# ALPHA-FETOPROTEIN AND IMMUNOTHERAPY FOR HEPATOCELLULAR CARCINOMA

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**Abstract**

***Background and Aims***:

Hepatocellular carcinoma (HCC) often presents at a late stage which limits the use of curative therapy. Hence the pressing need for increasing research into newer therapies such as immunotherapy. Alpha-Fetoprotein (AFP) is an oncofetal antigen elaborated in most HCCs and is a tumour rejection antigen in animal models for HCC making it a target for the development of T cell based immunotherapy. The effect of AFP on anti-tumour immune responses in patients with HCC has not been explored in depth. We aimed to study the effects of AFP on the immune system cells including dendritic cells (DCs).

In man, naturally occurring anti-AFP CD8 T cell responses have been detected in patients with HCC. One vital step for the design of epitope-based therapeutic vaccines is the identification and characterization of T-cell epitopes on AFP. Several AFP-derived peptides have been identified and T cells recognizing these epitopes have been studied in patients with HCC. However, the role of anti-AFP CD4+ T cell responses (Th1 cells or regulatory T cells) in HCC patients has not been studied. Also, our aim was to study the role of Th1 cells in HCC patients and investigate any possible association between the expansion of these cells with clinical features of the disease such as stage of disease, serum AFP levels and tumour invasiveness.

***Results and Conclusions***:

In our first study, the treatment of monocyte-derived DCs with AFP led to DC dysfunction as detected by the down-regulation of surface molecules (CD40 and CD86) and inhibition of their T cell-stimulatory capacity. In addition, AFP treatment induced apoptosis of DCs and reduced their ability to produce TNF-alpha and IL-12. Our *ex vivo* results showed that the ability of monocytes, isolated from patients with elevated levels of serum AFP (>1,000 ng/ml), to produce TNF-alpha was impaired.

In the second study we identified an AFP-derived T cell epitope that was recognized by circulating CD4+ T cells from patients with HCC and normal or mildly elevated AFP level in the early stage of the disease. This response was absent in healthy donors and in patients with chronic liver disease, which suggested that this response had been previously expanded *in vivo* in response to the tumour. The induction or activation of regulatory T cells (T-regs) by tumours or pathogens may suppress protective immunity.

In the third study, we demonstrated that AFP contained an epitope which activated the expansion of inducible TGF-beta producing T-regs.

In our fourth study we revealed that CD4 T-cell response expanded in the early stages of disease (Child–Pugh A score), which is usually associated with low concentrations of serum AFP. Furthermore, CD8 T cell response was largely detected in HCC patients with a Child–Pugh B or C score. Necrosis of tumour cells can activate both innate and adaptive antitumor immunity. In a further study by our group, development of higher frequencies of AFP-specific CD4+ T cells after embolisation therapy was noted. Necrosis produced by Transarterial Chemoembolization (TACE) or Chemoembolization (TAE) unmasks tumour rejection Antigen-specific T cell responses. This represented a method of *in situ* immune response induction that could be combined with immunotherapy to increase the frequency of AFP-specific T cells with the aim of controlling tumour growth and improving survival. Also, two further HLA-DR-restricted AFP-derived CD4+ T cell epitopes were detected.

From our studies thus far, we concluded that predictive factors for detecting an AFP-specific Th1 response in patients with HCC included a serum AFP of <1000 ng/ml, Okuda stage 1 and treatment with TACE/TAE.

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| | |
|---|---|
| AFP | Alpha-Fetoprotein |
| AASLD | American Association for Study of Liver Diseases |
| APC | Antigen Presenting Cells |
| BCLC | Barcelona Clinic Liver Cancer |
| BM | Body Mass Index |
| CT | Computerised Tomography |
| CTLA4 | Cytotoxic T Lymphocyte–associated Antigen 4 |
| CTL | Cytotoxic Lymphocyte |
| CP | Child-Pugh |
| DC | Dendritic Cells |
| EASL | European Association for Study of Liver disease |
| ELISPOT | Enzyme-Linked Immunospot |
| GITR | Glucocorticoid-Induced TNF receptor–Related protein |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| GPC3 | Glypican-3 |
| HBV | Hepatitis B virus |
| HCC | Hepatocellular Carcinoma |
| HCV | Hepatitis C virus |
| HH | Hereditary Haemochromatosis |
| HFE | Human haemochromatosis protein |
| IGF | Insulin-like Growth Factor |
| IFN | Interferon |
| IL | Interleukin |
| LDLT | Living Donor Liver Transplant |
| LR | Liver Resection |
| LT | Liver Transplant |
| MDSCs | Myeloid-Derived Suppressor Cells |
| MELD | Model for End stage Liver Disease |
| mAbs | Monoclonal Antibodies |
| MRI | Magnetic Resonance Imaging |
| NASH | Non Alcoholic Steatohepatosis |
| NK | Natural Killer Cells |
| PBC | Primary Biliary Cirrhosis |
| PEI | Percutaneous Ethanol Injection |
| PIVKA-II | Protein Induced by Vitamin K Absence or Antagonist-II |
| PTEN | Phosphatase and Tensin homolog deleted on chromosome ten |
| RFA | Radiofrequency Ablation |
| SHARP | Sorafenib HCC Assessment Randomized Protocol |
| TAA | Tumour Associated Antigens |
| TACE | Transarterial Chemoembolisation |
| TAE | Transarterial Embolisation |
| TAM | Tumour Associated Macrophages |
| TCR | T Cell Receptor |
| TNF | Tumour Necrosis Factor |
| TGF | Tumour Growth Factor |
| TRAIL | Tumour necrosis factor Related Apoptosis Inducing Ligand |
| US | Ultrasound |
| VEGF | Vascular Endothelial Growth Factor |

**THESIS PLAN**

This thesis highlights the potential role of AFP in the emergence of novel immunotherapy based treatment for HCC.

The **First Chapter** gives a detailed account of the current management of HCC including epidemiology, risk factors, diagnosis, surveillance, staging and expert recommendations pertaining to different treatment modalities. The role of inflammation and cytokines produced by the innate immune system in the promotion or inhibition of cancer is discussed in the **Second Chapter**. An overview of the different hypothesis relating to the development of cancer including the immunosurveillance and the immunoeditting hypothesis is discussed in **Chapter Three,** along with the mechanisms by which tumours escape immunological control.

