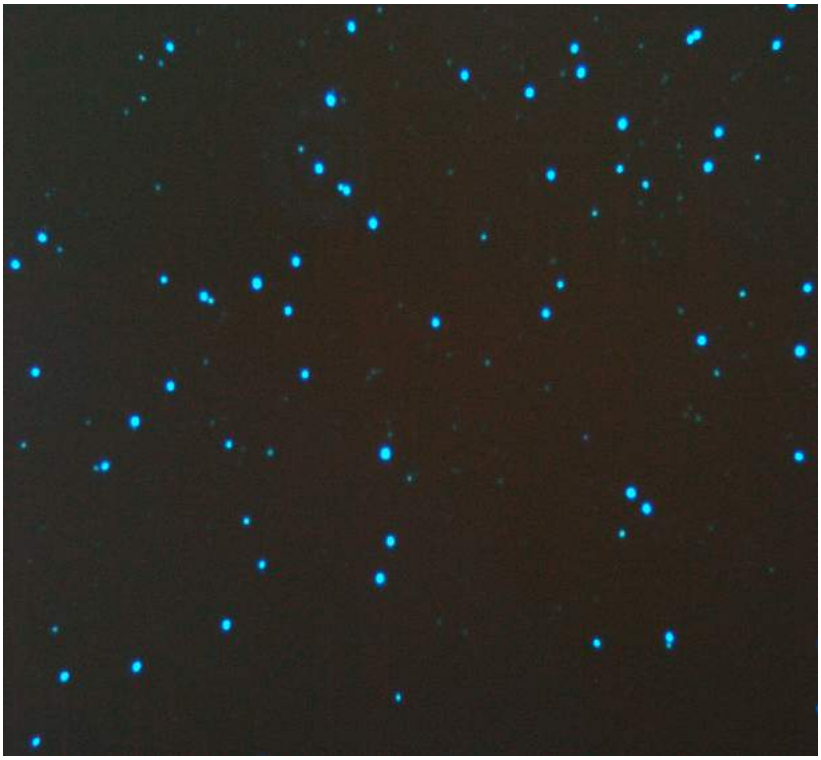

Figure 2-4 CellTrace violet labelled responder T-cell

Table 2-3 Proliferation assay

Number of responder T cells, regulatory T cells (Treg) and Treg Suppression Inspector beads per well.

Ratio Responder cells: Treg	Responder cells	Tregs	Treg suppression inspector beads
1:0	$5x10^4$	-	$5x10^4$
0:1	-	$5x10^4$	$5x10^4$
1:1	$5x10^4$	$5x10^4$	$10x10^4$
2:1	$5x10^4$	$2.5x10^4$	$7.5x10^4$
4:1	$5x10^4$	$1.25x10^4$	$6.25x10^4$
8:1	$5x10^4$	$0.625x10^4$	$5.625x10^4$
Control 1:0	$5x10^4$	-	$5x10^4$
Control 0:1	-	$5x10^4$	$5x10^4$
Total cells/bead	$3x10^5$	$3.57x10^5$	$6.57x10^5$
Total cells/beads per 1 assay (duplicates)	$6x10^5$	$5.1\ x10^5$	$11.25\ x10^5$

Following 3 days of culture (5 days for allogeneic T-cell proliferation assay) flow cytometry was carried out to determine responder cell division. In selected experiments, the ability of $CD8^+$Treg to suppress responder T-cells was studied in the presence or absence of neutralising monoclonal antibodies/inhibitor against IL-10 (5μg per mL, R&D; clone 23738), TGFβ (10μg per mL, R&D; polyclonoal) CTLA-4 (10 μg per mL, Thermo Scientific: clone AS32), CD39 (10 μg per mL, Serotec; clone A1) and ARL67156 (Sigma). The requirement for cell-cell contact in suppression was studied by co-culturing $CD8^+$Treg and responder T-cells in an 96-well culture plate. The cells were separated by the presence of a transwell insert (0.4μm pore size; Corning) (Figure 2-5).

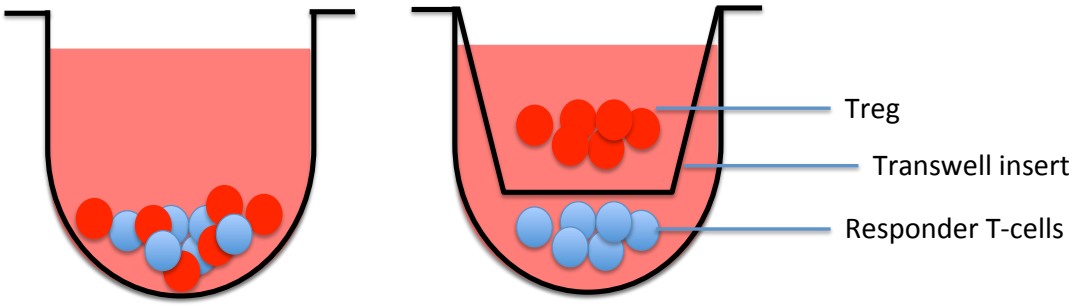

Figure 2-5 Suppression assay preformed using transwell insert

To determine the need of cell-to-cell contact in the suppressive function of Treg (red), responder T-cells (blue) were cultured with or without the presence of a transwell insert.

2.9 Generation of monocyte-derived Dendritic Cells

$CD14^+$ monocytes isolated from PBMC were incubated at $2x10^6$ cells per mL of RPMI-1640 containing 1000IU/mL of both granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech) and interleukin-4 (IL-4; Peprotech) in RPMI-1640, 10% FCS, 1% GPS in a 24-well plate (BD). Cells were washed every second day by centrifugation at 300 x *g* for 10 minutes and discarding 50% of the medium before adding the same volume of fresh medium. The cells were incubated at 35^oC, 5%, CO_2 for 7 days to induce differentiation of $CD14^+$ monocytes to DC. DC phenotype was confirmed by demonstrating expression of HLA-DR and absence of $CD14^+$ expression.

2.10 Priming of Naïve $CD8^+$T-cells with tissue conditioned medium

Conditioned medium generated from HCC tumour and matched non-tumour tissues was used to prime $CD8^+$T-cells. Naïve $CD8^+$ T-cells ($CD8^+$, $CD45RA^+$) were isolated using a negative selection kit as previously described. Isolated naïve $CD8^+$T-cells were cultured in RPMI-1640 only, tumour or matched non-tumour conditioned medium. Conditioned medium were diluted with different ratios of RPMI-1640, 10% FCS and incubated for 1, 3 and 5 days respectively. The phenotypes of primed $CD8^+$T-cells were assessed by flow cytometry for surface expression of CD25, CD127 and intracellular staining for FOXP3. In selected experiments, CD3/CD28 activation beads (Invitrogen) were added to induce T-cell proliferation.

2.11 Priming of Dendritic cells with tissue conditioned medium

Conditioned medium generated from HCC tumour and matched non-tumour tissues were used to prime moDC. Following differentiation from PBMC derived $CD14^+$ monocytes, moDC were washed in PBS and centrifuged twice (300 x *g*, 5 minutes). For the priming of moDC, tumour or matched non-tumour conditioned medium were diluted at a ratio of 1:5 with RPMI-1640, 10%, FCS and incubated with for 24 hours. The primed moDC were washed three times and centrifuged (300 x *g*, 5 minutes) prior to use in further experiments. In selected experiments, in addition to the tissue conditioned medium, neutralising antibodies against IL-10 (R&D), TGF (R&D), IL-6 (R&D) and IL-6-receptor (R&D) were added to the culture at the start of moDC priming. The phenotypes of moDC were assessed by flow cytometry for surface expression of HLA-DR, HLA-A/B/C, CD80 and CD86. Function of moDC was assessed by intracellular cytokine detection and allogeneic T-cell proliferation assay, as previously described, when, following culture in conditioned medium for 24 hours, the primed moDC were washed three times and centrifuged (300 x *g*, 5 minutes) before the presence of IL-10 and IL-12 was demonstrated by multi-colour flow cytometry (see Table 2-2 for antibodies). For allogeneic T-cell proliferation assays, CD3/CD28 activating beads were replaced with conditioned primed DC. MoDC and $5x10^4$ violet cellTrace labelled responder T-cells ($CD25^{low}$) were cultured at a ratio 1:10 for 5 days in a 96 well plate. Following incubation, the proliferation capacity of responder T-cells was analysed by flow cytometry.

2.12 Co-culture of Naïve CD8 T-cells and moDC

Naïve $CD8^+$ T-cells ($CD8^+$, $CD45RA^+$) were isolated using a negative selection kit (StemCell Technologies) from PBMC obtained as previously described from whole blood. Naïve $CD8^+$ T-cells were seeded at $2.5x10^5$ per well in 96-well, round bottomed plates, in RPMI-1640 supplemented with 10% FCS and 1% GPS. moDC that had been primed with conditioned medium were added into the well at a 10:1 ratio (T-cells:moDC) before incubation for 7 days in 35^oC, 5% CO_2 . Cells were centrifuged on day 2 and the medium exchanged with fresh RPMI-1640 supplemented with 10% FCS, 1% GPS and 1000IU/mL of IL2 (Peprotech). T-cell phenotype was assessed for the expression of CD3, CD8, CD25, CD127, FOXP3, CTLA-4 and CD39 by flow cytometry. Function of $CD8^+$T-cells following co-culture was assessed by intracellular cytokine staining or by suppression assay as previously described. In selected experiments the ability of moDC to induce $CD8^+$Treg was studied in the presence and absence of neutralising antibodies or inhibitor against IL-10 (R&D), TGF (R&D), inducible nitric oxide synthase (iNOS) (Calbiochem), argainase (Sigma-Aldrich), and indoleamine 2,3-dioxygenase (IDO) (Sigma-Aldrich).

The requirement for cell-cell contact for the induction of $CD8^+$Treg by moDC was assessed by culturing moDC and $CD8^+$Treg in a 96-well culture plates with the addition of a transwell insert (0.4μm pore size; Corning) to separate cells.

2.13 Live cell imaging

The interaction of conditioned medium primed or LPS stimulated moDC with $CD8^+$ T-cells was assessed by the Cell IQ imager (ChipMan Technologies). Naive $CD8^+$ T-cells were first labelled with carboxyflurescein succinimidyl ester (CFSE)(Invitrogen) by incubating $1x10^6$ cells with 2µL of CFSE dye in 1 mL of MACS buffer for 10 min at 37°C. 5 mL of ice-cold buffer was added to the cells before incubation for a further 5 minutes. The cells were washed three times in MACS buffer and centrifuged at 350 x *g* for 5 minutes. The CFSE labelled cells were re-suspended at $2.5x10^4$ per mL in RPMI-1640 supplemented with 10% FCS, 1% GPS. The labelled cells were incubated with conditioned medium primed moDC at a 10:1 (T-cells:moDC) ratio in a 24-well culture plate. The cells were cultured for 3 days at 35°C, 5% CO_2 within the cell-IQ incubator. Phase contrast and fluorescent images were taken at 5 minutes intervals after which a movie was generated from the images by the Cell IQ analyser software. The interaction time of moDC and $CD8^+$T-cells were obtained manually by analysing each individual CD8 T-cell (fluorescently labelled) interaction with moDC (unlabelled cells) in a given field. The total time of each $CD8^+$T-cell-moDC interaction can be obtained by counting the number of frames each individual $CD8^+$T-cell remains in contact with a moDC. To obtain the overall interaction time, the contact time of 10 T-cells were counted in two separate fields and an average taken. Contact of each individual $CD8^+$T-cell with a new moDC were counted as a new interactions.

2.14 Freezing of cells

Cells were suspended in freezing medium (FCS plus 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich)), at a concentration of $1x10^7$ cells per mL and transferred into a cryovials. The cells were placed in a CoolCell alcohol-free cell freezing container (Biocision) and transferred into a -80^oC freezer for 48 hours after which cryovials are stored in liquid nitrogen until use. The cells were thawed by removal from liquid nitrogen, followed by immediate washing with warm RPMI-1640 and centrifuged at 300 x *g* for 5 minutes. The cells were re-suspended in RPMI-1640 supplemented with 10 %FCS, 1% GPS and 50IU/mL IL2, overnight at a concentration of $5x10^6$ per mL. Viability of cells were confirmed using trypan blue exclusion the following day and the cells were passed though a 70μM sterile filter to remove debris and aggregates prior to use.

2.15 Cytokine array

To investigate the composition of conditioned medium, a human cytokine array kit (R&D system) was used to quantify cytokines, chemokines and acute phase proteins. The analytes detected by the kit are displayed in (Table 2-4).

Assays were performed according to manufacturer's instructions. Briefly, array membranes were first blocked with buffer provided for one hour. This was followed by the incubation of the membranes with the matched conditioned medium and antibody mix at 4^oC overnight. The membranes were rinsed with wash buffer and incubated with streptavidin-horseradish peroxidase (R&D system) for 30 minutes at

room temperature. To develop the membrane, each array was first rinsed with wash buffer twice and incubated with the Chemi Reagent Mix for 1 minute. The membranes were then exposed with enhanced chemiluminescence detection film (Amersham,UK) and developed using a Kodak X–Omat 1000 processor (University of Birmingham). The pixel densities on the developed film were analyzed by image-J.

Table 2-4 Analytes measured by the human cytokine array Kit A

C5a	IL-1 alpha
CD40 Ligand	IL-1 beta
G-CSF	IL-1ra
GM-CSF	IL-2
CXCL1/GRO alpha	IL-4
CCL1/I-309	IL-5
ICAM-1	IL-6
CXCL8/IL-8	IL-27
IL-10	IL-32 alpha
IL-12 p70	CXCL10/IP-10
IL-13	CXCL11/I-TAC
IL-16	CCL2/MCP-1
IL-17	MIF
IL-17E	CCL3/MIP-1 alpha
IL-23	CCL4/MIP-1 beta
Serpin E1/PAI-1	TNF-alpha
CCL5/RANTES	TREM-1
CXCL12/SDF-1	

2.16 Protein extraction

For the detection of IDO, protein was extracted from moDC. The cells of interest were centrifuged at 15000 x *g* in a 1.5 mL Eppendorf for 5 minutes to obtain a cell pellet. The cells were lysed by adding 125µL of lysis buffer (49.6µL CelLytic M; Sigma-Aldrich, 62.5µL completeULTRA [2x stock from 1 tablet in 5mL CelLytic M], 0.38µL DNase [10U/µL stock; Roche] and incubated at 40^{o}C for 30 minutes. The cells were centrifuged at 15000 x *g* for 1 minute and the supernatant containing the protein was transferred to a new Eppendorf. The concentration of protein was determined by the bicinchoninic acid assay (BCA; Sigma-Aldrich) according to the manufacturer's instructions and samples were stored at -80^{o}C until use.

2.17 Western Blot

To prepare the protein lysate sample, 5µL of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) sample buffer (200mM Tris pH6.8, 20% glycerol, 10% SDS, 0.05% bromophenol blue and 10 mM ß-mercaptoethanol) was added to 20µL of protein lysate (protein concentration of 1mg/mL) in a 1.5 ml Eppendorf and heated at 100^{o}C for 2 minutes and centrifuged at 15000 x *g* for one minute. The protein lysate was separated on 5-10% SDS polyacrylamide gel using the BioRad Mini Trans Blot Cell System (Biorad). Gels were by prepared (Table 2-5) by laying a stacking gel over a polymerized resolving gel between two glass plates and left to set. In the meantime, the gel apparatus was assembled and running buffer (30.3g Tris, 144g glycine, 10g SDS in 1 liter of ddH_2O) was then poured into the tank ensuring that the electrodes were fully covered. 20µL of each protein lysate sample was slowly

loaded onto each lane of the stacking gel. One line was reserved for a protein ladder (Amersham, GE Healthcare). Electrophoresis was performed at 200V for 30 minutes.

Table 2-5 Recipe for SDS-PAGE gels

	Stacking gel (5%)	Resolving gel (12%)
ddH2O	6.8mL	3.3mL
30% Acryamide mix (Biorad)	1.7mL	4.0mL
1.0M Trisma base pH 6.8 (Sigma-Aldrich)	1.25 mL	
1.5M Trisma base pH 6.8 (Sigma-Aldrich)		2.5mL
10% (w/v) SDS (Sigma-Aldrich)	0.1mL	0.1mL
10% (w/v/) ammonium persulfate (Sigma-Aldrich)	0.1mL	0.1mL
N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma-Aldrich)	40 µL	40 µL

To develop the blot, the resolving gel containing the protein lysate samples was transferred onto a nitrocellulose membrane (Amersham, GE Healthcare). This was performed by placing the resolving gel onto the nitrocellulose membrane before the gel and the membrane were further sandwiched between transfer buffer (6.0g Tris, 28.8g glycine, 400mL methanol, 1g SDS in 2 litres of ddH_2O) soaked filter papers and sponges. The stacked gel was inserted into a transfer block. To transfer the protein from the gel to the membrane, the transfer block was submerged into a transfer tank filled with transfer buffer and ice packs. Protein transfer was performed at 100V for 60 minutes. The transfer of protein was confirmed by staining of the membrane with Ponceau Red (0.1% Ponceau in 5% acetic acid; Sigma-Aldrich), which will reversibly stain protein bands on the membrane if transfer was successful. Once transfer had been confirmed, the membrane was washed for 5 minutes in water.

The protein-coated membranes were blocked with 5% w/v non-fat milk (Marvel) in PBS/0.02% Tween 20 for 60 minutes at room temperature, followed by incubation with primary IDO antibodies (ab55305 Abcam; mouse anti-human monoclonal IgG2b 5µg per mL) diluted in 5% w/v non-fat milk overnight at 4^{o}C on a rocking platform.

The membrane was washed three times with PBS-0.02% Tween 20 for five minutes, followed by incubation with a peroxidase-conjugated secondary antibody (P0161 Dako; rabbit anti mouse polyclonal 0.28 µg per mL) in 5% w/v non-fat milk for 60 minutes at room temperature on a rocking platform. The membrane was washed three times with PBS-0.02% Tween 20 for five minutes, followed by the final wash for 30 minutes with PBS-0.02% Tween 20. Chemiluminescence reagent (ECL plus:

Amersham, GE Healthcare) was added to the membrane for 5 minutes and the membrane was then exposed with enhanced chemiluminescence detection film (Amersham, GE Healthcare) and developed using a Kodak X–Omat 1000 processor (University of Birmingham). To semi-quantify the concentration of the protein of interest, Image J (version 1.46, NIH, USA) was used to measure differences in band densities.

2.18 Clinical study

A clinical study was devised to assess the regulatory immune response in patients with HCC undergoing local ablative treatment. Serial blood samples were collected from patients with HCC under going TACE on the day before treatment, followed by 3 days and 42 days after treatment. Blood samples were analysed to determine changes in regulatory T-cell subsets by flow cytometry as previously described. The function of regulatory T-cells was assessed by suppression assays before and after treatment. When possible, PBMC were frozen for later use for the measurement of tumour antigen-specific response by stimulating with overlapping peptide pools covering alpha-feto protein and glypican-3. The data were correlated with the patient's cross sectional imaging 42 days after TACE treatment and clinical response to treatment was defined by the modified response evaluation criteria in solid tumours (mRecist) (Table 2-6). All patients gave written informed consent before entering the study, and the study protocol, approved by the local ethical committee, conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Protocol and ethics application was written as part of this work. Ethical approval was obtained at the local ethics committee meeting (REC reference number: 11/WM/0135).

Table 2-6 mRECIST for HCC

The overall response of individual lesion after treatment was assessed using the modified Response Evaluation Criteria in Solid Tumors (mRECIST) criteria. The overall patient response is calculated by combining the assessment of target lesions, non-target lesions, and new lesions. [395]

Complete response (CR)	Disappearance of any intratumoral arterial enhancement in all target lesions
Partial response (PR)	At least a 30% decrease in the sum of diameters of viable (enhancement in the arterial phase) target lesions, taking as reference the baseline sum of the diameters of target lesions
Stable disease (SD)	Any cases that do not qualify for either partial response or progressive disease
Progressive disease (PD)	An increase of at least 20% in the sum of the diameters of viable (enhancing) target lesions, taking as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started

Target lesions	**Non-target lesions**	**New lesions**	**Overall response**
CR	CR	No	CR
CR	Incomplete response/SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or no	PD
Any	PD	Yes or no	PD
Any	Any	Yes	PD

CR; complete response , PR; partial response, SD; stable disease, PD; progressive disease.

2.19 Statistical analysis

Data were analysed using the Mann Whitney U test when comparing differences between two unrelated groups, and two–way Analysis of variance (ANOVA) analysis followed by Bonferroni's post test for comparison between more than two groups.

Statistical analysis was performed and presented using the GraphPad Prism software version 5.0. A value of $P \leq 0.05$ is considered statistically significant. Asterisks were inserted into figures to indicate statistical significance and data are expressed as follows: *≤0.05, **≤0.01, ***≤0.001.

3 Regulatory T-cells infiltrate human HCC

It is well recognised the immune system has the ability to recognise cancer cells. However, tumours also employ multiple mechanisms to avoid detection and elimination by the host's immune response. The idea of manipulating the host's immune system to tip the balance towards anti-tumour immune response has been the holy grail of tumour immunologists. An increase in our understanding of the tumour immune environment will hopefully provide us with the ammunition to develop such treatments.

3.1 T-cells infiltrating hepatocellular carcinoma

A number of groups have previously demonstrated the infiltration of immune cells into a wide array of human solid tumours, including human HCC [396]. To confirm the findings of these previous studies, immunohistochemistry staining was performed on HCC paraffin sections for the presence of T-cells by using antibodies raised against CD4 and CD8. The staining showed both infiltration of CD8$^+$T-cells (Figure 3-1) and CD4$^+$T-cells (Figure 3-2) in HCC tumours and matched non-tumour tissues. The distribution of T-cells was homogenous within tumour tissues, but when compared to matched non-tumour tissues, T-cells were confined mainly to the fibrotic bands with very few cells infiltrating the liver parenchyma. To confirm that CD8$^+$T-cells and CD4$^+$T-cells were equally distributed within tumours, dual immunohistochemistry staining was used to demonstrate both CD4 and CD8 expression simultaneously. This confirmed that both subsets of T-cells are present in the same compartments within HCC tumours (Figure 3-5).

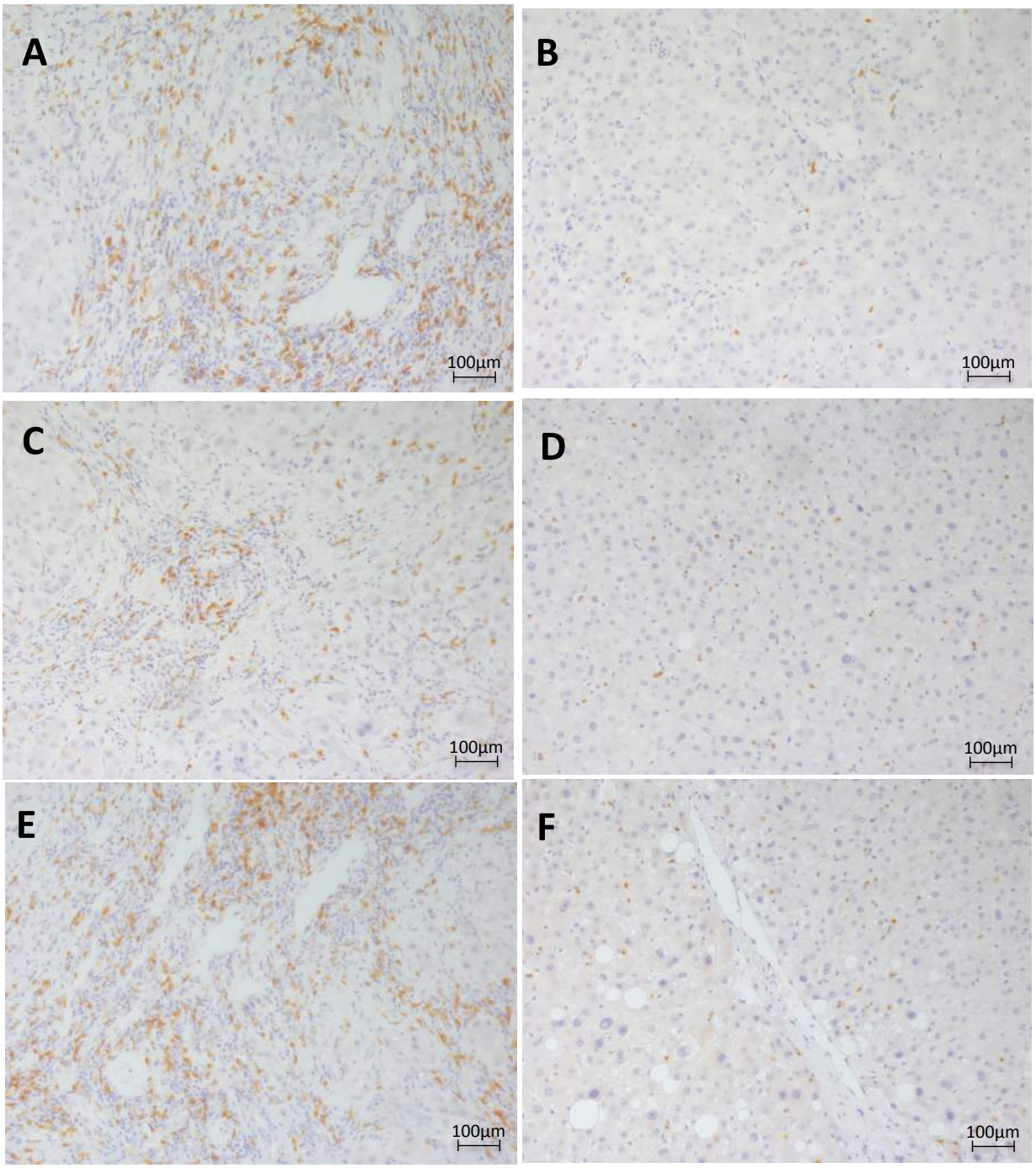

Figure 3-1 CD8$^+$T-cells in human HCC tumour and matched non-tumour tissue. Immunohistochemistry staining for CD8 expression in (A, C, D) human HCC and (B, D, E) matched non-tumour tissue. Images from 3 separate matched samples.

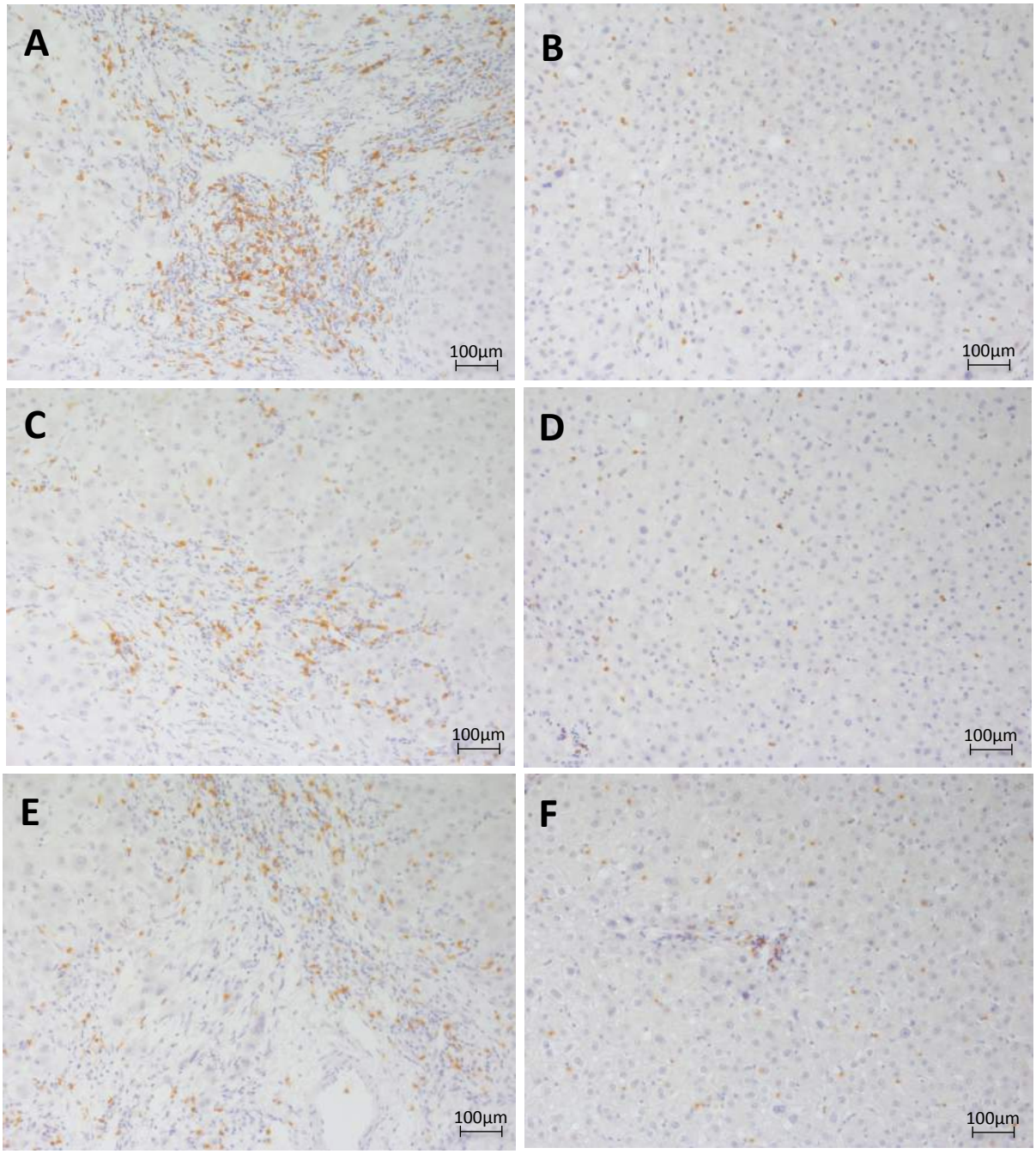

Figure 3-2 CD4$^+$T-cells in human HCC tumour and matched non-tumour tissue. Immunohistochemistry staining for CD4 expression in (A, C, D) human HCC and (B, D, E) matched non-tumour tissue. Images from 3 separate matched samples.

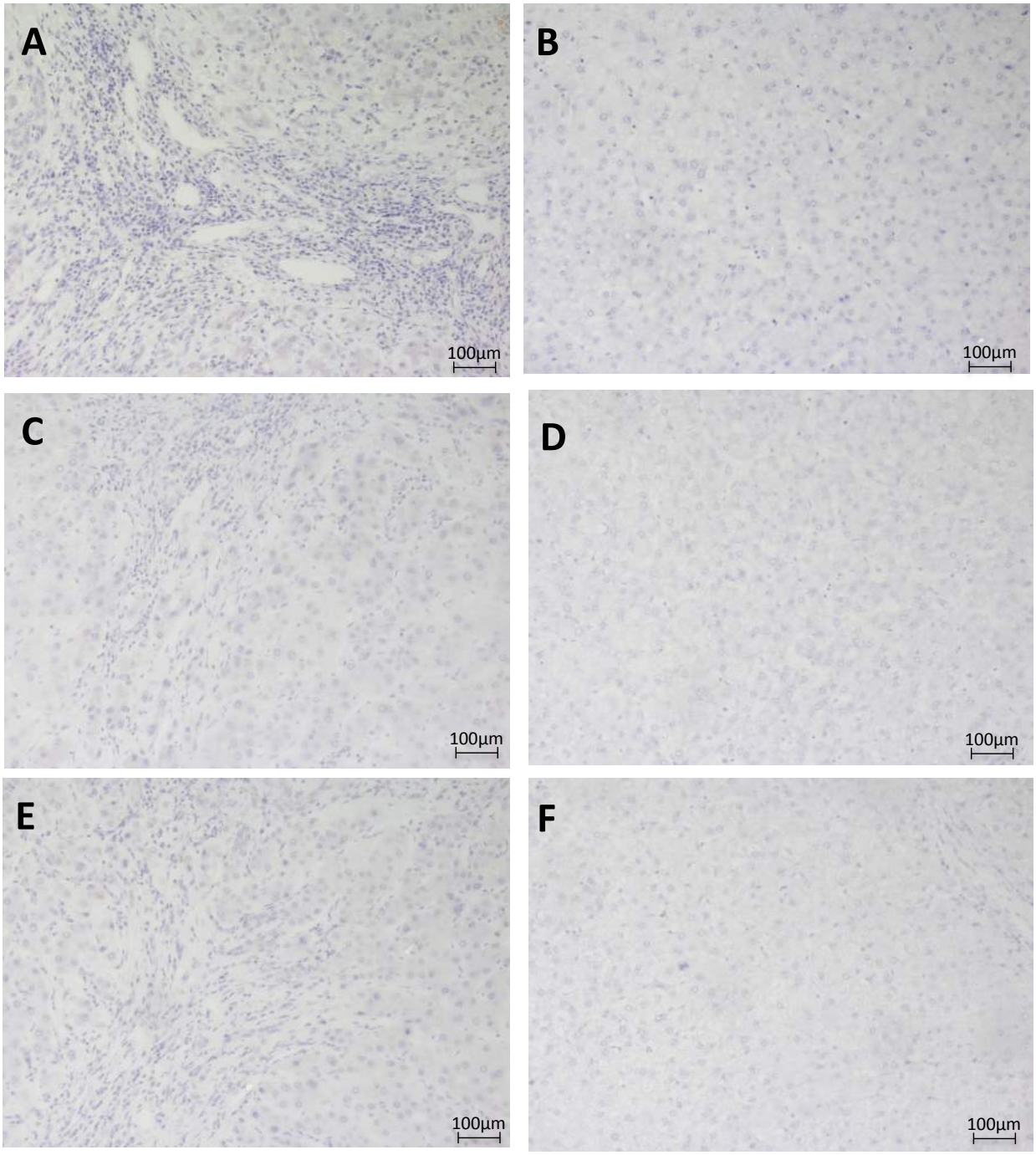

Figure 3-3 IgG^1 isotype control staining in human HCC tumour and matched non-tumour tissue.

IgG^1 isotype control staining (A, C, D) human HCC and (B, D, E) matched non-tumour tissue. Images from 3 separate matched samples

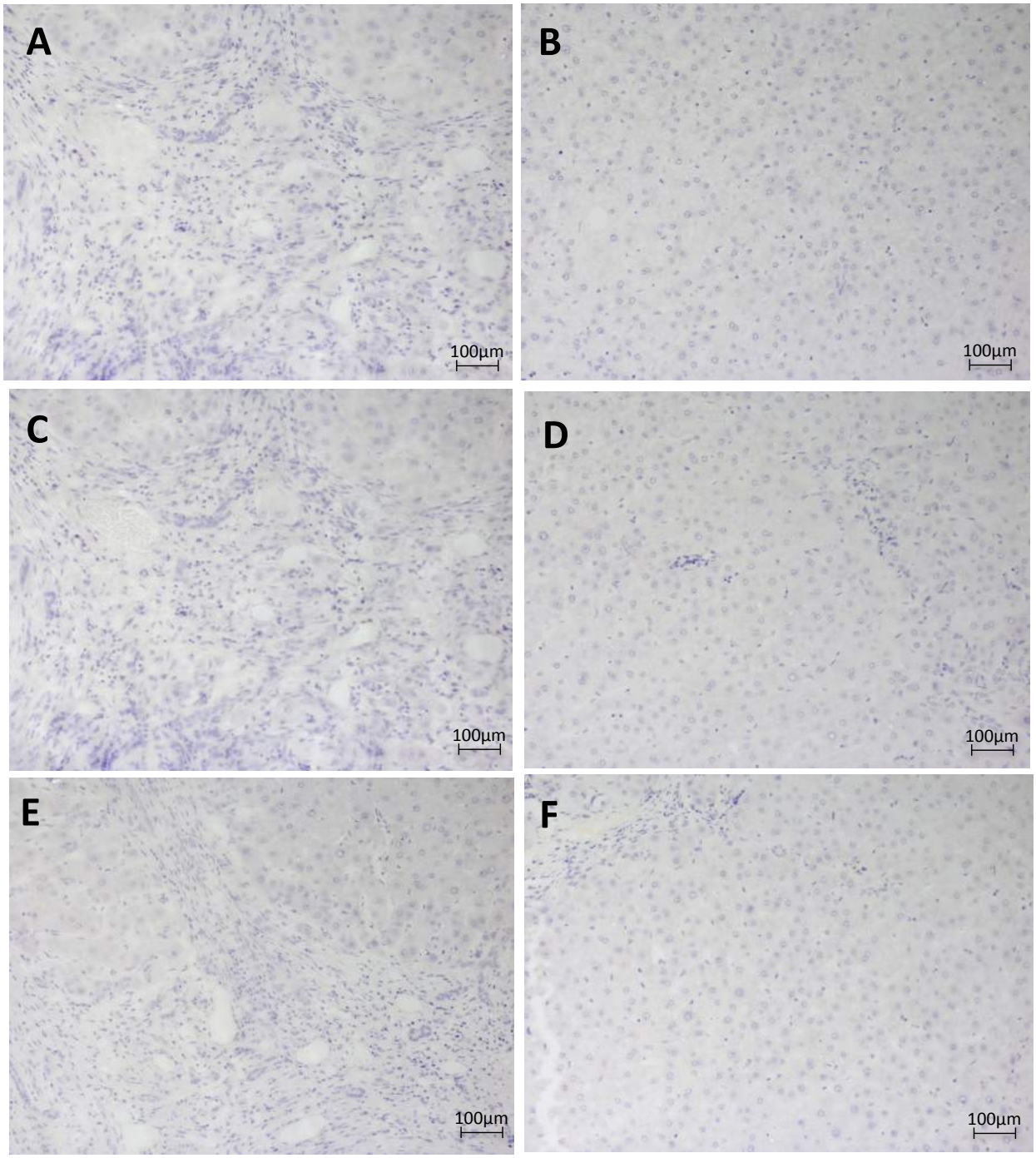

Figure 3-4 IgG^{2a} isotype control staining in human HCC tumour and matched non-tumour tissue.

IgG^{2a} isotype control staining (A, C, D) human HCC and (B, D, E) matched non-tumour tissue. Images from 3 separate matched samples.

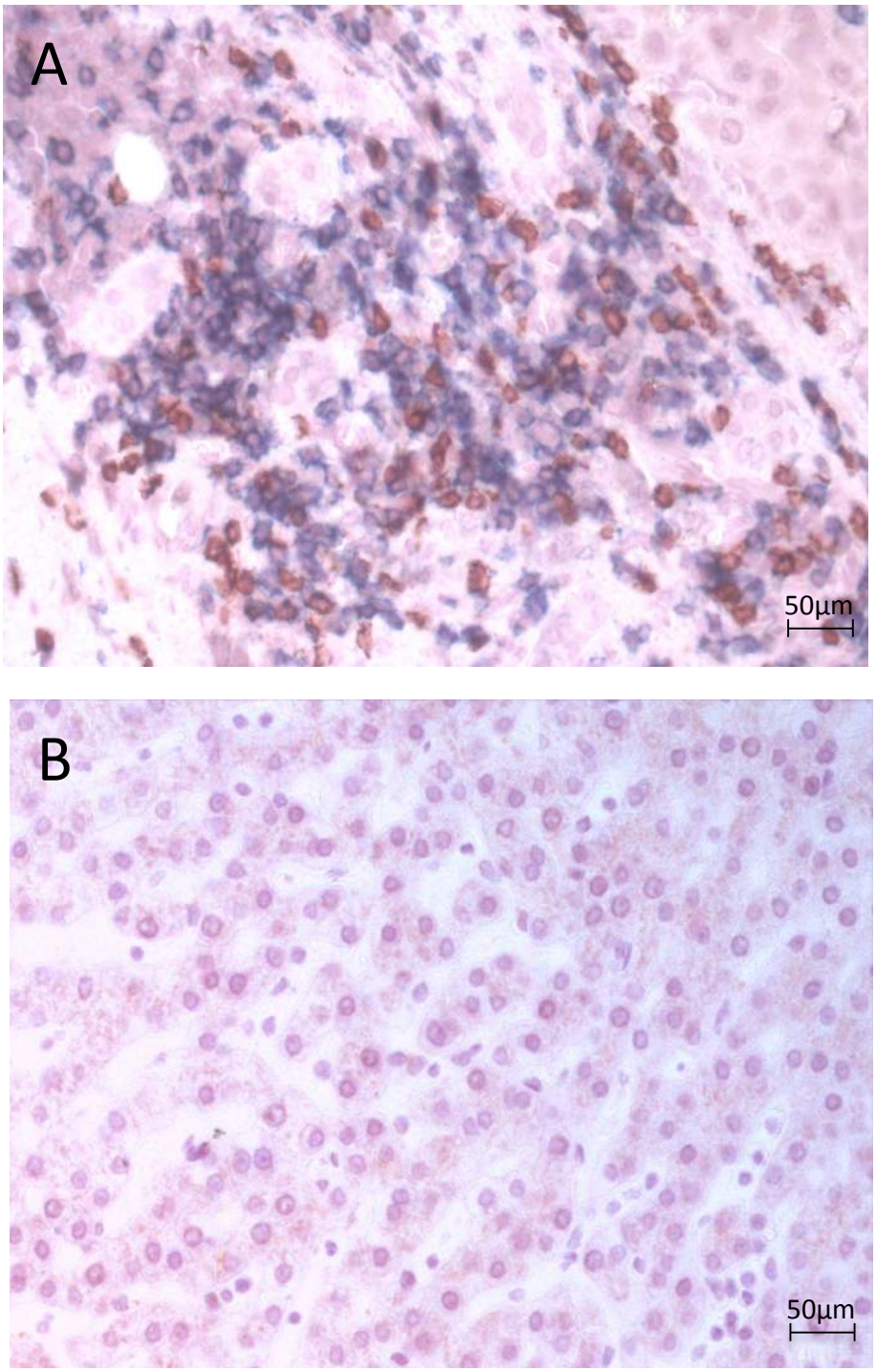

Figure 3-5 CD8$^+$T-cells & CD4$^+$T-cells localisation in HCC tumour

Dual immunohistochemistry staining for co-expression of CD8 (blue) & CD4 (red) in (A) human HCC. (B) Staining with isotype control. Representative images from 3 samples.