An outline of the different modalities of Cancer immunotherapy with an emphasis on T cell based immunotherapy is discussed in **Chapter Four**. In **Chapter Five,** an account of the role of cell mediated immunity in HCC, highlighting the role of tumour associated antigens and AFP-specific CD8 & CD4 T cell responses. A current review of the literature on immunotherapy in HCC is presented in **Chapter Six.** This is followed in **Chapter Seven** by an account of the different immunosuppressive factors involved in tumour progression and evasion from immune control. Our group's published articles relating to the role of AFP in HCC are then presented in **Chapters Eight to Eleven**. The role of AFP in the impairement of APC function is discussed in **Chapter Eight**. Following on in **Chapter Nine**, an account of our discovery of novel AFP derived epitopes in HCC patients capable of producing a peptide specific CD4+ T cell response and others capable of inducing a subset of CD4+ cells to produce TGF-beta **as described in Chapter Ten**. The expression of anti-AFP Th1 and Tcl resposes in HCC patients was found to occur in different stages of the disease and is discussed in details in **Chapter Eleven** taking into consideration the account of various other published findings in the literature. In **Chapter Twelve,** the various salient findings discussed in this thesis are summarized.

# CHAPTER 1: THE CURRENT MANAGEMENT OF HEPATOCELLULAR CARCINOMA

## 1.1 Introduction

As the fifth most common cancer worldwide, hepatocellular carcinoma (HCC) constitutes a significant global disease burden. There is increasing interest in the advancement of new diagnosis and treatment modalities for HCC. This section aims to encompass the most recent advances in the management of HCC, summarizing the transatlantic recommendations for diagnosis and surveillance, as well as incorporating established and developing therapies.

## 1.2 Epidemiology

HCC is the third leading cause of cancer-related death worldwide, according to most recent World Health Organisation report data [4]. Incidence varies across the globe, with highest rates in Asia, followed by Western Africa, with intermediate risk in the United Kingdom and the United States. The recent rise in incidence reported in the West is attributed to increased immigration from Hepatitis B virus (HBV) endemic countries and increasing numbers of chronic Hepatitis C virus (HCV) infection [5-6].

Although the incidence of HCC rises with increasing age, reaching its peak in those aged above 65 years [7-8] and more commonly in men [4], over the past two decades there has a shift in incidence towards a younger age group [9].

Despite the increased incidence in HCC, the number of HCC related deaths appears to be stabilising, or even decreasing in some Asian population studies [10], a result of improved surveillance and treatment regimens together with aggressive HBV vaccination programmes. In contrast, there has been a reported increase in mortality rates in the western world, for example in the United States, where middle aged men in particular are most affected [11].

## 1.3 Risk Factors

Liver cirrhosis is the main common finding in the majority of cases of HCC and HBV-related chronic hepatitis is the most common cause of HCC in the world [7]. The development of HCC in patients with chronic HCV occurs mostly in a cirrhotic liver in 95% of the time whilst the figure is close to 60% in Chronic HBV [5]. A number of underlying risk factors are recognized in the development of HCC; some are considered well established whilst the role of others remains controversial.

**1.3.1** HBV

HBV is the commonest risk factor for the development of HCC, a fact particularly relevant to HBV endemic areas, where vertical transmission is responsible for the majority of HBV acquisition [12]. In such patients HCC can develop before the onset of cirrhosis as a result of the direct oncogenic effects of HBV. With the increasing global implementation of HBV immunization, it is anticipated that the incidence of HCC will decline as a consequence of the reduced burden of HBV infection [13]. In a prospective study, HBV viral load $>10^5$ copies/ml was associated with increased mortality [14]. New data is emerging on the significance of HBV genotypes in HCC development, with evidence to suggest that in Asia, genotype C, independent of the viral load, predisposes to HCC, whilst in the West it is genotype D [15] .

Several factors are implicated in the pathogenesis of HBV related HCC which includes the repeated liver damage by HBV causing necroinflammation and consequent regeneration of hepatocytes. The other is the direct oncogenic property of HBV which integrates itself into the hepatocyte DNA, producing cis- or transactivation of the cellular oncogenes **[6].**

The Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (REVEAL-HBV) Study was the largest and longest study to evaluate the relationship between HCC and serum HBV DNA level. This prospective cohort trial included 3653 persons with untreated hepatitis. The main conclusion of this study was that uncontrolled HBV replication leads to poor clinical outcomes and a direct correlation between HBV DNA level and the risk of HCC. Over an average of 11.4 years, there were 164 new cases of HCC. The risk of HCC was directly related to HBV DNA level at baseline. After 13 years of follow-up, 15% of the patients with the highest levels of HBV DNA (≥1 million copies/mL) developed HCC (hazard ratio = 6.1), compared with only 1.3% of those with undetectable HBV DNA. The persistence of high HBV load led to the highest HCC risk. They recommended long-term monitoring of HBV viral load as essential requirement for the management of chronic HBV infection [16].

### **1.3.2** HCV

Chronic HCV infection is a major risk factor for HCC development. Once cirrhosis is established, the risk of malignant transformation is high with an HCC incidence ranging between 2-8% per year [17]. The significance of HCV genotype as a risk factor for HCC is the subject of debate but in a recent meta-analysis HCV genotype 1b was implicated as key in HCC development, particularly in the context of early stage liver disease [18].

### **1.3.3** Alcohol

Prolonged heavy alcohol intake leading to cirrhosis represents one of the common risk factors for HCC. Although it has not been established whether alcohol has a direct carcinogenic effect on the liver [19], new reports add to the growing body of evidence for such an effect [20]. Positive synergism exists between alcohol intake, HBV and HCV infections in escalating HCC risk [19]. The presence of concomitant HCV infection in heavy alcohol consumers has been observed to produce an additional 2-fold increase in the risk of HCC development when compared to HCV-negative alcohol consumers [21].

### **1.3.4** Haemochromatosis

Patients with cirrhosis secondary to hereditary haemochromatosis (HH) have a higher risk of HCC development, with a relative risk of around twenty [22]. Furthermore, the carcinogenic role of iron itself in the absence of HH has recently been shown to be associated with HCC in patients with end-stage liver disease [23]. Data examining the proportion of HCC cases with concomitant HFE gene mutations is limited and has been estimated at 3-5% in small case series [24-25]. In large meta-analyses involving 202 studies, the link between haemochromatosis genotypes and various disease end points was analysed and it demonstrated an associations of C282Y/C282Y with hepatocellular carcinoma and diabetes mellitus in Northern Europeans [26].

### **1.3.5** NASH

The First case of nonalcoholic steatohepatitis (NASH)-associated HCC was described by Powell et al [27], but since, numerous number of case series of NASH-associated HCC have been reported in the the literature [28-29]. The prevalence of nonalcoholic fatty liver disease (NAFLD) in adults is increasing in the Western world and Japan

probably as a result of the rise in obesity and diabetes mellitus [30]. NASH represents the advanced disease progression stage in the spectrum of NAFLD and as much as 20% of NASH may progress to cirrhosis as well as end stage complications such as HCC [31-32].

NASH-associated HCC was reported to be linked with liver cirrhosis in the majority of studies [33-34], however, in a recent Japanese case series the majority of cases were accompanied by non-cirrhotic liver [35] and further similar case reports were published [28, 35-36]. Direct evidence linking non-alcoholic NASH to HCC development is lacking. However, HCC has been reported to develop in patients with pre-existing NASH [40].

It has been reported that large proportion of patients with Cryptogenic Cirrhosis (CC) have clinical features consistent with NASH making it the lead cause of CC, but a diagnosis of NASH could not be confirmed by histology as a result of the finding of possible "burnt out NASH" [37-39].

The relationship between NASH and HCC is supported by the notion that HCC develops in patients with obesity and diabetes when both of which are positively associated with NASH.

### **1.3.6** Obesity

Obesity is a predisposing factor to insulin resistance, a recognized contributor to the development of hepatic steatosis [41], its progression to steatohepatitis [42] and ultimately liver fibrosis [43]. A raised body mass index (BMI) has itself been associated with HCC development. A case control study reported a significant relationship between a BMI $\geq 30$ and HCC, with allowance for known risk factors [44].

### **1.3.7** Diabetes

The role of diabetes mellitus as an independent risk factor for HCC has been suggested in data obtained from a population based case control study in the United States [45]. In a recent case control study, the incidence of HCC in diabetic patients was shown to be as high as double the incidence in non-diabetic patients [46]. The presence of diabetes has also been associated with more advanced liver lesions and an increase in mortality at one year when compared with non-diabetic HCC counterparts [47]. A systematic review of epidemiologic studies on the aetiologies of HCC in Southern Europe,

demonstrated data which supported a role of diabetes and obesity as single agents or preferably co-factors in causing HCC [48].

**1.3.8** Aflatoxin B1

Areas of high aflatoxin B1 exposure correspond to a high prevalence of HCC [9]. Aflatoxin B1 has been newly linked with a somatic mutation at codon 249 in TP53 (R249S) and studies are emerging to suggest that the detection of serum R249S months before the clinical diagnosis of HCC may permit early diagnosis and facilitate targeted treatment through the measurement of this biomarker [49-50]. Areas of high aflatoxin B1 exposure correspond to a high prevalence of HCC [51]. Aflatoxin B1 has been newly linked with mutations with A G to T transversion at the third position of codon 249 of the *p53* gene ($249^{ser}$) as a common finding in HCC. A large meta-analysis study from southern Guangxi region in China found an association between the ($249^{ser}$) mutation and increasing levels of aflatoxin exposure 19. Studies are emerging to suggest that the detection of serum ($249^{ser}$) months before the clinical diagnosis of HCC may permit early diagnosis and facilitate targeted treatment through the measurement of this biomarker [49] [50] Some reports suggested that ($249^{ser}$ ) mutation is more common in HBV-positive tumors than in HBV-negative tumors [52-53].

**1.3.9** OCP

The oral contraceptive pill (OCP) has been linked to malignant tumours including HCC, amongst other hepatobiliary carcinomas [54]. However Maheshwari et al's meta-analysis of epidemiological data failed to demonstrate any such association with HCC development and thus the relationship between the two remains unclear [55].

### 1.4 Diagnosis of Hepatocellular Carcinoma

The European Association for Study of Liver disease (EASL) have proposed recommendations for the diagnosis of HCC, which are crucial for both early detection and the implementation of appropriate treatment.

A known cirrhotic patient presenting with a liver lesion on ultrasound >2cm in diameter has a greater than 95% chance of having HCC [56]. An AFP level >200ng/ml, as well as radiological features consistent with HCC (e.g. hypervascularity) obtained on two dynamic imaging modalities such as Computerised Tomography (CT) and Magnetic Resonance Imaging (MRI), provides the diagnosis of HCC and negates the need for liver biopsy [57]. Conversely, if the AFP is <200ng/mL and the characteristic vascular profile is not visualized on imaging then liver biopsy is indicated to confirm the diagnosis.

For lesions of 1-2cm in diameter a guided liver biopsy should be performed, regardless of their vascular profile [57]. However, Bruix et al highlight the technical difficulty of biopsying such small lesions and the discrepancy that occurs between pathologists in discriminating between dysplasia and well-differentiated HCC. Thus they argue that lesions measuring between 1-2 cm should first be imaged with two dynamic studies and if the findings are not characteristic of HCC, only then proceed to biopsy [58].

Liver lesions smaller than 1cm in diameter are far less likely to be malignant in nature, especially on a background of cirrhosis and even less so if they fail to take up contrast on dynamic imaging [59]. However, the potential for malignant transformation of even tiny nodules over time still remains [60-61] and it is therefore prudent to continue ultrasound follow-up every 3-6 months in order to pre-empt HCC development [58]. A lack of increase in size over a period greater than 1-2 years permits return to the routine surveillance programme [58].