3.2 FOXP3 positive cells infiltrating hepatocellular carcinoma

Despite the infiltration of immune cells into the tumour microenvironment, this is often ineffective at disease control and the tumour continues to progress. One possibility for the inability of the immune system to destroy tumour cells may be due to the induction or recruitment of regulatory immune cells that are able to suppress the effector immune response, resulting in disease progression. Treg have been implicated in human diseases, including tumour immune escape. The identification of the master regulatory gene FOXP3 as an essential transcription factor found in both murine and human Tregs has boosted interest in research in regulatory immune cells. The availability of antibodies against FOXP3 has enabled this to be used as a surrogate marker for the demonstration of Tregs in a wide array of tissues. By using immunohistochemistry, the presence of FOXP3 expression was confirmed within both tumour and matched non-tumour tissues (Figure 3-6). These FOXP3 positive-cells are likely to represent Treg. Similarly to T-cells, FOXP3 positive cells were distributed homogenously within tumour tissues, but mainly confined to the fibrotic bands within matched non-tumour tissues.

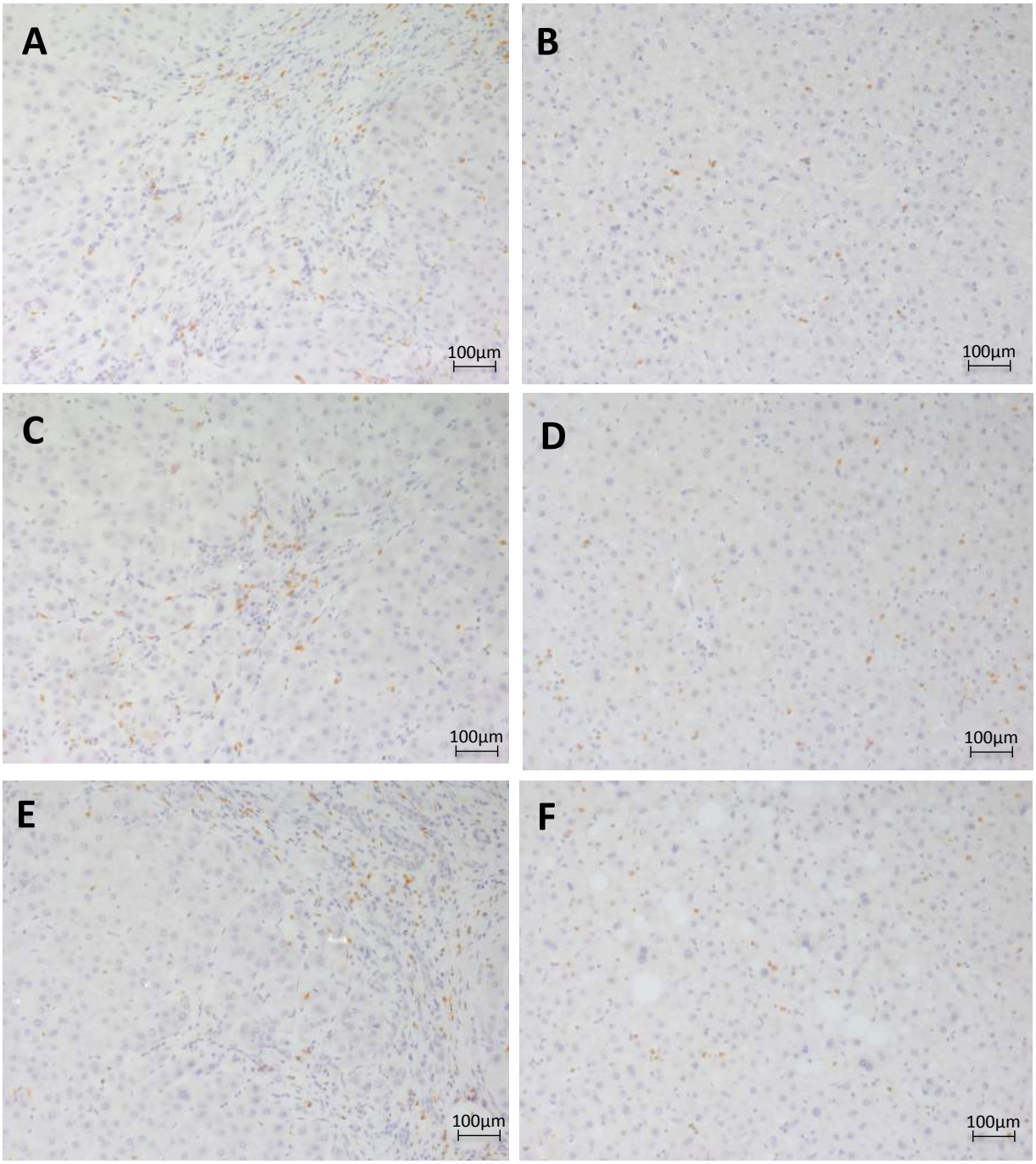

Figure 3-6 Infiltration of FOXP3$^+$cells in HCC tumour and matched non-tumour tissue.

Immunohistochemistry staining for FOXP3 expression in (A, C, D) human HCC and (B, D, E) matched non-tumour tissue. Images from 3 separated matched samples.

3.3 CD8$^+$FOXP3 positive cells infiltration in hepatocellular carcinoma

The majority of studies on regulatory T-cells have focused mainly on CD4$^+$Treg. However, there is increasing evidence to suggest regulatory T-cells expressing the surface marker CD8 may also play an important role in the pathogenesis of human diseases. Using FOXP3 as a marker of Treg, only one previous study had confirmed the presence of CD8$^+$FOXP3$^+$ Treg by immunohistochemistry [198]. To confirm this original study, CD8$^+$FOXP3$^+$ Treg in HCC were demonstrated by dual immunohistochemistry staining (Figure 3-7). To further appreciate the presence of these CD8$^+$FOXP3$^+$ expressing cell, immunofluorescence staining was performed on frozen HCC tissue sections to demonstrate CD8$^+$ and FOXP3$^+$ cells (Figure 3-7). Once again cells co-expressing both CD8 and FOXP3 can be detected within tumour tissue. It is this population of CD8$^+$FOXP3$^+$ cells that will be studied in greater detail in the latter sections of this thesis.

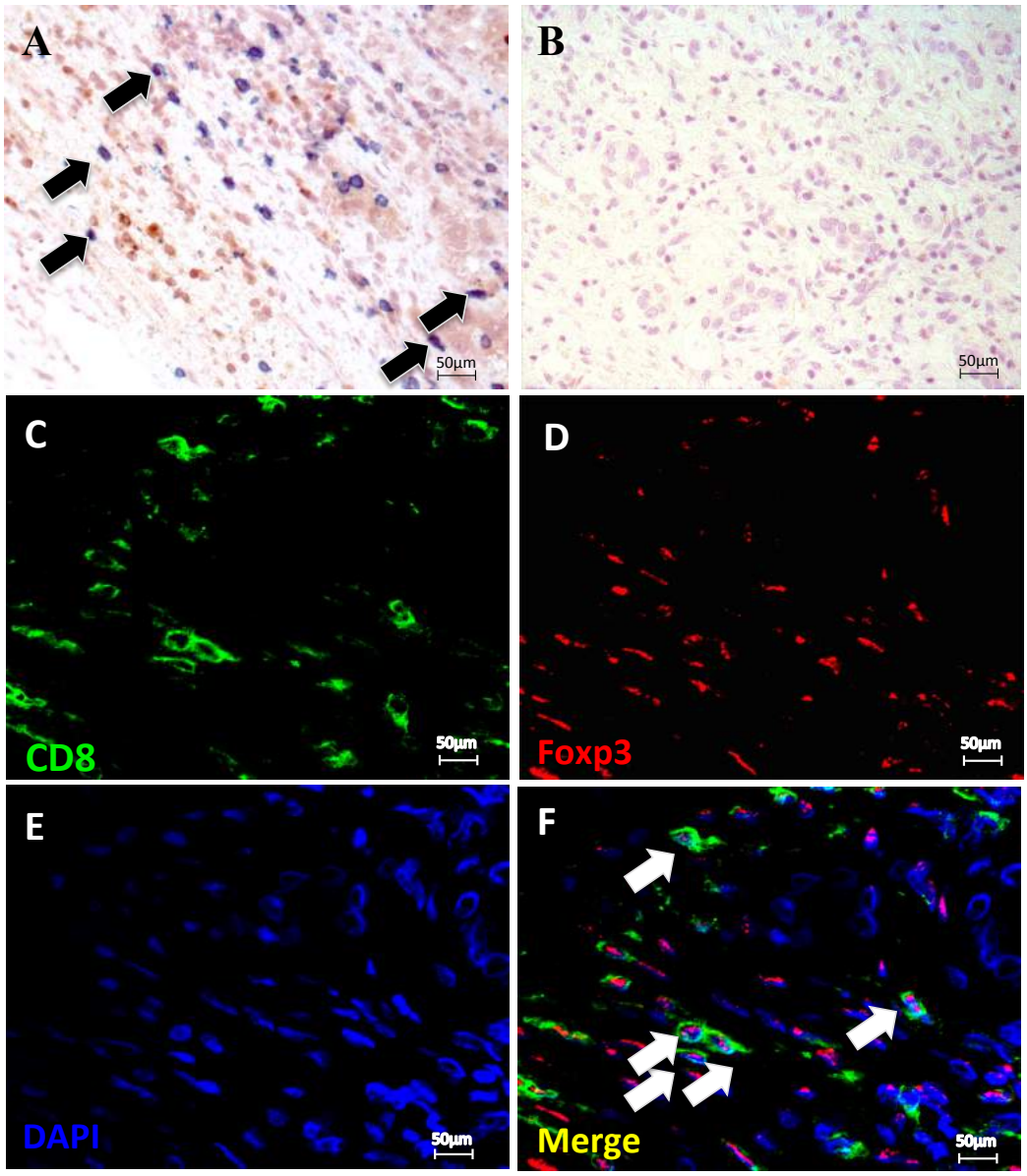

Figure 3-7 Infiltration of cells co-expressing CD8 & FOXP3 in HCC tissue. Immunohistochemistry staining for co-expression of CD8 (blue) and FOXP3 (red) in (A) human HCC tumour. (B) Staining with isotype control. Immunofluorescence staining on HCC was carried out on snap frozen acetone fixed tissues and analysed using confocal microscopy. (C-F) Triple staining indicates that CD8 and FOXP3 are co-expressed on the same cells in HCC. Arrow indicating CD8 and FOXP3 co-expressing cells. Representative staining from 3 samples.

3.4 Quantification of CD8$^+$Treg infiltration within HCC

Immuno-staining is an invaluable method for the assessment of immune cells, especially when the availability of tissues is limited, such as in the case of human tumours. However, analysis of staining data can often be subjective and is limited by the number of simultaneous markers you can detect on any one sample. The use of multi-colour flow cytometry has provided me with the tool to overcome some of these problems. Flow cytometric analysis allows the objective quantification of cells of interest, with the added benefit of analysing multiple markers on the same sample. Lastly, only a small number of cells are needed for meaningful analysis using flow cytometry.

To further quantify the presence of CD8$^+$Treg in fresh tumour and matched non-tumour tissues, MNC were isolated from explanted or resected liver tissue and assessed by multiple-colour flow cytometry. The immediate isolation and processing of cells from tissue is essential to increase cell viability and limit changes on receptor expression such as CD25. The isolated MNC are labelled with the appropriate conjugated antibodies or isotype control and analysed by flow cytometry unfixed. CD8$^+$Treg were identified as CD3$^+$CD8$^+$CD25^{high}CD127^{low} expressing cells, which we will refer to as CD8$^+$Treg in the rest of this thesis and CD4$^+$Treg as CD3$^+$CD4$^+$CD25^{high}CD127^{low} expressing cells. Dead cells and debris were excluded using a live/dead marker (Figure 3-8).

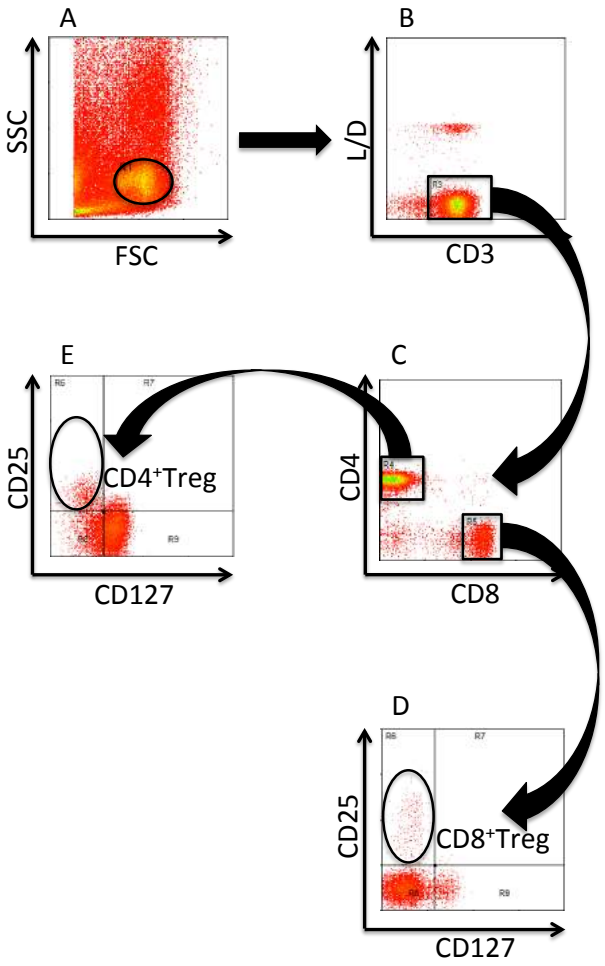

Figure 3-8 Representative flow cytometric gating strategy for $CD8^+$Treg and $CD4^+$Treg isolated from human HCC

(A) Tumour derived mononuclear cells (MNCs) were identified on forward scatter (FSC) and side scatter (SSC). (B) The MNC were gated against CD3 and Live/Dead marker (L/D) to identify viable T-cells. (C) Viable $CD3^+$T-cells were gated against CD8 and CD4 to identify $CD3^+CD8^+$ T-cells and $CD3^+CD4^+$ T-cells. (D) $CD3^+CD8^+$ T-cells are gated against CD25 and CD127 to identify $CD8^+$Tregs ($CD3^+CD8^+CD25^{high}CD127^{low}$cells). (E) $CD3^+CD4^+$T-cells are gated against CD25 and CD127 to identify $CD4^+$Tregs ($CD3^+CD4^+CD25^{high}CD127^{low}$ cells).

A total of 28 patients provided matched tumour and non-tumour tissues for flow cytometric analysis. T-cells from multiple tumours consisted of a significantly higher proportion of $CD8^+$Treg (2.47%±3.53), compared to matched non-tumour tissues (1.36%±2.42) (Figure 3-9). The percentage of $CD4^+$Treg was also significantly higher within the tumour (6.38%±5.10), compared to matched non-tumour tissues (2.90%±2.92). The proportion of $CD4^+$Treg appeared to be higher when compared to $CD8^+$Treg, irrespective of tissue compartment.

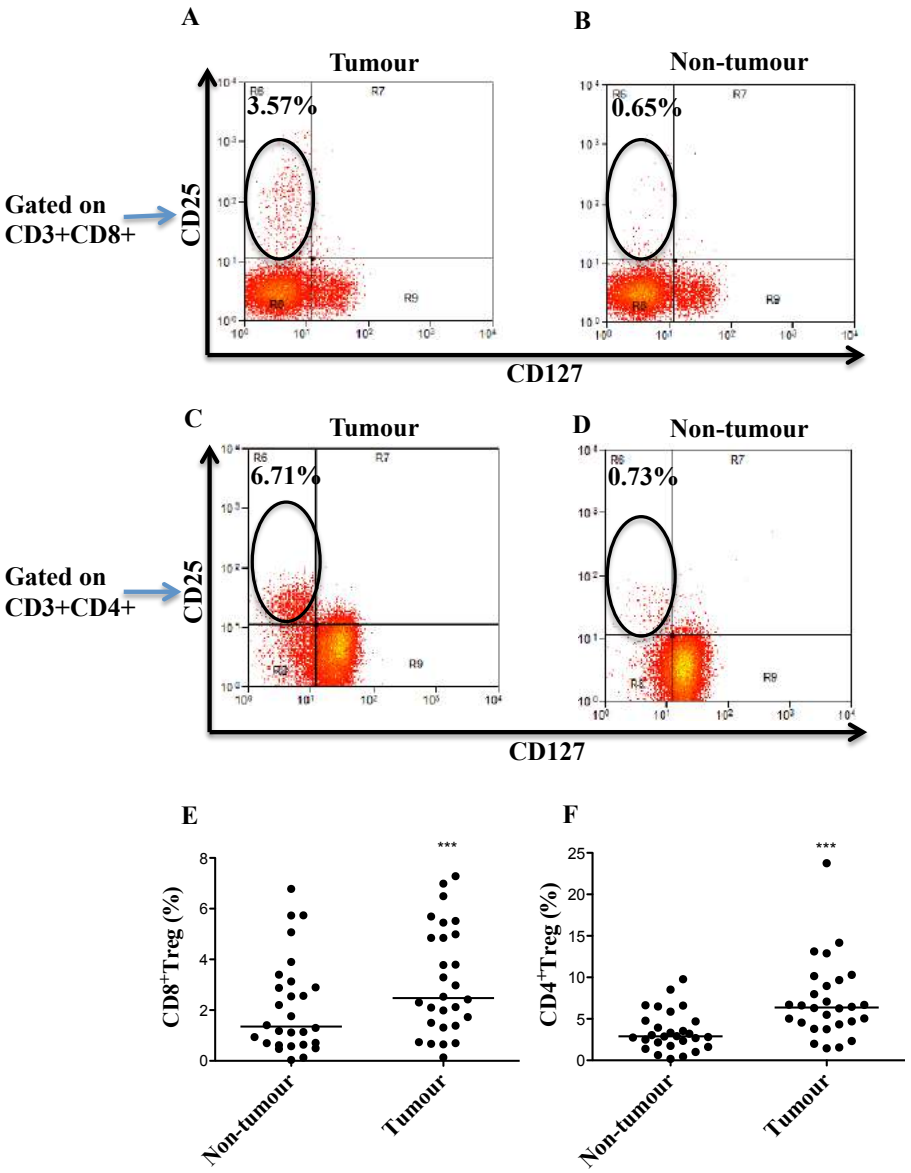

Figure 3-9 The proportion of CD8⁺Treg & CD4⁺Treg in human HCC

Representative dot plots of MNC isolated from matched tumour and non-tumour tissues. (A) Showing the percentage of CD8⁺Tregs in the tumour compared to (B) matched non-tumour tissue. (C) CD4⁺Tregs within the tumour and (D) matched non-tumour tissue is shown as a comparison. (E) The percentage of CD8⁺Treg in HCC was compared to matched non-tumour tissue in consecutive matched samples. (F) The percentage of CD4⁺Treg in HCC tumour and matched non-tumour tissue are shown as a comparison. Data are shown for 28 matched samples, statistical significance was tested using two-tailed Wilcoxon matched pairs test. ***p<0.001

3.5 CD8$^+$Treg infiltration in disease stage.

A number of studies have suggested the infiltration of selected subsets of immune cells with the potential to use this as a predictor of disease prognosis and staging of disease [397]. To ascertain whether the percentage of CD8$^+$Treg or CD4$^+$Treg infiltration correlated with tumour volume or disease stage in human HCC, the total macroscopic tumour volume at the time of histological examination or the disease stage was plotted against the percentages of CD8$^+$Treg or CD4$^+$Treg tumour infiltration as measured by flow cytometry. No correlation between the percentages of tumour infiltrating CD8$^+$Treg or CD4$^+$Treg with total tumour volume was detected (Figure 3-10). Patients were further classified according to their disease stage using the Barcelona Cancer Liver Clinic (BCLC) system (Figure 1-2). In total 9 patients had stage A disease and 19 patients had stage B disease. The disease stage did not correlate with Treg infiltration, with the percentage of tumour infiltrating CD8$^+$Treg being comparable between both stage A disease (2.10%±4.47) and stage B disease (2.23%±3.60%). Similarly, no significant differences were seen in tumour infiltrating CD4$^+$Treg percentages between stage A disease (6.46%±6.39%) and stage B disease (6.30%±5.14%) (Figure 3-10).

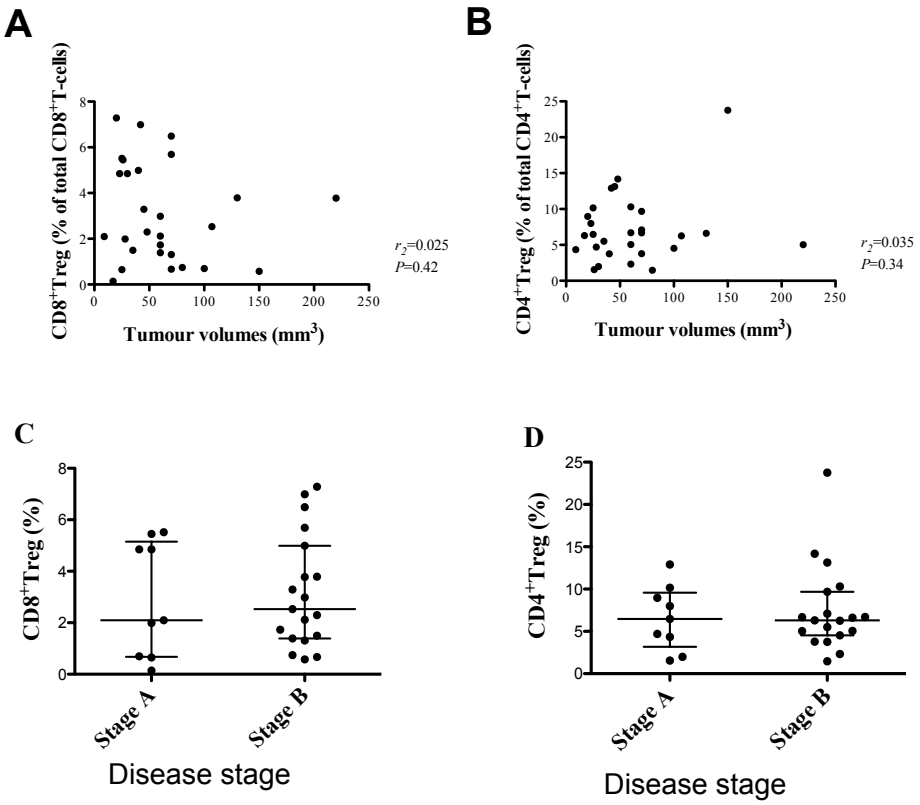

Figure 3-10 Correlation between $CD8^+$Treg or $CD4^+$Treg tumour infiltrations with tumour volume or disease stage.

(A) Percentage of tumour infiltrating $CD8^+$Treg and (B) $CD4^+$Treg were plotted against total tumour volume. Patients were classified according to the BCLC staging system and plotted against (C) tumour $CD8^+$Treg and (D) $CD4^+$Treg infiltrations. Data are expressed as median and interquartile range and statistical significance was tested using Spearman's correlation and Mann-Whitney test. r_2 and p values are indicated. (n=28)

3.6 Effect of Viral liver disease on CD8$^+$Treg infiltration seen in HCC

CD8$^+$Treg have been implicated in the pathogenesis of chronic viral infection such as HCV which may have an effect on Treg infiltration in tumour and non-tumour tissues [215]. To identify the possible effects of chronic viral infection on CD8$^+$Treg infiltration, samples were grouped according to their underlying chronic liver diseases into viral (n=13) or non-viral (n=15) groups and frequency of CD8$^+$Treg infiltration analysed. The percentage of CD8$^+$Treg infiltration remained higher in the tumour, when compared to matched non-tumour tissues in both viral and non-viral groups (Figure 3-11). However, this only reached statistical significance in the non-viral group. The frequency of CD4$^+$Treg infiltrating the tumour remained significantly higher when compared to matched non-tumour tissues in both viral and non-viral groups (Figure 3-11). Interestingly, no significant differences were seen in CD8$^+$Treg or CD4$^+$Treg infiltrating in non-tumour tissue between viral and non-viral disease.

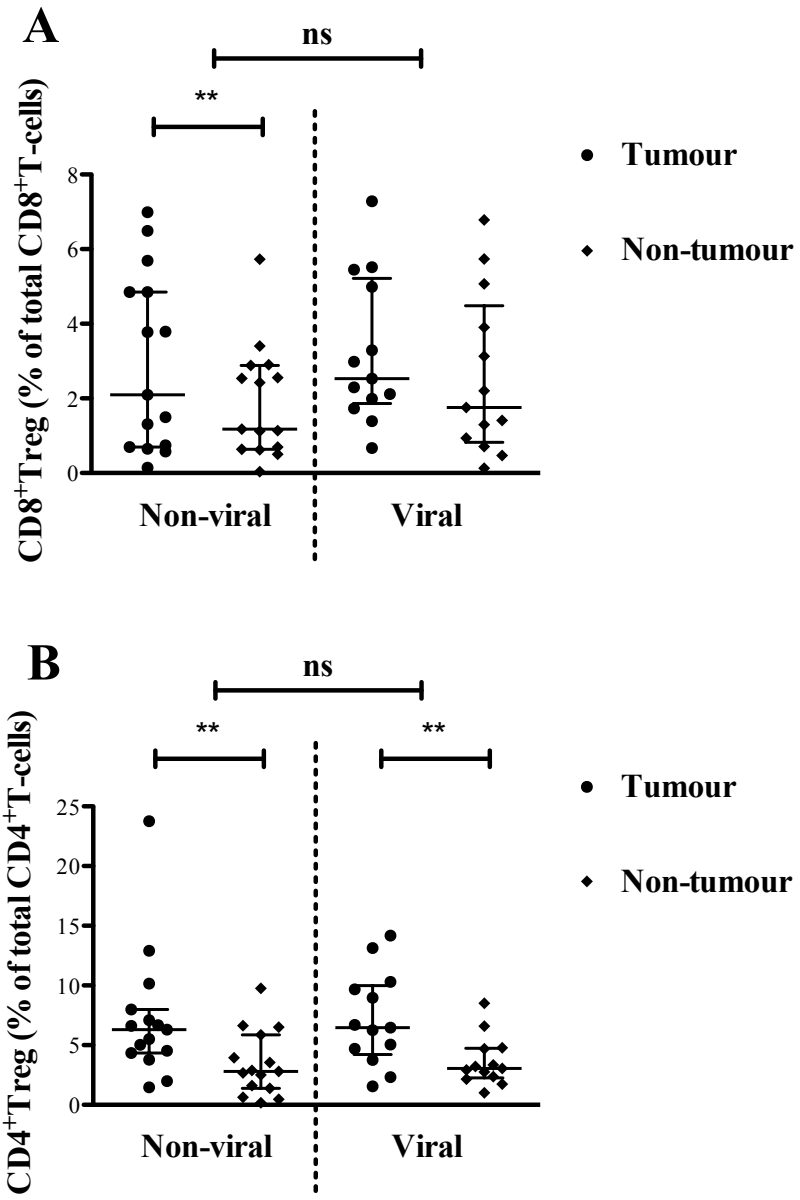

Figure 3-11 CD8⁺Treg frequency in HCC tissues, stratified for non-viral and viral disease background.

Samples were separated into non-viral or viral groups according to the underlying chronic liver disease. The frequency of (A) CD8⁺Treg and (B) CD4⁺Treg infiltrating HCC and non-tumour tissues were analysed. Data are expressed as median and inter-quartile range and statistical significance was tested using the Mann-Whitney test for non-viral vs. viral or two-tailed Wilcoxon matched pairs test for tumour vs. matched non-tumour. ns=non-significant, **p<0.01 (n=28)

3.7 CD8$^+$Treg phenotype

Previous studies have begun to define the phenotype of CD8$^+$Treg [398], but research in this area are much more scarce when compared with CD4$^+$Treg. Assessment of expression of proteins that had been previously detected on both CD8$^+$Treg and CD4$^+$Treg subsets were used to further ascertain the precise phenotype of the CD8$^+$Treg isolated from HCC. Freshly isolated tumour derived MNC were labelled with a range of monoclonal antibodies to define surface and intracellular phenotype. Cells were determined as CD8$^+$Treg or CD8$^+$Non-Treg by the expression of $CD3^+CD8^+CD25^{high}CD127^{low}$ or $CD3^+CD8^+CD25^{low}$ respectively. Increased expression of FOXP3 (76.3%±7.37% vs. 3.66%±3.66%), CTLA-4 (72.3%±6.1% vs. 3.4%±0.77%) and CD39 (72.4%±28.45% vs. 8.15%±2.32%) was detected in CD8$^+$Treg from HCC when compared to CD8$^+$Non-Treg. Both CD8$^+$Treg and CD8$^+$Non-Treg expressed a high level of CD28 (66.69%±11.97% vs. 69.29%±8.46%). However, in contrast to previous studies, Treg associated expression of GITR (6.18%±5.98% vs. 3.93%±1.45%) and CD103 (1.60%±0.17% vs. 2.53%±1.78%) were only expressed at a low level on CD8$^+$Treg isolated from tumour tissues and the expression was comparable to CD8$^+$Non-Treg (Figure 3-12).

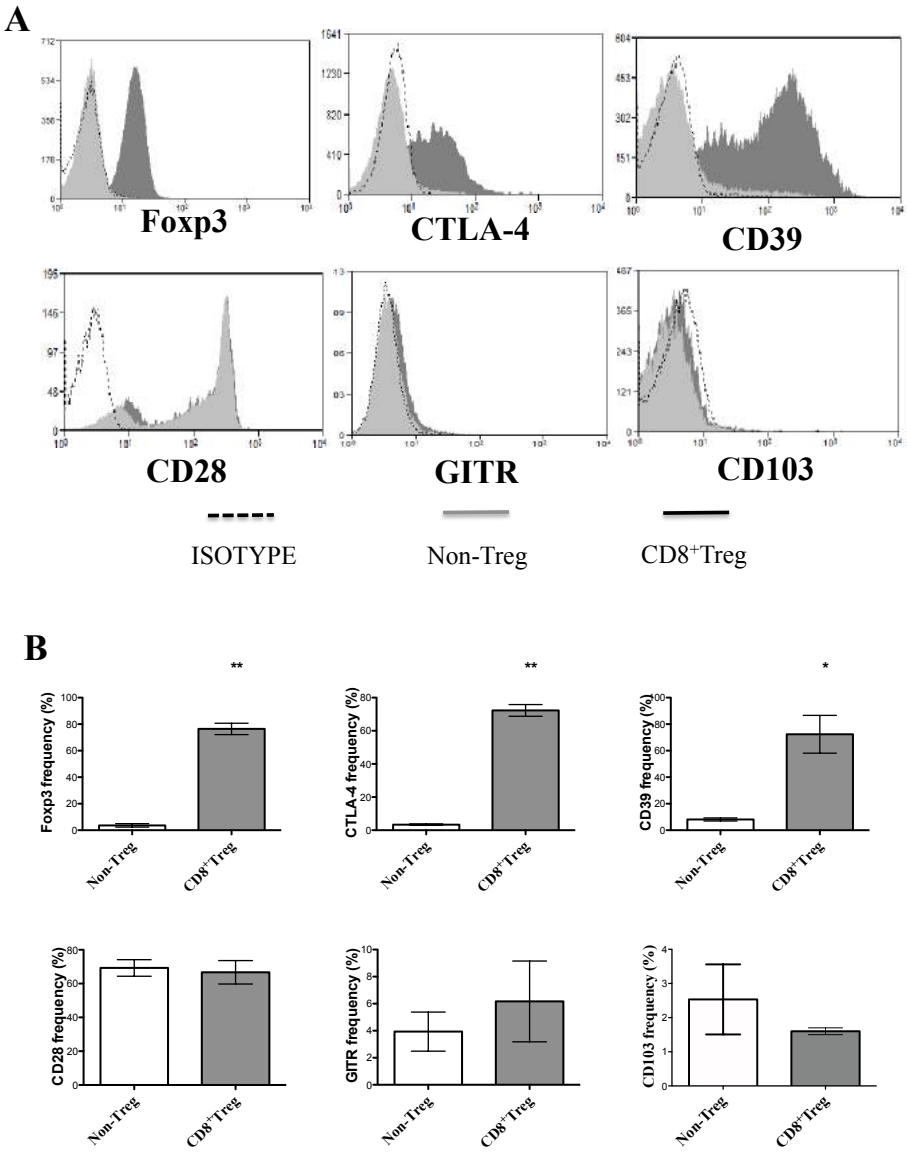

Figure 3-12 Regulatory phenotype of isolated CD8$^+$Treg from human HCC.

(A) Representative histograms showing, expression of FOXP3, CTLA-4, CD39, CD28, GITR and CD103 on CD8$^+$Treg and CD8$^+$Non Treg cells isolated from human HCC compared to isotype control. (B) The frequency of FOXP3, CTLA-4, CD39, CD28, GITR and CD103 expression were compared between CD8$^+$Treg and CD8$^+$Non-Treg in tumour samples. Data are expressed as mean and statistical comparison was made with paired students t-test *$p<0.05$, **$p<0.01$.

3.8 CD8$^+$Treg level in the blood of HCC patients or healthy donor

Previous studies on CD8$^+$Treg have suggested these cells are likely to be induced by the local immune environment and as a result are only present at a low frequency in blood [399]. This is compared to CD4$^+$Treg, which can occur naturally or though induction from non-Treg cells [400]. To analyse the level of CD8$^+$Treg in different tissue compartments, matched samples of blood, tumour and non-tumour were obtained for the demonstration of CD8$^+$Treg. The frequencies of tumour infiltrating CD8$^+$Treg were significantly higher (3.57%) when compared to either non-tumour tissue (0.65%) or blood (0.95%) from the same patients (Figure 3-13). However, the distribution of CD4$^+$Treg slightly differs from CD8$^+$Treg. Not only was the frequency of CD4$^+$Treg much higher within the tumour (6.71%) when compared to matched non-tumour tissue (0.73%), a concomitant elevation in CD4$^+$Treg numbers (5.33%) in the blood from the same patient was recorded, to a comparable level as was seen in the tumour. To further analyse the presence of circulating CD8$^+$Treg, blood from 50 patients with HCC, prior to treatment with TACE, and 10 healthy donors, was obtained for the analysis of CD8$^+$Treg. The frequency of blood CD8$^+$Treg was detectable but at consistently very low levels and there were no significant differences between HCC patients (1.30%±1.30%) and healthy donor (0.94%±1.8%) (Figure 3-13). Again, a different pattern was seen with CD4$^+$Treg compared with CD8$^+$Treg, in which the CD4$^+$Treg level from the blood of HCC patients (6.01%±6.65%) was much higher when compared to healthy donors (3.03%±3.33%).

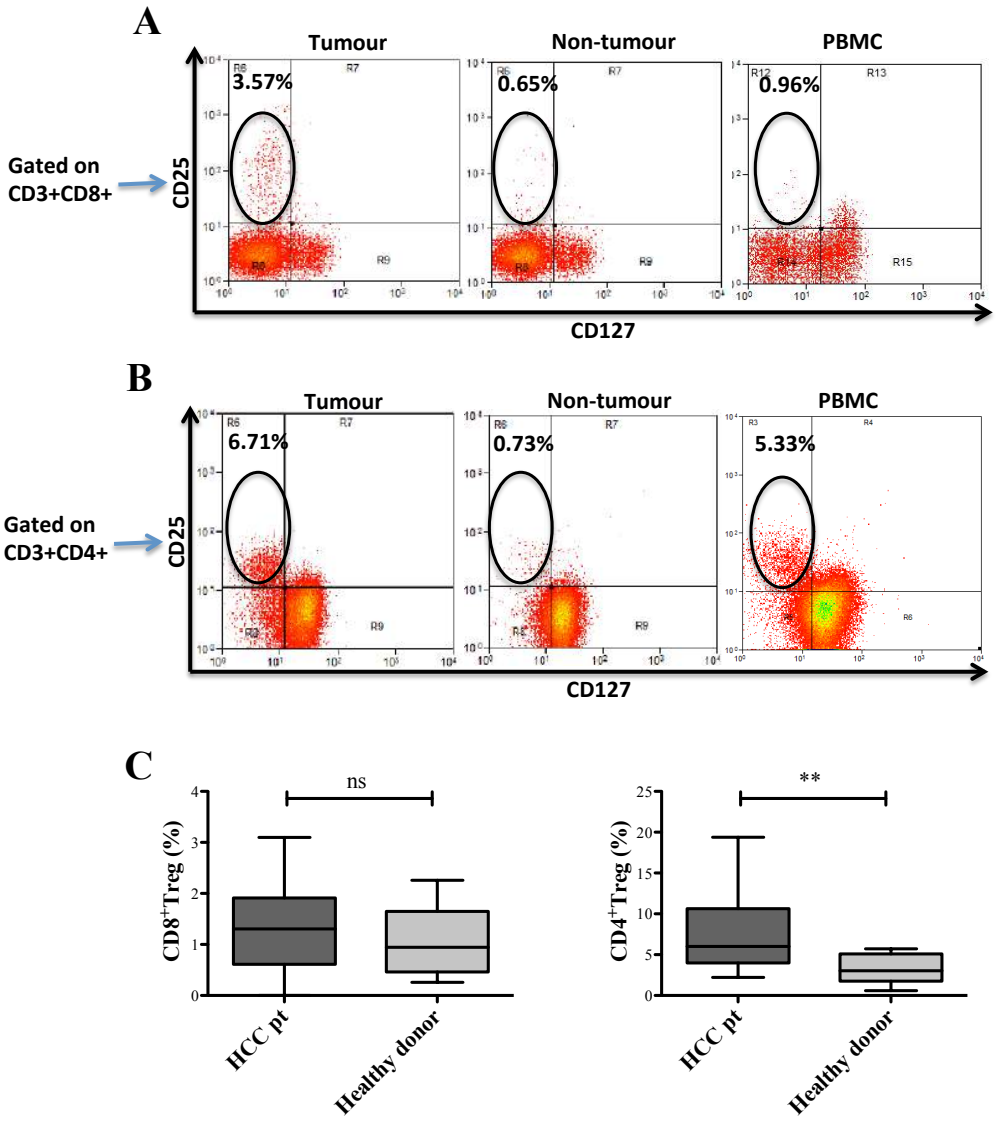

Figure 3-13 CD8$^+$Treg level in blood of HCC patients

Flow cytometric analysis of (A) CD8$^+$Treg and (B) CD4$^+$Treg from matched tumour, non-tumour and blood obtained from a patient with HCC. CD8$^+$Treg and CD4$^+$Treg are shown in black circles and cells were gated on CD3$^+$CD8$^+$ and CD3$^+$CD4$^+$ respectively. The level of blood CD8$^+$Treg or CD4$^+$Treg from HCC patients (n=50) was compared to healthy donors (n=10). Data expressed as median and interquartile range and statistical comparison was made using the Mann-Whitney test where ns=non-significant, **p<0.01.

3.9 Discussion

Research on suppressive T-cells has focused mainly on regulatory T-cells that are characterised by the surface expression of CD4, but increasing evidence has suggested a role for other subsets of regulatory T-cell in the generation of a suppressive tumour microenvironment. Our understanding of different subsets of suppressive immune cells will allow us to identify possible targets for immunotherapeutic intervention and it is likely that the targeting of multiple suppressive cell types will be needed for the development of an effective anti-tumour immune response.

To increase the accuracy in identifying CD8$^+$Treg, a number of different techniques were used in this current work. The use of a multifactorial panel of Treg markers increases the likelihood of identifying the correct cell type. This work has used the now accepted expression pattern of CD25^{high}CD127^{low} to define regulatory cells in addition to CD8 to define the subset of T-cells of interest. These cells expressed the regulatory markers FOXP3, CTLA-4, CD39 and CD28, but only possessed limited expression of CD103 and GITR. Therefore, the cells that have been described differ from CD8$^+$CD28$^-$ and CD8$^+$CD103$^+$ regulatory T-cells described previously by others [191, 192, 217-219], but expresses a similar phenotype to previously identified CD8$^+$CD25$^+$Treg [143, 220-225]. In comparison to some studies on CD8$^+$CD25$^+$Treg, I was not able to detect any significant expression of the tumour necrosis factor receptor (TNFR) superfamily member, GITR, identified as key to the induction of CD4$^+$ Treg [401]. However, variations in the expression of regulatory markers are common. One possible explanation may stem from the location and disease from

which the CD8$^+$Treg have been isolated. The microenvironment of different diseases and tumours varies enormously and it is likely such differences may influence the CD8$^+$Treg phenotype. Such variation in phenotype could also reflect the heterogeneity of CD8$^+$Treg, suggesting that CD8$^+$Treg are unlikely to be a stable population of T-cells, but rather a T-cell subset, which can be altered by the local immune environment. The presence of CD4$^+$Treg in HCC has been confirmed by a number of studies and this current work offers further evidence for the important role CD4$^+$Treg have on the tumour microenvironment. In addition, the majority of study looking at Treg, often focus on one particular tissue/blood compartment. In particular, it is not uncommon to assume results generated from isolated peripheral cells are the same as what occur within the tumour. Hence, for future study it maybe useful to analyse CD8$^+$Treg from tumour and peripheral and compare their phenotype and function. The importance of the origin of the cells and its effect on its phenotype was elegantly demonstrated by Scurr et al [402]. In which distinct T-cell populations isolated from the blood, tumour and colon tissue of colorectal cancer patients demonstrated differences in the expression of common regulatory marker such as CTLA-4 and CD39. The differences in phenotype correlated with where the cells were isolated from.

CD8$^+$CD25^{high} Treg have been demonstrated to infiltrate human HCC in one previous study [239]. This study analysed the phenotype of T-cells infiltrating HCC and healthy donor liver tissues. The group demonstrated the presence of a subset of CD8$^+$T-cells with a high expression of the regulatory markers CD25, FOXP3, GITR, CTLA-4 and a low expression of CD127. The frequency of this subset of cells was

significantly higher in HCC tissue when compared to healthy donor tissues. A limitation however of this study is the lack of confirmation that this subset of CD8$^+$T-cells possessed regulatory function. In addition, the study demonstrated a positive correlation between CD8$^+$Treg numbers and advanced tumour stage. This is different from my current findings.

At the time of writing, due to the limited number of studies in CD8$^+$Treg no other group had investigated the relationship between CD8$^+$Treg infiltration and HCC tumour stage. However, a study on colorectal cancer concluded a subset of CD8$^+$CD25$^+$Treg with suppressive function correlated with tumour stage [228]. In a more recent study, Chen et al., demonstrated the percentage of circulating CD8$^+$CD28$^-$Treg correlated with the pathological stage in non-small cell lung cancer and tumour burden [403] .