In a large USA case-control study of 836 patients (50% with cirrhosis and 50% with HCC) comparing different tumour markers for early HCC diagnosis, AFP had a sensitivity of 66% and specificity of 81%, at a new cut off of 10.9 ng/mL [62].

The serum biomarkers Protein Induced by Vitamin K Absence or Antagonist-II (PIVKA-II) and glypican-3 (GPC3) have recently been assayed in patients with HCC. The combination of AFP with PIVKA-II has been demonstrated to increase diagnostic

accuracy in ALD and NAFLD associated HCC [63] and furthermore, PIVKA-II, and not AFP has been proposed as a more valuable prognostic indicator in HBV associated HCC [64]. The percentage of AFP binding to Lens culinaris agglutinin (AFP-L3%) has been explored as a diagnostic and prognostic marker for HCC. In patients with total AFP of 10-200 ng/ml, AFP-L3% greater than 35%, had a reduced sensitivity of 33%, but an increased specificity of 100% for diagnosis of HCC [65]. AFP-L3 was found to be associated with poorly differentiated HCC and portal vein invasion [66-67].

Serum PIVKA-II was found to have a sensitivity of 48-62% and a specificity of 81-98% in the diagnosis of HCC 25 and as a marker of vascular invasion similar to AFP-L3 [66-67]. It was also noted to be utilised as a marker of recurrent HCC following surgical resection [68].

### 1.5 Molecular Mechanisms of Hepatocarcinogenesis

There has been an increased interest in exploring the molecular pathogenesis of HCC with the aim of developing newer targeted molecular therapies. In their recent review article El-Serag et al propose an interplay of several factors in the induction of hepatocarcinogenesis [19]. The activation of several oncogenic pathways has been proposed. Activation of the Wnt β-catenin pathway in HCC has been reported as high as one third of cases studied [69]. Up to 30% of HCC exhibit an over-expression of IGF2 (insulin-like growth factor 2) [70]. Furthermore, mutations in the IGF2 receptor, IGF2R, have been demonstrated in over 60% of dysplastic hepatic nodules and HCC [71]. Activation of the P13/Akt pathway and impairment of the Akt regulator, phosphatase and tensin homolog (PTEN), occurs in approximately 50% of HCC [72] with worsened prognostic outcome [73]. The derangement of apoptotic pathways such as p53 mutations have been noted to account for up to 40% of HCC [74]. Llovett et al, proposes vascular endothelial growth factor (VEGF) as a key factor in HCC tumour neo-angiogenesis [69]. The value of VEGF as a prognostic marker has recently been assessed and high serum and tissue levels of VEGF were shown to correlate with decreased survival [75].

### 1.6 Alpha-fetoprotein

Alpha-fetoprotein (AFP) which is a tumor-associated fetal protein, has been utilized as a marker for fetal defect and to monitor fetal distress as well as being a tumour marker for HCC and other germinal cell tumours [76-77] [78]. AFP is synthesized in the fetal yolk sac endoderm and liver during fetal development [79]. This was confirmed by messenger RNA for AFP analysis studies [80]. After birth, AFP concentrations in maternal serum remain high in the newborn rat for about 3–4 weeks and continues with the ongoing proliferation of hepatocytes until it is completed at about 3 weeks after birth, when AFP level is reduced sharply [81]. In response to injury to the liver, hepatocytes respond by proliferation, producing AFP and releasing it into the circulation [82]. Similar to albumin, serum AFP is known to bind and transport several ligands such as bilirubin, fatty acids and various drugs [83]. AFP is classified as a member of an albuminoid gene family, which consists of: albumin (ALB), vitamin D-binding (Gc) protein (DBP), AFP, and alpha-ALB (αALB), termed afamin in humans[84] [85]. It is structurally characterized by cysteine residues which are folded into layers forming loops dictated by disulfide bridging, resulting in a triplet domain and a U-shaped molecular structure. The albuminoid genes in humans lie in tandem on chromosome 4 within the 4q11-q22 region, encompassing 15 exons and 14 introns [86]. Genetic variants of the adult form of human AFP (HAFP) exist as it might be derived from multiple RNA transcripts (i.e., 2.2, 1.7, and 1.6 kb). It is well established that HAFP detected in most assays is 69,000- to 70,000-kD (2.2 kb) polypeptide. Cell surface receptors for AFP have been described with both endothelial components and epithelial cell surface membrane receptors [87-90]. They have been localized on monocytes as well cells belonging to the reproductive and immune systems, in addition to tumor cells, mainly HCC. HAFP could be conjugated with anti cancer drugs such as doxorubicin, and cis-platinum [91] as it can bind and enter tumour cells via a receptor-mediated endocytotic pathway [91]. Cloning of HAFP gene has permitted the use of specific peptides as therapeutic targets. Reports have confirmed that AFP could be an inducer of growth enhancement and inhibition. This is achieved by means of apoptosis induction in various tumor cell culture lines, protein-to-protein interaction during the cytoplasmic signaling cascades or interruption of G-coupled signal transduction [92-93].

## 1.7 Surveillance

The main objective of HCC surveillance is to offer better survival benefit. The use of ultrasound (US) every 6 months with or without serum AFP levels is the widely accepted recommendation for the surveillance of HCC [57-58]. Patients at high risk for developing HCC should be entered into surveillance programs. This includes patients with cirrhosis secondary to HBV, HCV, alcohol, HH and primary biliary cirrhosis (PBC). Chronic hepatitis B carriers, depending on their age, race and viral DNA level should also be included [58]. Additionally, patients on the waiting list for liver transplant (LT) require continued surveillance in light of the possible implications on priority listing should HCC be discovered and the potential for bridging therapies such as embolisation or ablation. Prospective studies of the usefulness of AFP in HCC surveillance indicated a sensitivity of 39-64% with specificity of 76-91%, and positive predictive value of 9-32% [57].