Further evidence on the association of Treg frequency with tumour stage and prognosis has been provided by work carried out on CD4$^+$Treg. A number of studies in HCC and other human tumours have concluded the enrichment of CD4$^+$Treg correlated with more advanced stage of disease and is associated with a poorer prognosis [126, 404-409]. However, similar to my current work, conflicting results are emerging arguing against such findings. Studies in breast cancer did not detect correlation between tumour stage or circulating CD4$^+$Treg frequencies with prognosis [410, 411]. Work in haematological cancer, head and neck cancer and more recently in colorectal cancer has even suggested increased CD4$^+$Treg infiltration might be beneficial to survival, through the inhibition of inflammatory driven tumour

progression by Treg [412-414]. These discrepancies between studies emphasise the complexity of the tumour microenvironment.

The inconsistencies in such results may be explained in a number of ways. The methods used to identify tumour-infiltrating Treg differ widely between studies, such as the use of immunohistochemistry, flow cytometry and polymerase chain reaction (PCR), making direct comparison between studies difficult. Similarly, the clinical end points used to assess correlation with Treg infiltration differ between studies, e.g. the use of different tumour staging systems and survival end points. As already discussed, early difficulties in Treg research had been the result of a lack of specific markers. This of course can also be translated into interpretation of Treg studies. The makers used to define Treg vary enormously between these studies, with some early studies confining to the use of single marker to identify their Treg population. In addition, the lack of functional data in some of these works may further hamper the robustness of the studies. Lastly, the source of the cells being analysed also differs between studies, with some studies using blood Treg and others using tumour-infiltrating Tregs, marking comparison of the actual data troublesome.

In my current work, the tissues analysed were obtained from patients with no prior treatment for their HCC. The use of adjuvant treatment, such as local ablation in HCC may affect the analysis of immune cells infiltration. Following successful local ablation treatment, the tumour often undergoes necrosis followed by a reduction in viable tumour cell numbers, but this does not directly translate into a reduction in

tumour size. Hence, caution is needed when interpretation result of Treg infiltration against tumour volume or disease stage.

Unlike other solid tumours, HCC usually occurs on the background of a wide range of different chronic liver diseases. The underlying chronic disease is likely to play an important role in the generation of the tumour microenvironment and may in part affect the number and type of immune cell infiltrating the tumour. In addition, increasing evidence derived from genetic profiling of human tumours, including HCC has supported the notion of variation in biological properties between tumours [415, 416].

The frequency of CD8$^+$Treg and CD4$^+$Treg were comparable between non-tumour tissues obtained from patients with underlying non-viral and viral disease. In addition, the frequency of CD8$^+$Treg and CD4$^+$Treg remained higher in tumour tissues when compared to matched non-tumour tissues irrespective of the underlying liver disease. The frequency of CD8$^+$Treg infiltration was not statistically significant between tumour and matched non-tumour tissues in viral disease, but this is likely to be the result of a reduction in sample numbers (n=13). However, this doesn't exclude the possible effect of underlying liver disease on the tumour microenvironment.

Numerous studies have looked into the incidence of recurrent HCC after liver transplantation, with the common surrogate endpoint being 5 years recurrence free survival [417]. Unfortunately, my work has been conducted only in the last three years with a mean follow-up period of 562 days (range 57-1027 days), which is too

short for any meaningful analysis of survival. At the time of writing 3 patients had developed recurrent tumour following their surgery.

The differences seen in the distribution of CD8$^+$Treg and CD4$^+$Treg in tissues and blood was noteworthy. Whilst CD4$^+$Treg frequencies in the blood of HCC patients seem to mirror the increased level of CD4$^+$Treg in the tumour, this wasn't the case for CD8$^+$Treg, which are present at a reduced frequency in the blood of both HCC patients and healthy donors. This may suggests the generation of the two subsets of Treg are rather different and gives further support to the notion that increased level of CD8$^+$Treg in tissues are the result of induction from non-Treg cells within the tumour microenvironment. However, the precise mechanism on how CD8$^+$Treg are induced remains unclear.

In summary, a novel subset of CD8$^+$CD25^{high}CD127^{low} T-cells has been identified in HCC. They express some of the common regulatory markers, previously described in CD8$^+$Treg and CD4$^+$Treg. CD8$^+$Treg appear to account for a very small number of circulating Treg in health and disease.

4 The induction of CD8$^+$Treg

The previous chapter described the presence of a subset of CD8$^+$T-cells in HCC expressing some of the common markers for regulatory T-cells. We hypothesised that these CD8$^+$T-cells likely represent a novel subset of CD8$^+$Treg. However, it remains unclear whether these cells occur naturally or arise via induction by the tumour-microenvironment. This chapter will illustrate how DC are able to induce CD8$^+$Treg.

4.1 Circulating CD8$^+$Treg in HCC patients

CD8$^+$Treg (CD8$^+$CD25^{high}CD127^{low}) appear to only be present at a low frequency in the peripheral blood of HCC patients when compared to tumour tissues. In addition, the frequencies of circulating blood CD8$^+$Treg are no different in HCC patients or healthy donors (Chapter 3). However, the percentages of tumour infiltrating CD8$^+$Treg were significantly higher when compared to non-tumour tissues. The increase in CD8$^+$Treg appears to be confined to within the tumour, with no effect on circulating CD8$^+$Treg. This observation points towards the possible role of the tumour microenvironment in the induction CD8$^+$Treg. To further support this concept, disease progression and therefore increase tumour volume doesn't appear to have an effect on circulating CD8$^+$Treg. In a cohort of 10 patients who had progressive disease despite TACE treatment, the percentages of circulating CD4$^+$Treg (CD4$^+$CD25^{high}CD127^{low}) were significantly elevated as disease progressed (Figure 4-1). In comparison, despite disease progression the percentage of circulating CD8$^+$Treg remained static. These data further support for the possible role of

induction of CD8$^+$Treg by the tumour environment and not from recruitment of naturally occurring CD8$^+$Treg from a distal site.

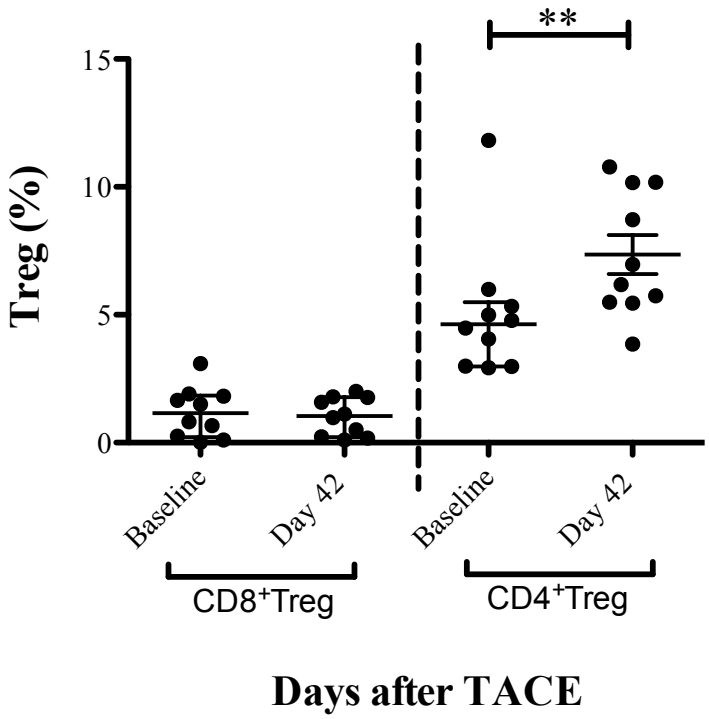

Figure 4-1 Measurement of circulating Treg during HCC progression

10 patients had progressive HCC despite TACE as defined by mRECIST and blood samples were analysed for change in circulating CD4$^+$Treg and CD8$^+$Treg by flow cytometry. In patients with HCC the frequency of circulating CD8$^+$Treg did not change with disease progression, in comparison a significant increase was seen in circulating CD4$^+$Treg frequencies. Data are expressed as median and inter-quartile range and statistical significance was tested using the two-tailed Wilcoxon matched pairs test. **$p<0.01$ ($n=10$)

4.2 Direct effect of tumour condition medium on CD8$^+$Treg induction

A number of suppressive cytokines have been proposed to be involved in the direct induction of Treg and some of these cytokines have been shown to be elevated within tumours, including HCC [418-421]. To ascertain whether soluble factors produced by HCC are involved in the induction of CD8$^+$Treg, tumour-conditioned medium from fresh HCC tissue and matched non-tumour-conditioned medium were generated. Naïve CD8$^+$T-cells isolated from healthy donors were cultured in conditioned medium at different concentrations for 24 hours, 3 days and 5 days. The cells were assessed for induction of CD3$^+$CD8$^+$CD25^{high}CD127^{low}T-cells using flow cytometry. Surprisingly, following incubation of CD8$^+$T-cells in condition medium, no induction of CD8$^+$Treg was detected at any of these time points (Figure 4-2). The use of different concentrations of tumour-conditioned medium did not appear to alter the result.

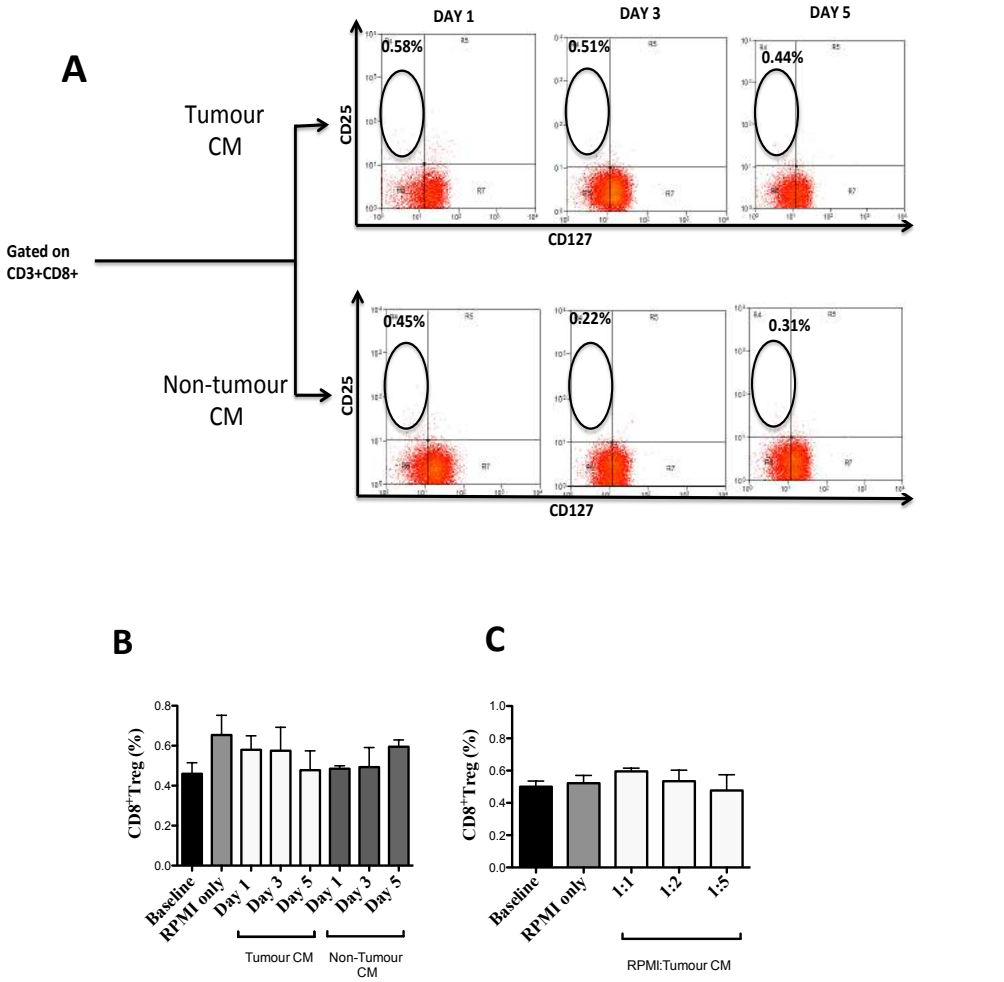

Figure 4-2 The effect of tumour conditioned medium on CD8$^+$Treg induction

Naïve CD8$^+$T-cells were cultured in RPMI alone, tumour or matched non-tumour conditioned medium for 1-5 days. (a) The expression of CD25 and CD127 were determined by flow cytometry on day 1, 3 and 5. Cells were gated on CD3$^+$CD8$^+$ expression. (B) Representative dot plots of CD25^{high}CD127^{low} cells (black circle) following culture with tumour condition medium **(top panel)** or matched non-tumour condition medium **(bottom panel)** at 3 different time points. (C) Results from 3 independent experiments to demonstrate induction of CD8$^+$Treg following incubation with conditioned medium at three different time points, compared to baseline and RPMI only. Results from 2 sets of experiments to demonstrate induction of CD8$^+$Treg with different ratios of tumour conditioned medium compared to baseline and RPMI after 3 days of incubation. Data were expressed as median and interquartile range. Statistical comparisons were made with Mann-Whitney test.

In the setting of $CD4^+$Treg induction, evidence has suggested TCR activation is required in the addition of suppressive cytokines for the generation of induced Treg [422-424]. To recapitulate this here, naïve $CD8^+$T-cells were incubated in the presence of conditioned medium (1:1 CM:RPMI ratio) plus CD3/CD28 activation beads to provide TCR engagement. Indeed a population of $CD25^{high}CD127^{low}$ cells were induced, however, this same phenotype was generated even in the absence of conditioned medium (Figure 4-3 & Figure 4-4). To further ascertain whether these $CD25^{high}CD127^{low}$ cells are truly Treg, the induced $CD25^{high}CD127^{low}$ cells were stained for the expression of FOXP3. In addition, the induced cells were isolated by flow cytometric sorting and used in a suppression assay to confirm their functional capacity. These cells were confirmed to be FOXP3 negative and importantly did not possess suppressive capacity against responder T-cells (Figure 4-3 & Figure 4-4). These data suggest this population of cells represents recently activated T-cells and not Treg.

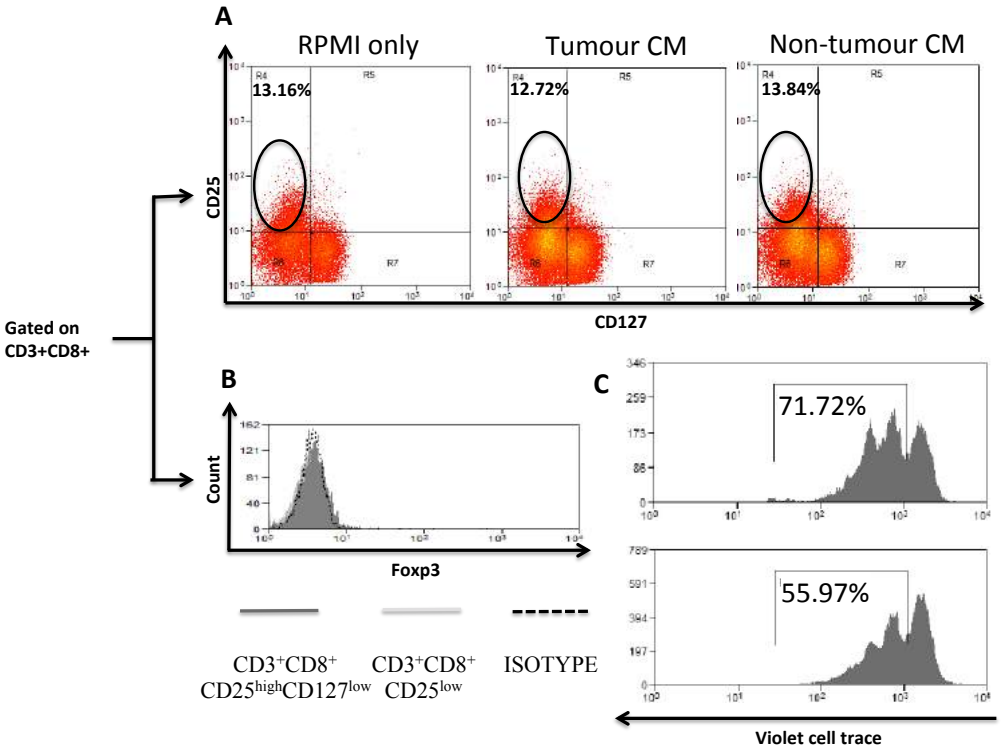

Figure 4-3 Representative flow cytometry data on the phenotype & function of $CD8^+CD25^{high}CD127^{low}$T-cell induced in the presence of conditioned medium.

Naïve $CD8^+$T-cells were cultured in the presence of RPMI alone, tumour or matched non-tumour conditioned medium with the addition of CD3/CD28 activating beads at 1:1 beads to cells ratio for 3 days. The expression of CD25, CD127 and FOXP3 were determined by flow cytometry. $CD25^{high}CD127^{low}$ cells were isolated by flow cytometric cell sorting and used in an allogenic T-cell suppression assay. Cells were gated on $CD3^+CD8^+$ cells. (A) Representative dot plots of $CD25^{high}CD127^{low}$ cells **(black circle)** induced following incubation with RPMI alone, tumour or matched non-tumour conditioned medium with the addition of CD3/CD28 activation. (B) Histogram showing the expression of FOXP3 on induced $CD25^{high}CD127^{low}$ T-cells compared to $CD25^{low}$ T-cells and isotype control. (C) Representative histograms showing allogeneic responder T-cells ($CD25^{low}$) proliferation, as determined by dilution of violet cell trace, in the presence of induced $CD25^{high}CD127^{low}$ T-cells **(top panel)**, or alone **(bottom panel)**.

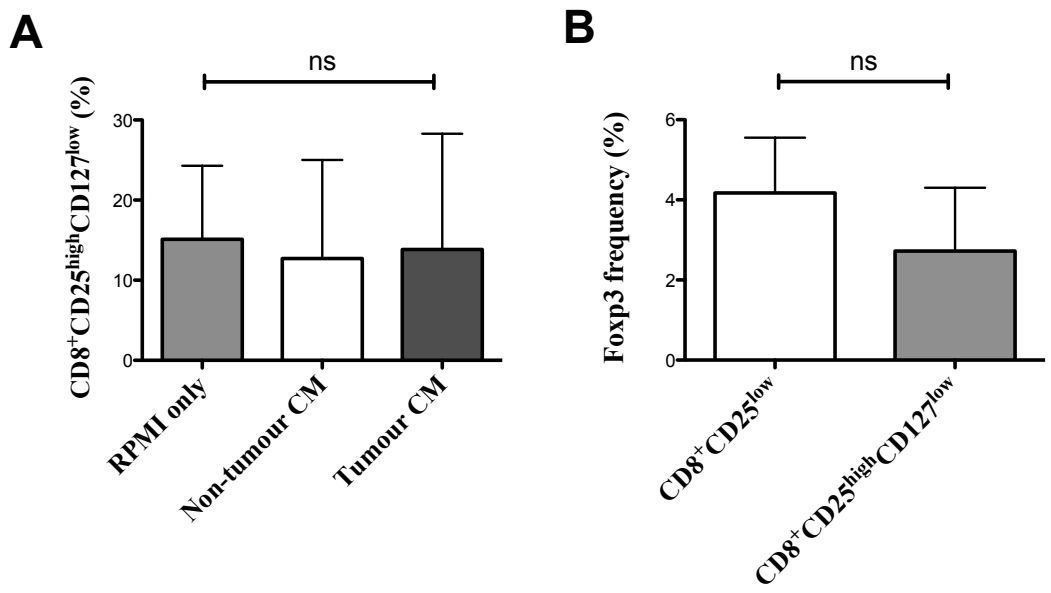

Figure 4-4 Phenotype & function of $CD8^+CD25^{high}CD127^{low}$T-cell induced in the presence of conditioned medium.

(A) The frequency of $CD8^+CD25^{high}CD127^{low}$ T-cells following incubation with RPMI alone, tumour or matched non-tumour conditioned medium with the addition of CD3/CD28 activation were compared. (B) The frequency of FOXP3 expression were compared between $CD8^+CD25^{high}CD127^{low}$ and $CD8^+CD25^{low}$ T-cells following incubation with tumour conditioned medium. Data were expressed as median and interquartile range. Statistical comparisons were made with Mann-Whitney test. Representative data from 3 independent experiments. CM; conditioned medium.

4.3 Effect of HCC tissue conditioned medium on DC phenotype

Tumour-conditioned medium, either alone or used in combination with TCR activation, was not able to induce a suppressive phenotype on CD8$^+$T-cells. In an attempt to induce CD8$^+$Treg, a more physiological method was tested. DCs are the prototypic professional antigen-presenting cell, characterised by their ability to process antigens and generate immune responses. However, it has also been recognised that DC play an important role in immune regulation. An increasing number of studies have demonstrated the presence of immature/tolerogenic DC, which on interaction with T-cells, result in the induction of Treg [73, 425]. Previous data from our lab [426] has demonstrated the presence of tolerogenic DC infiltrating human HCC. Hence, HCC interaction with DC may result in the generation of tolerogenic DC and in addition may have the potential to induce CD8$^+$Treg.

The frequency of DC in liver tissue is variable and their use in downstream experiments therefore unpredictable. Accordingly, an alternative approach was needed to assess the possible effect the tumour environment may have on DC phenotype and function. Using a well-established approach, moDC were generated *ex vivo* by the differentiation of CD14$^+$ monocytes in the presence of IL-4 and GM-CSF to provide a source of cells for further experiments. To investigate the effect of HCC on DC maturation, the ex-vivo generated moDC were primed with conditioned medium obtained from either human HCC tumour tissue or matched non-tumour tissues for 24 hours.

Following priming, moDC were assessed for their expressions of DC maturation markers by flow cytometry (Figure 4-5). Expression of DC maturation markers was compared on moDC primed in the presence of tumour-conditioned medium or matched non-tumour conditioned medium. Priming in HCC condition medium consistently lead to reduced expression of ; MHC Class I (21.5 IQR 17.3-30.8 vs. 53.8 IQR 34.3-57.1, $p<0.05$), MHC Class II (160.5 IQR 121.9-185.6 vs. 266 IQR 235.1-352, $p<0.01$), CD80 (6.3 IQR 4.5-7.7 vs. 7.8 IQR 4.3-9.6, $p<0.05$) and CD86 (15.4 IQR 10.4-24.9 vs. 21.9 IQR 12.6-43.3, $p<0.05$) (Figure 4-6). The results reflected the groups earlier finding that primary isolated DC from tumour tissue displayed an immature phenotype when compared to DC from matched non-tumour tissues [426].

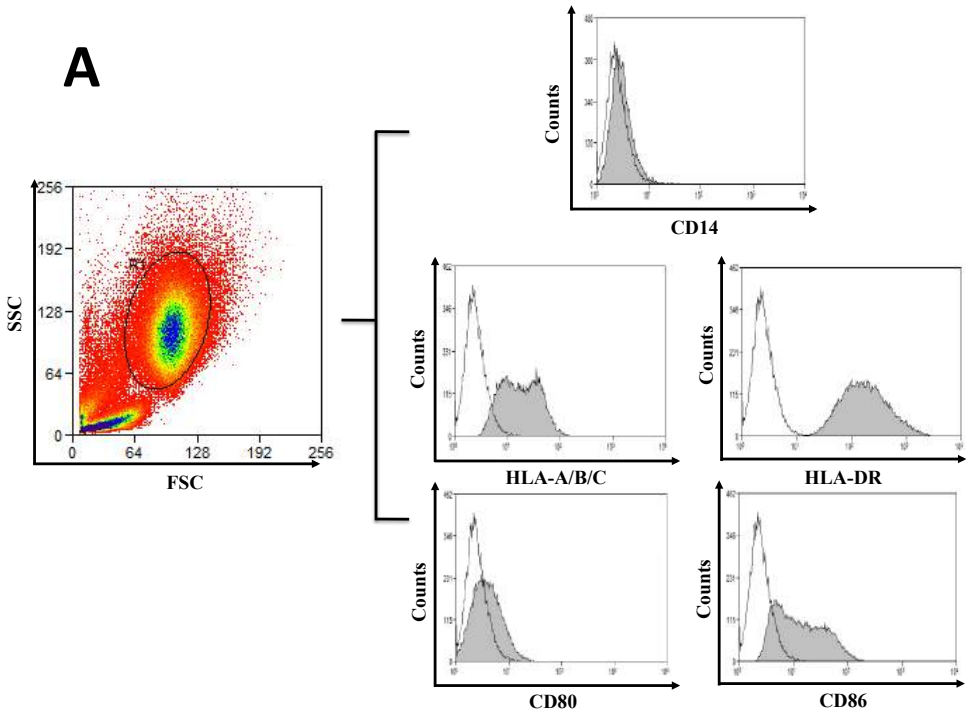

Figure 4-5 Representative flow cytometric for DC phenotype

MoDC were differentiated from circulating CD14$^+$ monocytes and primed by either tumour or matched non-tumour conditioned medium for 24 hours. Following priming, moDC were analysed for the expression of DC maturation markers by flow cytometry. MoDC were identified by FSC and SSC and determined to be CD14 negative. Representative histogram displaying the expression of MHC-I, MHC-II, CD80 and CD86 on moDC (grey) compared to isotype control (white).

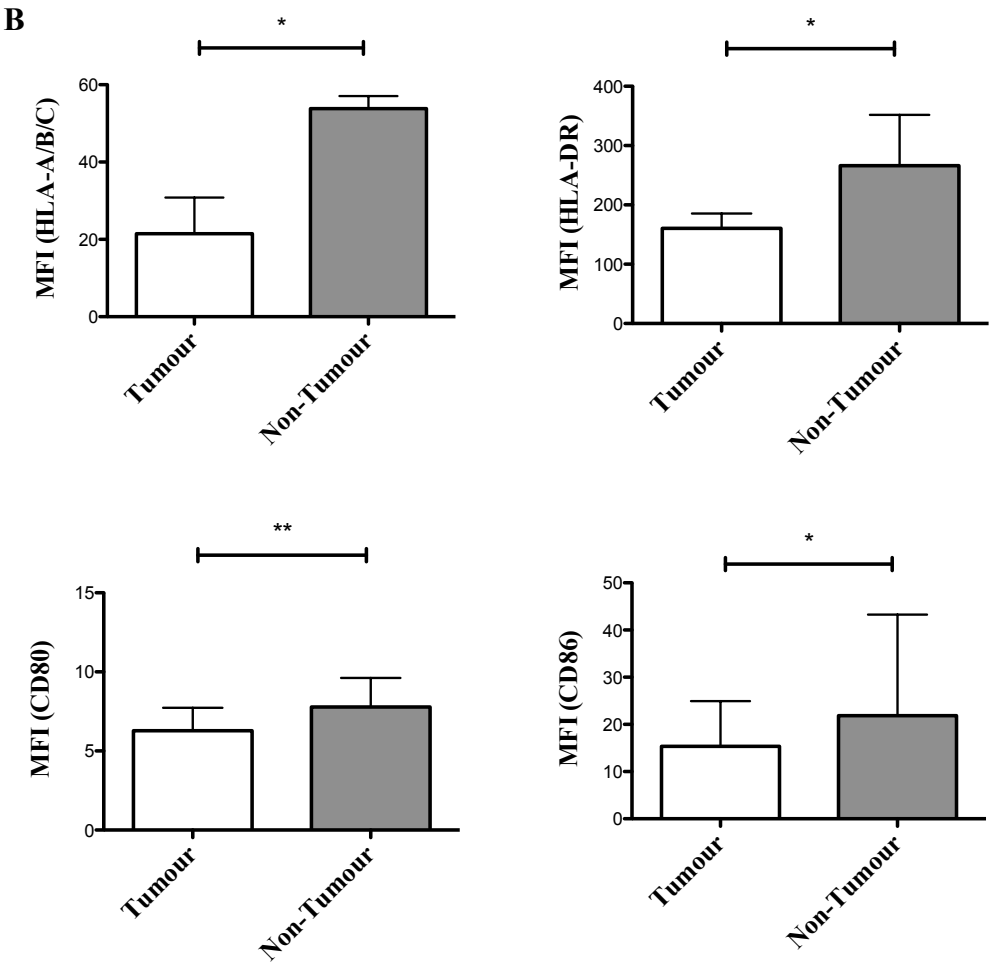

Figure 4-6 moDC phenotype following priming with HCC conditioned medium.

Comparison of DC maturation markers expressions between moDC primed with tumour or matched non-tumour conditioned medium. Results from 5 replicate experiments with each experiment carried out in duplicate. Data are expressed as median and inter-quartile range and statistical significance was tested using the two-tailed Wilcoxon matched pairs test. *$p<0.05$, **$p<0.01$. MFI; median fluorescence intensity

4.4 Effect of tumour-conditioned medium on the functions of DC

With the ability to generate adequate number of moDC, attempts were made to ascertain weather the reduction in DC maturation markers following priming with tumour-conditioned medium translates into dysfunction of these cells. MoDC primed with conditioned medium were assessed for the production of IL-12, which is normally secreted by activated DC to induce activation of T-cells. By using flow cytometry it was shown moDC that had been primed by tumour-conditioned medium produced less IL-12, when compared to moDC that had been primed with matched non-tumour conditioned medium (Figure 4-7). In addition, previous studies had suggested the production of the suppressive cytokine IL-10 may be important in the induction of T-cells into Treg and its secretion can act as a marker of their tolerogenic phenotype [427]. However, IL-10 was not detected by flow cytometry in moDC following priming by either tumour or non-tumour conditioned medium (Figure 4-7).

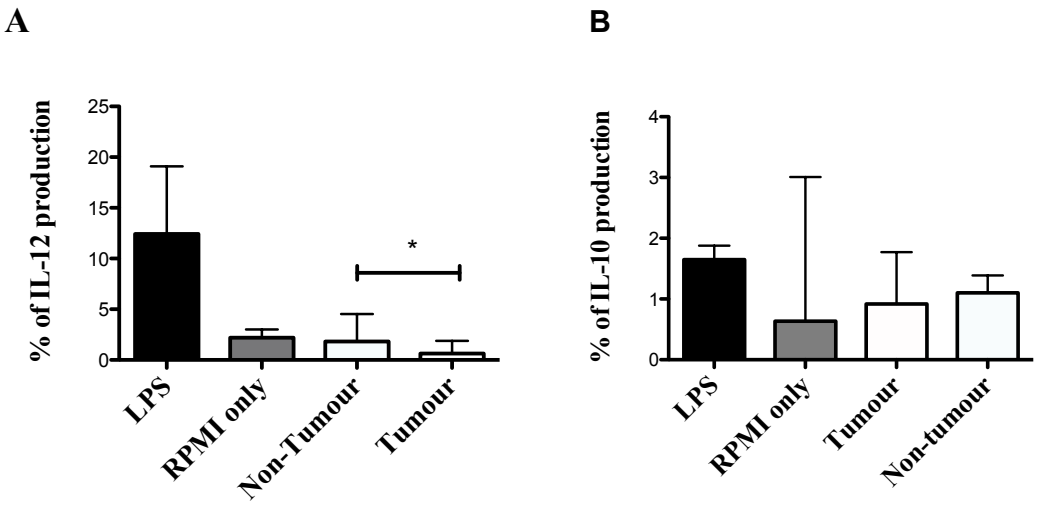

Figure 4-7 The effect of tumour conditioned medium

on DC cytokines production

MoDC that had been primed by tumour or matched non-tumour conditioned medium cells were analysed for their production of cytokines by intracellular staining. RPMI and lipopolysaccharide (LPS) were used as a negative and positive control respectively. Results from 3 replicate experiments, showing the production of (A) IL-12 and (B) IL-10 by tumour and matched non-tumour condition medium primed moDC. Data are expressed as median and inter-quartile range and statistical significance between tumour and non-tumour primed cells was tested using the two-tailed Wilcoxon matched pairs test. *$p<0.05$

As professional APC, the key function of DC is to interact with T-cells and prime their recognition of cognate antigen and to initiate T-cell proliferation. The induction of T-cell proliferation was used to assess the ability of moDC primed with conditioned medium to function as APC using an allogeneic T-cell proliferation assay, in which primed moDC are co-cultured with naïve CD8$^+$T-cells for 5 days. The ability of CD8$^+$T-cells to proliferate was assessed by flow cytometry. MoDC that had been primed with tumour-conditioned medium had a significant reduction in their ability to induce T-cell expansion, compared to moDC that had been primed by matched non-tumour conditioned medium, or RPMI alone. However, this did not reach statistical significant (Figure 4-8).

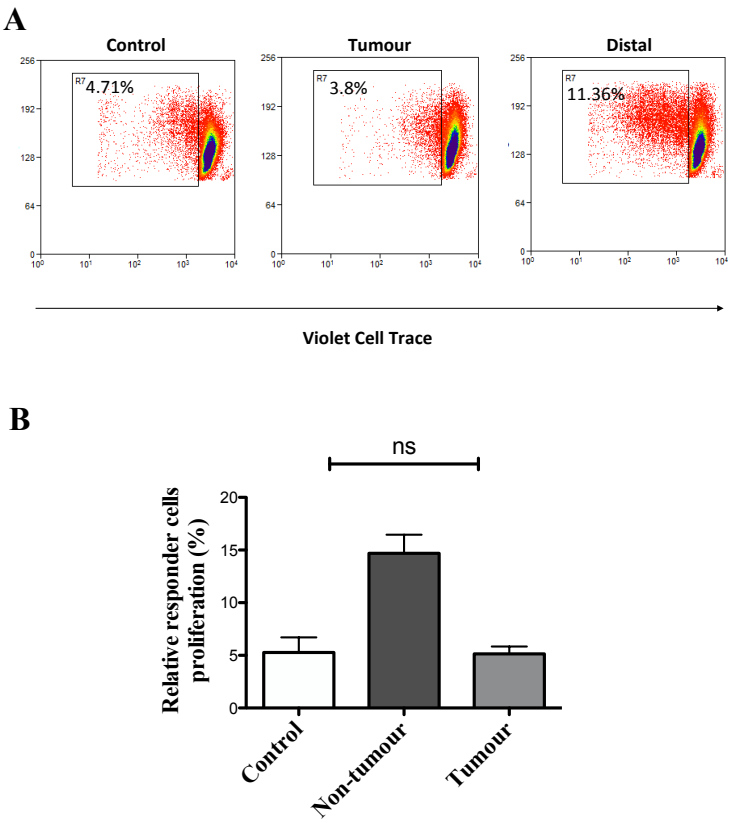

Figure 4-8 The effect of tumour conditioned medium on DC induced T-cell proliferation.

MoDC were cultured in tumour or matched non-tumour conditioned medium and cells cultured in RPMI were used as the control. After 24 hours, the moDC were harvested and co-cultured with naïve allogeneic CD8$^+$T-cells (labelled with violet cell trace tracking dye) for a further 5 days. The ability of moDC to stimulate the proliferation of CD8$^+$T-cells was assessed by the dilution of violet cell trace. (A) Representative dot plots showing the percentages of CD8$^+$T-cell proliferation. (B) The ability of moDC cultured in control medium, tumour or non-tumour matched conditioned medium to induce CD8$^+$T-cells proliferation were compared. Data are expressed as mean and standard deviation. Statistical comparisons were made with Wilcoxon signed ranked test. Data were obtained from 3 replicate experiments. ns; non-significant.

4.5 The effect of DC on CD8$^+$Treg induction

HCC conditioned medium appears to effect both DC maturation and function. To assess whether tumour primed DC are able to induce CD8$^+$Treg, primed moDC were co-cultured with allogeneic naïve CD8$^+$T-cells for 5-7 days. Following, the culture period, the expression of common regulatory markers on CD8$^+$T-cells were analysed by flow cytometry. Co-culturing of naïve allogeneic CD8$^+$T-cells with tumour primed moDC induced a higher percentage of CD3$^+$CD8$^+$CD25^{high}CD127^{low}T-cells when compared to CD8$^+$T-cells that had been cultured with moDC that had been primed with matched non-tumour conditioned medium or RPMI alone (Figure 4-9).

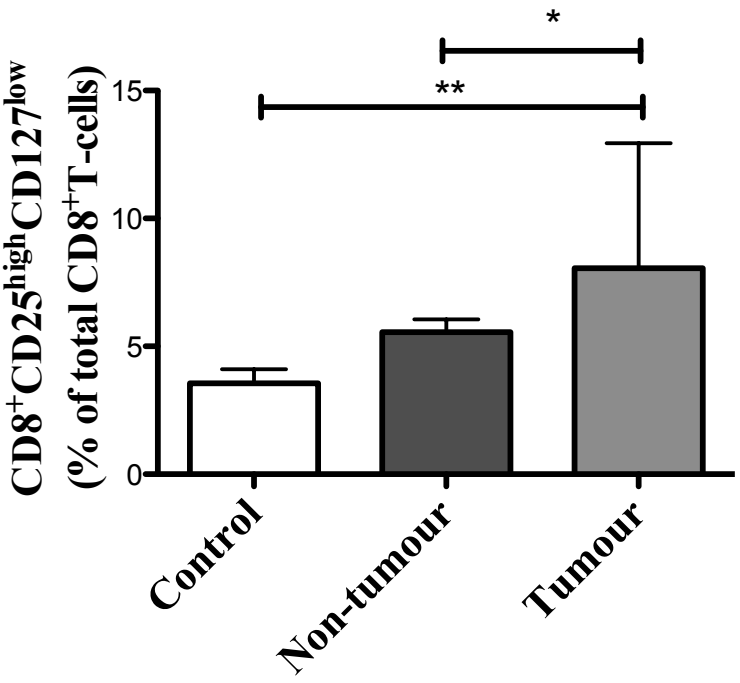

Figure 4-9 The effect of tumour conditioned medium primed moDC on the induction of $CD8^+CD25^{high}$ $CD127^{low}$T-cells

MoDC were cultured in tumour or matched non-tumour conditioned medium and cells cultured in RPMI alone were used as control. After 24 hours, the moDC were washed and co-cultured with naïve allogeneic $CD8^+$T-cells for a further 5 days. At the end of the culture period, the expression of CD25 and CD127 on $CD8^+$T-cells was analysed by flow cytometry. The results from 5 replicate experiments are displayed. Data are expressed as median and IQR, statistical significance was tested using the two-tailed Wilcoxon matched pairs test. *$p<0.05$, **$p<0.01$.

Having demonstrated the induction of CD8$^+$CD25^{high}CD127^{low} T-cells by conditioned medium primed moDC in an allogeneic co-culture assay, I wanted to more accurately mirror the *in vivo* tumour microenvironment. Therefore, this assay was repeated using autologous naïve CD8$^+$T-cells. Indeed, the same induction of Treg was recorded when co-culturing naïve autologous CD8$^+$ T-cells with tumour primed moDC wherein a higher proportion of induced CD8$^+$CD25^{high}CD127^{low}T-cells were recorded when compared to autologous CD8$^+$ T-cells cultured with matched non-tumour primed moDC or medium alone (Figure 4-10).

Importantly unlike CD8$^+$CD25^{high}CD127^{low}T-cells that had been cultured with tumour conditioned medium with or without TCR activation by CD3/CD28 beads, but in the absence of MoDC, the CD8$^+$CD25^{high}CD127^{low}T-cells induced by tumour-primed moDC expressed high levels of the regulatory marker FOXP3 in addition to CTLA-4 and CD39. This further evidence strengthens the hypothesis that these cells are induced CD8$^+$Treg (iCD8$^+$Treg) (Figure 4-11 & Figure 4-12).

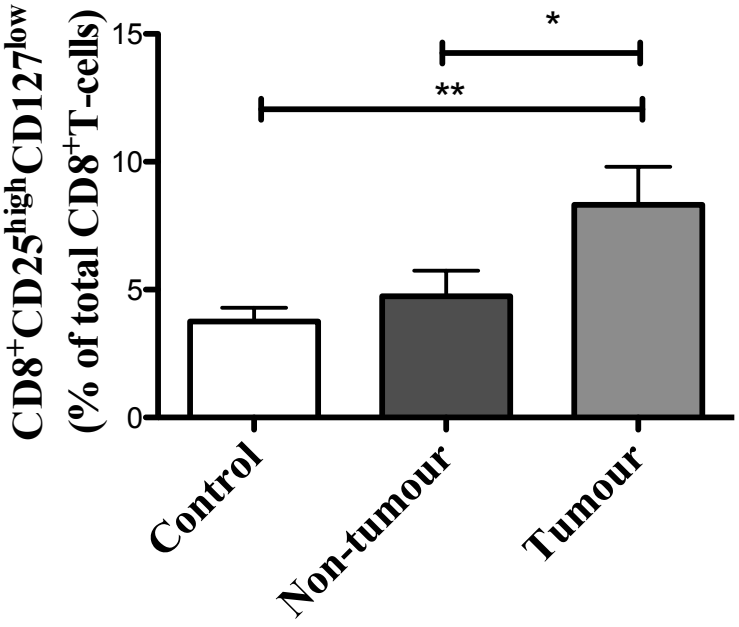

Figure 4-10 The ability of tumour conditioned medium primed DC to induce autologous CD8$^+$Treg

Autologous naïve CD8$^+$T-cells were cultured in RPMI alone, or with moDC that had been primed by tumour or matched non-tumour conditioned medium for 5 days. The expression of CD25 and CD127 on CD8$^+$T-cells was analysed by flow cytometry. The results from 5 replicate experiments are displayed. Data are expressed as median and inter-quartile range and statistical significance was tested using the two-tailed Wilcoxon matched pairs test. *$p<0.05$, **$p<0.01$.