An AFP level greater than 20 ng/ml offers a sensitivity of only 60% in detecting HCC and thus is inadequate as a surveillance test, however, it can still serve as a diagnostic marker. In high risk cirrhotic patients, a persistently elevated AFP is a known risk factor for HCC [94] and if accompanied with a liver mass, an AFP greater than 200ng/mL has a high positive predictive value for HCC [95]. In a recent meta-analysis the utility of ultrasound in surveillance was reported to have a pooled sensitivity of 94%, but the efficacy in detecting early HCC was reduced, unless performed at six monthly intervals [96]. The limitations as a result of lack of detection of circulating AFP in almost 40% of patients with HCC, may restrict the use of AFP as a TAA in designing immunotherapy vaccine in this group. However, this percentage may be smaller as even in patients with HCC and undetectable circulating AFP, the tumour microenvironment may be rich in AFP secretion.

### 1.8 Staging of Hepatocellular Carcinoma

The Barcelona-Clinic Liver Cancer (BCLC) staging system was developed based on a retrospective analysis of various HCC studies and incorporated patients with early, intermediate, and advanced terminal disease [97]. The BCLC system identifies prognostically relevant variables for each group [97] and is currently the most widely acceptable staging system for HCC [98-101].

In contrast to other staging systems, the BCLC not only recommends treatment modalities but also offers prognostic estimates. Patients are stratified into four groups: early, intermediate, advanced, and end-stage. Patients with a Child-Pugh (CP) score A and B or those with a solitary lesion or up to 3 lesions ≤3cm in size are classified as having early stage disease. In these cases, the recommended treatment includes liver resection (LR), liver transplantation (LT) or radiofrequency ablation (RFA), offering a 5-year survival rate of 50-75%. Intermediate stage disease encompasses those with CP score A, B and those with large/multifocal HCC in the absence of cancer related symptoms, macrovascular invasion or extra-hepatic spread. The survival without therapy at 3 years will not surpass 50% and therefore these patients are favorable candidates for transarterial chemoembolisation (TACE). In advanced stage disease, where cancer symptoms, vascular invasion and/or extrahepatic spread are present, predicted survival at 1 year is 50%, and thus these patients are considered as candidates for therapeutic clinical trials. Patients with a CP score of C, extensive tumours and a performance status >2 are considered to have end-stage disease [101]. Symptomatic treatment should be provided for this group, in whom the median survival is less than 3 months.

A recent study of patients in USA identified a number of factors as predictors of survival in cirrhotic patients with HCC [102]. These included hepatic function (score according to the Model for End stage Liver Disease: MELD), performance status, tumour characteristics (size and portal vein thrombosis) and the effects of treatment. Taking these factors into consideration, they compared seven prognostic staging systems for HCC, and indicated that the BCLC system provided the best independent predictor of survival. These findings were further validated by an Italian study which focused on patients with asymptomatic, early to intermediate HCC and concluded that the BCLC system provided the most precise estimates of survival [103].

## 1.9 Treatment

A number of different treatment modalities, dependent on the disease stage, are recognized as possible options for the management of HCC and offering varying prognoses. The concept of combining therapies has also been considered to improve survival.

### **1.9.1** Liver Resection

Liver resection (LR) is the treatment of choice for HCC in non-cirrhotic patients. However, only 10-30% of HCC patients are suitable candidates at presentation [104]. LR is best offered to the following patient groups: patients with a single nodule ≤5cm or up to 3 nodules ≤3cm; Okuda stage 1 or 2; Child-Pugh A or B; or a performance score of 0. They must also possess preserved liver function, a normal bilirubin level, and absence of portal hypertension (hepatic vein pressure gradient <10mmHg). These parameters represent the best predictors of post-LR outcomes corresponding to a limited risk of liver failure post operatively and a 5-year survival greater than 70% [105-106]. The MELD score has been reported as a useful tool in predicting peri-operative mortality following LR, where a score over 9 correlates with a peri-operative mortality of 29%, in contrast with a mortality of 0% in those with a score less than 9 [107]. Following LR, patients with portal hypertension and elevated bilirubin levels have a 5 year survival less than 30%, regardless of their CP stage [105].

LR is contraindicated for BCLC intermediate-stage HCC with preserved liver function, according to the recommendations by American Association for Study of Liver Diseases (AASLD) [58] and EASL [57]. In the West, portal hypertension and bilirubin levels represent the main suitability criteria to be considered for treatment with LR [108]. Significant portal hypertension can be deduced from the presence of oesophageal varices, splenomegaly and a platelet count lower than 100×109/L, without the need to resort to hepatic vein catheterization. Contrastingly, in Japan, there is dependence on indocyanine green (ICG) testing as a quantitative measure of liver function [109].

The most powerful predictors of disease recurrence following LR are the presence of microvascular invasion and/or additional tumour sites besides the primary lesion [110-111]. In view of this recurrence risk, the role of adjuvant therapy before and after LR has been studied. A recent case control study concluded that TACE may worsen survival if offered pre-LR [112]. Another approach is to perform portal vein embolisation of the tumour-containing hepatic lobe to increase the size of the

unaffected lobe reducing the risk of small for size syndrome. However, this has been shown to add no survival benefit [113]. A review of randomized control trials evaluating adjuvant therapies described no benefit from postoperative TACE or chemotherapy and concluded that no adjuvant therapy is available to reduce recurrence rates [114]. However, a study has proposed adjuvant intra-arterial Iodine-131-lipiodol as beneficial in producing disease-free survival and prolonging overall survival post-LR [115].

Liver resection provides the opportunity to identify markers of increased risk of disease-recurrence, such as vascular invasion and satellite lesions. Once discovered, enlistment for salvage transplantation is advocated by some centres [116-117].

There is also emerging evidence of the gene expression profiles associated with metastatic HCC. A recent investigation of genome-wide expression profile via DNA microarray found a total of 90 clones to be correlated with intrahepatic metastasis of HCC [118]. Another group identified genes that were relevant to metastasis and patient survival [119]. In addition, proteomics technology has become increasingly popular for identifying biomarkers for early detection of tumour and metastatic potential. A recent study identified expressed proteins in the serum that could represent potential markers of HCC [120].

**1.9.2** Liver Transplantation

Following the landmark studies of Mazzaferro [121] and Bismuth [122] where liver transplantation was shown to produce excellent results in patients with solitary HCC <5cm or with up to 3 nodules <3cm, the widely accepted Milan criteria were established [123-124]. The 5-year survival of these early stage patients was shown to exceed 70% and the Milan criteria were endorsed by the AASLD [58].