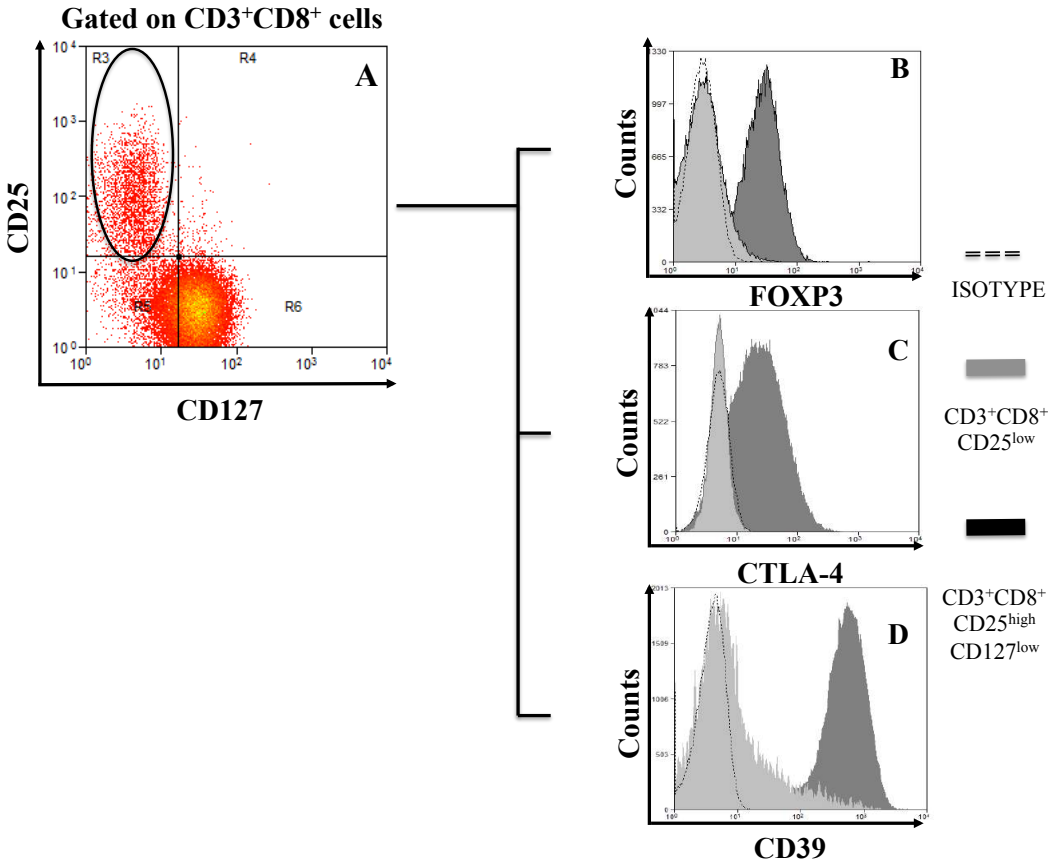

Figure 4-11 Phenotype of DC induced $CD8^{+}CD25^{high}CD127^{low}$T-cells

$CD8^{+}$CD25highCD127lowT-cells that had been induced by tumour-conditioned medium primed moDC were analysed for their expressions of the regulatory markers FOXP3, CTLA-4 and CD39 by flow cytometry. Representative dot plot showing the induction of CD25 and CD127 expressions (black circle) following culture with tumour-conditioned medium primed moDC (A). Histograms showing the expressions of FOXP3 (B), CTLA-4 (C) and CD39 (D) on CD25highCD127low cells (dark grey), compared to CD25low cells (light grey) and isotype control (dotted line).

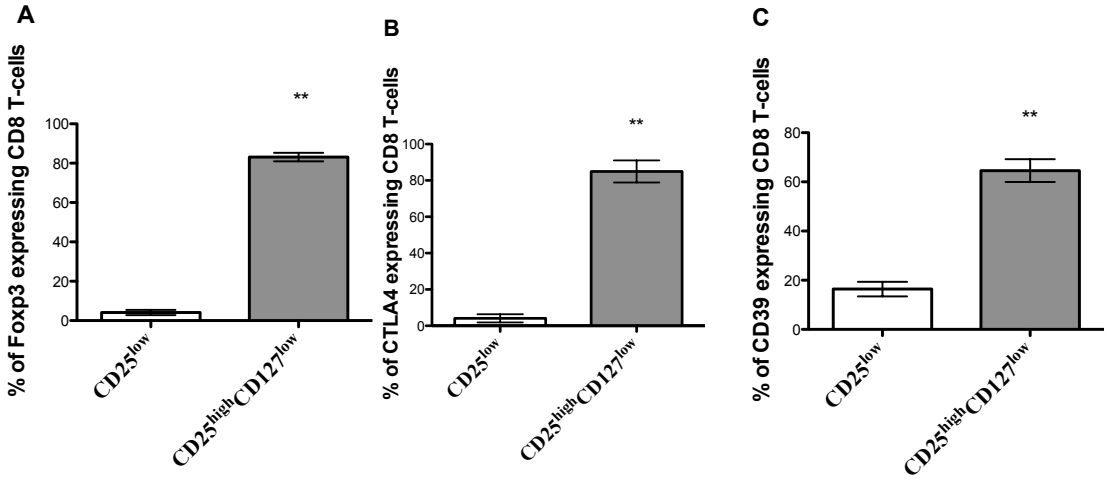

Figure 4-12 Expression of regulatory phenotype on induced CD8$^+$T-cells

The frequency of FOXP3, CTLA-4 and CD39 expression on CD8$^+$CD25^{low} cells and CD8$^+$CD25^{high}CD127^{low} cells, following induction with tumour-conditioned medium primed moDC were compared in three replicate experiments. Data are expressed as mean and standard error. Statistical comparison was made with paired students t-test. **$p<0.01$.

4.6 Cells-contact dependent induction of CD8$^+$CD25^{high}CD127^{low}T- Cells

Given the evidence that HCC conditioned DC are able to induce a subset of CD8$^+$CD25^{high}CD127^{low}T-cells that express distinct markers of regulatory T-cells, a number of proposed mechanisms were considered. In the absence of cell-cell contact, HCC conditioned DC may result in the generation of Treg via the production of suppressive cytokines. Alternatively, in the presence of direct cell-cell contact, inadequate co-stimulation due to down regulation of CD80/CD86 or the direct interaction of DC with T-cells via previously un-described mechanisms may result in the observed effect [277, 278, 427].

Tumour primed moDC do not produce the suppressive cytokine IL-10 (see previous section), suggesting that the induction of Treg maybe more likely to be related to contact–dependant mechanisms. To further ascertain that the induction of CD8$^+$Treg by tumour primed moDC required close cell-to-cell contact, naïve CD8$^+$T-cells were co-cultured with tumour-conditioned medium primed DC with or without the inclusion of a 0.4 μM transwell inserts to prevent cell contact. The cells were co-cultured either side of the transwell insert for 5 days and the expression of CD25 and CD127 on CD8$^+$T-cells were analysed by flow cytometry. The induction of CD25^{high}CD127^{low} T-cells was entirely abolished by the prevention of cell-cell contact between T-cells and moDC (Figure 4-13) suggesting the induction of CD8$^+$Treg is dependent on direct cell-cell contact.

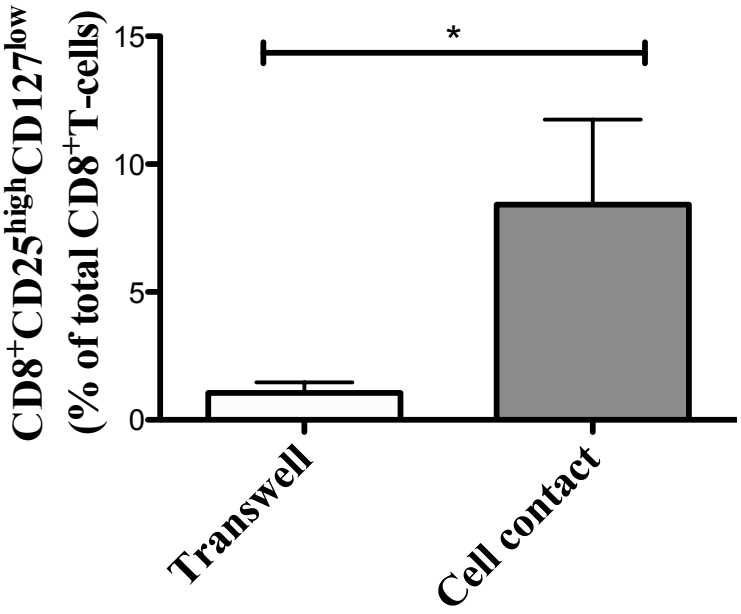

Figure 4-13 The effect of transwell on the induction of CD8$^+$Treg

The requirement of cell-contact dependent induction of CD8$^+$CD25^{high}CD127^{low}T-cells by moDC was assesed by culturing naïve CD8$^+$T-cell and tumour conditioned medium primed moDC together or seprated by a 0.4 μm transwell insert. The percentage of induced CD8$^+$CD25^{high}CD127^{low}T-cells was determined by flow cytometry. Results from 3 replicate experiments are displayed. Data are expressed as median and inter-quartile range and statistical significance was tested using the two-tailed Wilcoxon matched pairs test. *$p<0.05$.

4.7 Localisation of DC and CD8$^+$T-cells within HCC

Having demonstrated that cell-cell contact is important for the induction of CD8$^+$Treg, the presence of this phenomenon *in vivo* was considered. I used immunohistochemical staining of paraffin embedded human HCC tissues to demonstrate the location of DC and CD8$^+$T-cells. Using the expression of CD11c and CD8 as markers of DC and CD8$^+$T-cells respectively, DC and CD8$^+$T-cell can be seen co-localised within the tumour environment (Figure 4-14).

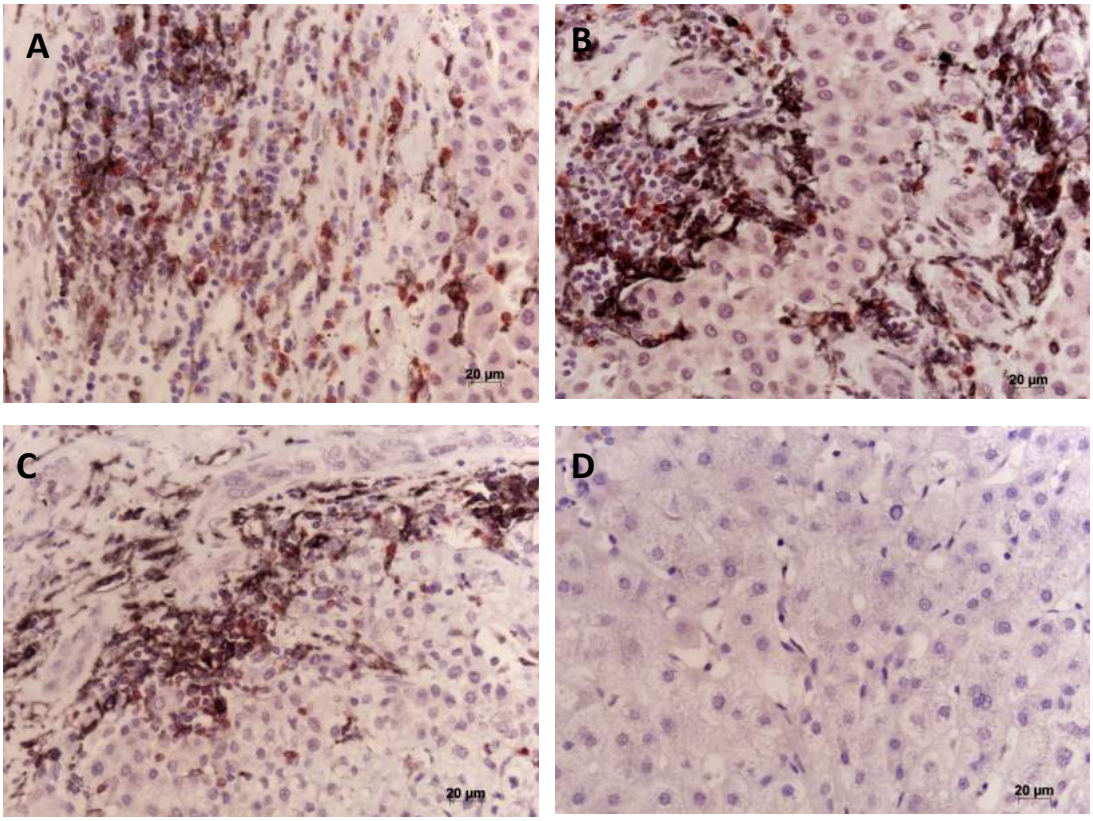

Figure 4-14 Localisation of DC and CD8$^+$T-cells within human HCC

(A,B,C) Dual immunohistochemistry staining for CD8 (red) and CD11c (black) expression in human HCC tumour. (D) Staining with isotype control. Scale bar represents 20 µm. Representative experiment of 3 replicates.

4.8 The effect of contact dependent DC-CD8$^+$T-cells interactions

For DC to activate T-cells, multiple signals need to be transmitted, including activation of the TCR, co-stimulatory signals and secretion of pro-inflammatory cytokines. In addition, the length of time DC spend in contact with T-cells also determine the fate of the T-cell [428]. To determine whether the amount of time tumour primed DC interacts with T-cells is critical for the induction of CD8$^+$Treg, the interactions of moDC with T-cells were assessed using a fluorescent live cell imaging technique. Similar to previous experiments, moDC were primed by either tumour-conditioned medium or matched non-tumour conditioned medium for 24 hours. In addition, a positive control of LPS activated moDC was used. Naïve CD8$^+$T-cells were isolated and labelled with CFSE to distinguish them from moDC. Following their priming, moDC were co-cultured with CFSE labelled naïve CD8$^+$T-cells and their interactions over 24 hours recorded using a Cell IQ imager. Images were captured at 5 minutes intervals and contact time between individual T-cells and moDC were calculated. Of note, differences in the microscopic appearance between the tumour and non-tumour conditioned medium primed moDC were immediately obvious. MoDC primed in matched non-tumour medium or LPS appeared to have the classical features of matured/activated DC, characterised by numerous surface protrusions (Figure 4-15). In comparison, moDC that had been primed by tumour-conditioned medium remained round, suggesting a more immature phenotype.

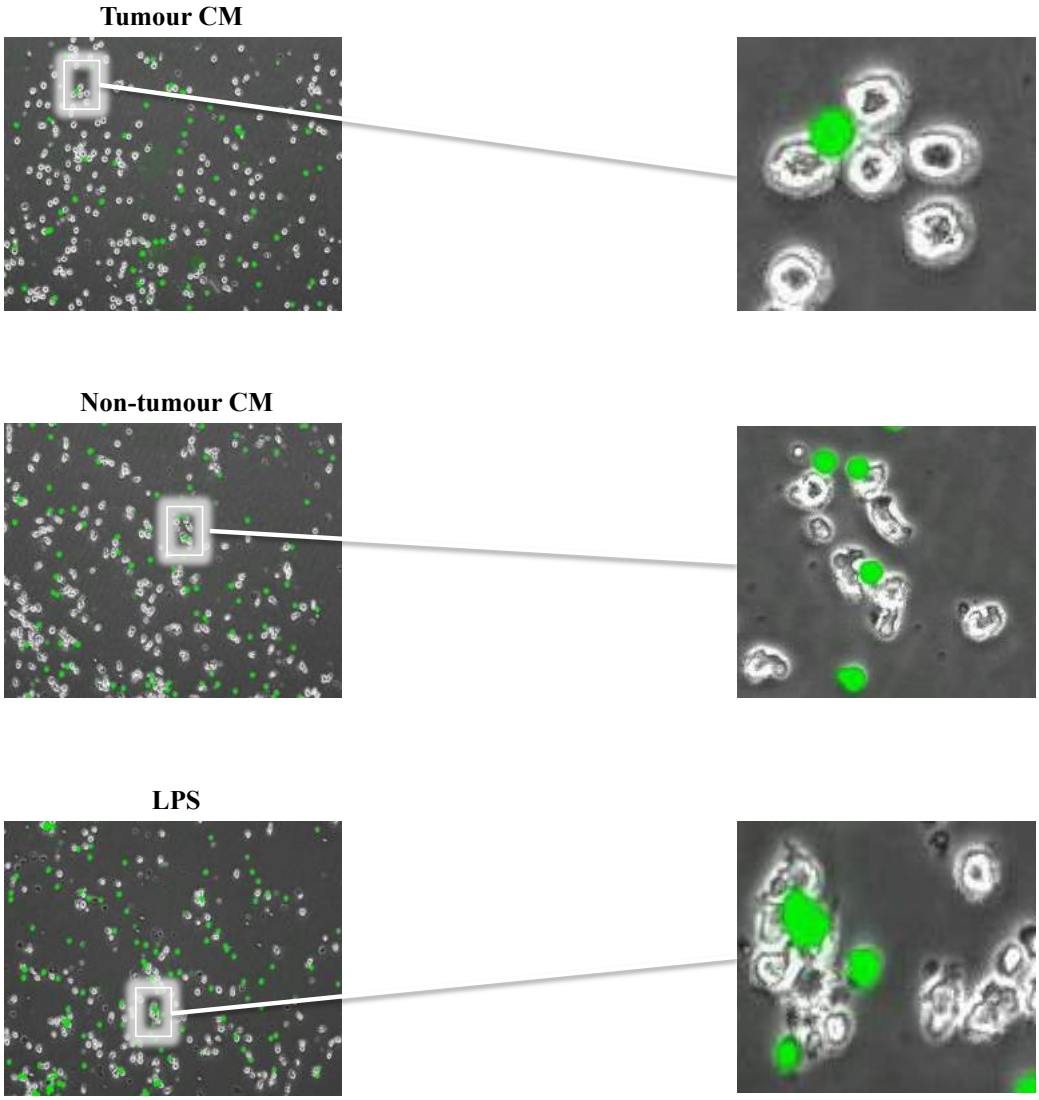

Figure 4-15 Analysis of DC-T-cell interactions using the cell IQ imager

Naïve CD8$^+$T-cells were labelled with CFSE **(green cells)** and co-cultured with tumour or matched non-tumour conditioned medium (CM) primed moDC or LPS primed moDC **(grey cells)**. Images were captured using the cell IQ imager at 5 minutes intervals. Representative images from two experiments showing T-cell-moDC interaction and their microscopic appearance at 1 hour post culture.

On analysing the time moDC spend in contact with naïve CD8$^+$T-cells, LPS primed moDC had a significantly higher contact time with T-cells (Figure 4-16). This is in comparison to moDC that had been primed by either tumour-conditioned medium or matched non-tumour conditioned. Importantly, no differences were seen in the interaction time of CD8$^+$T-cells with moDC that had been primed with tumour or matched non-tumour conditioned medium. Hence, interaction time between moDC and CD8$^+$T-cells appear not to be involved in the induction of CD8$^+$Treg.

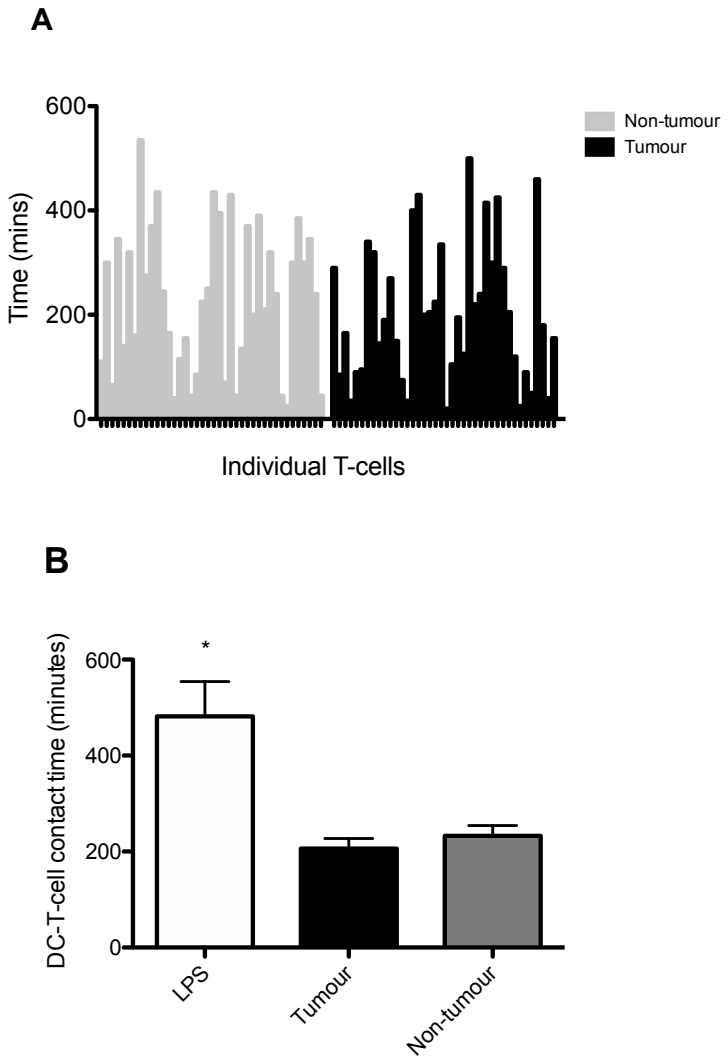

Figure 4-16 Interaction time between DC and CD8$^+$T-cells

Naïve CD8$^+$T-cells were labelled with CFSE and co-cultured with moDC that had been primed by tumour or matched non-tumour conditioned medium for 24 hours. The interaction time between individual CD8$^+$T-cells and moDC were captured at 5 minutes intervals over a 24 hours period and analyse. (A) Graph showing individual DC-T-cells contact time. (B) Data from a total of 120 observed cell-to-cell interactions in two separate experiments. Data are expressed as mean and standard error and statistical significance was tested using the Mann Whiney U test *$p<0.05$.

4.9 Indoleamine 2,3-dioxygenase-dependent induction of CD8$^+$Treg

Having demonstrated that cell-to-cell contact appears to be involved in the induction of CD8$^+$Treg, but that the interaction time between CD8$^+$ T-cells and DC is not critical. The expression of co-stimulatory molecules (CD80, CD86) on DC seems to be also affected by HCC tumour conditioned medium. However, DC that had been cultured in medium alone also displayed reduced expression of co-stimulatory molecules, but did not possess the same ability to induce CD8$^+$Treg, suggesting tumour primed DC must exploit additional factors for the induction of CD8$^+$Treg. Studies of a wide range of human tumours have implicated multiple regulatory molecules in the generation of a suppressive tumour environment [429], including iNOS, agrinase, IDO, TGF-β and IL-10. It is likely some of theses molecules are involved in the induction of CD8$^+$Treg seen within HCC tumours and in the experimental models used in this study. To investigate the involvement of theses molecules, moDC primed with tumour-conditioned medium were co-cultured with naïve CD8$^+$T-cells in the presence of neutralising antibodies or inhibitors of each of theses candidate molecules. The effects on the blocking of theses molecules on CD8$^+$Treg induction was assessed by flow cytometry. Inhibition of each of the candidate molecules did not affect the ability of tumour-primed moDC to induce CD8$^+$Treg, except for IDO. When naïve CD8$^+$T-cells were cultured in the presence of tumour primed moDC and the IDO inhibitor 1-methyl-tryptophan (1-MT), the percentage of CD8$^+$CD25^{high}CD127^{low}T cells induced was significantly reduced and in a dose dependent manner (Figure 4-17).

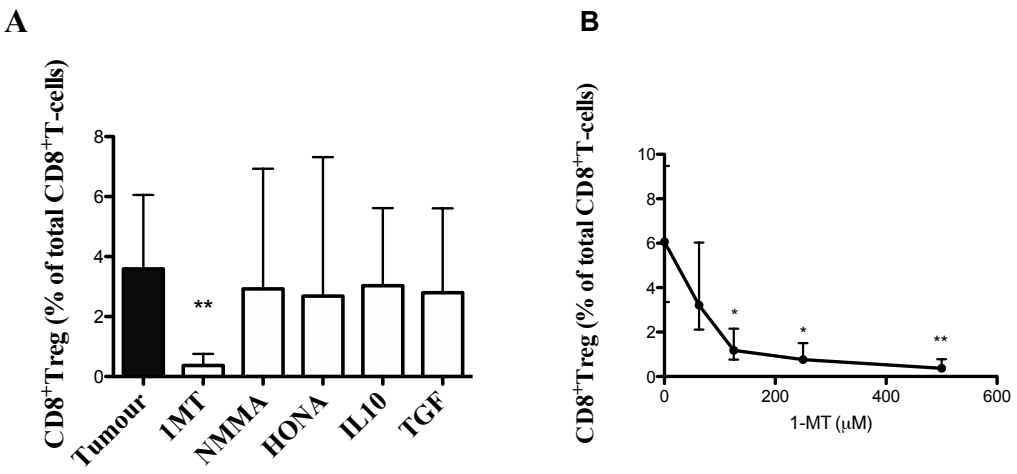

Figure 4-17 The effect of IDO on the induction of $CD8^+CD25^{high}CD127^{low}$T-cells by moDC

(A) Naïve $CD8^+$T-cells were cultured with tumour conditioned medium primed moDC in the presence or absence of neutralising monoclonal antibodies/inhibitor against IL-10 (5μg per mL), TGFβ (10μg per mL), against IDO by 1-MT (500 μM), iNOS by NMMA (300μM) and arginase by HONA (500μM). (B) The role of IDO in the induction of $CD8^+CD25^{high}CD127^{low}$T-cells by tumour conditioned medium primed moDC was confirmed to be dose dependent by titration of the inhibitor 1-MT. Representative data from five replicate experiments. Data are expressed as median and inter-quartile range and statistical significance was tested using the Mann Whiney U test vs. tumour conditioned primed moDC. *$p<0.05$, **$p<0.01$. Indoleamine 2,3-dioxygenase; IDO, 1-MT; 1-methyl-tryptophan, iNOS; inducible nitric oxide synthases, interleukin-10; IL-10, Transforming growth factor beta; TGF-β, nonselective nitric oxide synthase inhibitor; NMMA, N-hydroxy-L-arginine; HONA.

4.10 Presence of IDO in HCC

The enzyme indoleamine-2,3-dioxygenase (IDO) has been increasingly recognized to play an important role in immune modulation [430]. In particular, IDO has been linked to tumour progression through the inhibition of effector immune cells [431, 432] and recruitment and induction of CD4$^+$Treg [433]. The expression of IDO has been shown to be elevated within the tumour environment in a number of different human cancers and has been associated with disease stage [434, 435]. To confirm the role of IDO in human HCC, the expression of IDO within tumour and matched non-tumour tissue were compared at a protein level by Western blot. Assays were performed with protein obtained from snap frozen matched tumour and non-tumour HCC tissues. Although IDO was shown to be expressed in tumour and non-tumour tissues, semi-quantification using Image J software demonstrated increased IDO expression in tumour tissues when compared to matched non-tumours (Figure 4-18).

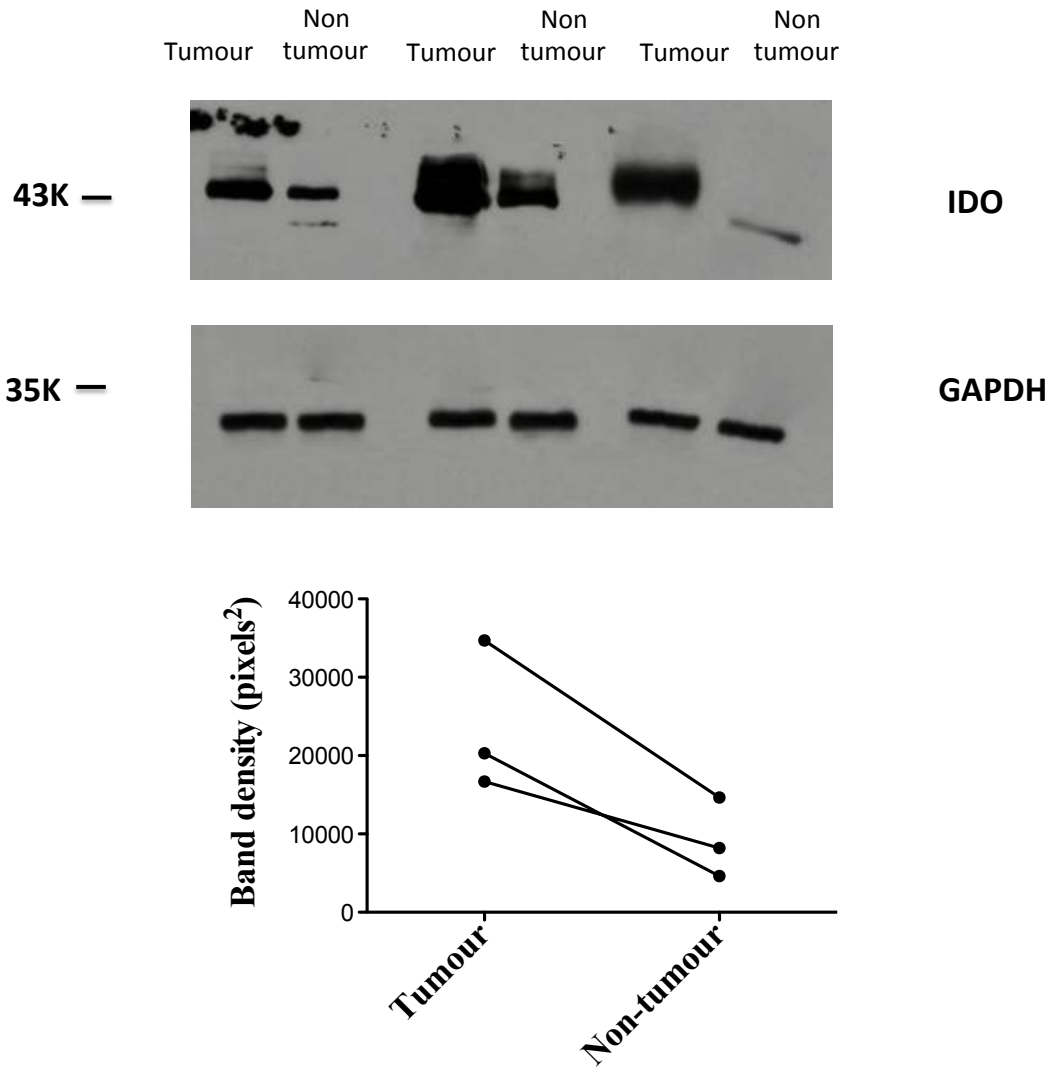

Figure 4-18 Expression of IDO in HCC

Protein lysates were isolated from HCC and matched non-tumour tissues and analysed for the presence of IDO by western blot. GAPDH was used as control. (n=3)

Given the demonstration of increased expression of IDO within HCC tissues when compared to non-tumour tissues we went on to determine if this could be attributed, at least in part, to production by tumour infiltrating DC. Multi-colour immunofluorescence staining was used to analyse the presence of IDO in tumour infiltrating DC. Co-localisation of the DC marker CD11c and IDO was demonstrated on tumour infiltrating DC, in human HCC tissues (Figure 4-19). To further quantify the effect of HCC on IDO production by DC, the expression of IDO on tumour or matched non-tumour primed moDC were compared by intracellular staining and flow cytometry. The expression of IDO was increased in moDC following priming with tumour-conditioned medium when compared to moDC primed with matched non-tumour conditioned medium (Figure 4-20).

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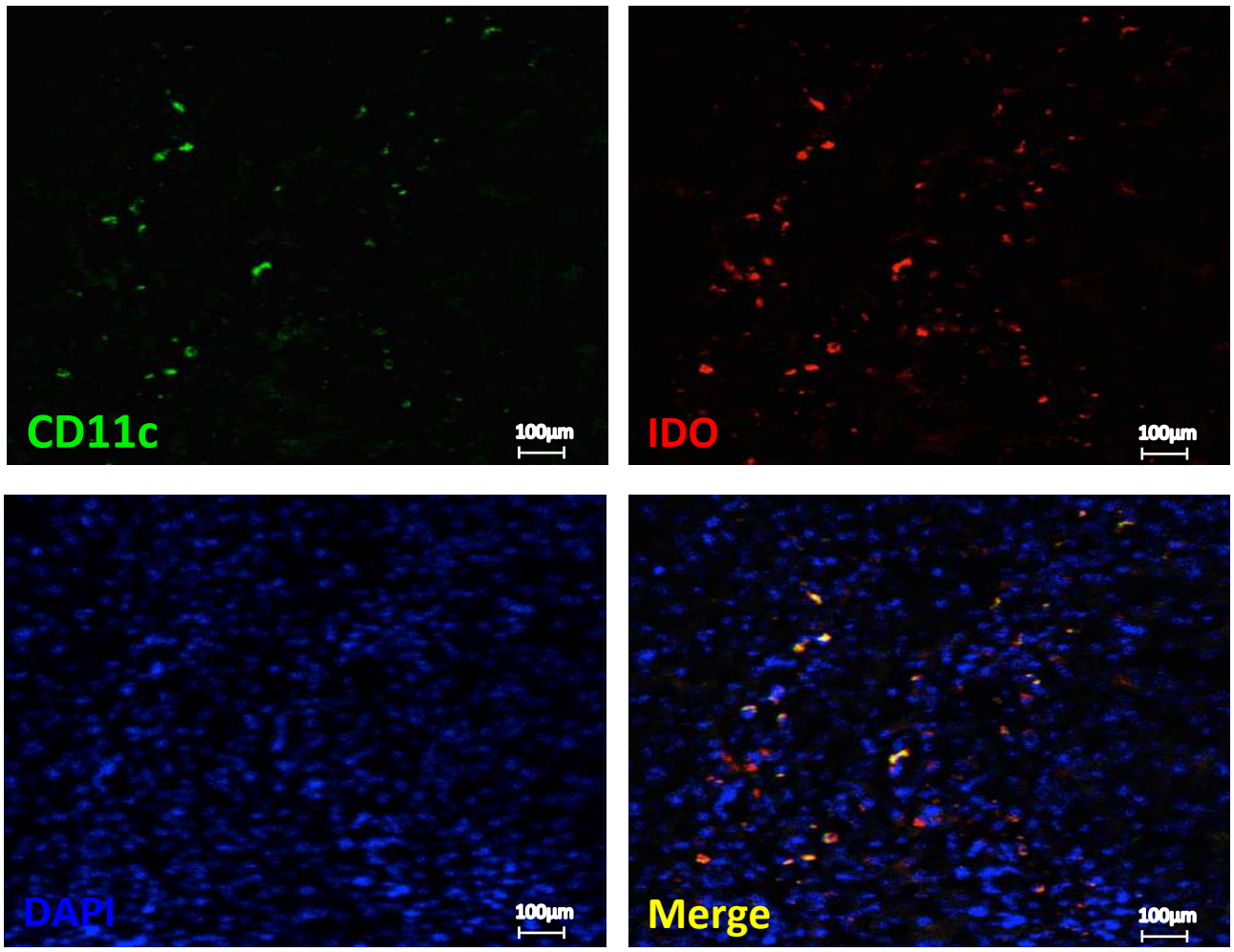


Figure 4-19 Staining of IDO in human HCC

Immunofluorescence staining of snap frozen HCC tumour tissue for co-expression of CD11c (green), IDO (red) and nucleus (DAPI:blue) in human HCC tumour sections**.** Scale bar represents 100 μm. Representative data from 3 experiments.

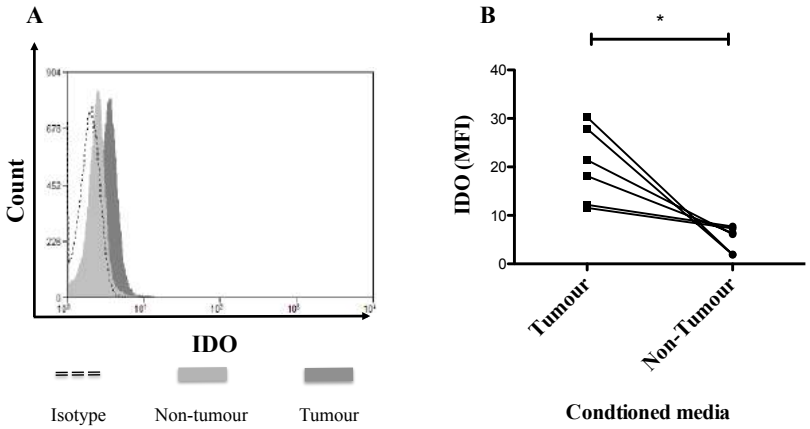


Figure 4-20 IDO expression on tumour conditioned DC

MoDC were primed with tumour or matched non-tumour conditioned medium for 24 hours. Followed by the analysis for they expression of IDO by intracellular staining and flow cytometry. MoDC were identified by forward (FSC) and side scatter (SSC) and determined to be CD14 negative. (A) A representative histogram on the expression of IDO by moDC. (B) Data from five separate experiments, showing the expression of IDO on tumour or matched non-tumour primed moDC. Data are expressed as median fluorescent intensity and statistical significance was tested using the two-tailed Wilcoxon matched pairs test. *$p<0.05$.

4.11 IL-6 level in tumour-conditioned medium.

Having confirmed a role for IDO in the induction of CD8$^+$Treg by tumour-conditioned medium primed moDC, we next assessed the content of conditioned medium for the presence of other soluble factors that may influence DC phenotype.

To define possible candidate soluble factors involved in the modulation of DC, a cytokine array kit was used to measure differences in relative levels of multiple cytokines, chemokines, and acute phase proteins between tumour and non-tumour conditioned medium.

IL-6 was noted to be significantly elevated in tumour-conditioned medium when compared to matched non-tumour conditioned medium (Figure 4-21). Suggesting IL-6 may be a possible target that may be responsible for the modulation of DC.

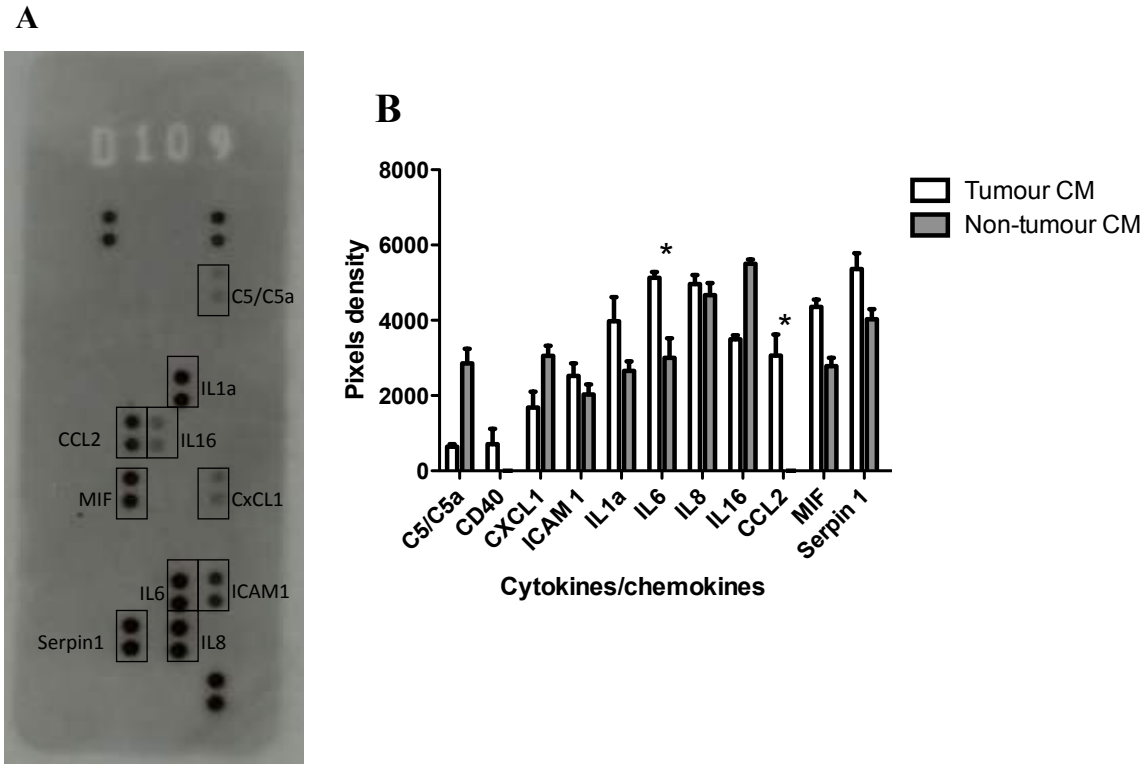


Figure 4-21 Measurement of IL-6 in tumour conditioned medium

The presence of 36 different cytokines, chemokines, and acute phase proteins were measured by a commercial cytokine array kit in tumour and matched non-tumour conditioned medium samples. (A) A representative cytokine array film developed following incubation with tumour conditioned medium. (B) The spot density was measured by image-J in 2 sets of matched tumour and non-tumour condition medium samples. Data are expressed as median and inter-quartile range and statistical significance was tested using the two-tailed Wilcoxon matched pairs test. *$p<0.05$.

4.12 IL6 is not critical for the generation of tolerogenic DC

To ascertain whether IL-6 was contributing to the priming of tolerogenic DC, moDC were cultured in the presence of tumour-conditioned medium plus the addition of increasing concentrations of an IL-6 neutralising antibody. The expression of maturation markers on tumour primed DC were measured by flow cytometry. The addition of IL-6 blockade did not statistically alter the expression of MHC-I, MHC-II, CD80 or CD86 (Figure 4-22).

IL-6 is a complex cytokine; it can be present as a soluble form or surface bound to its receptors [436] and can induce the differentiation of immune cells along two defined pathways. The classical pathway involves the activation of membrane bound IL-6 receptor. Alternatively, IL-6 can exert is action via the trans-signalling pathway, though the activation of soluble IL-6 receptor. Importantly, the trans-signalling pathway has been shown to be a significant component of inflammation driven tumourgenesis [437, 438]. Consequently, the blocking of IL-6 alone may not be adequate to fully neutralise its effect. With this in mind, an additional series of experiments was performed utilising an IL-6 receptor neutralising antibody to block the activity of IL-6. DC were cultured in the presence of tumour-conditioned medium plus the addition of increasing concentration of an IL-6 receptor neutralising antibody. The expression of maturation markers on tumour primed DC were once again measured by flow cytometry (Figure 4-23). Inhibition of IL-6 receptor had no statistically additional effect on the expression of maturation markers on tumour primed DC, suggesting the presence of IL-6 in tumour conditioned medium is not a critical component for the induction of tolerogenic moDC.

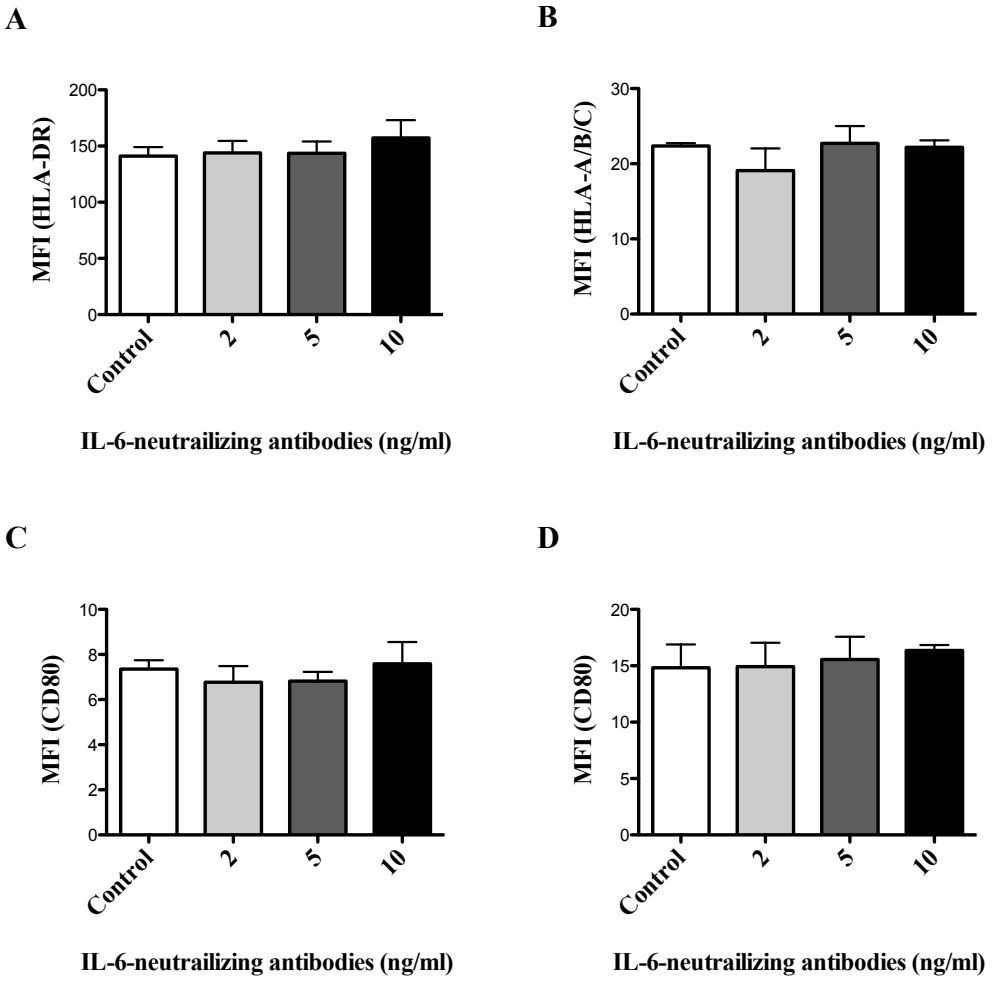


Figure 4-22 The effect of IL-6 blockade on tumour conditioned medium primed moDC

MoDC were primed with either tumour conditioned medium in the absence or presence of increasing concentrations of IL-6 neutralizing antibodies for 24 hours. The expression of (A) MHC-I, (B) MHC-II, (C) CD80 and (D) CD86 were analysed by flow cytomtery. Results are from 3 replicate experiments and data are expressed as median and inter-quartile range. Statistical significance was tested using the Mann Whiney test compared to control (tumour conditioned primed moDC). MFI; median fluorescence intensity.

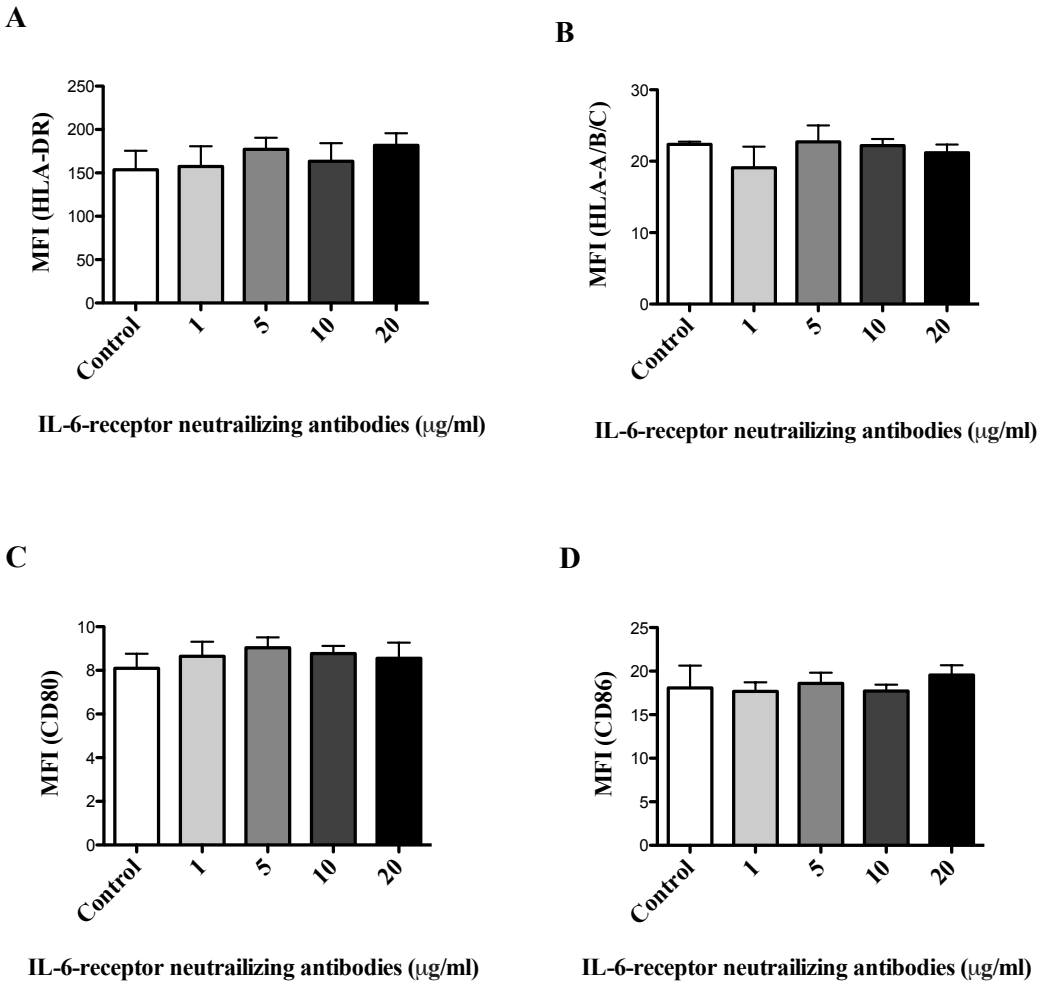


Figure 4-23 The effect of IL-6 receptor blockade on tumour conditioned medium primed moDC

MoDC were primed with either tumour conditioned medium in the absence or presence of increasing concentrations of IL-6 receptor neutralizing antibodies for 24 hours. The expression of (A) MHC-I, (B) MHC-II, (C) CD80 and (D) CD86 were analysed by flow cytomtery. Results are from 3 replicate experiments and data are expressed as median and inter-quartile range. Statistical significance was tested using the Mann Whiney test compared to control (tumour conditioned primed moDC). MFI; median fluorescence intensity.

4.13 Discussion

The CD4$^+$Treg population is formed of both naturally occurring and induced regulatory T-cells [439-441]. In comparison, most previous studies have suggested induction as the predominant route for CD8$^+$Treg development [229, 230]. The low frequency of CD8$^+$Treg present within the peripheral blood and non-tumour tissues, compared to those seen in tumour tissues of patients with HCC, plus the static level of circulating CD8$^+$Treg despite HCC progression, points towards an active role of the tumour microenvironment in the generation of CD8$^+$Treg.

A number of different mechanisms have been proposed to be involved in the induction of CD8$^+$Treg. In the current study, attempts were made to induce CD8$^+$Treg by culturing naïve CD8$^+$T-cells with HCC conditioned medium with or without TCR activation with CD3/CD28 beads. However, the resulting population was predominately composed of newly activated effector cells. Initially, this result was unexpected as multiple studies have previously demonstrated the ability to generate CD4$^+$Treg and CD8$^+$Treg *ex-vivo* by a combination of TCR activation with the presence of tumour supernatant or suppressive cytokines such as IL-10 and TGF-β [231, 442]. However, unlike the current study, the majority of these studies employed sub-physiological concentrations of cytokine to induce the Treg. This may explain the inability in the current study to induce CD8$^+$Treg directly with tumour-conditioned medium alone, which more accurately recapitulates the physiological microenvironment.

The data in this chapter highlights the importance of DC interaction with tumour cells within HCC in the generation of CD8$^+$Treg. The interaction of APC with T-cells has been proposed by others to be involved in the induction of CD8$^+$Treg [204, 228]. *In vitro* studies designed to assess the modulating effect of HCC on immune cells has shown DC that have been incubated with human hepatoma supernatant were able to induce a subset of CD4$^+$Treg [274]. Clinical studies in patients with solid organ cancers, including HCC, demonstrated the frequencies of DC with a tolerogenic phenotype correlated with an increase in circulating CD4$^+$Treg [443, 444]. Importantly, the role of CD8$^+$Treg induction in tumours has recently been demonstrated in colorectal and ovarian cancer [204, 228]. Wei et al., demonstrated that DC isolated from malignant ascitic fluid removed from ovarian cancer patients was able to induce CD8$^+$T-cells that secreted IL-10. Importantly, these induced CD8$^+$T-cells were able to suppress effector cell proliferation. However, the study lacked data on CD8$^+$Treg phenotype.

Despite increasing evidence suggesting the possible role of DC in Treg induction, the precise mechanisms involved remain unclear. DC are essential in the generation of appropriate immune responses against a wide array of harmful pathogens and cancer cells. However, data from previous studies has suggested tumours are able to disrupt the function of DC and may even have to ability to 'hijack' DC to aid immune evasion and Treg induction. A possible mechanism involved is the disruption of DC function though the down regulation of co-stimulatory molecules on DC and a reduction in pro-inflammatory cytokine production [73]. This leads to inadequate stimulation of T-cells resulting in the induction of Treg. Support for such a hypothesis

stems from the identification of blood derived and tumour infiltrating DC from HCC patients that lack maturation markers such as MHC molecules and co-stimulatory molecules [270-272, 426]. These DC are impaired in their inability to induce T-cell proliferation and produce pro-inflammatory cytokines [274]. In addition, DC isolated from HCC patients acquire tolerogenic properties though the production of suppressive cytokines such as IL-10 and TGF-β [445, 446]. Data from this chapter demonstrated similar results, with the ability of HCC to interact with DC, resulting in the lack of expression of maturation markers and production of the pro-inflammatory cytokine IL-12. However, no significant up-regulation of IL-10 or TGF-β production was recorded. Also transwell experiments designed to prevent cell-cell contact demonstrated cell contact is essential in the induction of CD8$^+$Treg. Taken together, this data suggests soluble factors are unlikely to play a key role in CD8$^+$Treg induction in HCC, although there may still be a role for membrane bound IL-10 or TGF-β to induce their effects on naïve T-cells.

Increasing interest has been focused on the importance of the physical interaction between DC and T-cell in the initiation of immune responses. On engagement with T-cells, DC form an immunological synapse in which a wide array of signals (MHC molecules, TCR, co-stimulatory molecules) are involved in the activation of T-cells [447]. In addition, interaction of adhesion molecules present on the surface of both DC and T-cells (CD11a-ICAM-1) also plays an important role in maintaining the interaction between cells, leading to T-cell activation [448]. The contact time between DC and T-cells has been suggested to play a role in their functional outcome [428, 449]. However, in the current study, the length of interaction time between DC and T-

cell does not appear to differ between tumour or non-tumour primed DC and is therefore unlikely to be important in the generation of $CD8^+$ T-reg.

The enzyme IDO has been increasingly recognized to play an important role in immune modulation [430] via its contribution to the degradation of the essential amino acid tryptophan into is metabolite kynurenine. IDO has been suggested to affect immune responses in three distinct ways. Firstly, the degradation of tryptophan results in the disruption of a number of molecular stress response pathways, such as the general control nonrepressed-2 (GCN-2) and the mammalian target of rapamycin (mTOR) pathways, both of which are involved in immune cell activation. The disruption of such pathways by IDO, results in the inhibition of effector cell activation and importantly has been shown to induce Treg [450, 451]. Secondly, the accumulation of kynurenine, a major ligand for aryl hydrocarbon receptor (AhR), can induce Treg via the promotion of FOXP3 expression and priming of tolerogenic DC [452]. Lastly, IDO has recently been shown to provide a direct intracellular signal to DC leading to the triggering of regulatory pathways independent of tryptophan breakdown or kynurenine accumulation. However, despite increasing study suggesting the possible role of IDO in aiding tumour progression, conflicting evidences are emerging.

IDO can be detected in a wide array of tissues and cells including those associated with immune function and other non-immune cells. In particular IDO is highly expressed in a wide range of human tumours, including HCC, and importantly has been demonstrated to correlate with cancer patient's disease prognosis [453-458].

Contrary to such findings, a recent study in patients with HCC demonstrated that the presence of IDO within the tumour environment correlated positively with progression-free survival. Importantly, it was show that the cytotoxic activities of peripheral mononuclear cells isolated in these patients was directly proportionate to the level of IDO present and this was suppressed by blocking with 1-MT [459]. Similarly, the presence of IDO within human renal cell cancer correlated with better progression free survival [460]. In addition, further contradicting effect of IDO can be seen in study looking at the direct effect of IDO on tumour cell survival. It has been shown that the effect of IDO, can have a direct effect on prolonging tumour cells survival though tryptophan depletion and the built up of kynurenine metabolite. However, studies have demonstrated the opposite effect. In which IDO can inhibit tumours cells proliferation [461-463]. However, such studies often have their limitation, mainly due to association studies being a poor reflection on actual causation.

As stated previously, IDO can modulate the immune response though a number of different pathways and the current study has highlighted the potential for Treg induction in response to production of IDO by professional APC such as DC [452]. Importantly, recent work investigating the activation of IDO in tumour cells has suggested a reciprocal role of Treg. Mainly through the induction of tolerogenic DC by Treg expression of CTLA-4, resulting in the expression of IDO on DC [464]. In addition, a recent clinical study demonstrated IDO expression in tumour stromal tissues correlated with better response in patients treated with a CTLA-4 blocking antibody [465], further supporting the possible interaction between Treg and IDO.

A direct result of the increasing evidence demonstrating the role of IDO in the disruption of anti-tumour immune response is the development of studies focusing on the therapeutic potential of IDO blockade as a cancer treatment. Early rodent studies suggest IDO blockade using the enzyme inhibitor 1-MT is able to delay tumour growth [431, 454]. Several IDO inhibitors are now in early phase clinical trials as treatments for human cancer, including molecules which have a higher IDO blocking ability compared to 1-MT [466]. Studies supporting the use of IDO blockade in the attempt to reverse tumour suppression appear promising. However, our current understanding on how IDO affect tumour immune response remains limited. It is proposed that the blockade of IDO may in effect reverse the immune suppression seen in tumour progression. Such hypothesis was based on one of the pivotal study, demonstrating IDO induced decrease in tryptophan resulted in inhibition of T-cell proliferation [467]. However, once again conflicting data exists, in which one study demonstrated that despite total depletion of trytphan, no suppression of immune cell proliferation was seen [468]. It has been agreed that such differences seen in these studies can been related to the differences in experimental condition. It is plausible that in-vitro experiments are unlikely to mimic one seen in in-vivo experiments. In which the tryptophan level is unlikely to be depleted to a similar level when compared to in-vitro condition. Recent data has emerged questioning the actual involvement of IDO in tumour suppression. As already mentioned increasing interest has been put on the use of 1-MT as a potential anti-cancer treatment. It is hypothesis the blockage of IDO by 1-MT results in the reversal of tumour immune suppression. However, recent studies has suggested the anti-tumour effect seen in the use of 1-MT, may actually be

independent of IDO. Agaugue et al. demonstrated in a murine model that the affect of 1-MT on DC function was depending on the quality of DC maturation through TLR and the effects of 1-MT were independent to IDO activity [469]. In addition, the DC matured through TLR4 pathway in the presence of 1-MT resulted in the generation of IL5 and IL13 secreting T-cells and not IFN. Such effects maybe detrimental in the setting of anti-tumour response due to the skewing towards a Th2 response. It was suggested by the authors, the IDO independent effect of 1-MT might be the result of disruption of trytphan uptake or metabolism by DC. However, further studies are required to unravel such findings. To further add uncertainty into the actual mechanism of how 1-MT may reverse tumour immune suppression, a biochemical identical enzyme of IDO; tryptophan-2,3-dioxygenase (TDO) are also present in human cancer [470]. However, TDO cannot be inhibited by 1-MT. Hence, adding further evidence to suggest that the affect of 1-MT maybe independent on is inhibition on IDO.

Putting the data against the role of IDO on the generation of a suppressive tumour environment aside. The suitability of 1-MT as a treatment for cancer has also been questioned. In addition to the potential Th2 skewing effect of 1-MT on DC mentioned above, the actual potency of 1-MT inhibition on IDO has also been questioned. 1-MT, a competitive inhibitor of IDO exists in two isomers: L-1MT and D-1MT. The two isomers appear to act differently depending on the cells types and cell compartment [471]. The main effect of L-1MT appears to be against cell free IDO activity and IDO secreting cell lines. While D-1MT appears to inhibit tryptophan degradation on IDO expressing mDC. Due to the effect of D-1MT on immune cells, it has been commonly

used as the isomer of chose for clinical studies. However, with the advances in science much potent inhibitor of IDO has been developed. In particular the use of siRNA to suppress IDO expression at a genomic level. The use of siRNA has enormous advantage over chemical inhibitor such as 1-MT. Mainly through the avoiding of off target effect as seen in the use of 1-MT as discussed above.

Additional studies have proposed the suppression of anti-tumour immune responses may involve other enzymatic pathways. The enzyme arginase-1 has been implicated mainly in the suppression and function of MDSC [472], but recent evidence has supported a role for this molecule in the induction of tolerogenic properties in DC [473]. Similarly to IDO, arginase-1 results in the activation of GCN-2 via the degradation of the amino acid arginine, leading to the disruption of effector cell activation and induction of regulatory T-cells [474]. Arginase-1 has been shown to be present on DC following culture with retinoic acid leading to the induction of regulatory T-cells [473, 475]. The possible role of arginase-1 in the induction of regulatory T-cells was further supported by the demonstration of FOXP3 positive cells with co-expression of arginase [476]. Similarly, the enzyme nitric oxide synthase, though the production of nitric oxide, has been implicated as another potential candidate in the induction of regulatory T-cells [477]. However, unlike IDO neither of these enzymes was shown to be involved in CD8$^+$Treg in the current study.

After confirming the involvement of IDO in the induction of CD8$^+$Treg, an attempt was made to investigate what component of tumour-conditioned medium resulted in the generation of tolerogenic DC. Using a cytokine array, increased secretion of IL-6

was identified, which, given its wider effects may have been responsible for generating the tolerogenic phenotype and function of tumour primed DC. IL-6 has been described to be associated with the pathogenesis of HCC and an increased risk of HCC development [78, 79, 83, 478]. More recently IL-6 has been shown to be involved in the disruption of DC maturation [80, 479, 480]. In addition, a study of human colorectal tumours demonstrated the serum concentration of IL-6 correlated with the presence of tumour infiltrating CD8$^+$Treg [228]. Importantly, the study demonstrated the ability to induce CD8$^+$Treg *ex-vivo* in the presence of IL-6. However, no assessment of the suppressive function of the induced CD8$^+$Treg was performed.

In the current study, despite blocking both soluble IL-6 and its receptor, the imprinting of a tolerogenic phenotype on DC by tumour-conditioned medium was not affected. This contradicted the afore mentioned studies which demonstrated the immune-tolerogenic effect of IL-6 on DC and with the confirmation of an elevated level of IL-6 in tumour conditioned medium. Whilst the demonstration of IL-6 involvement in the induction of CD8$^+$Treg would have been a satisfactory explanation for the modulating effect of HCC, the inability to identify the components of a secretome responsible for DC disruption, emphasises the complexity of the tumour microenvironment. This also highlights the limitation of the use of tumour-conditioned medium to mimic what occurs within tumours. However, steps were incorporated into the experimental design, in an attempt to minimize the possible confounding factors, commonly seen in the use of conditioned medium. Firstly, conditioned medium was obtained by culturing tissues in serum free conditions to

minimise the recognition of serum components and constituents of the secretome [481]. Secondly, fresh human tumour slices and not cell lines were used to generate conditioned medium. This has the advantage of investigating the interaction between different cells within the tumour microenvironment and not just a single cell. Lastly, studies of HCC are often confounded by the presence of underlying liver disease, in particular viral liver diseases. It is therefore difficult to distinguish between the effects of the viral disease and those resulting from the tumour. To limit such effect, conditioned medium was generated only from tumour samples obtained from patients with non-viral liver disease. There are however other factors that were not possible to control, such as hypoxic conditions. In addition, a positive control wasn't added to the experiments to confirm the potency of the neutralising antibodies used. Finally, the concentration of the conditioned medium may not mirror the *in-vivo* picture where the interplay between different organ systems will result in a complex secretome.

In addition to IL-6, the chemokine CCL2 was also presence at a higher level within the tumour supernatant were compared to matched non-tumour supernatant. CCL2 also commonly know as monocyte chemoattractant protein-1 (MCP-1), is an important chemokines involved in the trafficking of monocytes and macrophages [482]. CCL2 expression was shown to be present in human HCC and has been suggested to correlate with prognosis [483]. Is effect has been suggested to be related to the tumour ability to recruit suppressive immune cells such as TAM and MDSC into the tumour environment. Hence, preventing anti-tumour immune cells response in the tumour [484, 485]. One of the main limitation of this current work, relate to lack of further experiments looking at the involvement of CCL2 on DC modulation or

Treg induction. With the development of therapeutic targeting against the CCL2 pathway, future work in this area is very attractive.

5 Functional capacity of CD8$^+$Treg

The data so far have demonstrated the presence of a subset of CD8$^+$Treg infiltrating human HCC, displaying a regulatory phenotype similar to other previously identified CD8$^+$ regulatory T-cells in human diseases, including cancer. In addition, DC has been demonstrated to interact with tumour cells in human HCC, in the induction of CD8$^+$Treg from naïve CD8$^+$T-cells.

It is, however, important to recognise that the current arrays of phenotypic markers we used to define regulatory cells are not 100% specific. Indeed, it is becoming increasingly accepted that some of these markers can be expressed on non-regulatory cells dependent on the microenvironment in which they are induced. To further confirm the regulatory role of the CD8$^+$Treg identified in the current study, their suppressive function needs to be confirmed.

5.1 Cytokine production of HCC infiltrating CD8$^+$Tregs

To confirm that the CD8$^+$Treg population identified in HCC tissue are true regulatory T-cells, their suppressive capacity was assessed. A number of previous studies examining regulatory CD8$^+$T-cells have demonstrated their ability to secrete suppressive cytokines and this has been proposed to be central to their suppressive function [486]. To enable the assessment of cytokine production in tumour infiltrating T-cells, mononuclear cells from human HCC were freshly isolated and stimulated by TCR activation. The ability of the T-cells to produce cytokine was demonstrated by

intracellular flow cytometry. Following stimulation, the cytokine production profile differed markedly between T-cell subsets isolated from tumours. CD8$^+$Treg secreted predominately the suppressive cytokine IL-10 and only produced a low level of the pro-inflammatory cytokine IFN-γ. This is in comparison to the CD8$^+$CD25^{low} T-cells (non-Treg), which secreted predominately IFN-γ and minimal IL-10 (Figure 5-1B & C). The production of the suppressive cytokine TGFβ was also measured on different subsets of CD8$^+$T-cells but this did not differ significantly between CD8$^+$Treg and non-Treg cells (Figure 5-1D & E) (Figure 5-2).

This suggests, in addition to the induction of CD8$^+$Treg, the tumour environment may enhance Treg function whilst at the same time adversely affect the function of responder cells.

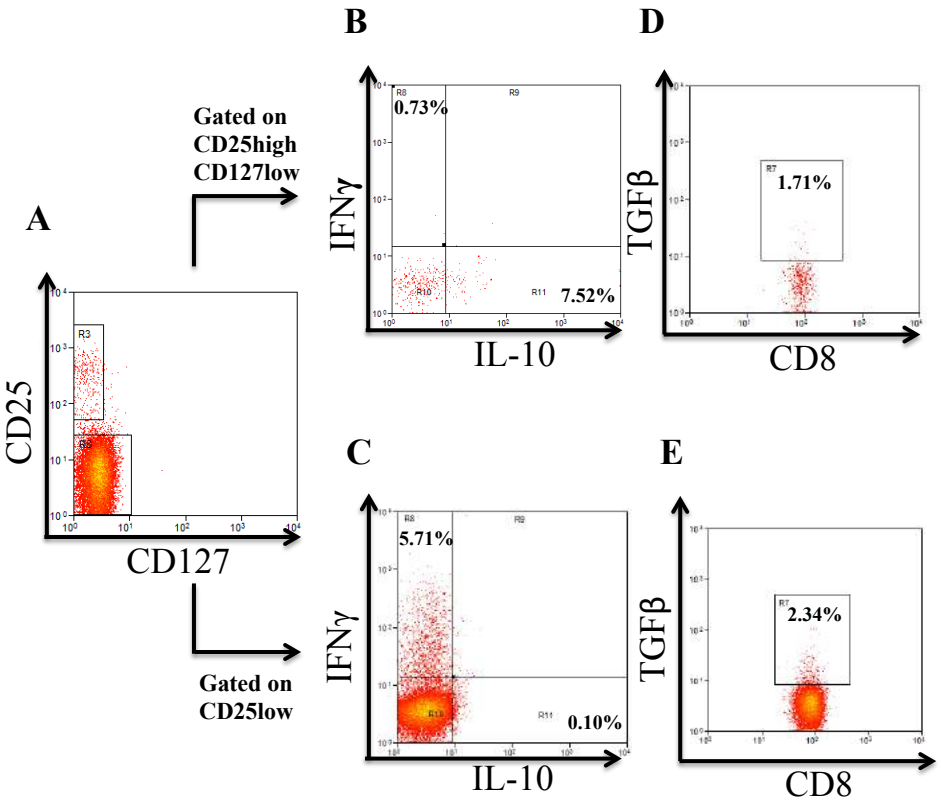


Figure 5-1. Flow cytometric analysis of cytokine production from HCC-infiltrating CD8$^+$T-cells

Mononuclear cells were isolated from HCC tissues and stimulated by TCR activation (Cytostim) before the expression of IL-10, IFN-γ and TGFβ on T-cell subsets was analysed. (A) Cells were gated on CD3$^+$CD8$^+$ and defined as either regulatory or effector based on expression of their CD25 and CD127. (B) CD8$^+$Treg produced IL-10 in preference to IFN-γ whilst in (C) CD8$^+$CD25^{low} T-cells (non-Treg) this pattern was reversed. TGF-β production did not differ significantly between either (D) CD8$^+$Treg or (E) CD8$^+$CD25^{low} T-cells. Representative data from 5 replicate experiments. Interleukin-10; IL-10, interferon gamma; IFN-γ, transforming growth factor beta; TGF-β.

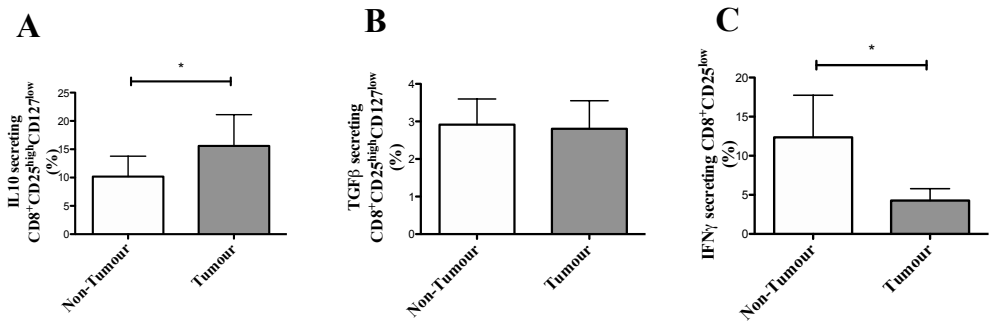


Figure 5-2 Measurement of cytokine secretion in HCC infiltrating CD8$^+$Treg by flow cytometry

Cytokine secretion by T-cells was assessed in cells isolated from three independent samples of HCC tumour and matched non-tumour tissue. MNC were isolated from matched tumour and non-tumour tissues and stimulated with cytostim. The expression of IL-10, IFN-γ and TGF-β on T-cell subsets was assessed by flow cytometry. Cells were gated on CD3$^+$CD8$^+$. CD8+Treg isolated from tumour tissue produced the suppressive cytokines (A) IL-10 and (B) TGFβ. Comparison of cells isolated from matched tumour and non-tumour samples demonstrated that tumour resident CD8$^+$Treg produced more IL-10 whilst no difference was seen in cells producing TGF-β. Conversely, the expression of the pro-inflammatory cytokine (C) IFN-γ by CD8$^+$CD25^{low} T-cells (non-Treg) was increased in non-tumour samples when compared with matched tumour samples. Data from 5 replicate experiments. Data are expressed as median and interquartile range. Statistical comparisons were made with Wilcoxon signed ranked test where *P<0.05. Mononuclear cells; MNC, interleukin-10; IL-10, interferon gamma; IFN-γ, transforming growth factor beta; TGF-β.

5.2 Optimization of a suppression assay

The hallmark of Treg is their ability to suppress the function and proliferation of responder T-cells (CD25^{low}T-cells). To assess the direct suppressive capacity of tumour infiltrating CD8$^+$Treg, a well-established suppression assay was employed. This assesses the ability of responder T-cells to proliferate in the present of regulatory T-cells. However, in a 'classical' suppression assay, a large number of regulatory T-cells are often required [487] which can be a major obstacle in research focussing on recovery of T-cells from tumour. This is due to the low frequency of cells isolated from tissues. For this reason, the assay was optimised for the use in situations when the number of regulatory T-cells is limited.

Suppression assays require the isolation of regulatory T-cells. When the number of cells used in the assay is small, the purity of the isolated cell population is essential, because contamination by other cells may have a significant effect on the final result. In the current study, to ensure the purity of the isolated tumour CD8$^+$Treg, cells were obtained by high-speed flow cytometric cell sorting **(**Figure 5-3**)**. This allows the isolation of highly purified populations of viable tumour infiltrating CD8$^+$Treg (>90%), whilst at the same time allowing the acquisition of phenotype data, hence maximising the use of limited HCC tissues.

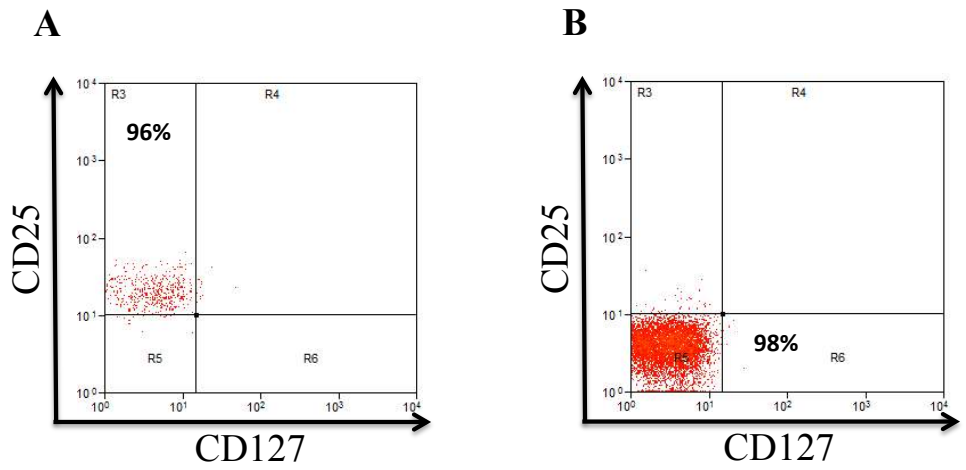


Figure 5-3 Cells isolated by flow cytometric sorting.

Representative dot plots showing cell purity on isolated (A) CD8$^+$Treg and (B) CD8$^+$CD25^{low} T-cells (non-Treg) following cell sorting.

The numbers of cells isolated from each tumour were variable, but usually low (between 5000-20,000 CD8$^+$Treg per gram of tissue). For a classical suppression assay, an average of 400,000 cells are often quoted to be required. In an attempt to increase the number of CD8$^+$Treg, the isolated cells were expanded using protocols developed for peripherally isolated CD4$^+$Treg [488, 489]. However, no significant expansion of tumour isolated CD8$^+$Treg was seen and the cultured CD8$^+$Treg often died following prolonged expansion (14 days). In an attempt to maintain viability, MNC isolated from tumour were expanded directly without prior isolation of CD8$^+$Treg, but again the numbers and viability of recovered cells remained low.

Due to the difficulties increasing the number of tumour infiltrating CD8$^+$Treg, attention was turned towards optimising the suppression assays for use with low numbers of cells. It is commonly quoted, 50,000 responder T-cells are required per well of a 96-well plate for suppression assays. Reduction in the number of responder T-cells will therefore directly reduce the number of Treg required. To ensure, a reduction in responder T-cells numbers will not effect T-cell proliferation, the proliferation capacity of different concentrations of responder T-cells was compared. Responder T-cells from healthy donors were first isolated by magnetic antibody cell selection methods and labelled with a cell trace dye to enable visualisation of proliferation by flow cytometry. Either 25,000 or 50,000 responder T-cells were transferred into each well of a 96-well round bottomed plate (to maximise the contact between CD8$^+$Treg and responder T-cells) before the cells were activated with CD3/CD28 beads (Dynal) or Treg suppressor beads (Miltenyi Biotec) to induced proliferation. Following 3 days of culture, the proliferation of T-cells was assessed by

flow cytometry. When compared, the proliferation of 25,000 responder cells was similar to the usual 50,000 cells (Figure 5-4), suggesting the reduction in responder T-cell number does not compromise the ability to proliferate and therefore the accuracy of the assay.

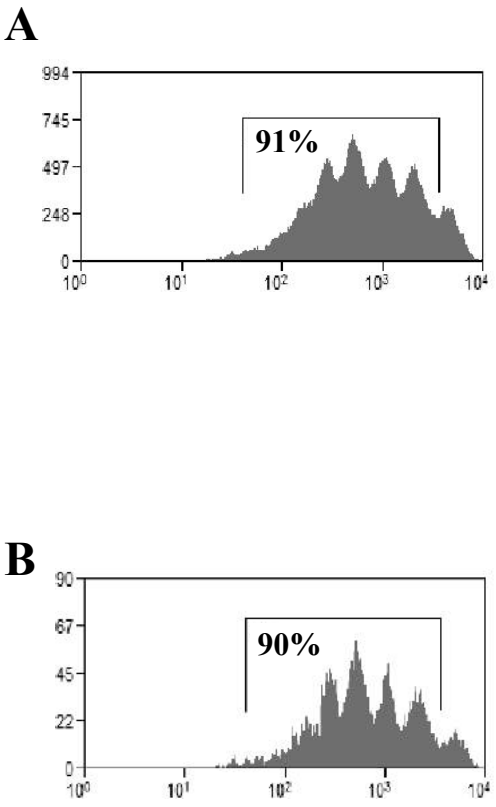


Figure 5-4 Optimisation of suppression assay

Responder T-cells ($CD3^+CD25^{low}$) were isolated by flow cytometric cell sorting from PBMC and labelled with Violet cell trace. The labelled cells were cultured in the presence of CD3/CD28 activating beads to induce proliferation for 3 days and proliferation was determined by dilution of violet cell trace by flow cytometry. Representative histograms showing responder T-cells cultured at (A) 50,000 cells per well and (B) 25,000 cells per well. Cell proliferation are shown as percentage. Representative of 3 replicate experiments.

The initial optimisation of the suppression assay using CD3/CD28 Dynal beads to induce T-cell proliferation was unsuccessful (Figure 5-5 A & C). No suppression of responder T-cells was seen even at a 1:1 Treg to responder T-cells ratio. Dynal beads are commonly used to induce T-cell expansion and activation for downstream experiments and are large (4.5 μm) particles, which in this setting seem to induce intense supra-physiological stimulation of responder T-cells, which appeared to overcome Treg suppression.

In an attempt to overcome the problem of excessive stimulation/activation and the inability to suppress T-cell proliferation, when Dynal beads was used to drive responder T-cell proliferation, an alternative method was assessed. Treg inspector beads (Miltenyi Biotec) have been specifically developed for use in suppression assays to induce T-cell proliferation. The beads consist of a cocktail of CD3, CD2 and CD28 activating antibodies adsorbed onto 50 nm particles. Using Treg inspector beads, it was possible to demonstrate the suppressive capacity of peripherally isolated CD4$^+$Treg. However, this did not reach statistical significant (Figure 5-5).

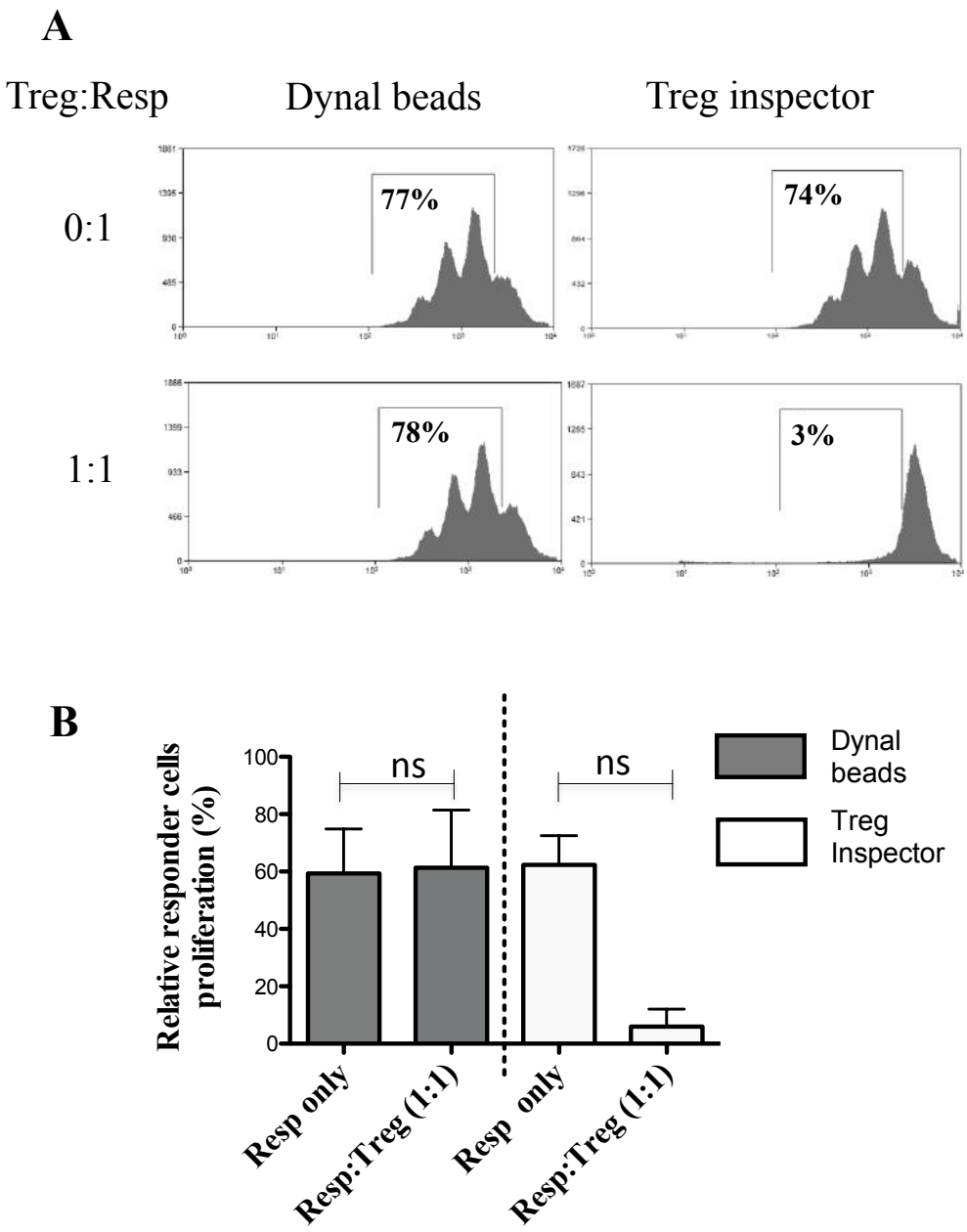


Figure 5-5 Comparison of Dynal beads and Treg inspector

$CD4^+$Treg ($CD4^+CD25^{high}CD127^{low}$ T-cells) and responder T-cells ($CD3^+CD25^{low}$) were isolated by magnetic antibody cell sorting from PBMC. Responder T-cells were labelled with Violet cell trace and cultured in the presence of Dynal CD3/CD28 activating beads or Miltenyi Biotec Treg inspector beads at an 1:1 bead to cell ratio to induce proliferation for 3 days. Cells were cultured either alone or in the presence of

CD4$^+$Treg at a 1:1 (responder:Treg) ratio. Proliferation of responder T-cells was determined by dilution of violet cell trace. Cells undergoing proliferation is shown as a percentage. Representative flow cytometric histograms showing responder T-cell proliferation cultured alone with either dynal beads or Treg inspector or in the addition of Treg at a 1:1 Treg:responder ratio. (B) The suppression capacity of Treg was compared in the presence of either dynal beads or Treg inspector. Data are expressed as mean and standard deviation. Statistical comparisons were made with Wilcoxon signed ranked test. Data were obtained from 3 replicate experiments.

5.3 CD8$^+$Treg isolated from HCC are functionally suppressive

Having finalised the optimisation of the suppression assay, the suppressive function of tumour infiltrating CD8$^+$Treg was assessed. Following isolation by flow cytometric cell sorting, HCC derived CD8$^+$Treg were cultured with allogenic responder T-cells at a 1:8 Treg:responder ratio in the presence of Treg inspector beads for 3 days. In the presence of tumour infiltrating CD8$^+$Treg, the proliferation capacity of responder T-cells was significantly reduced (Figure 5-6A). The suppressive ability of tumour infiltrating CD8$^+$Treg was comparable to tumour infiltrating CD4$^+$Treg isolated from the same tumours (Figure 5-6B) confirming tumour-infiltrating CD8$^+$CD25^{high}CD127^{low}T-cells are true regulatory T-cells.