Yao et al.'s group from the University of California San Francisco (UCSF) expanded the selection criteria for LT [125]. They proposed that solitary tumours ≤6.5cm, the presence of 3 or fewer nodules (with the largest lesion ≤4.5cm), or a total tumour diameter ≤8cm, without evidence of gross vascular invasion should be included in the criteria. These recommendations were based on tumour characteristics noted on explanted tumours, rather than those observed preoperatively. The 5 year survival for patients meeting the UCSF criteria was reported as 75.2%. A recent retrospective study showed no significant difference between 5 year post-transplant survival in patients meeting the Milan versus the UCSF criteria based on preoperative imaging findings

(79% vs. 64% respectively) [126]. Despite these reports, the AASLD did not advocate expanding the listing criteria beyond the standard Milan Criteria.

The decision whether to treat patients with small HCC and preserved liver function with either LR or LT is a topic of controversy. It has been suggested that LR impairs future transplantability of patients [127], however, evidence does exist demonstrating comparable overall survival rates between the two treatment options, and that survival after secondary salvage transplantation for postresection recurrence is also comparable to that of primary transplantation [128-129]. Hence, most clinicians advocate a strategy of LR and salvage transplantation for disease recurrence. This is further supported by the limited availability of liver donors for cadaveric transplant.

To help reduce waiting list drop out rates which may occur as a result of tumour progression, AASLD guidelines promote a variety of bridging therapies, including TACE [130], local ablative therapy, or a combination of both, especially if the waiting list exceeds six months [58, 131]. Newer strategies include the use of virally infected livers in patients with HBV or HCV related HCC, amyloid domino LT and living donor liver transplant (LDLT).

LDLT offers the advantage of early surgery and may allow a more flexible and expanded inclusion criteria but there are limited data on LDLT. Nonetheless there are a number of retrospective studies indicating a comparable survival between LDLT and LT for patients with HCC [132-133]. However, an increased rate of HCC disease recurrence in LDLT recipients compared with cadaveric donor transplantation recipients has been reported [134-136]. Of note, in centres where the MELD score is implemented for prioritising patients on the waiting list, a reduction in LDLT has been observed, as patients with HCC are allocated a higher priority and thus are candidates for earlier LT surgery [132].

The concept of down-staging the HCC tumour by means of ablative or embolisation therapy for patients who fall outside the conventional criteria has been explored, however, further research into this area is required [137].

The recent advances in gene expression profiling studies may allow improved HCC recurrence predictability [138] and could improve the pre-LT HCC staging. A distinctive pattern of gene expression was noted in early and advanced HCC which could be utilized in improving the management of HCC [139]

### **1.9.3** Percutaneous Ablation

Percutaneous ablation is considered the treatment of choice for patients with early stage HCC, who are not candidates for LR or LT. This treatment modality can also be utilized as a bridge to transplantation, in accordance with AASLD recommendations [58]. The advantage of RFA over Percutaneous Ethanol Injection (PEI) in the treatment of HCC has been demonstrated by recent studies [140-141]. Furthermore, RFA offers improved tumour response and long-term survival. Studies of ablation therapies have reported a 50% survival at 5 years in CP score A patients [142-143], which compares well with the outcome of LR.

In a randomized control trial of patients with a solitary HCC ≤5cm, PEI was found to be as effective as LR and offered the advantage of being less invasive [144]. The efficacy of RFA relies on heat delivery which can be hampered by tumour hypervascularity, thus some authors have proposed performing embolisation first and then to be followed by RFA [145].

### **1.9.4** Embolisation

The AASLD recommends Transarterial Embolisation (TAE) and TACE for patients unsuitable for LR, LT and RFA, provided they have maintained portal blood flow and in the absence of extrahepatic tumour spread [58]. TAE/TACE is reported to offer 5-year survival rates exceeding 50%. Patients with advanced liver disease and/or evidence of liver decompensation should not be considered for embolisation therapy due to the increased risk of liver failure and death.

The evidence for significant impact on survival rates following treatment with embolisation has been demonstrated by a number of studies [146-147] including a recent cumulative meta-analysis [148]. The most recent development with TACE is the use of doxorubicin-eluting beads. In a Phase I/II trial of this method, it was found to be a safe and effective treatment, suggesting the need for phase III randomized trials [149].

### 1.10 Molecular Therapies

Sorafenib is a multikinase inhibitor that both blocks tumour cell proliferation and exerts antiangiogenic effects. This is achieved through inhibition of the Raf/mitgen-activated protein kinase/extracellular signal-regulated kinase (Raf/MEK/ERK) signaling pathway and action against the VEGF-2, VEGF-3 and PDGF receptors respectively. In the recent landmark SHARP phase III trial (Sorafenib HCC Assessment Randomized Protocol), sorafenib was demonstrated to increase overall survival from 7.9 months to 10.7 months and prolonged the median time to disease progression when compared with the control group [150]. Cheng et al. demonstrated similar findings in their Asian-Pacific population phase III trial [151]. These findings represent a noteworthy breakthrough in the ongoing management of HCC, where no other chemotherapy options have been shown to provide such survival benefit.

Currently, there are newer molecular therapies which have been tested in phase II trials and although survival benefits have been reported, none have as yet been shown to produce the same therapeutic impact as sorafenib [69]. These therapies include: (1) anti-EGFR agents e.g. erlotinib and cetuximab; (2) anti-angiogenic agents e.g. bevacizumab and sunitinib; (3) P13K/Akt/mTOR inhibitors e.g. everolimus; and (4) other less well-developed agents such as those inhibiting the Wnt pathway and IGFR1. The concept of ‘combination molecular therapy’ may provide a promising treatment option in the future.

### 1.11 Novel Therapies

A number of novel treatment modalities are currently under development for HCC, including radioactive agents and extracorporeal energy therapies. Radionuclide Yttrium-90 is a pure β-emitter and, in the form of microspheres can be administered into the hepatic artery, delivering localised tumour radiation. It appears to be well tolerated and reports of increased survival in patients with unresectable HCC are emerging [152-154]. Extracorporeal energy therapy incorporates conformal radiotherapy, proton beam therapy and high intensity focus ultrasound (HIFU). A study assessing the efficacy of HIFU, showed significant improvement in the symptom scores, AFP levels and survival rates of patients with unresectable HCC [155].