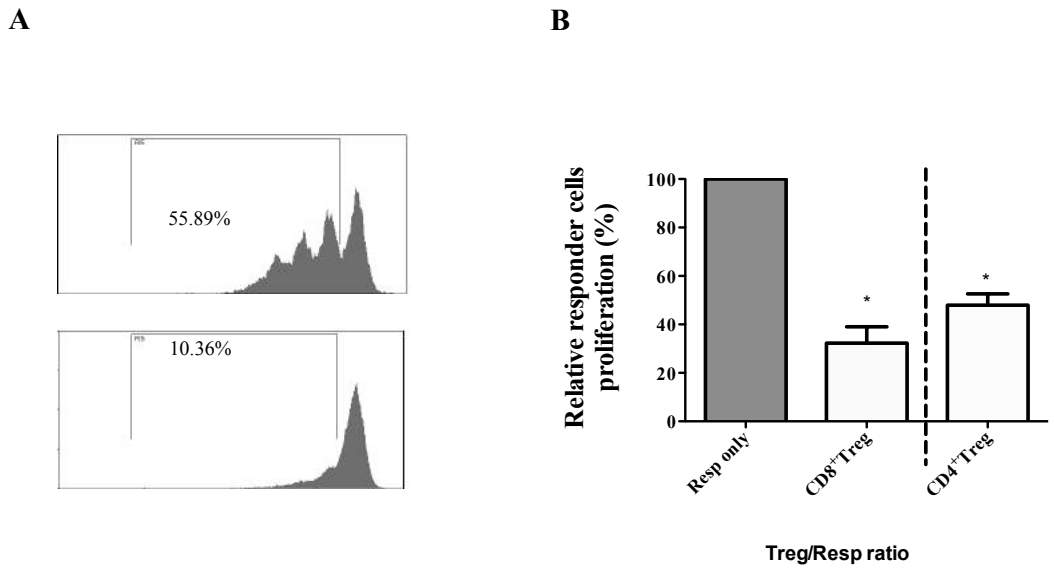


Figure 5-6 Suppressive function of HCC infiltrating CD8$^+$Treg

To assess the suppressive ability of HCC infiltrating CD8$^+$Treg, violet cell trace labelled allogeneic responder T-cells (CD3$^+$CD25^{low}) were cultured in the absence or presence of CD8$^+$Treg. Responder T-cells were stimulated with Treg inspector beads at 1:1 ratio to induce proliferation. (A) After 3 days of culture, T-cell proliferation was analysed by determining the percentage of violet cell trace dilution. Representative flow cytometric histograms showing responder T-cell proliferation cultured alone (**top**) and in the presence of HCC infiltrating CD8$^+$Treg (**bottom**) at a 1:8 Treg:responder ratio. (B) The suppression capacity of tumour infiltrating CD8+Treg was compared to CD4$^+$Treg (CD4$^+$CD25^{high}CD127^{low}) obtained from the same tumours. Data are expressed as mean and standard deviation. Statistical comparisons were made with Wilcoxon signed ranked test where *P<0.05. Data were obtained from 4 replicate experiments.

5.4 Suppressive function of induced CD8$^+$Treg

In the previous chapter, tumour primed DC was demonstrated to induce a subset of CD8$^+$T-cells resembling the phenotypic characteristic of HCC infiltrating CD8$^+$Treg. Hence, we continued to investigate whether these induced cells possess regulatory function. Assays were performed to assess their cytokine production capability and suppression function. Cytokine production of induced Treg-cells was assessed by stimulating naïve CD8$^+$T-cells that had been cultured with tumour primed DC with a TCR linking antibody (cytostim). The expression of IL-10 and IFN-γ by CD8$^+$T-cells subsets was determined by flow cytometry. Similar to HCC infiltrating CD8$^+$Treg, tumour primed DC induced CD8$^+$Treg capable of producing the suppressive cytokine IL-10, but not IFN-γ (Figure 5-7). Similarly, non-Treg cells produced predominately IFN-γ and not IL-10.

To assess the suppression capacity of iCD8$^+$Treg, T-cells, which had been co-cultured with condition medium primed DC were isolated using flow cytometric cell sorting to separate CD8$^+$CD25^{high}CD127^{low} and CD8$^+$CD25^{low} T-cells. Different ratios of sorted iCD8$^+$Treg cells were incorporated into a suppression assay. In the presence of iCD8$^+$Treg, the proliferation ability of responder T-cells was significantly reduced in a dose dependent manner (Figure 5-8). To ensure the suppressive function was only confined to iCD8$^+$Treg, sorted CD8$^+$CD25^{low} T-cells cells cultured with tumour primed DC acted as a control in the suppression assay. CD8$^+$CD25^{low} T-cells were not able to suppress the proliferation of responder T-cells.

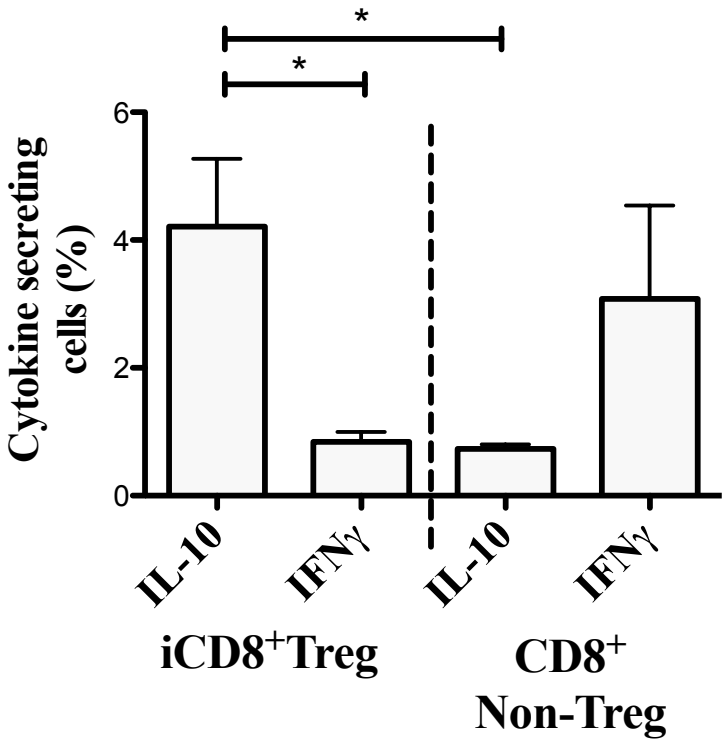


Figure 5-7 Measurement of IL-10 & IFN-γ in HCC induced $CD8^+$Treg

Navie $CD8^+$T-cells were cultured with tumour conditioned medium primed DC for 5 days and assessed for their cytokine production by TCR-linking stimulation and flow cytometry. The cells were gated on $CD3^+CD8^+$ and iCD8+Treg were defined as $CD8^+CD25^{low}CD127^{low}$ T-cells, whilst $CD8^+$Non-Treg was defined as $CD8^+CD25^{low}$ T-cells. The number of cytokine producing cells was stated as percentage of total $CD8^+$T-cells. In common with $CD8^+$ Treg isolated from tumours, iCD8+Treg produced predominantly IL-10 whilst non-Treg cells produced IFN-γ. Data are expressed as mean and standard deviation. Statistical comparisons were made with Wilcoxon signed ranked test where *$P<0.05$. Data were obtained from 4 replicate experiments.

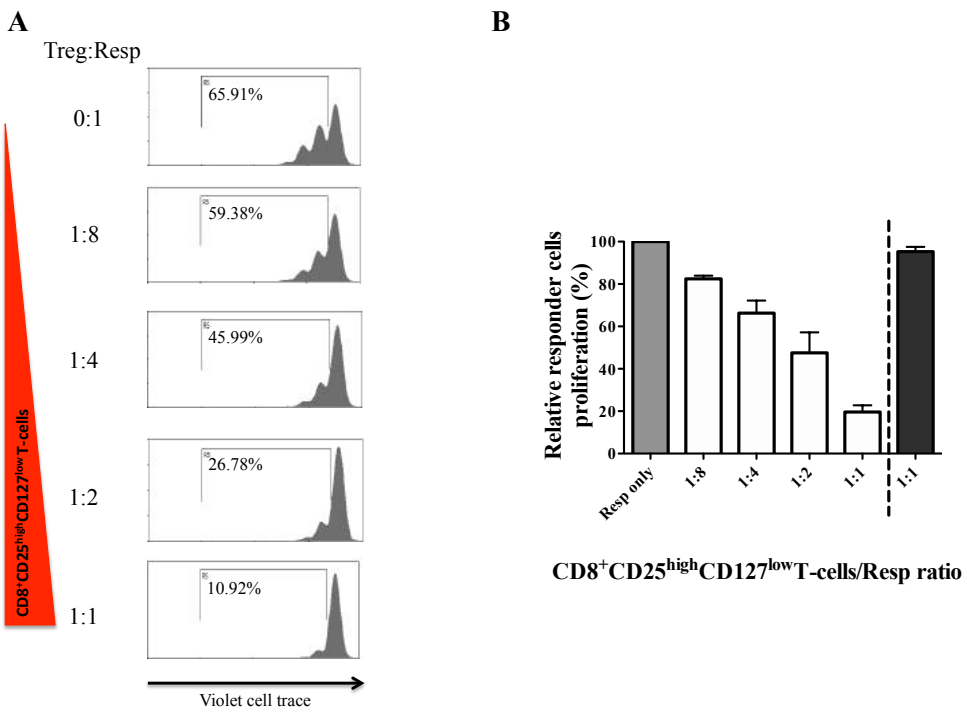


Figure 5-8 Assessment of suppressive function in induced CD8$^+$Treg

Naïve CD8$^+$T-cells were cultured with tumour-conditioned medium primed DC for 5 days. Following culture with tumour primed DC, iCD8$^+$Treg were isolated by flow cytometric sorting. The suppressive ability of iCD8$^+$Treg was assessed by labelling allogeneic responder T-cells with violet cell trace and cultured in the absence or presence of different ratios of iCD8$^+$Treg. CD3$^+$CD2$^+$CD28$^+$ beads (Treg inspector) were added to induce responder T-cell proliferation. After 3 days of culture, T-cell proliferation was analysed by determining the percentage of violet cell trace dilution, demonstrating the capacity of iCD8$^+$Treg to supress effect T cell proliferation. (A) Representative flow cytometric histograms showing T-cell proliferation in the presence of an increasing ratio of iCD8$^+$Treg. (B) Suppression assays from 3 replicate experiments, the black bar represents the use of CD8$^+$CD25^{low} cells instead of iCD8$^+$Treg. Data are expressed as means and standard deviation.

5.5 The suppressive function of CD8$^+$Treg is mediated thought CD39

Functional assessments of Treg isolated from tissue are often limited due to the difficulty in obtaining adequate numbers of cells. However, with the ability to generate CD8$^+$Treg on demand by culturing tumour conditioned-medium primed moDC with naïve CD8$^+$T-cells, further characterisation of the mechanisms involved in the suppressive capacity of CD8$^+$Treg were performed.

As shown in previous chapters, CD8$^+$Treg isolated directly from HCC or induced from tumour primed DC, secreted IL-10, TGF-β, expressed CTLA-4 and CD39. All of these molecules have been shown in previous studies to be involved in the suppressive functions of Tregs [228, 229, 486, 490]. To further ascertain weather any of these factors are involved in the suppressive capacity of CD8$^+$Treg, increasing concentrations of neutralising antibody against IL-10, TGF-β, CTLA-4 and CD39 were added into an allogeneic suppression assay.

The blockade of IL-10, TGFβ and CTLA-4 did not affect the suppression capacity of iCD8$^+$Treg on responder T-cell proliferation (Figure 5-9). However, the addition of a CD39 neutralising antibody was able to reverse the suppressive capacity of induced CD8$^+$Treg in a dose dependent manner.

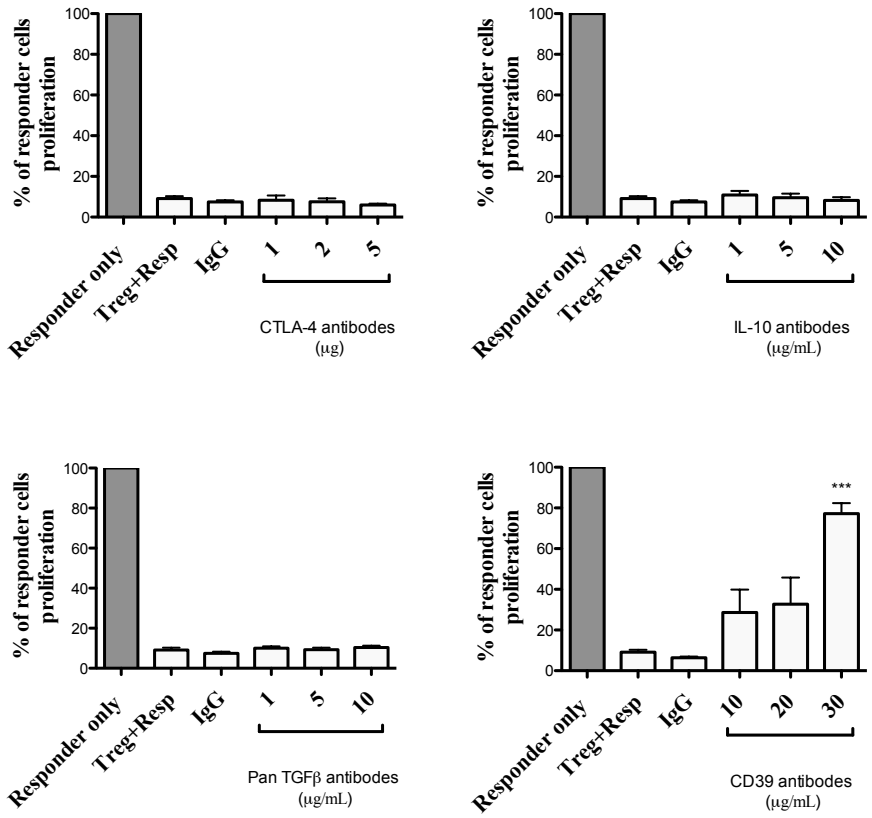
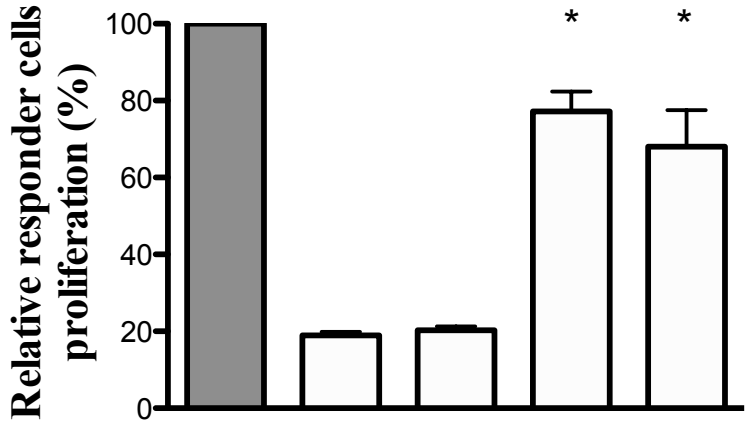


Figure 5-9 The effect of CTLA-4, IL-10, TGF-β & CD39 blockade on CD8$^+$Treg function

Naïve CD8$^+$T-cells were co-cultured with moDC primed with HCC tissue-conditioned medium and then isolated by flow cytometric sorting based on CD3$^+$CD8$^+$CD25^{high}CD127^{low} expression. The ability of induced CD8$^+$Treg to suppress responder T-cell proliferation was assesed by suppression assays. Responder T-cells were labelled with violet cell trace and stimulated with CD2$^+$CD3$^+$CD28$^+$ activating beads (Treg inspector) and cultured with induced CD8$^+$Treg at a 1:1 ratio in the presence or absence of increasing concentrations of neutralising antibodies against IL-10, TGF-β, CTLA-4, CD39 or isotype control. Responder T-cells cultured alone acted as a positive control. After 3 days of culture, T-cell proliferation was analysed by determining the percentage of violet cell trace dilution. Data are expressed as mean and standard deviation. Statistical comparisons were made with Mann-Whitney U test where ***P<0.001 vs. Treg+Resp. Results are from 3 replicate experiments.

A study by Bastid *et al* suggested that CD39 is involved in the suppressive mechanism of Treg though the generation of adenosine from ATP/ADP [491]. To further confirm the importance of CD39 in the suppressive capacity of CD8$^+$Treg, A selective ecto-ATPase inhibitor; diethyl-b-c-dibromomethylene-D-adenosine-50-triphosphate trisodium salt hydrate (ARL67156) was used to block the CD39 adenosine pathway. Similar to the addition of CD39 neutralizing antibody, the suppressive capacity of CD8$^+$Treg was partially reversed by the disruption of CD39 by ARL6715 (Figure 5-10).



Responder + beads	+	+	+	+	+
Treg	-	+	+	+	+
IgG	-	-	+	-	-
CD39	-	-	-	+	-
ARL67156	-	-	-	-	+

Figure 5-10 The effect of CD39-adenosine pathway blockade on CD8$^+$Treg function

The ability of CD8$^+$Treg to inhibit responder T-cell proliferation was assesed by suppression assay in the presence or absence of the ATPase inhibitor diethyl-b-c-dibromomethylene-D-adenosine-50-triphosphate trisodium salt hydrate (ARL67156) or neutralising CD39 antibodies. Responder T-cells cultured alone acted as the positive control. Data are expressed as mean and standard deviation and result are from 3 replicate experiments. Statistical comparisons were made with Mann-Whitney U test where *P<0.01 vs. Treg

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5.6 CD8$^+$Treg suppress responder T-cells though cell contact

The localization of CD39 within lipid rafts of cells has been demonstrated previously [492], suggesting close cell-cell contact is likely to be required to support its function. To ascertain weather cell-cell contact is critical for CD8$^+$Treg to effect their suppressive capacity, suppression assays were carried out in the presence of transwell inserts to prevent contact between responder T-cells and CD8$^+$Treg. In this setting it was possible to demonstrate that suppression of effector T cell expansion by CD8$^+$Treg is cell contact dependent, as the special separation of both cell populations, whilst maintaining free movement of soluble factors, abolishes their suppressive capacity (Figure 5-11).

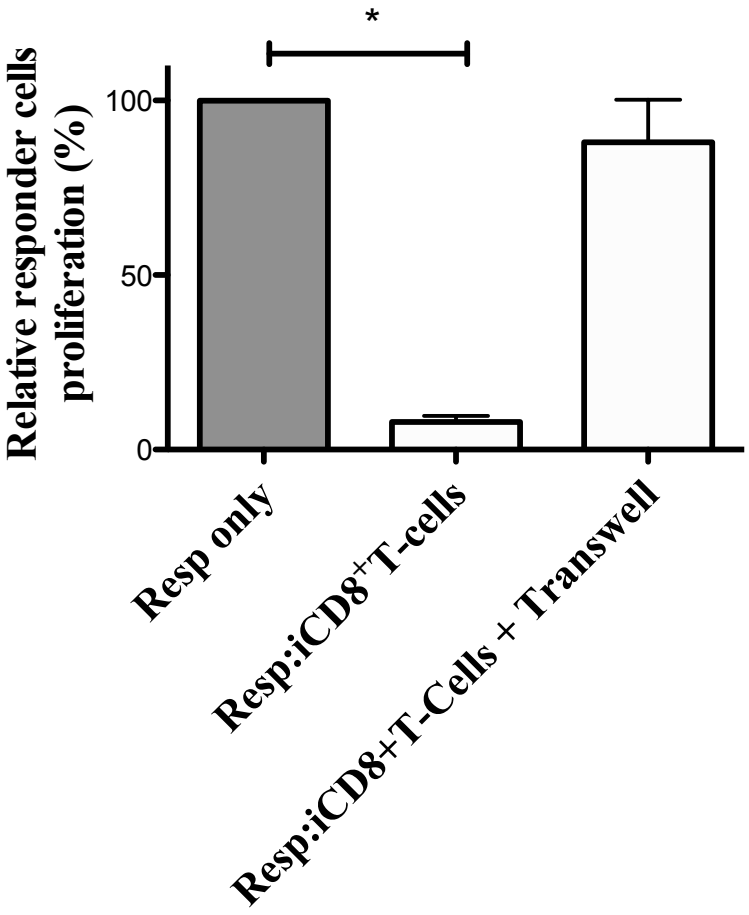

Figure 5-11 The effect of transwell on $CD8^+$Treg suppression

The requirement of cell-contact dependent suppression by induced $CD8^+$Treg ($iCD8^+$T-cells) was assesed by culturing the flow cytometric sorted induced cells with responder T-cells, together or separated by a 0.22μm transwell insert in the presence of $CD2^+CD3^+CD28^+$ activating beads (Treg inspector) at a 1:1 ratio. After 3 days of culture, T-cell proliferation was analysed by determining the percentage of violet cell trace dilution. Data are expressed as mean and standard deviation and result are from 3 replicate experiments. Statistical comparisons were made with Mann-Whitney U test where *P<0.05 vs. responder only

5.7 CD8$^+$Treg do not suppress via cytotoxic pathways

An alternative mechanism for CD8$^+$Treg suppression may involve direct cell killing of the responder cells [493]. To assess whether CD8$^+$Treg can suppress by direct cell cytotoxicity, iCD8$^+$Treg were co-cultured with responder T-cells in a suppression assay. To assess for possible killing of responder T-cells, the viability stain 7-ADD (a membrane dye that is excluded from viable cells) was added prior to flow cytometry. Dead or dying cells are unable to export the dye out of the cells and appear as fluorescently labeled cells on flow cytometry. Despite effective suppression of responder cell proliferation by CD8$^+$Treg no increase in 7-AAD positive cells was detected, suggesting CD8$^+$Treg does not exert their function in a cytolytic manner.

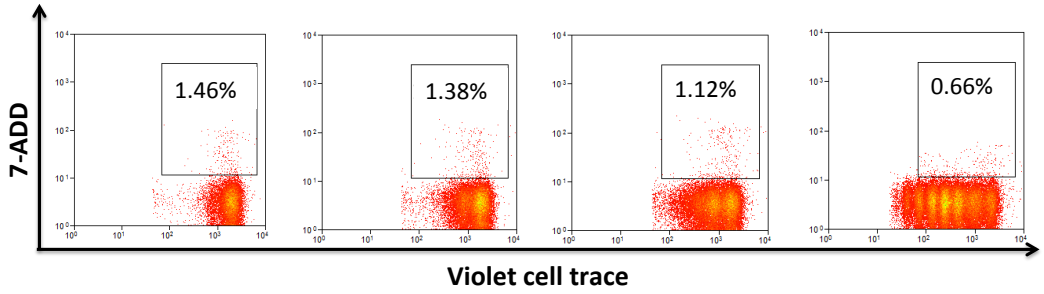

Figure 5-12 Assessment of Cytotoxic activates in CD8$^+$Treg

CD8$^+$Treg were cultured with violet cell trace labelled responder T-cells at increasing ratios and in the presence of CD2$^+$CD3$^+$CD28$^+$ beads (Treg inspector) to stimulate responder T-cell proliferation. Following 3 days of culture, the cells were stained with 7-ADD for 5 minutes at room temperature prior to flow cytometry. Responder T-cell proliferation was determined by dilution of violet cell trace and dead or dying cells was defined as 7-ADD positive cells. Representative dot blot from 3 replicate experiments

5.8 Discussion

A number of mechanisms have been suggested to explain the suppressive function of regulatory CD8$^+$T-cells, although many of these remain contentious. The production of suppressive cytokines by regulatory CD8$^+$T-cells has been proposed by a number of studies to play an important role in their suppressive ability with IL-10 being one of the key cytokines involved in this and the control of a wide range of effector immune cells [494]. Comparable to the current study, IL-10 expressing regulatory CD8$^+$T-cells has been identified in the blood of patients with chronic infection and cancer. Importantly, a positive association between the frequency of IL-10 producing regulatory CD8$^+$T-cells and disease progression has been demonstrated [204, 208, 211]. In addition, the importance of IL-10 in mediating regulatory CD8$^+$T-cell suppression gained further support by studies demonstrating the reversal of effector cell dysfunction following the *in vitro* blockade of IL-10 secreting regulatory CD8$^+$T-cells [211]. Likewise the cytokine TGF-β has an important role in the maintenance of immune tolerance by controlling the proliferation, differentiation and apoptosis of a plethora of immune cells [495]. Presence of TGF-β producing regulatory CD8$^+$T-cells has been associated with a wide range of human diseases [216]. However, at present, there are no data to show the involvement of TGF-β producing regulatory CD8$^+$T-cells in human cancer.

Despite the detection of IL-10 and TGF-β expressing regulatory CD8$^+$T-cells, I was unable to demonstrate a role for either of these cytokines in the suppressive function of CD8$^+$Treg. Recent studies have also questioned the importance of suppressive cytokines for the inhibitory function of regulatory T-cells [496]. In a study of HCV,

the *in vitro* blockade of IL-10 had no effect on the suppressive function of IL-10 expressing regulatory CD8$^+$T-cells [210]. In a separate study on human prostate cancer, the neutralization of both IL-10 and TGF-β did not prevent regulatory CD8$^+$T-cells suppression [497].

Further evidence to discount the effect of soluble factors such as IL-10 and TGF-β in the suppressive function of regulatory CD8$^+$T-cells comes from studies linking the requirement of cell-contact dependant mechanisms for maintenance of regulatory function. These studies demonstrated the prevention of cell-contact between responder T-cells and regulatory CD8$^+$T-cells entirely abolished the suppressive capacity of regulatory cells [203, 210].

Despite not being able to demonstrate the effect of IL10 and TGF-β in the current work, is worth considering they may still be involved. Such hypothesis stem from the shortfall of the current experiment. The functional assays on cytokine blockade were conducted on peripheral isolated T-cells that have been induced by tumour-conditioned DC. These iCD8$^+$Treg processes identical phenotypical markers to the one found in human HCC. However, due to the limited number of tumour infiltrating CD8$^+$Treg, it was not possible to confirm if blocking of IL10 or TGF-β effects the functionality of tumour derived CD8$^+$Treg. This is especially important as previously discussed in chapter 3. This is because despite expressing the same regulatory marker, the functional capacity of Treg appears to be determined by their location. Hence, even if induced peripheral CD8$^+$Treg doesn't appear to suppress through either IL-10 or TGF-β, it is still possible that tumour infiltrating CD8$^+$Treg maybe

able to suppress via soluble factors. To answer such question, we would need to perform functional assays using matched CD8$^+$Treg from the blood, tumour and non-tumour tissue. This will be currently challenging due to the limited number of CD8$^+$Treg we can isolate from these compartments.

Continued debate on the role of soluble factors in regulatory CD8$^+$T-cell function, has given rise to a number of novel mechanisms as possible candidates to explain the suppressive capacity of these cells. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a glycoprotein expressed on T-cells. It is homologous to the receptor CD28 and shares the same ligands (CD80 & CD86) that are essential for effector T-cell activation [299]. The important role of CTLA-4 in immune regulation was first recognised in animal models in which CTLA-4 deficient mice exhibit lethal lymphoproliferative disorder and multi-organ lymphocyte infiltration [300, 301]. However, the precise mechanism in which CTLA-4 modulates the immune system remains controversial.

The observation of high CTLA-4 expression on regulatory T-cells gave credit to the concept of its possible involvement in their suppressive function. Early *in vivo* experiments supported such a hypothesis by demonstrating the neutralisation of CTLA-4 inhibited CD4$^+$Treg suppression [498, 499]. Further evidence of a role for CTLA-4 in the function of regulatory T-cells were provided by studies showing defective CD4$^+$Treg function in CTLA-4 deficient mice [498, 500]. A possible role for CTLA-4 in regulatory CD8$^+$T-cell function has also been reported. In a study by Olson *et al*., an infiltrating population of antigen specific regulatory CD8$^+$T-cells were identified in tumour samples taken form patients with prostate cancer. These

regulatory cells were also positive for CTLA-4, and their suppressive activity was diminished by the blockade of CTLA-4 [497]. However, in the current work, despite the high expression of CTLA-4 seen on regulatory CD8$^+$T-cells, its blockade did not result in the reversal of responder T-cell suppression. However, these findings do not differ from those in some other studies that also argued against the role of CTLA-4 in regulatory T-cell function, with failure to reverse their suppression through CTLA-4 blockade [496, 501]. Despite this conflicting evidence for a role of CTLA-4 in regulatory T-cell function, the involvement of this molecule in other immune modulating roles has emerged. These include the induction of the suppressive molecules TGF-β and IDO on APC, the restriction of CD28-CD80/CD86 signalling, the direct inhibition of APC cells and disruption APC-T-cell activation via inhibition of cell adhesion [302-307].

Another candidate involved in the suppressive mechanism of regulatory CD8$^+$T-cells is CD39, shown to be present on CD4$^+$Treg and detected on regulatory CD8$^+$T-cells by others [502, 503]. CD39 (ectonucleoside triphosphate diphosphophydrolase-1) is an ectoenzyme that is proposed to have immune suppressive function by hydrolyzing ATP and ADP, respectively to AMP. AMP is further processed by the ectoenzyme CD73 ecto-5'-nucleotidase, which drives the conversion of AMP into adenosine. Whilst adenosine is usually present at a low concentration in the extracellular compartment, it has been shown to be increased in a number of human tumours and has a direct suppressive effect on a wide range of effector immune cells [504].

Adenosine can trigger multiple pathways via the activation of membranous adenosine receptors, classified as A1, A2A, A2B and A3 receptors. The A2A receptor is present on a wide range of immune cells and is up regulated on TCR activation in T-cells. The binding of adenosine on T-cell expressed A2A receptor results in a rapid increase of intracellular cAMP leading to inability to proliferate. The reversal of T-cell suppression by the blocking of CD39 in the current study is consistent with the reported importance of the CD39-adenosine pathway in immune evasion. However, despite the demonstration of increased expression of CD39 in both primary and induced regulatory $CD8^+$T-cells, it was not possible to detect the presence of the ecto-enzyme CD73 on $CD39^+CD8^+$Treg. CD73 expression has been reported on both immune cells and stromal cells and is required downstream of CD39 in the conversion of ATP/ADP to AMP. However, similar to my work, other groups have also demonstrated the lack of CD73 and CD39 co-expression on regulatory T-cells. Alternatively, $CD39^+$ regulatory T-cells may still be able to maintain their suppressive function in the absence of CD73 co-expression via interaction with CD73 from a paracrine source such as responder cells. Furthermore, the lack of CD73 co-expression on regulatory T-cell may be a technical limitation of flow cytometry, where relatively high concentrations of soluble factors are required for effective demonstration.

In addition to the direct regulatory effect of CD39 expressing Treg on effector T-cells, the CD39-adenosine pathway had also been reported to be involved in immune modulation of other immune cell subsets and increased levels of adenosine result in

the trafficking of a wide range of immune cells in to the tumour microenvironment to aid its progression [505].

Additionally, adenosine has the ability to disrupt the function of effector immune cells, in some cases converting them towards a tolerogenic phenotype [491]. NK cells have been shown to play an important role in the anti-tumour response and adenosine had been demonstrated to inhibit the function of these cells by reducing their ability to produce pro-inflammatory cytokines such as IFN and TNF and impairing their cytolytic function [506]. In addition, NK cells that bear the T-cell receptor, NKT cells, which constitutively produce IFN, have been shown to produce IL-4 and TGF in response to adenosine. Furthermore, macrophages are also affected by adenosine, both by inhibition of its phagocytic function [507] as well as by switching M1 macrophages into an M2 phenotype. This results in the production of suppressive cytokines such IL-10, but also the pro-angiogenic cytokines VEGF, which may further enhance tumour growth.

More recently, CD39 has been proposed to play a role in the tolerogenic capacity of liver DC. It has been demonstrated in an animal liver transplant model, the presence of CD39 provided protection against ischemic/reperfusion damage, when compared to CD39$^{-/-}$ mice [508].

Only partial reversal of responder T-cell suppression by CD8$^+$Treg was possible by blockade of CD39, either with a neutralising antibody or the inhibitor ARL. This is perhaps unsurprising given the multiple mechanisms employed by regulatory T-cells

to suppress immune cells. One such additional mechanism involves the immunomodulating molecule indoleamine-2,3-dioxygenase (IDO). In addition to its role in the induction of regulatory CD8$^+$T-cells (chapter 4), IDO has been linked to the direct inhibition of effector cells via the degradation of the essential amino acid tryptophan [509]. IDO has also been shown to be present on regulatory CD8$^+$T-cells and disruption of IDO though a single nucleotide polymorphism (SNP) in the IDO gene resulted in regulatory CD8$^+$T-cell dysfunction in patients with systemic sclerosis [510].

In addition, the limitation of the use of ARL as an inhibitor can also affect the result seen. 6-N,N-diethyl-D-b-g-dibromomethylene adenosine triphosphate or better know as ARL 67156 was described as a selective inhibitor of ecto-ATPase [511]. Since the discovery of ARL 67156 our understanding of ecto-ATPase has increased, with the identification of additional ecto-ATPase. Which includes the ecto-nucleoside triphosphate diphosphohy- drolase (ENTPDase) family, that consists of CD39 (NTPDase1), CD39L1 (NTPDase2), CD39L3 (NTPDase3) and hepatic ATPDase (NTPDase8). Two members of the ecto- nucleotide pyrophosphatases/phosphodiesterases (E-NPP) family, NPP1 and NPP3 were also recently discovered to confer ecto-ATPase activities [512-515]. It has been recognised that the main target of ARL 67156 appear to be against selected ecto-ATPase, mainly, CD39, CD39L3 and NPP1 [516]. Hence, the partial inhibitor effect seen in the current study can be the result of the inability of ARL 67156 to block other active ecto-ATPase. To complicate the matter, our understanding of the distribution of the ecto-ATPase in different tissue compartments and cancers remains poor. Hence, a

better understanding of ecto-ATPase together with the development of a broader inhibitor of ecto-ATPase is needed.

One of key recent observations that lead to the design of this study to investigate regulatory $CD8^+$T-cells, was evidence suggesting that they may be more potent when compared to $CD4^+$Treg. In a study by Chaput et al., $CD8^+CD25^{high}$T-cells isolated from colorectal cancer not only suppressed effector T-cell proliferation, but also more importantly appear to have a higher suppressive capacity when compared to $CD4^+$Treg isolated from the same source [228]. Similarly, in a study of allogeneic bone marrow transplantation, regulatory $CD8^+$T-cells were noted to be more potent when compared to $CD4^+$Treg and importantly protected the host from GVHD [227]. However, in this study, the suppressive capacity of regulatory $CD8^+$T-cells and $CD4^+$Treg isolated from HCC were similar. The differences seen between my data and previous works may be due to heterogeneity in the method used to assess Treg suppression. Previous studies on regulatory $CD8^+$T-cells had often used H^3-thymidine to determine cellular proliferation of responder T-cells. In such assays, H^3-thymidine is incorporated into new strands of chromosomal DNA during mitotic cell division. The extent of cell proliferation can be determined by obtaining the radioactivity in cultured cells. However, the use of H^3 thymidine can be limited by is inability to differentiate between responder T-cells and regulatory T-cell proliferation. For example, the superior regulatory $CD8^+$T-cells suppression seen over $CD4^+$Treg can be the result of $CD4^+$Treg proliferation, which can be misinterpreted for responder T-cell proliferation. This can lead to a false impression of an increased suppression by regulatory $CD8^+$T-cells over $CD4^+$Treg. In the current work, flow cytometry has been

chosen as the method to assess cell proliferation. Fluorescence is only incorporated into responder T-cells and their proliferation can be determined by the dilution of the fluorescence by flow cytometric analysis, eliminating the problem of regulatory T-cell proliferation contributing to a false read out.

One of the limits to the current work on the assessment of CD8$^+$Treg function had been the inability to compare the suppressive function of isolated peripheral and tumour isolated cells. However, such experiments would have been technically challenging due to the low number of CD8$^+$Treg present in the blood of HCC patients (chapter 3). Nevertheless, some comparison can be made by looking at the suppressive capacity of circulating CD4$^+$Treg from patients with HCC. When assessed using the same responder to CD4$^+$Treg ratio, it appears tumour infiltrating CD4$^+$Treg are more potent in their suppressive capacity when compared to CD4$^+$Treg isolated from blood. However, we have to be cautious in such interpretation as these samples are taken from different patients. Full evaluation of this phenomenon would require blood samples being taken from patients undergoing resection or transplantation for HCC such that direct comparison of Treg function could be made. Similarly, despite induced CD8$^+$Treg displaying phenotypic features similar to primary isolated CD8$^+$Treg obtained from HCC, the suppression capacity of induced cells appears to be less when compared to primary isolated cells. One possible reason for such differences could be down to the additional effect the tumour environment has on the function of CD8$^+$Treg. HCC tumours consist of multiple components in addition to tumour cells, such as stromal cells and immune cells. Increasing evidence has suggested the different components of the tumour may all play an important role

in the generation of the suppressive tumour environment. For example tumour fibroblasts have been shown to have the ability to present antigen to immune cells and produce a wide array of suppressive cytokines [54, 517, 518]. It was beyond the scope of the current study to determine any affect stromal cells my play in the induction of CD8$^+$Treg. Additional evidence to support the modulating effect of the tumour microenvironment on T-cells can be seen from the assessment of their cytokine production. CD8$^+$Treg from the tumour produced a higher percentage of the suppressive cytokine IL-10 compared to cells isolated from matched liver tissue. Similarly, CD8$^+$CD25^{low} T-cells (non-Treg) cells isolated from the tumour produced less IFN-γ when compared to cells isolated from non-tumour tissue.

Research into immune cell function in human disease is often limited by the inability to isolate adequate numbers of cells and this has become increasingly problematic with the increased complexity we use to define rarer subsets of immune cells. In Treg research, attempts to overcome such limitations lead to the use of expanded T-cells. However, I was unable to expand tumour derived CD8$^+$Treg using established protocols developed for CD4$^+$Treg expansion. It has been proposed that the survival of immune cells within the liver may involve constant communication between them and liver stromal cells. This may explain the inability to induce tumour CD8$^+$Treg in the absence of the supportive network found in the tumour/liver environment. This hypothesis is supported by work carried out in our own laboratory and others demonstrating the importance of hepatic factors in the survival of liver immune cells [519] [520].

Not withstanding these limitations, the ability to assess the function of primary isolated cells has a major advantage over expanded cells. Expansion of any cell population is largely dependent on the purity of the starting population; even minor contamination by non-Treg cell can affect the final expanded cell population. In addition, current methods used for the expansion of Treg have often resulted in an unstable subset of cells, with limited survival potential and occasionally even conversion into an effector T-cell phenotype[521]. It is also critical to take care when designing assays to assess the suppressive capacity of Treg where a number of different methods have been employed to stimulate responder T cell proliferation. Historically, professional antigen presenting cells, such as DC have been used in combination with soluble anti-CD3 antibody to provide a more physiological method to induce cell proliferation. However, the addition of a third population of cells into the assay will only add to the complexity of interpretation and is likely to result in more variability. In the current work, CD3/CD28 beads were used to stimulate T-cell, as these should provide a consistent proliferative signal to the responder cell population. However, it was apparent that the size and antibody loading of these beads is critically important to ensure assay sensitivity. This was evidenced by the excessive stimulation provided by the large beads sourced from Dynal which resulted in a degree of proliferation in responder cells that could not suppressed by the addition of Treg, a problem that was overcome by using smaller nano-particles to drive proliferation. Indeed, the importance of reagents used in suppression assays was elegantly demonstrated by Oberg et al., showing how bead concentration may effect the ability of Treg to suppress in-vitro.[522]

6 TACE modulate regulatory $CD4^+$T-cells

To this point, the current work has emphasised the importance of Tregs in the generation of a tolerogenic tumour environment. Treatments that lead to their depletion or disruption are likely to enhance immunological rejection of malignancy. Given the increasing evidence that "classical" cytostatic therapy might also have the potential to enhance anti-tumour immune responses, it was hypothesise that local ablative treatment such as TACE, may have the ability to counteract tumour induced immune suppression by the disruption of Tregs.

Treatment options for incurable HCC depend on the tumour stage. For patients with limited disease confined to the liver, local ablative treatments such as TACE have been shown to be beneficial [523]. TACE involves the intra-hepatic arterial infusion of a chemotherapeutic agent such as doxorubicin, followed by embolization of the blood vessels supplying the tumour with a gelatine sponge or other embolic agents. This leads to a combination of cytotoxic and ischaemic induced tumour cell death. Increasing evidence suggests that control of HCC can also be mediated indirectly by activation of anti-tumour immune responses during local ablative therapy [524], but the precise mechanism remains unclear. Therefore, investigating the immunological mechanisms, in particular focusing on the regulatory response, that take place following local ablative treatment for HCC, may provide more information on the kinetics and nature of the immune response. This information will allow us to target particular components of the anti-tumour immune response by enhancing immunotherapy and thereby assisting in the development of new treatments for HCC.

To investigate the effect of TACE on the regulatory response, the frequency and function of circulating Tregs in HCC patients, together with TAA response, at the time they were receiving TACE. Was studied the immunological results were correlated with clinical response to treatments (

Figure 6-1**).**

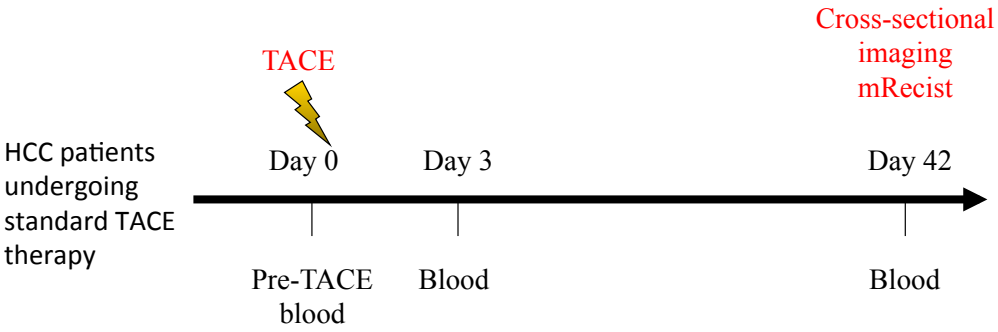

Figure 6-1 Study design.

Study time line, showing time point for obtaining blood for immune response measurement before and after treatment.

6.1 Patient's characteristics

50 patients were recruited into the study over a period of 2.5 years and a total of 150 blood samples were obtained during that time. HCC was diagnosed based on histology or cross sectional imaging as defined by the European Association for Study of the Liver (EASL) guidelines [525]. All patients underwent TACE with lipiodol and doxorubicin and 12 patients had previous treatment for their HCC (2 resection, 2 RFA, 8 TACE) prior to inclusion into the study. The studied cohort was typical of a Western centre, consisting of mainly male patients (n= 42; 84%) with liver cirrhosis (n=48; 96%) as a result of predominately ALD (n=13; 26%), NAFLD (n=10; 20%) and HCV (n=9; 18%). The average age of patients at the time of TACE was 65 (range 45-83). All patients had stable Child's A liver disease with a mean MELD score of 9.1 (range 7.5-11.4). The mean AFP at baseline was 5ng/mL (range 3-22.5). The mean tumour volume was 50mm^3 (range 15-166) with the number of lesions being treated ranging from 1 to 5. All patients gave written informed consent prior to taking part in the study.

Pt.	Age	Gender	Disease	Total tumour size (mm)	No. of lesions	CPS	MELD
1	58	M	HBV/HIV	64	1	10	14.6
2	78	M	HBV	18	1	5	9.4
3	67	M	HFE	58	4	5	3.9
4	66	M	HBV	35	1	5	11.1
5	77	M	ALD	103	5	5	10.4
6	50	M	HBV	44	1	5	15.8
7	60	M	HCV	48	1	5	6.4
8	70	M	ALD	22	1	5	8.5
9	64	F	ALD	28	1	5	7.7
10	61	M	HFE	75	3	5	6.4
11	70	M	ALD	19	5	5	7.5
12	75	M	NAFLD	60	1	5	10.4
13	64	M	ALD	48	1	5	6.8
14	83	M	Non-cirrhotic	55	1	7	15.6
15	67	M	HBV	92	3	5	6.4
16	65	M	ALD	54	1	8	12.5
17	76	M	HBV	27	1	5	9.7
18	75	F	HCV	64	2	8	15.0
19	65	M	HBV	20	1	7	12.0
20	67	M	ALD/NAFLD	32	1	5	7.5
21	71	M	HCV	30	1	5	6.4
22	55	M	HCV	37	1	7	12.0
23	73	M	NAFLD	20	1	5	10.3
24	63	M	HCV	49	3	5	7.5
25	80	M	ALD	22	1	5	11.4
26	69	M	ALD	100	1	5	7.5
27	64	M	ALD	90	2	5	6.4
28	69	M	ALD/HBV	18	1	5	11.9
29	64	M	ALD	17	1	5	13.6
30	65	F	NAFLD	19	1	5	6.4
31	53	F	HCV	17	1	5	6.4
32	72	M	NAFLD	166	2	5	13.5
33	58	M	ALD/HCV	116	4	6	9.6
34	60	M	ALD	53	3	5	9.4
35	61	F	HBV	17	1	5	7.5
36	69	M	ALD	18	1	5	7.5
37	50	M	HCV	54	1	5	8.2
38	65	M	NAFLD	66	3	5	9.2
39	62	M	Non-cirrhotic	35	3	5	9.5
40	66	M	NAFLD	60	1	7	11.2
41	66	F	HCV	35	2	5	7.5
42	83	M	HHC	15	1	5	8.7
43	72	M	NAFLD	49	1	5	7.5
44	54	M	NAFLD	112	1	6	12.9
45	45	M	HCV	30	1	5	11.9
46	54	F	NAFLD	36	1	5	9.1
47	68	M	NAFLD	64	1	5	8.4
58	68	F	PSC	100	3	7	8.9
59	59	M	Crytogenic	58	1	5	9.6
50	66	M	ALD	35	2	5	8.5

F, Female; M, Male; HBV, Hepatitis B Virus; HCV, Hepatitis C Virus; HHC, hereditary haemochromatosis; HIV, Human Immunodeficiency Virus; NAFLD, Non-Alcoholic Fatty Liver Disease; ALD, Alcoholic Liver Disease; PSC, Primary Sclerosing Cholangitis; CPS, Child-Pugh Score; MELD, Model for End-Stage Liver Disease.

6.2 Treg in the blood of patients with HCC

To confirm the presence of Treg in the peripheral blood of patients suffering from HCC and to define baseline frequencies, PBMC were isolated from the blood samples of HCC patients on the day prior to their TACE-treatment and analysed by flow-cytometry. CD4$^+$Treg and CD8$^+$Treg were defined as CD3$^+$CD4$^+$CD25^{high}CD127^{low} cells and CD3$^+$CD8$^+$CD25^{high}CD127^{low} cells respectively. Both CD4$^+$Treg and CD8$^+$Treg can be detected in the blood of HCC patients prior to treatment (Figure 6-2A-E).

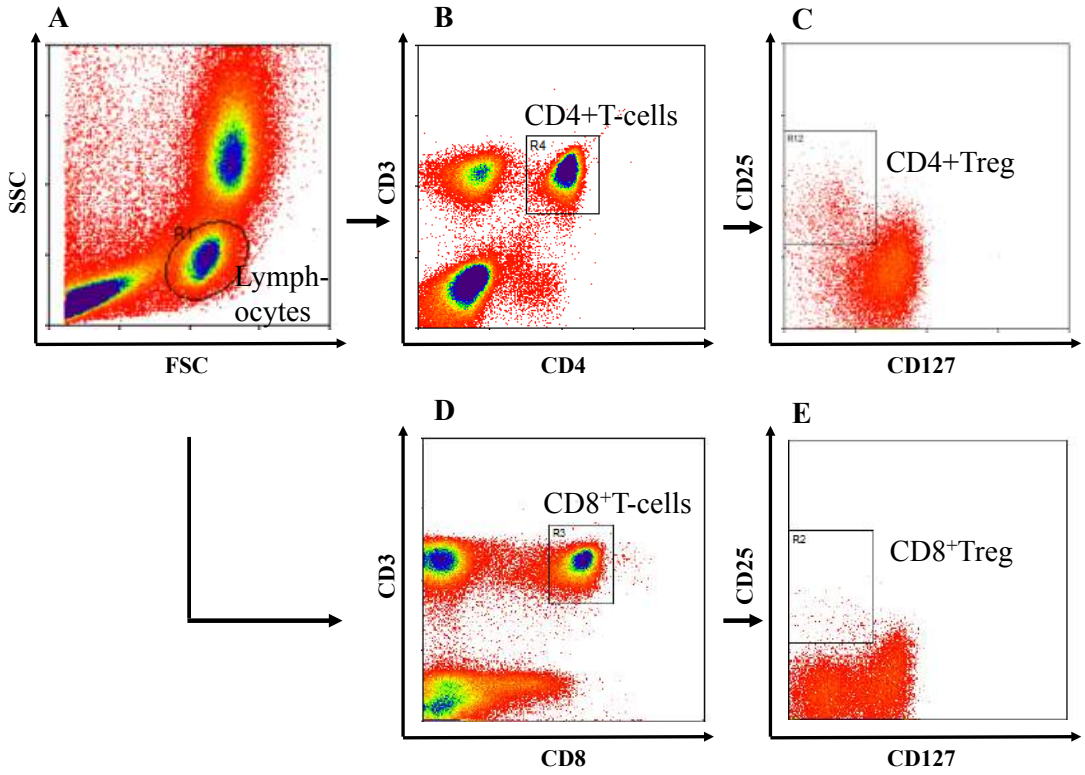

Figure 6-2. Gating strategies for the identification and characterization of CD4$^+$Treg & CD8$^+$Treg

(A) Representative dot plots of flow cytometric analysis for the demonstration of Treg in the peripheral blood of HCC patients. PBMCs were gated on forward scatter (FSC) and side scatter (SSC). (B) CD4$^+$T-Cells were identified by surface expression of CD3$^+$CD4$^+$. (C) CD4$^+$Treg are represented as CD3$^+$CD4$^+$CD25^{high}CD127^{low} cells. (D) CD8$^+$T-Cells were identified by surface expression of CD3$^+$CD8$^+$. (E) CD8$^+$Treg are represented as CD3$^+$CD8$^+$CD25^{high}CD127^{low} cells. Representative of 150 samples.

6.3 Changes in circulating CD4$^+$Treg during treatment

To assess the effect of TACE on different subsets of circulating Treg, blood was collected from HCC-patients and assessed using flow cytometry before treatment, with follow up 3 days and 42 days after treatment. Figure 6-3 and Figure 6-4 show representative dot plots of CD4$^+$Treg and CD8$^+$Treg respectively from two HCC patients undergoing TACE. Patient-17 was defined as a complete responder following TACE treatment in accordance to the mRECIST criteria with a decrease in circulating CD4$^+$Treg percentage 42 days after TACE, but no change in the frequency of circulating CD8$^+$Treg. Treg frequency was expressed as a percentage of total CD4$^+$ T-cells or CD8+T-cells respectively. In comparison, patient-43 who had progressive disease despite TACE, showed an increase in the percentage of CD4$^+$Treg 42 days after TACE whereas the percentage of circulating CD8$^+$Treg remained stable.

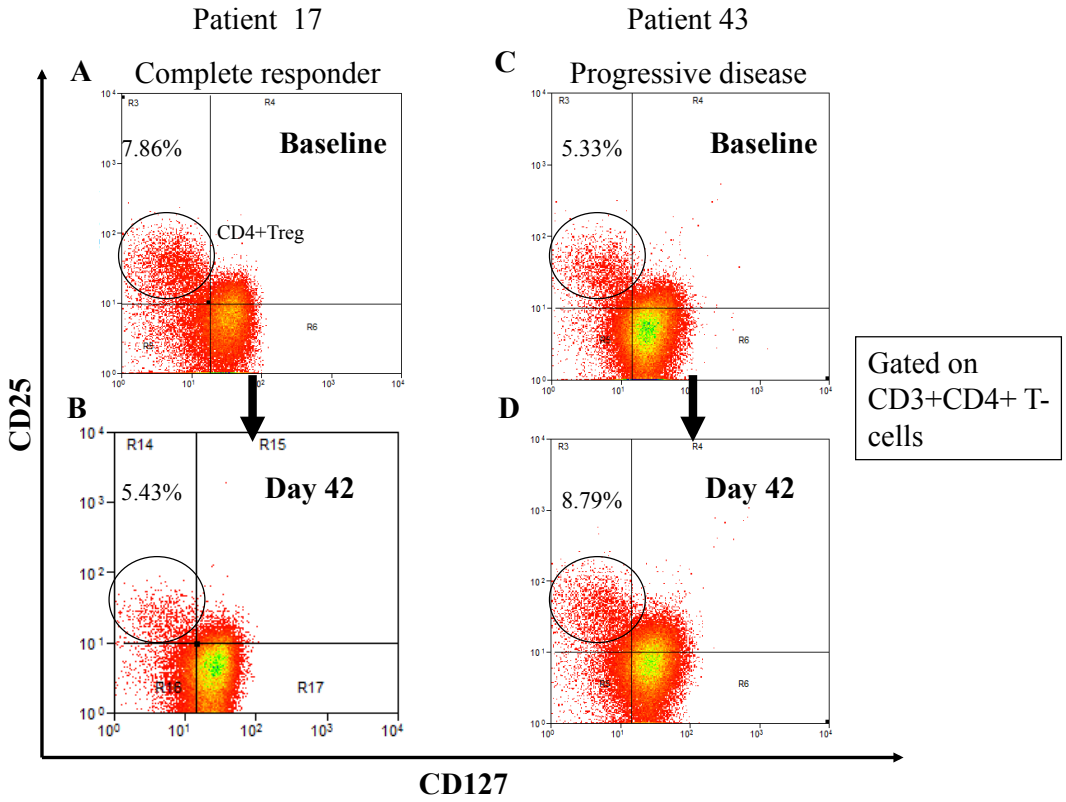

Figure 6-3 Changes in circulating CD4^{+}Treg following TACE

Representative flow cytometry dot plots demonstrating changes in CD4^{+}Treg at baseline and 42 days after TACE treatment in two patients. Cells were gated on CD3^{+}CD4^{+} cells and CD4^{+}Treg are shown as CD25^{high}CD127^{low} cells **(black circle)** and the percentage of CD4^{+}Treg in relation to total CD4^{+}T-cells are displayed. (A&B) Patient-17 is a complete responder and (C&D) Patient-43 had progressive disease.

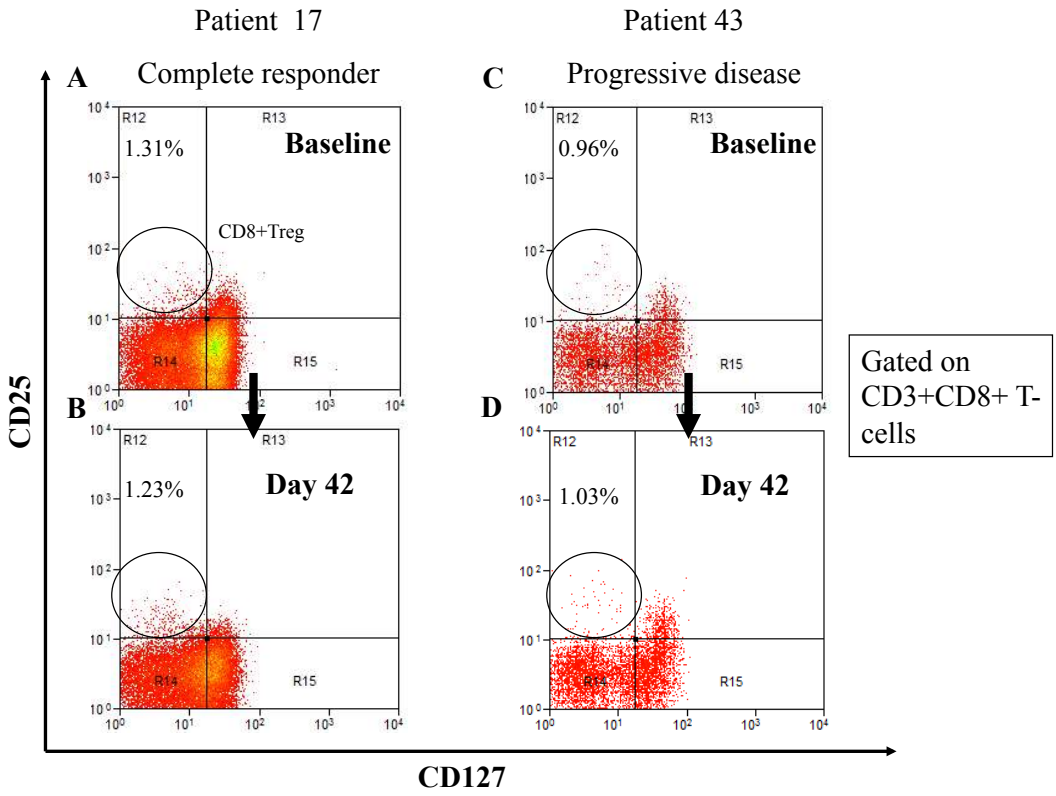

Figure 6-4 Changes in circulating $CD8^{+}$Treg following TACE

Representative flow cytometry dot plots demonstrating changes in $CD8^{+}$Treg at baseline and 42 days after TACE treatment in two patients. Cells were gated on $CD3^{+}CD8^{+}$ cells and $CD8^{+}$Treg are shown as $CD25^{high}CD127^{low}$ cells **(black circle)** and the percentage of $CD8^{+}$Treg in relation to total $CD8^{+}$T-cells are displayed. (A&B) Patient-17 is a complete responder and (C&D) Patient-43 had progressive disease.

Blood samples were obtained from 50 patients at all three time points. The percentage of circulating CD8$^+$Treg remained stable following TACE treatment (baseline; 2.1%±2.1%, day 3; 1.8%±1.7%, day 42; 1.6%±2.3%) (Figure 6-5). In comparison, the percentage of circulating CD4$^+$Treg were significantly reduced in patients following TACE when compared to baseline and this effect can be seen as early as day 3 after treatment (n=50; baseline; 6.0%±6.6%, day 3; 4.8%±5.5%, day 42; 5.5%+5.2%) (Figure 6-6).

Stratifying patients according to treatment efficacy, the percentages of CD4$^+$Treg were significantly reduced following TACE in both the complete-responder-group (n=23; baseline; 6.3%±5.58%, day 3; 4.4%±4.5%, day 42; 4.3%±5.7%) and the partial-responder-group (n=17; baseline; 9.3%±8.8%, day 3; 8.6%±7.1%, day 42; 6.0%±5.3%). Contrary to this observation, a significant increase of circulating CD4$^+$Treg (n=10; baseline; 4.6%±2.5%, day 3; 5.1%±2.4%, day 42; 6.6%±4.7%) was seen in the peripheral blood of patients who were non-responders following TACE-treatment. In order to account for any variability in baseline Treg frequency, both CD4$^+$ and CD8$^+$ Treg were analysed for fold change from baseline (Figure 6-6E).

Similar to the pattern of changes seen in the percentage of circulating CD4$^+$Treg, the fold change of CD4$^+$Treg was significantly different following stratification according to treatment response. The frequency of circulating CD4$^+$Treg in the peripheral blood of complete responders decreased on day 3 and day 42, -1.79±1.83 and -1.31±3.38 fold decrease compared to baseline respectively. In the partial responder/stable disease group a -0.63±1.96 and -1.72±2.88 fold decrease in circulating CD4$^+$Treg on

day 3 and day 42 respectively compared to baseline was observed. In the progressive disease group, a -0.01±1.48 decrease in circulating CD4$^+$Treg on day 3 compared to baseline was observed. Interestingly, a 1.12± fold increase in circulating CD4$^+$Treg on day 42 compared to baseline was observed in the non-responder-group.

To assess rather comparable changes are seen in the absolute number of Treg following TACE. Flow cytometry was performed using counting beads in 30 patients. Similarly to the percentage change, there was a statistically significant difference in the absolute number of CD4$^+$Treg in patients following TACE (Figure 6-8). However, no significant different was seen in the absolute number in CD8$^+$Treg before and after TACE treatments (Figure 6-8).

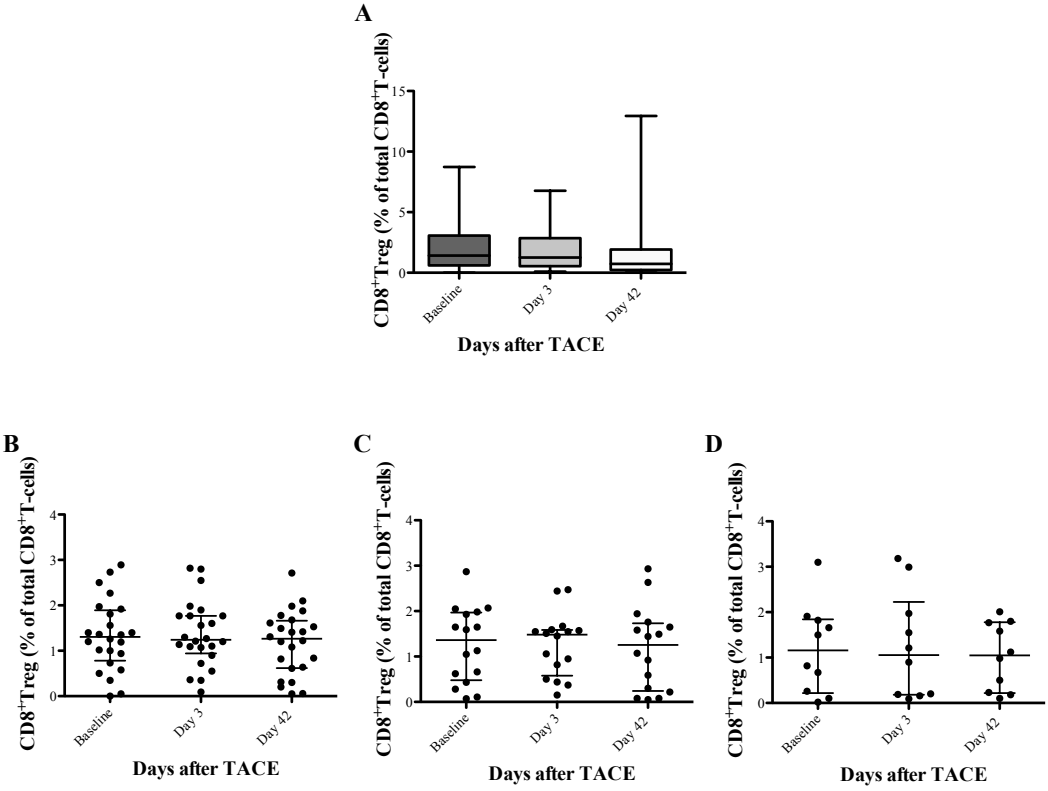

Figure 6-5 CD8$^+$Treg level following treatment with TACE

The frequency of CD8$^+$Treg were analysed in 50 patients at baseline, day 3 and day 42 following TACE and correlated to treatment response according to mRECIST criteria by cross-sectional imaging. (A) Changes in circulating CD8$^+$Treg pecentage in entire cohort (n=50), (B) complete responder (n=23), (C) partial responder/stable disease (n=17), (D) non-responders (n=10) at baseline, day 3 and day 42 following TACE. Data are expressed as median and interqurtile range. Statstiscal comparisons were made with Wilcoxon signed ranked test.

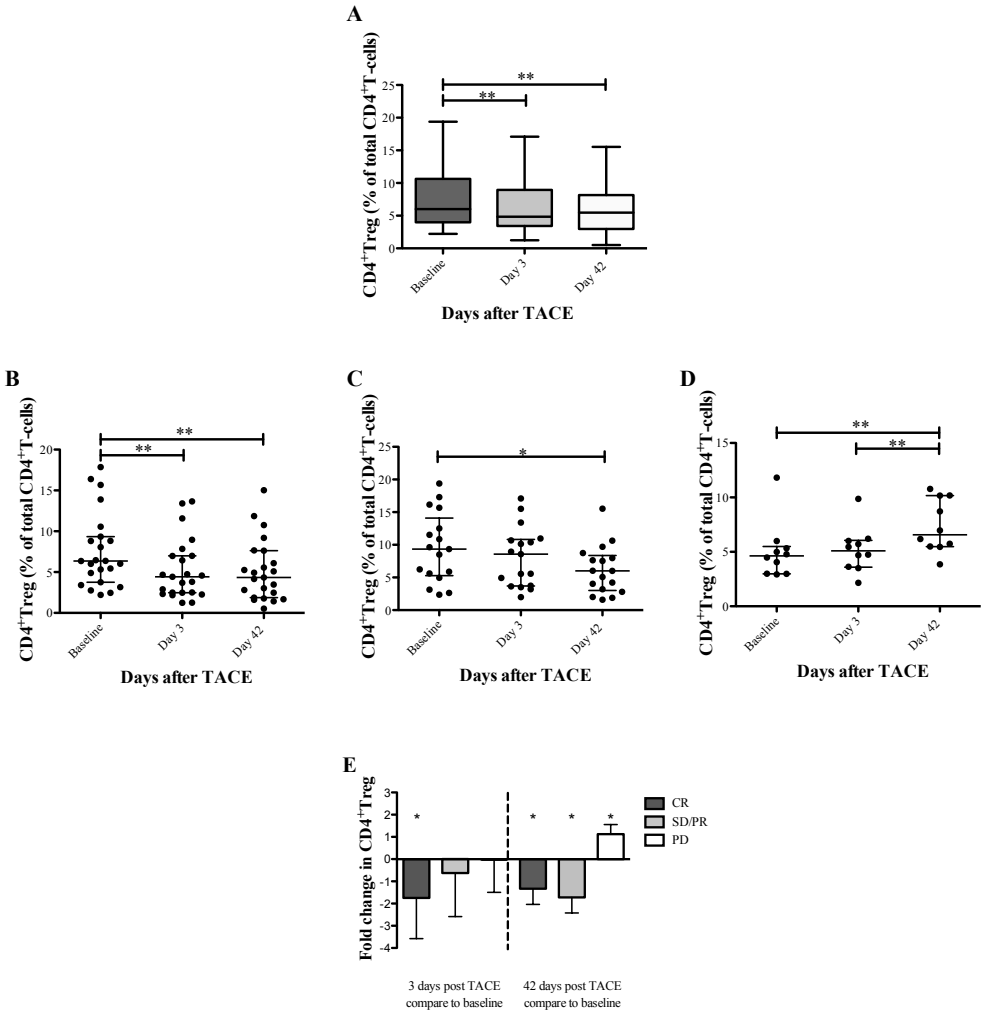

Figure 6-6 CD4$^+$Treg level following treatment with TACE

The percentage of circulating CD4$^+$Treg were analysed in 50 patients at baseline, day 3 and day 42 following TACE and correlated to treatment response according to mRECIST criteria by cross-sectional imaging. (A) Changes in circulating CD4$^+$Treg pecentage in entire cohort (n=50), (B) complete responder (n=23), (C) partial responder/stable disease (n=17), (D) non-responders (n=10) at baseline, day 3 and day 42 following TACE. (E) Comparison in fold change in circulating CD4$^+$Treg at day 3 and day 42 after TACE were also made. Data are expressed as median and interqurtile range. Statstiscal comparisons were made with Wilcoxon signed ranked test where *P<0.05, **P<0.01, vs. baseline.

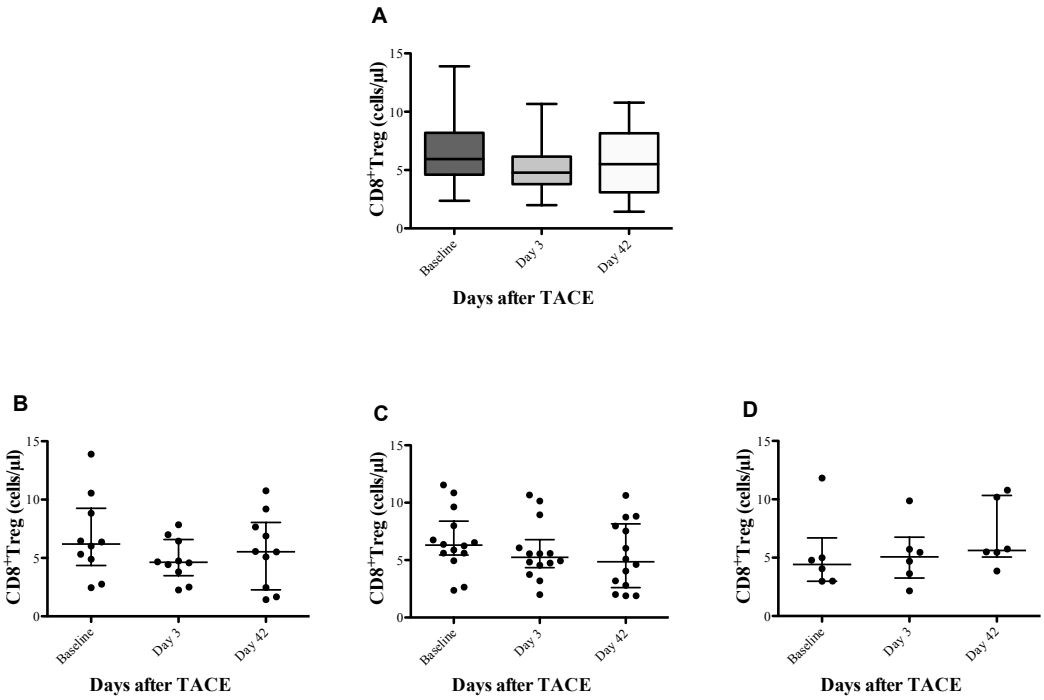

Figure 6-7 CD8$^+$Treg absolute number following treatment with TACE

The absolute number of circulating CD8$^+$Treg were analysed in 30 patients at baseline, day 3 and day 42 following TACE and correlated to treatment response according to mRECIST criteria by cross-sectional imaging. (A) Changes in circulating CD8$^+$Treg number in entire cohort (n=30), (B) complete responder (n=10), (C) partial responder/stable disease (n=14), (D) non-responders (n=6) at baseline, day 3 and day 42 following TACE. Statstiscal comparisons were made with Wilcoxon signed ranked test..

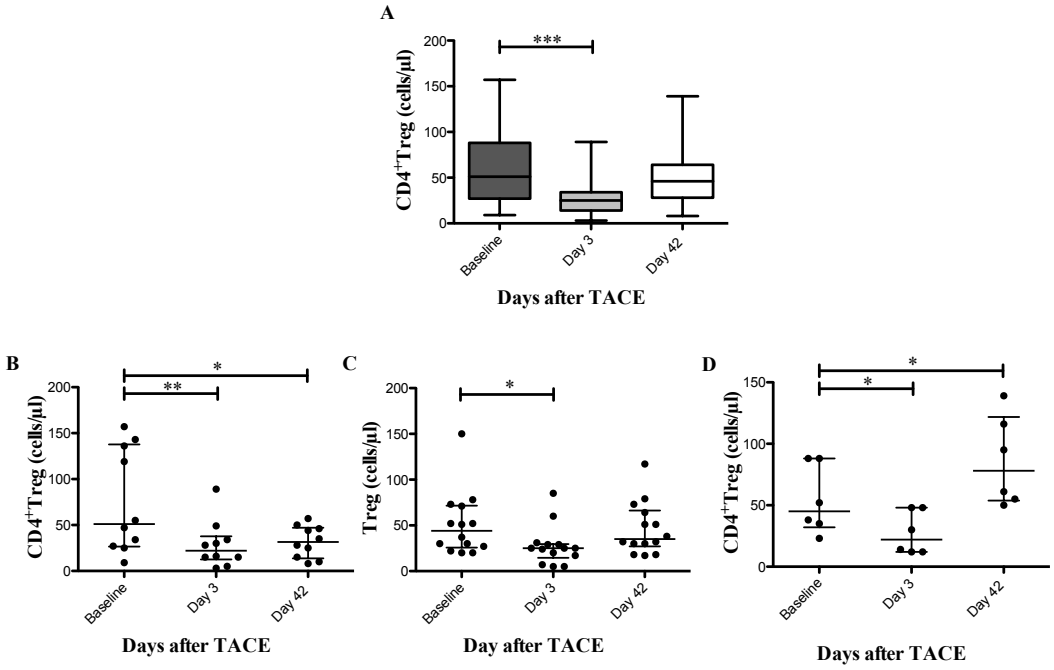

Figure 6-8 CD4$^+$Treg absolute number following treatment with TACE

The absolute number of circulating CD4$^+$Treg were analysed in 30 patients at baseline, day 3 and day 42 following TACE and correlated to treatment response according to mRECIST criteria by cross-sectional imaging. (A) Changes in circulating CD4$^+$Treg number in entire cohort (n=30), (B) complete responder (n=10), (C) partial responder/stable disease (n=14), (D) non-responders (n=6) at baseline, day 3 and day 42 following TACE. Statstiscal comparisons were made with Wilcoxon signed ranked test where *P<0.05, **P<0.01, ***P<0.001 vs. baseline.

6.4 Frequency of circulating Treg in disease stage and age

A number of studies had previously confirmed the presence of Treg in the circulation of patients with different tumours and the frequency of Treg had been suggested to be predictive of disease stage and prognosis. Having already demonstrated the lack of correlation between tumour infiltrating Treg and tumour volume or disease stage in the current study (Chapter 3). A repeat analysis based on circulating Treg was performed. Similarly, circulating CD8$^+$Treg or CD4$^+$Treg did not correlate with tumour volumes in this study (CD4$^+$Treg; r^2=0.034, CD8$^+$Treg: r^2=0.032) (Figure 6-9). However, 10 patients had prior local ablative treatment (8 had TACE, 2 had RFA) before inclusion into the study, which may confound the interpretation of the reported results. In addition, analysis was performed on the frequency of circulating CD4$^+$Treg and CD8$^+$Treg according to patient's age. However, no correlation was found (CD4$^+$Treg; r^2=0.01, CD8$^+$Treg: r^2=0.041) (Figure 6-10).

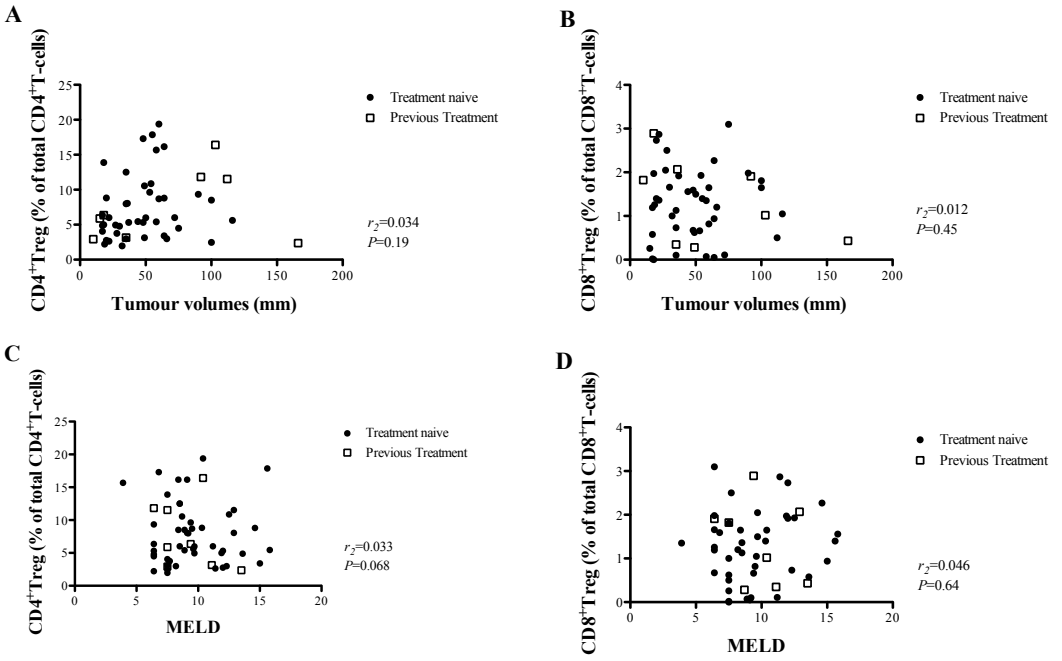

Figure 6-9 The effect of tumour volumes or severity of liver disease on circulating CD4$^+$Treg or CD8$^+$Treg level

The baseline percentage of (A&C) circulating CD4$^+$Treg or baseline percentage of (B&D) circulating CD8$^+$Treg of individual patients was plotted against total tumour volume (mm^3) or severity of liver disease according to their MELD score. Patients who were treatment naïve are displayed as black circles and patients who had previous local ablative treatments are displayed as white squares. Statstiscal comparisons were made with Spearman's correlation r^2 and P value are indicated. MELD; modified end-stage liver disease.

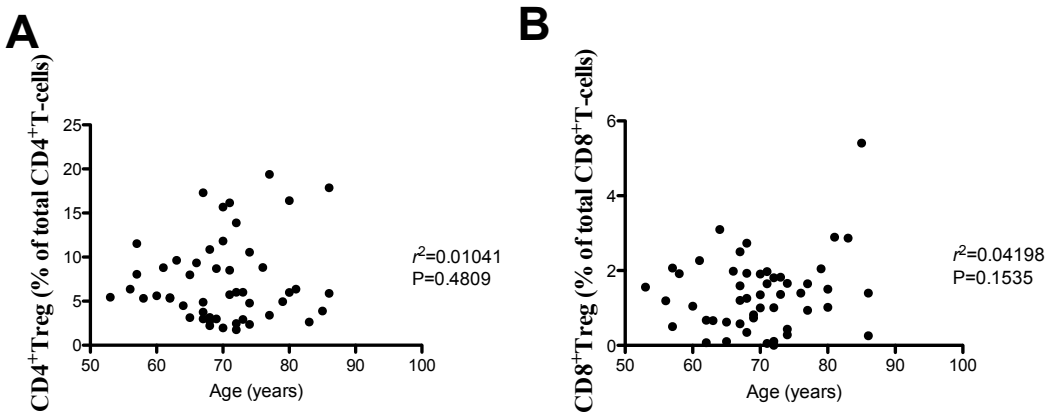

Figure 6-10 The effect of age on circulating CD4$^+$Treg or CD8$^+$Treg level

The baseline percentage of (A) circulating CD4$^+$Treg or baseline percentage of (B) circulating CD8$^+$Treg of individual patients was plotted against age. Statstiscal comparisons were made with Spearman's correlation r^2 and *P* value are indicated.

6.5 Effect of chronic viral liver disease on CD4$^+$Treg following TACE

As no variation in the frequency of circulating CD8$^+$Treg following TACE was identified, the study focused on circulating CD4$^+$Treg.

As the development of HCC occurs invariably on the background of underlying chronic liver disease (in particular viral liver disease) it is important to consider the effect the viral immune response may have on the results seen. Following adjustment of the obtained data by censoring of patients with viral disease (n=16) in the analysis, the changes in circulating CD4$^+$Treg after TACE displayed a similar trend to the original cohort (viral + non-viral) (Figure 6-11). A statically significant reduction in circulating CD4$^+$Treg percentage in both complete-responder (n=16; baseline; 6.15%±8.04%, day 3; 4.62%±4.54%, day 42; 5.01%±9.30%) and partial responder/stable disease groups (n=14; baseline; 9.48%±7.28%, day 3; 8.75%±6.4%, day 42; 6.78%±5.24%) was seen.

The changes seen in patients with progressive disease were also similar, with an increase in circulating CD4$^+$Treg percentage following TACE (n=4; baseline; 3.73%±1.89%, day 3; 4.71%±1.89%, day 42; 6.57%±2.62%), but this did not reach statistical significance. The stratification of treatment response in the viral group, also demonstrated a trend toward a reduction in circulating CD4$^+$Treg percentage in the complete-responder (n=7; baseline; 5.32%±3.2%, day 3; 2.49%±4.27%, day 42; 3.02%±3.83%) and partial responder/stable disease group (n=3; baseline; 4.94%±2.47%, day 3; 3.64%±3.56%, day 42; 3.19%±2.32%), and an increase in

circulating CD4$^+$Treg percentage in patients with progressive disease (n=6; baseline; 4.85%±3.16%, day 3; 5.59%±3.86%, day 42; 7.95%±5.27%). However, none of the stratified viral group reached statistical significance. Interestingly, patients with viral disease consisted 60% (6/10) of the progressive disease group despite forming only 30% (16/50) of the entire cohort.

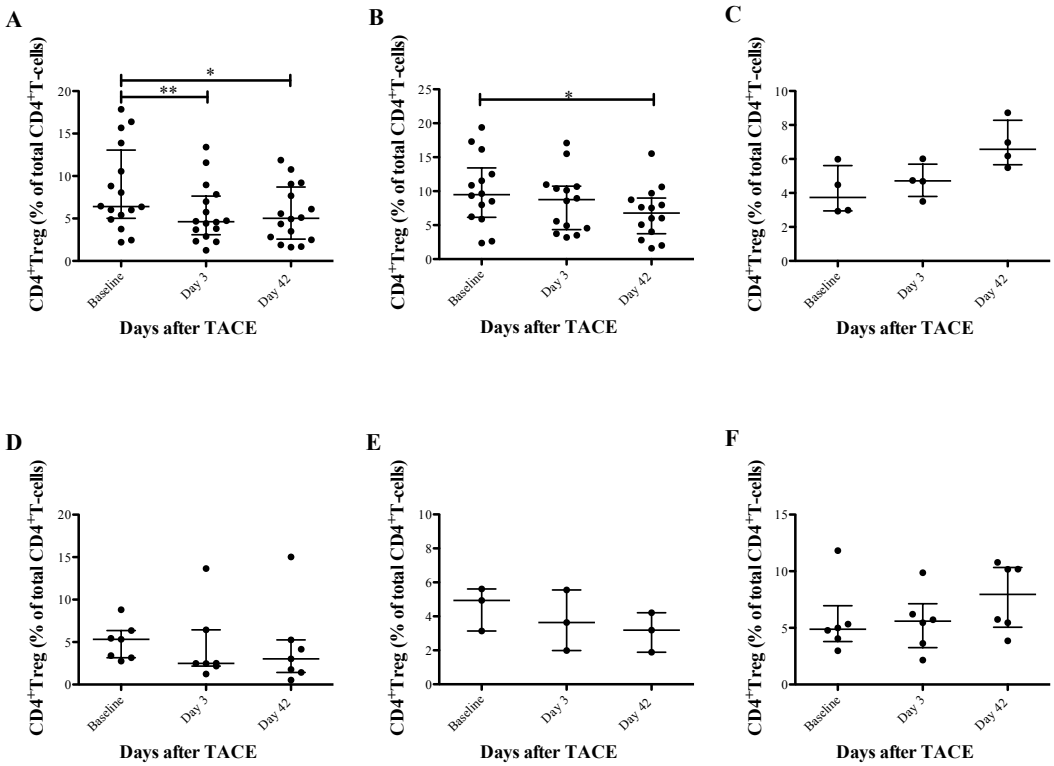

Figure 6-11 Changes in circulating CD4$^+$Treg with treatment response

The percentage of circulating CD4$^+$Treg was analysed according to the underlying chronic liver disease (non-viral disease (n=34) and viral disease (n=16)) at baseline, day 3 and day 42 following TACE and correlated to treatment response according to mRECIST criteria by cross-sectional imaging. Changes in the percentage of circulating CD4$^+$Treg in the non-viral group accoding to response; (A) complete responder (n=16), (B) partial responder/stable disease (n=14), (C) non-responders (n=10) and in the viral group; (D) complete responder (n=4), (E) partial responder/stable disease (n=3), (F) disease progressor (n=6). Data are expressed as median and interqurtile range. Statstiscal comparisons were made with Wilcoxon signed ranked test where *P<0.05, **P<0.01, vs. baseline.

6.6 CD4$^+$CD25^{high}CD127^{low} T-cells phenotype following TACE

To ensure that the circulating CD25^{high}CD127^{low} cells I had identified are bono fide CD4$^+$Treg, I extended the phenotypic analysis to define expression of some of the accepted regulatory markers previously described in CD4$^+$Treg. CD4$^+$Treg isolated from blood of HCC patients demonstrated high expression of FOXP3, CTLA-4, CD39 and CCR4 (Figure 6-12).