# CHAPTER 2: Inflammation, innate immunity and cancer

## 2.1 The link between infection, inflammation and cancer:

Infection and inflammation can trigger and recruit the innate immune cells which produce cytokines capable of stimulating tumor growth and expansion, as can mediators released from tumour cells. Furthermore, inflammatory cells may stimulate the release of immunoprotective cytokines. There is a growing evidence that many malignancies are initiated by infections [156-157] and over15% of malignancies worldwide can be attributed to infections. Recent data from mouse models, have established that inflammation is a critical component of both tumour promotion and tumour progression [158-159] . The inflammatory environment is occupied by cells such as macrophages, Dendritic Cells (DCs), Natrural Killer cells (NK), Tumour-associated macrophages (TAMs) and T cells, with the latter two as being most prominent [160]. The infiltrated immune cells at the site of inflammation can exert contradictory effects during cancer development (Figure 2-1)[161]. Pro-inflammatory mediators present chronically are thought to contribute to tumour promotion and progression. In the tumour microenvironment, there is a subtle balance between pro-inflammatory activity, which weakens antitumour immunity and anti-tumour activity. When tumour-mediated immunosuppressive activity is stronger than antitumour immunity, tumour cells undergo immune escape and grow rapidly with local invasion and metastasis [162-163].

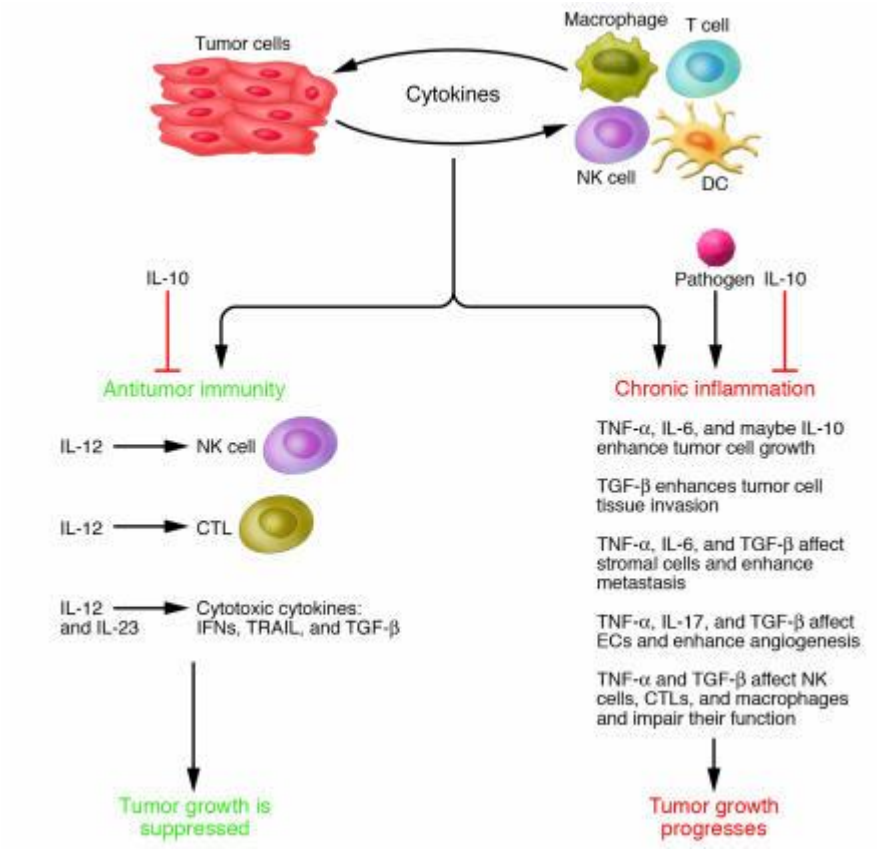



Figure 2-1: The diagram shows two outcomes of interactions between tumor cells and infiltrating inflammatory and/or immune cells in the tumor microenvironment [1]

## 2.2 The various cytokines produced by the innate immune cells and implicated in the promotion or inhibition of tumor development:

### **2.2.1** TNF-alpha

TNF-alpha is produced by tumor cells or inflammatory cells in the tumour microenvironment. The role of TNF-alpha in chronic inflammatory diseases and tumor-promoting effects is well recognized [164-166], as well as the role in promoting tumour cell survival through the induction of genes encoding NF-κB–dependent antiapoptotic molecules [166]. Other actions which may enhance tumour progression include; promotion of angiogenesis, metastasis and impairment of immune surveillance by suppressing T cell responses as well as cytotoxic activity of activated macrophages [167]. Genetic polymorphisms which enhance TNF-alpha production are associated with increased risk of HCC as well as other tumours such as multiple myeloma, bladder carcinoma, gastric carcinoma and breast carcinoma. Overall, TNF-alpha is an

important factor involved in initiation, proliferation, angiogenesis, and metastasis of various types of cancers (Figure 2-2).

### **2.2.2** IL-6

IL-6 is a potent pleiotropic inflammatory cytokine considered as a key growth-promoting and anti-apoptotic factor [168] (Figure 2-2).

### **2.2.3** IL-17

IL-17 induces many pro-inflammatory factors, e.g TNF-alpha, IL-6, and IL-1β, suggesting an important role in localizing and amplifying inflammation [169]. A new T cell subset named "Th17," was identified and characterized by the production of IL-17 [170]. IL-17 has tumour-promoting effect by promoting angiogenesis [171] (Figure 2-2).