It is possible TACE may have the potential to induce the activation of effector T-cells resulting in the up-regulation and expression of markers such as CD25. To ensure the changes in CD4$^+$CD25^{high}CD127^{low} seen in HCC patients truly reflect changes in CD4$^+$Treg and not activated T-cells, I analysed the expression of the master transcription regulator FOXP3 on CD4$^+$T-cells before and after treatment. The expression of FOXP3 on CD4$^+$CD25^{high}CD127^{low} T-cells was maintained at baseline (77.33%±10.07%), day 3 (73.33%±16.07) and day 42 (79.3%±11.93%) after TACE treatment (Figure 6-12).

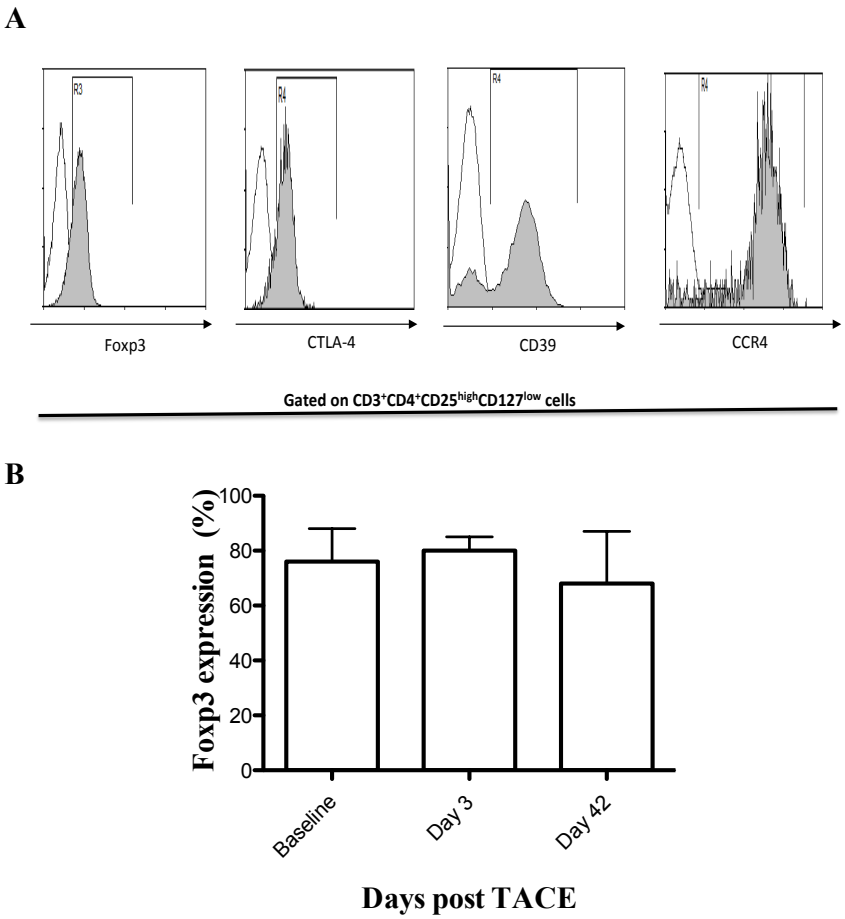

Figure 6-12 The effect of TACE on circulating CD4$^+$Treg regulatory phenotype

(A) Representative flow cytometic histograms showing the expression of FOXP3, CTLA-4, CD39 and CCR4 on CD4$^+$CD25^{high}CD127^{low} T-cells **(grey)** compared to isotype controls **(white)**, from the blood of HCC patients. (B) The expression of FOXP3 expressed on CD4$^+$Treg was analysed at baseline, day 3 and day 42 following TACE. Data are expressed as median and interqurtile range. Statstiscal comparisons were made with Wilcoxon signed ranked test vs. baseline. Representative of 3 samples.

6.7 The suppressive capacity of circulating CD4$^+$Treg following TACE

To further confirm that the circulating CD4$^+$CD25^{high}CD127^{low} T-cells are CD4$^+$Treg, I isolated CD4$^+$CD25^{high} T-cells from the blood samples of HCC patients prior to their TACE. CD4$^+$CD25^{high} T-cells were isolated by magnetic bead isolation and their regulatory function was analysed using a suppression assay as previously described (n=8). CD4$^+$Treg isolated from HCC patients were suppressive at baseline, as demonstrated by their ability to inhibit CD3$^+$CD28$^+$ activated responder T-cell proliferation in a dose dependant manner (Figure 6-13). These findings confirm that CD4$^+$CD25^{high}CD127^{low} T-cells detected in HCC patients are functional CD4$^+$Treg.

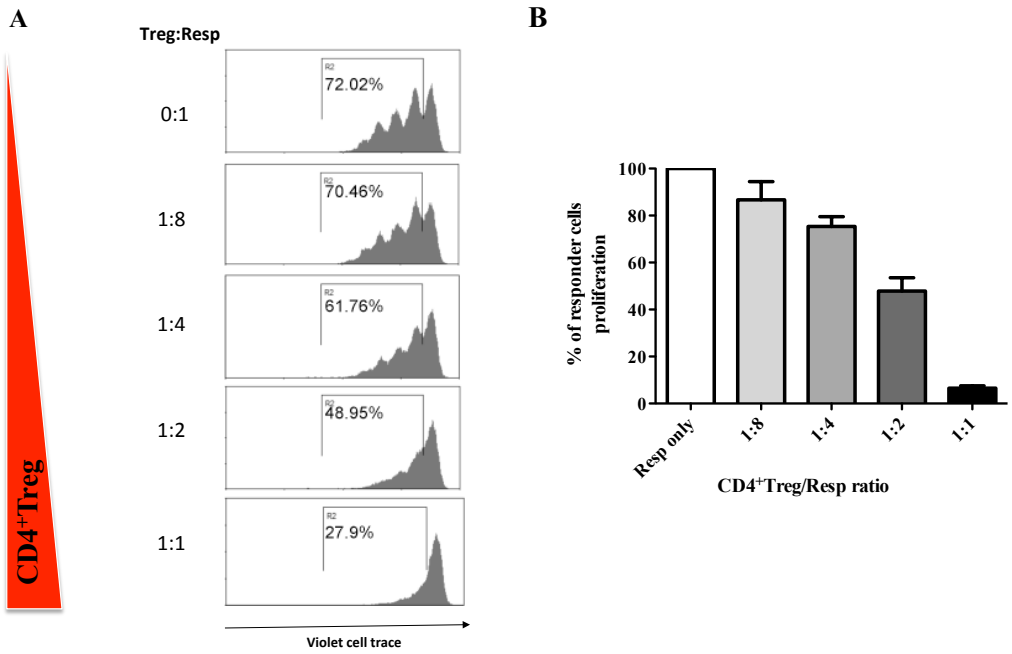

Figure 6-13 The assessment of circulating $CD4^+$Treg suppressive function from HCC patients

The suppressive ability of $CD4^+$Treg was assessed by labelling allogenic responder T-cells with fluorescent violet cell trace and cocultured with Treg in decreasing ratios following comparison to stimulated control. Responder T-cells were activated with $CD3^+CD28^+$ beads to induce responder T-cell proliferation. After 3 days of culture, T-cell proliferation was analysed by determining violet cell trace dilution using flow-cytometry. (A) Representative flow cytometric histogram showing T-cell proliferation in the presence of increasing ratio of $CD4^+$Treg from an HCC patient. (B) Replicate data presented in a table format. Data are expressed as mean and standard deviation. Representative of 24 samples.

To identify weather TACE-treatment has any effect on the function of $CD4^+$Treg, their suppressive capacity was compared before and after treatment. $CD4^+$Treg maintained their suppressive capacity despite TACE-treatment, with comparable suppressive ability before and after TACE irrespective of response to treatment (Figure 6-14). The suppressive capacity of $CD4^+$Treg showed inter-individual differences, despite standardising the assay by using responder T-cells from the same healthy donor.

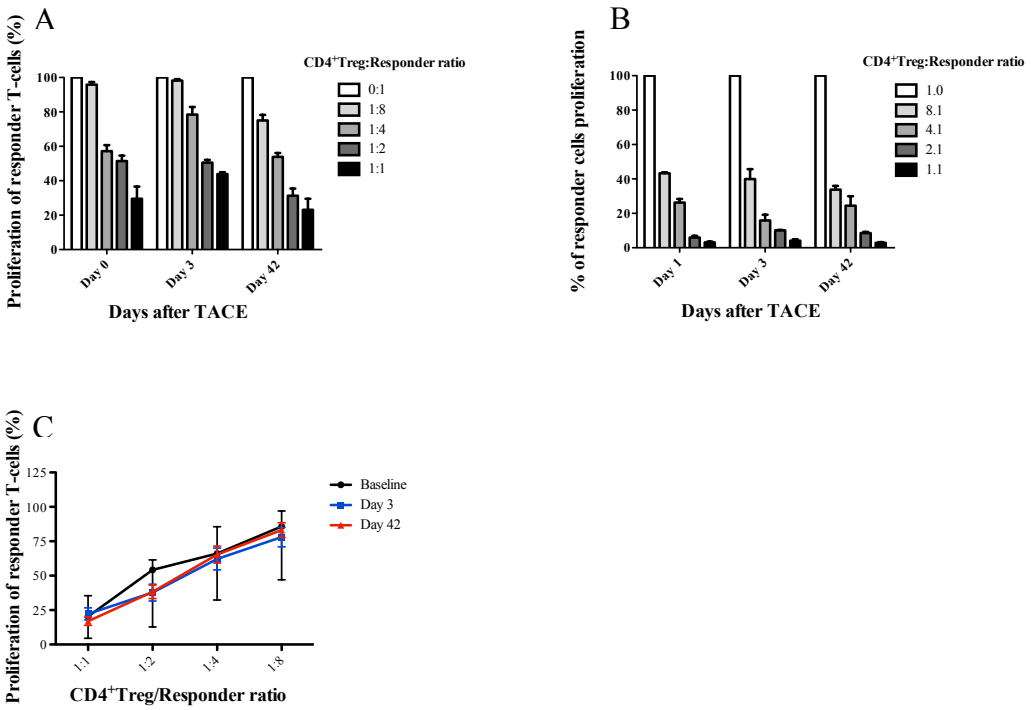

Figure 6-14 The assesment of suppressive ability of CD4$^+$Treg before and after TACE-treatment.

Data from two representative allogenic T-cell-proliferation-assays from (A) patient-31 and (B) patient-45 at baseline, day 3 and day 42 following TACE. (C) The suppression of CD4$^+$Treg at baseline, day 3 and day 42 following TACE were assessed in 10 patients. Data are expressed as means ± standard deviation.

6.8 Changes in circulating CD4$^+$Treg with progression free survival

To assess whether the prognosis of patients with HCC undergoing TACE treatment was associated with changes in circulating CD4$^+$Treg, analysis of any correlation between progressions free survival and changes in circulating CD4$^+$Treg was performed. Patients were separated into two groups according to either an increase or decrease in CD4$^+$Treg, at day 3 or day 42 after TACE treatment compared to baseline. Patients were censored if they proceeded to liver transplantation. Patients with a decrease in the circulating CD4$^+$Treg at day 42 following TACE had a significant prolonged progressive free survival (291 days) when compared to patients with an increase in CD4$^+$Treg (84 days), $p<0.05$. However, the change in circulating CD4$^+$Treg percentage at day 3 following TACE did not demonstrate such pattern, with both groups showing similar progression free survival time (increased; 291 days vs. decreased; 229 days).

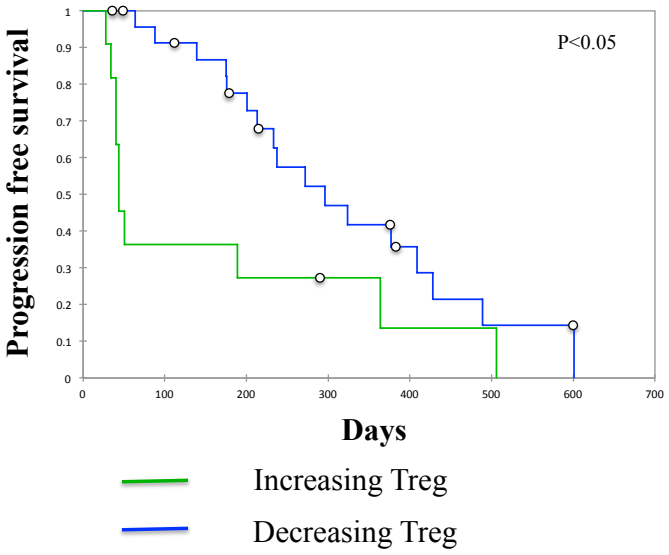

Figure 6-15 Correlation of progression free survival with circulating CD4$^+$Treg

Patients undergoing TACE-treatment were separated into two groups, according to the change in their circulating CD4+Treg percentage 42 days after treatment. Progression free survival when compared between the two groups. Kaplan-Meier survival curve illustrating the progression-free survival difference between patients that had an increase in CD4$^+$Treg (green) or decrease in CD4$^+$Treg (blue). Statstiscal comparisons were made with log rank test. $p < 0.05$.

6.9 Discussion

TACE has been shown to be an effective palliative treatment in patients with HCC, resulting in improvement in patient's survival [523]. The effectiveness of TACE is a result of directed local-regional delivery of chemotherapy and the disruption of blood supply to the tumour. However, increasing evidence suggests ablative treatment may enhance anti-tumour immune responses in addition to direct tumour cell killing. Such hypotheses derive from the observation that the destruction of tumour cells results in the release of large quantity of immune-active molecules (e.g. heat shock proteins and pro-inflammatory cytokines such as IL-17 and VEGF), which may have a potential role in inducing an immune response [391-394].

Further evidence supporting this hypothesis comes from studies demonstrating the activation of a wide range of immune cells following local ablative treatment [526]. Importantly, patients who demonstrated enhanced immune responses following such treatments had better outcomes when compared to patients who lacked evidence of immune responses [385]. In a study by Zeribini et al., circulating NK cells were increased following RFA in patients with HCC and this was associated with activation of NK cells with enhanced cytotoxicity and IFN-γ production. In a study investigating the presence of TAA-specific CD8$^+$T-cells before and after local ablative treatment in patients with HCC [386], the number of TAA-specific CD8$^+$T-cells was significantly associated with prolonged tumour free survival. In a similar study, T-cell-responses to autologous tumour lysate were assessed before and after ablative treatment in patients with HCC. Response to autologous tumour lysate was

significantly increased following treatment [387]. In a novel study addressing the effect of local ablative treatment on APC, ex-vivo generated moDC were stimulated with tumour tissue obtained from HCC patients at the time of RFA. MoDC stimulated with RFA treated tumour tissue displayed a more mature phenotype when compared to moDC stimulated with non-treated tumour tissue or matched non-tumour tissue. In addition, moDC stimulated with RFA treated tissues demonstrated an enhanced ability to produce IL-12, induce T-cell proliferation and direct the expansion of TAA-specific T-cells [387].

The enhancement of anti-tumour immune responses in patients undergoing local ablative treatment has often been explained by the release of tumour antigen into the circulation or the release of pro-inflammatory cytokines as a result of tumour cell death. However, the precise mechanisms how anti-tumour the immune response is enhanced following local ablative treatment remains unclear. Results from my current work suggest the modulation of the suppressive tumour environment by local ablative treatment may be a possible mechanism. The disruption of Treg by TACE may remove the inhibition on effector immune cells, leading to re-establishment of an anti-tumour immune response as suggested by the increase in TAA responses following TACE seen in my current work. Further support for the possible contribution of local ablative treatment on the disruption of the suppressive tumour environment derives from a recent study by Mizukoshi et al. Here, they reported that TAA-specific T-cell responses were inversely correlated with the presence of circulating suppressive MDSC (CD14-HLR-DR^{low}) in patients undergoing RFA treatment for HCC. [388]. In addition, in a study of 33 patients undergoing TACE for HCC, changes in circulating

CD4$^+$Treg were observed to correlate inversely with poorer prognosis [389]. However, the study was limited to the use of CD25 expression as a sole marker for CD4$^+$Treg and with no functional data to confirm the suppressive capacity of the CD4$^+$Treg population. In a study of HCC patients undergoing local ablative treatment with cryotherapy [390], 31 patients were monitored for changes in circulating CD4$^+$Treg as defined by a combination of CD25^{high}CD127^{low} and FOXP3 expression before and after treatment. Patients were stratified according to their response to treatment as defined by RECIST, in the 17 patients whom responded to treatment a correlation between a significant decrease in CD4$^+$Treg at day 48 and response was recorded. In comparison, 14 patients who had progressive disease had an increase in their circulating CD4$^+$Treg following treatment. Interestingly, compared to my work, the study observed an increase in CD4$^+$Treg suppressive capacity following treatment in the tumour progression group as compared to the responder group. Such differences in the results may be explained by variation in methods used to assess Treg suppression. In particular this study used [3H]-thymidine labelled cells to detect proliferation of responder cells which lead to an overestimation of the suppressive capacity of Treg (chapter 5) and, in addition, the study used responder T-cells from different healthy donors, with inherent variation in T-cell proliferation, which can be mistaken for differences in CD4$^+$Treg suppression seen between patients.

In my work, the majority of the patients had a decrease in circulating CD4$^+$Treg subsets following TACE and this correlated with a prolonged progression free survival when compared to patients with an increase in CD4$^+$Treg. A potential explanation for the decrease in circulating CD4$^+$Treg may be the result of direct

tumour killing, leading to a reduction in tumour bulk. The reduction in tumour bulk is likely to disrupt tumour cells or tumour stromal cells associated with the induction or stimulation of CD4$^+$Treg, leading to a decrease in CD4$^+$Treg percentage. It is plausible that the reduction in circulating CD4$^+$Treg may be the result of pooling of CD4$^+$Treg into the tumour following TACE. However, the prolonged progression free survival seen in this group of patients is counterintuitive for such a hypothesis, as an increase in CD4$^+$Treg tumour infiltration has been shown to be a predictor of a worse outcome. To clarify this issue, changes in tumour infiltrating CD4$^+$Treg before and after treatment with concomitant analysis of changes in circulating CD4$^+$Treg would need to be performed. However, this would require the obtaining of serial tumour biopsies, which is unlikely to be ethically acceptable in the UK due the risk of tumour seeding and, more importantly, the increased risk of bleeding associated with tumour biopsy. In addition, the interpretation of the results may be hampered by the heterogeneity of the tumour after TACE. Following TACE, the tumour will consist of variable regions of necrotic and viable tumour tissue and the results of such a study would be highly dependent on the region the biopsy was obtained from. However, in a study conducted in China, tumour biopsies were obtained from 16 HCC patients before and after treatment with cryoablation. Using immunohistochemistry, the frequency of FOXP3 positive cells was reduced following treatment. This would argue against a possible role for Treg pooling following treatment as a reason for the reduction in circulating CD4$^+$Treg [390].

Another potential reason for the reduction in circulating Treg may be related to the immunomodulation of other subsets of immune cells. The tumour microenvironment

is highly infiltrated by a variety of immune cells such as tolerogenic DC and MDSC. Both of these subsets of cells have been shown to be involved in the generation of CD4$^+$Treg. In a study looking at primary isolated DC from the blood of HCC patients undergoing ablative treatment, the maturation of mDC and their ability to induce T-cell proliferation were significantly enhanced following treatment with RFA or PEI [527]. Hence, the stimulation of DC may indirectly inhibit the induction of CD4$^+$Treg. Unfortunately in the current study I had limited availability of material to permit analysis of the maturation status of DC following treatment.

At the other end of the spectrum, patients who had an increased circulating CD4$^+$Treg frequency following treatment were associated with progressive disease and shorter progression free survival. In such patients the disease progressed rapidly, more so than expected for the slow proliferation of most HCC. Such rapid progression may be the result of positive feedback from the remaining viable tumour cells. This could result in the production of an array of pro-tumorigenic molecules (IL-6, VEGF)[528], supporting tumour progression [529, 530]. Interestingly the elevation of VEGF has been proposed to be associated with CD4$^+$Treg induction [531].

Accurate interpretation of data is essential if we are to arrive at robust conclusions. The majority of the studies involving Treg have used the percentage of Treg as their main readout and similarly I used the same method for the analysis of my data. The affect of Treg on immune response is likely dependent on the balance between effector cells and regulatory cells. Hence an absolute change in Treg numbers may not reflect the actual kinetics of an immune response, as an increase in Treg number

may be counteracted by a comparable increase in effector T-cells. Nevertheless, the result derived from looking at the change in absolute Treg count at day 42 following TACE also correlated with treatment response. Interestingly, the absolute CD4$^+$Treg count was reduced in all patients irrespective of treatment response at day 3, which differs from changes in the proportion of CD4$^+$Treg in relation to other T cells. The reduction in absolute cell count is likely a reflection on the lymphopenic effect caused by the chemotherapy used in TACE. Evidence suggests that the immunomodulation potential of certain chemotherapeutic agents may be implicated in the selective depletion of Treg [379]. This may provide another possible mechanism for the reduction in circulating Treg following TACE seen in my current work.

The current study has illustrated the immunmodulatory effects of TACE, in particular in relation to the depletion of circulating CD4+Treg. However, despite undergoing TACE, a significant number of patients (n=10) did not display a decrease in circulating CD4$^+$Treg and, importantly, this was associated with a shorter time to progression-free survival. Bearing this in mind, the data presented here suggests this group of patients may benefit from additional treatment in order to reduce circulating CD4$^+$Treg. However, the effectiveness of such an approach would need to be tested in a clinical trial setting.

Further depletion of CD4$^+$Treg may provide further benefit for patients irrespective of the change in CD4$^+$Treg caused by TACE. The most promising agent that has been investigated for the depletion of CD4$^+$Treg comes in the form of the alkylating agent cyclophosphamide. Cyclophosphamide is a well-known agent used in a wide range of

human malignant and autoimmune diseases. However, it was observed that the administration of cyclophosphamide at a lower dose than is traditionally used might have a potential immunomodulating effect. The first recognition of its ability to enhance immune responses was by demonstrating a boosting of vaccination responses in humans [377]. This was followed by the hypothesis that the immunmodulating property of cyclophosphamide may be due to its ability to selectively deplete immune suppressive T-cells.

In a mouse model, administration of low dose of cyclophosphamide (2mg intraperitoneal) caused a modest decrease in splenocytes within 24 hours, a nadir of 50% by day 4 and full recovery by day 10. Throughout the nadir, there was selective depletion of $CD4^+CD25^+$ cells, functionally confirmed as Treg [378]. In a rat model of Treg-sensitive immune-mediated tumour rejection, only cyclophosphamide and methotrexate had an effect on Treg numbers, from a range of cytotoxic agents tested. Cyclophosphamide 30mg/kg IP resulted in selective depletion of Treg and release from immune suppression, which peaked at day 7-post injection. This single cyclophosphamide dose delayed tumour growth and enhanced the efficacy of anti-tumour vaccination [368]. Furthermore, the effect on $CD4^+CD25^+$ cells accounts for the long recognised effect of cyclophosphamide on skin contact sensitivity to dinitrochlorobenzene [532] and increases the potential for activation of high avidity anti-tumour $CD8^+$ responses [533]. Bolus cyclophosphamide treatment of animals has been reported to modify the cytokine release profile of T lymphocytes and to transiently reduce T-cell proliferation followed by a rebound expansion [534].

More importantly, in a clinical study of patients with metastatic solid tumours, a metronomic low dose cyclophosphamide regimen (50mg orally twice daily day 1-7 on 14-day cycles) reduced numbers of putative Treg. Consecutively, this led to a reversal of suppression of both natural killer lytic activity and T cell receptor-induced T cell proliferation [379]. In line with these findings a small pilot study in HCC showed that low-dose cyclophosphamide reduced circulating Treg numbers in patients with HCC [380].

Conversely to these low dose studies, high dose cyclophosphamide has been successfully adapted as part of conditioning ahead of autologous T cell infusion for melanoma [535]. It is apparent that vaccination of profoundly lymphopenic hosts during T cell recovery can result in greater immune responses than vaccination of normal hosts [536]. Whether a similar mechanism might enhance expansion of responders to vaccination following low dose cyclophosphamide is not clear.

With increasing evidence suggesting the possible benefit of selective Treg depletion by cyclophosphamide in the enhancement of anti-tumour responses, low dose cyclophosphamide has been incorporated into trials of DC-based cancer vaccines. Such studies have also included patients suffering from HCC and have demonstrated the efficacy and safety of combining cyclophosphamide with available DC vaccines. Hence, the addition of cyclophosphamide in conjunction with TACE may offer further benefit.

The assessment of response to local ablative treatment in HCC can often be challenging. In many solid cancers, the response to treatment such as chemotherapy is commonly assessed by the RECIST criteria, which is biased towards shrinkage of tumour lesion(s) following treatment. Whilst the use of the RECIST criteria has offered a robust method to standardise how we assess treatment response, its use in the assessment of TACE treatment in HCC-patients can often be problematic. The successfulness of TACE is dependent on its ability to induce tumour necrosis and the use of RECIST criteria to assess response to TACE-treatment may not reflect this mechanism, leading to underestimation of the response. However, due to the unique vasculature of HCC, viable tumour can be assessed by enhancement seen during different phases on cross sectional imaging. Using such properties a modified RECIST criteria was developed incorporating vascular enhancement in the treated tumour to aid in the assessment of response to treatment. As a result, my current work has employed the use of mRECIST over traditional RECIST criteria for the assessment of tumour response following TACE.

The association of HCC with viral hepatitis is well documented and the persistence of viral disease has been associated with escape from the host immune response, potentially by the induction of regulatory immune cells such as Treg. Hence, it is possible that the result of this study may be affected by the additional immunosuppression caused by the virus itself. However, the majority of results obtained remained significant (CR & PD group) even when analysis of patients who suffered from viral liver disease (n=16) was censored. Interestingly, out of the 10 patients with progressive disease following TACE, 6 of them had either HCV or HBV

infection. It maybe therefore be plausible to suggest that the additional immune suppression exerted by the viral infection may compound the inhibition of any anti-tumour response.

Similarly to tumour infiltrating Treg (chapter 3), circulating Treg from HCC patients prior to TACE treatment did not appear to correlate with tumour volume. The reason for such discrepancy compared to previous published studies had already been discussed in detail in previous chapters. Additionally, unlike the analysis of tumour infiltrating Treg which allows for the macroscopic analysis of the tumour, the comparison of tumour volume/disease stage with circulating Treg is often carried out using measurements obtained from cross sectional imaging. It is well known that tumour size can differ widely between imaging and macroscopic measurement, hence adding further bias when correlating tumour volume data with circulating Treg.

7 Overview

This study has confirmed the presence of a subset of HCC infiltrating $CD8^+$T-cells, that share many phenotypical features of the well-studied $CD4^+$Treg. The $CD8^+$ Treg identified are characterised by elevated expression of the IL2 receptor alpha chain, CD25, minimal expression of the IL7 receptor alpha chain, CD127 and a sustained expression of the master transcription regulator FOXP3. More importantly, tumour infiltraing $CD8^+CD25^{high}CD127^{low}$ cells were able to inhibit responder T-cell proliferation, hence, confirming their status as regulatory $CD8^+$T-cells. $CD8^+CD25^{high}CD127^{low}$Treg have been previously described in human disease [537], including one study in human HCC [239]. However, the current work is the first study incorporating functional data.

A review of the published literature reveals many different subsets of $CD8^+$T-cell with regulatory functions have been described, with varied phenotype, suppressive capacity and proposed mechanism of action [187, 190, 200, 222, 225, 229, 486, 538]. This extensive variation in the description of regulatory $CD8^+$T-cells is a reflection of the complexity of the immune system. Unlike $CD4^+$Treg, the many subsets of regulatory $CD8^+$T-cells that have been described are often reported to be induced from non-regulatory precursor cells [180, 442, 539, 540], a finding mirrored in my current work. Evolution of an immune system that possesses a diverse mix of Treg with varied phenotype and function can be related to the key role of suppressive immune cells. It is logical for the host to have multiple mechanisms to counteract against autoimmunity, having the ability to tailor specific subsets of regulatory cells

to counteract self-reactive immune responses. This may explain the heterogeneous phenotype identified in studies describing $CD8^+$Treg. Alternatively, the reported variation may be an experimental artefact induced by the inconsistency of experimental methods used in the study of Treg by different investigators. A key criticism of many studies is the lack of functional assessment of the cells of interest, which remains the gold-standard to confirm regulatory phenotype. This can largely be explained by the difficulties faced in obtaining adequate human samples.

Lack of tissue availability can be the result of tumour location, i.e. non-operative tumours within abdominal organs, and the need of diagnosis overriding the need for research. Also the increasing use of non-invasive techniques in the diagnosis of some cancers has contributed to the continued restriction of material for research. The current study did not differ and many of the same difficulties were encountered. In the West, HCC remains uncommon when compared to countries with an epidemic of HCC [4]. Consequently, obtaining tissue was unpredictable, as sources of fresh tumour were derived from explanted liver following liver transplantation. To this end, I had to adopt my research methods due to the limitation of tissue availability, for example, by optimising the suppression assay [541]. However, as technology advances, we will be better placed to overcome the limitation commonly seen in cancer immunology research. The development of more sophisticated flow cytometry platforms capable of detecting more antigens in any one sample with the additional ability to isolate cells by cell sorting will increase the repertoire of assays we are able to perform [542].

In an attempt to overcome the difficulties in obtaining human samples, it is often necessary that we turn towards animal models and tumour cell lines to further our understanding of tumour biology. However, no animal models or cell lines can fully recapitulate the human system. This is especially important when studying human immune-biology. This disparity between human physiology and animal models is typified by many of the common murine models used in HCC. The majority of which occur without chronic liver disease, a scenario rarely encountered in human HCC [543]. Patients with chronic liver disease often present with immunological symptoms, indeed most liver damage is a consequence of immune activation in response to some form of insult. Accordingly, the immune response in these patients is often compromised and, as such, the use of animal models of HCC lacking the background of liver inflammation is unlikely to reflect the human system.

The current work has demonstrated that HCC tumour-microenvironment can imprint DC which, when cultured with naïve T cells, induces a subset of $CD8^{+}$Treg. However, in common with other tumour immune studies, the current experiments had focused on interaction of only a few cell types in isolation. It is important to recognise that the tumour is a dynamic environment, consisting of many tumour cells (often with varying mutations), tumour stromal cells and immune cells[429]. The tumour microenvironment is defined by the complex interaction between these cells and yet most research in the field is reductionist and focusses on at best a few cell types. Clearly, devising and validating robust models to recapitulate this heterogeneous milieu will be both complex and time consuming. As a result of this, immune pathways are frequently treated as one directional processes, although increasing

evidence has suggested the importance of multi-directional cross-talk between different cell types within the tumour [544]. Of particular relevance to the current study, recent work has suggested that not only do Treg become activated and induced by DC, Treg themselves may also have the potental to direct DC towards a tolerogenic phenotype [545-547]. Thus, creating a positive feedback towards the generation of a suppressive tumour microenvironment. It is important therefore to consider multi-cell/direction interaction in an studies continuing on from my research.

The field of immunotherapy has developed significantly since it was first described. Early attempts at treating malignant disease by inducing an immune reaction were developed by William B Coley in the late 19^{th}/early 20^{th} Century and were met with scepticism [548]. However, contemporary data supporting his approach have validated these early claims and more recent studies demonstrating the infiltration of immune cells in a wide range of human tumours, including HCC, have provided support the concept of immunotherapy [549]. The first generation of modern-day immunotherapeutic treatments consisted of cytokine-based therapy and demonstrated promising results in cancer patients with improvement in recurrence-free survival [316-318]. This resulted in an increased interest in the field and gave rise to several studies using dendritic cells as a form of cellular vaccine against cancer, further encouraging the potential of immunotherapy [349].

However, despite the potential of early phase clinical trials, the overall result of immunotherapy has so far been disappointing. Whilst many studies have demonstrated an ability to enhance anti-tumour responses in patients with solid organ

tumours, few have translated to lasting clinical responses [550]. Most immunotherapy has focused on the amplification of the effector arm of the anti-tumour immune response, either by vaccination with antigen loaded DC (including in the first FDA approved cancer vaccine for use in prostate cancer [551]) or by expansion of tumour infiltrating T-cells [552]. However, as our understanding of the suppressive nature of the tumour microenvironment has increased, a greater understanding of the molecular pathways involved has enabled the development of drugs designed to target tumour suppression. Currently, data from the new generation of immunomodulatory agents, such as checkpoint blocking drugs is very encouraging [553]. A number of trials involving immune checkpoint inhibitors designed to block the effect of CTLA-4 and PD-1 have shown early promise, including a recent phase II clinical trial of CTLA-4 blockade with tremelimumab in patients with HCC. [335]

Data from the current work offer further support in the potential benefit of disruption of the suppressive tumour environment. Treatment responses to TACE correlated with a reduction in $CD4^{+}$Treg in patients with HCC, suggesting perturbation of the regulatory repertoire will be advantageous. Furthermore, such results may support the measurement of circulating Treg as a prognostic indicator of TACE treatment-response. Importantly, the ability of ablative treatment to modulate immune responses, in addition to the direct destruction of tumour cells, makes it an attractive adjuvant to current immunotherapeutic interventions. As immunotherapy is likely to be more effective on the background of reduced immunosuppression and reduced tumour bulk. Importantly, recent early phase clinical trials combining the use of transarterial embolization (TAE) with intra-tumoral infusion of DC vaccine for HCC

has appeared to be safe (Table 7-1), giving further support for the use of TACE as an adjuvant to immunotherapy in the next phase of clinical trials.

Table 7-1. Summary of DC vaccine trials involving trans-arterial embolization

Study	**Number of Patients**	**Vaccine**	**Injection route**	**Adverse reaction**
Nakamoto 2011 [554]	13	GM-CSF–IL-4 DCs and OK432	Intra-arterial	N/A
Nakamoto 2007 [555]	10	GM-CSF–IL-4 DCs	Intra-arterial	No grade 3 to 4
Mizukoshi 2009 [556]	33	GM-CSF–IL-4 DCs +/- OK432	Intra-arterial	No grade 3 to 4

GM-CSF, Granulocyte-macrophage colony-stimulating factor; IL-4, Interleukin-4; DC, dendritic cells.

However, the data generated in the current study suggest that despite the ability of TACE to disrupt $CD4^+$Treg frequency in a large majority of patients, a significant number (20% progressive disease) had little benefit. The observed increase in circulating $CD4^+$Treg in these patients suggests the addition of $CD4^+$Treg depletion treatment may be beneficial. Adjuvant treatment to disrupt the $CD4^+$Treg population could be achieved with the use of the alkylating agent cyclophosphamide, which, when used at low doses, has been shown to selectively deplete these cells [379].

Although early results from checkpoint inhibitor studies have shown great promise, it is unlikely that the targeting of individual suppressive pathways exploited by the tumour will by adequate to reverse tumour immune evasion in a majority of patients. There is however hope that an increased understanding of the interactions that occur

within the tumour microenvironment will allow for successes similar to those currently seen in the treatment of chronic infectious diseases. Much can be learnt from the novel treatments of HIV and HCV where the targeting of multiple pathways in these chronic viral infections has transformed the prognosis of patients with such diseases [557, 558]. As our understanding of the cancer proteome increases there is optimism that, by targeting multiple pathways, the future of cancer immunotherapy may mirror the progress made in viral diease.

The introduction of immunotherapy into routine clinical practice is still nascent and will require numerous iterations before a comprehensive arsenal of drugs is available. The UK government has made the development of cellular therapy a key goal. There remain, however, numerous obstacles to overcome. Firstly, due to the small scale of early phase clinical trials and the current methods used to manufacture most cellular immunotherapies, the production cost at present is high. Increased automation will likely bring these costs down and permit easier scale out of manufacturing processes to facilitate commercialisation. An additional consideration is the lack of consistency in the preparation of immunotherapeutic treatments between trials has resulted in a lack of clarity of what the best methods are to produce the most effective immunotherapy. This is typified well by the difficulty in defining an appropriate antigen to use in DC vaccines [559]. Continued research in this exciting field will hopefully yield reward in the foreseeable future and lead to effective cancer therapies with the potential to induce lasting remission.

With this in mind, I have been involved in the set-up of a new immunotherapy trial to be conducted by the University of Birmingham. ImmunoTACE: A randomised phase II clinical trial of conditioning cyclophosphamide and chemoembolisation with or without vaccination with dendritic cells pulsed with HepG2 lysate in vitro in patients with hepatocellular carcinoma opened to recruitment in 2015, with the first patients received DC vaccines in early 2016. The trial involves the use of an autologous ex-vivo generated DC pulsed with tumour cell line lysate and cyclophosphamide for the depletion of Treg in conjunction with TACE in patients with HCC. Both the design of this study and the techniques employed as part of the immune monitoring methods within the trial have evolved from the results generated in my current work. The ImmunoTACE clinical trial has been designed to integrate with the standard of care pathway for patients newly diagnosed with HCC whom do not meet the criteria for transplantation or surgical resection. This is an important consideration when developing immunotherapy-based studies to enable their delivery in a practical manner and hence facilitate their use in routine clinical practice.

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9 LIST OF ABBREVATIONS

g	Acceleration of gravity
AP-1	Activator Protein-1
ALD	Alcoholic Liver Disease
APC	Allophycocyanin
α	Alpha
AFP	Alpha-Feto Protein
7-ADD	7-aminoactinomycin D
ANOVA	Analysis of variance
APC	Antigen Presenting Cells
AhR	Aryl hydrocarbon Receptor
AASLD	Association for the Study of Liver Disease
BCLC	Barcelona Cancer Liver Centre
β	Beta
BCA	Bicinchoninic Acid Assay
CAF	Cancer Associated Fibroblast
CFSE	Carboxy Flurescein Succinimidyl Ester
cm	Centimeter
CCR	CC chemokine receptors
CPS	Child-Pugh Score
CD	Cluster of Differentiation
CSF-1	Colony-Stimulating Factor-1

CR	Complete Response
CTLA-4	Cytotoxic T-Lymphocytes-associated protein-4
δ	Delta
DC	Dendritic Cells
DNA	Deoxyribonucleic Acid
DAPI	4',6-diamidino-2-phenylindole
DEN	Diethylnitrosamine
DMSO	Dimethyl Sulfoxide
DPX	Di-N-Butyle Phthalate in Xylene
dH2O	Distilled Water
ER	Endoplasmic Reticulum
EDTA	Ethylene Diamine Tetraacetic Acid
EASL	European Association for Study of the Liver
EAE	Experimental Allergic Encephalomyelitis
ECM	Extra Cellular Matrix
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FDA	Food and Drug Administration
Foxp3	Forkhead Box P3
FSC	Forward Scatter
γ	Gamma
GCN-2	General Control Nonrepressed-2
GITR	Glucocorticoid-Induced TNFR-Related

GPS	Glutamine-Penicillin-Stretomycin
GVHD	Graft vs. Host Disease
g	Grams
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HSEC	Hepatic Sinusoid Endothelial Cells
HSC	Hepatic Stellate Cells
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HCC	Hepatocellular Carcinoma
HHC	Hereditary Haemochromatosis
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
H_2O_2	Hydrogen Peroxide
HIF-1α	Hypoxia Inducible Factor 1-alpha
IDO	Indoleamine 2,3-Dioxygenase
iCD8$^+$Treg	Induced CD8$^+$Regulatory T-cells
iNOS	inducible Nitric Oxide Synthase
IL	Interleukin
IQR	Interquartile Range
L	Litre
ICAM-1	Intercellular Adhesion Molecule-1
IFN	Interferons
IL	Interleukin

IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked
ILT	Immunoglobulin-like Transcript
IBD	Inflammatory Bowel Disease
LPS	Lipopolysaccharide
LAG-3	Lymphocyte-Activation Gene-3
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinases
mTOR	Mechanistic Target Of Rapamycin
MFI	Median fluorescent intensity
MAGE	Melanoma Associated Antigen
MSC	Mesenchymal Stem Cells
1-MT	1-Methyl-Tryptophan
µg	Microgram
µL	Microlitre
mL	Millilitre
MELD	Modified End Stage Liver Disease
mRECIST	Modified Response Evaluation Criteria In Solid tumours
mAb	Monoclonal antibodies
moDC	Monocyte Derived Dendritic Cells
MDSC	Monocyte Derived Suppressor Cells
MNC	Mono Nuclear Cells
MS	Multiple Sclerosis
NK	Natural Killer Cells

NY-ESO	New York-Esophageal Squamous Cell Carcinoma
NOHA	N-hydroxy-L-arginine
NAFLD	Non Alcoholic Fatty Liver Disease
NMMA	Nonselective nitric oxide synthase
NFkB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
n	Number (of subjects / cases)
PEI	Percutaneous Ethanol Injection
PS	Performance Status
PBS	Phosphate Buffer Saline
PE	Phycoerythrin
PDGF	Platelet-Derived Growth Factor
PD-1	Programmed cell death protein 1
PD	Progressive Disease
PSC	Progressive Sclerosing Cholangitis
PR	Partial Response
PAMP	Pathogen Associated Molecular Patterns
PRR	Pattern Recognition Receptors
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PCR	Polymer Chain Reaction
RAF-1	Rapidly Accelerated Fibrosarcoma-1
RFA	Radio Frequency Ablation
RCT	Randomized Controlled Trial

ROS	Reactive Oxygen Species
RAG	Recombination Activating Gene
RNA	Ribonucleic Acid
RPMI	Rosewell Park Memorial Institute
SCID	Severe Combined Immunodeficiency
SSC	Side Scatter
STAT-3	Signal Transducer & Activator of Transcription-3
SIV	Simian Immunodeficiency Virus
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SD	Stable Disease
SSX	Synovial Sarcoma X Breakpoint
SLE	Systemic Lupus Erythematosus
TSPY	Testis Specific Protein on Y chromosome
TIM	T-cell immunoglobulin and mucin-domain-containing-molecule-3
TCR	T-Cell Receptor
TLR	Toll-Like Receptors
TACE	Trans Arterial Chemoembolisation
TEMED	Tramethylethylenediamine
TGF	Transforming Growth Factor
TAA	Tumour Associated Antigen
TAM	Tumour Associated Macrophages
TNF	Tumour Necrosis Factor
Tregs	Regulatory T-cells

UCSF	University of California San Francisco
USS	Ultra Sound Scan
VEGF	Vascular endothelial growth factor
W/V	Weight/Volume