### **2.2.4** IL-10

In many experimental studies, IL-10 was found to exert antitumour activity as well as pro-tumorigenic action. IL-10 often exerts immunosuppressive and anti-inflammatory effects by inhibiting pro-inflammatory cytokines such as TNF-alpha, IL-6, and IL-12, thus limiting tumor development and progression [172]. Studies demonstrated a link between IL-10–dependent antitumor activity and $CD4^{+}CD25^{+}$ T regulatory cells (Tregs)[173]. Further studies suggested that IL-10 possesses immuno-stimulatory activity contributing to antitumour immunity [174]. It has also been shown to modulate apoptosis and suppress angiogenesis during tumour regression [175]. Furthermore, IL-10 may cause down-regulation of MHC class I expression, leading to enhanced NK cell–mediated tumour cell destruction. IL-10 ability to down-regulate VEGF, TNF-alpha and IL-6 production by TAMs might also account for its inhibitory effect on the tumour tissue. On the other hand, IL-10 might also promote tumour development through direct effects on tumour cells. In addition the ability of IL-10 to suppress adaptive immune responses has also been suggested to support tumour escape from immune surveillance. The opposing effects of IL-10 might depend on interactions with either cytokines or factors found in the tumour microenvironment, dictating its end effect [174] (Figure 2-2).

### **2.2.5** TGF-beta1

TGF-beta1 is a powerful immunosuppressive and anti-inflammatory cytokine but also has a central role for regulation of T-regs proliferation and function [176]. It might also enhance tumour progression, favor angiogenesis and inhibition of tumour-specific $CD8^+$ T cells [177-178] (Figure 2-2).

### **2.2.6** TRAIL

Tumour necrosis factor Related Apoptosis Inducing Ligand, is a recently identified member of the TNF family and is mainly produced by activated T cells and NK cells. TRAIL is able to induce apoptosis in various tumour cell types but has only negligible effects on normal cells and is one of the major mediators of antitumour immunity [179] (Figure 2-2).

### **2.2.7** IL-12 and IL-23

IL-12 and IL-23 belong to the IL-12 family of pro-inflammatory cytokines and are mainly produced by activated APCs, DCs and phagocytes**.** IL-12 is implicated in resistance to tumours and regression of existing tumours in mouse models. This is achieved by stimulating Th1 and Cytotoxic Lymphocyte (CTL) responses, producing IFN-gamma which has both direct toxic effect on tumour cells and anti-angiogenic activity. IL-12 use in cancer therapy is restricted as the high levels of IFN-gamma that it generates, has severe toxic side effects [180-181].

IL-23 can boost the proliferation of memory T cells and production by activated T cells of IFN-gamma and IL-12. Also, it can stimulate Th17-mediated responses, such as IL-17 production, thus promoting end-stage inflammation and tumour expansion [182] (Figure 2-2).

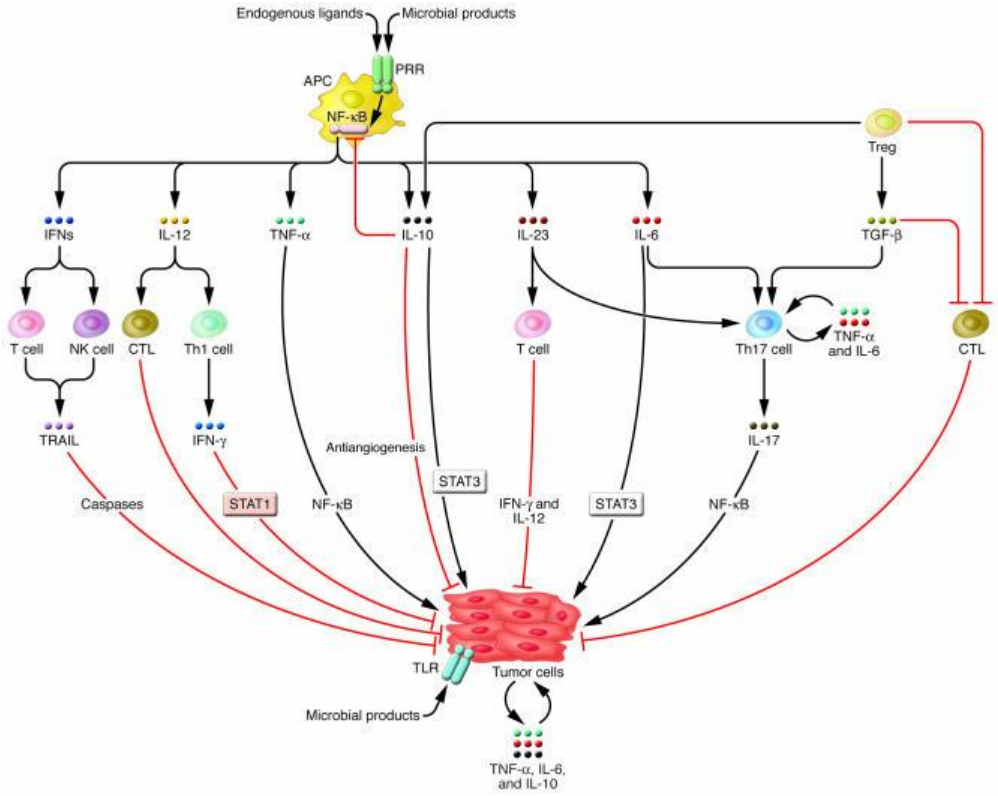



Figure 2-2: Interactions between various cell types in the tumour microenvironment determine the effects of cytokines on tumour development and progression [1].

The activation of innate immunity and inflammation results in the production of cytokines that can either stimulate or inhibit tumour growth and progression. Understanding of the molecular basis of inflammation-associated tumours can lead to novel approaches in the prevention and treatment of tumours.

## CHAPTER 3: CANCER IMMUNOSURVEILLANCE AND IMMUNOEDITING

### 3.1 Immunosurveillance

Ehrlich in 1909, first postulated that the immune system protects the host against tumour and Burnet and Thomas in the 1950's established the immunosurveillance hypothesis which posits that the immune system recognizes malignant cells as foreign agents and eliminates them through a "surveillance" function utilizing lymphocytes and cytokines which are important in this process [183-184]. However, the immunosurveillance hypothesis became unfavorable in the 1980's as tumours failed to develop more rapidly in nude mice lacking T cells and B cells, but not NK cells, than in wild-type mice. The immunosurveillance hypothesis was reconsidered in the 1990s, when evidence emerged that immunodeficient mice were at greater risk for spontaneous tumor development [185]. Endogenously produced IFN-gamma was shown to protect the host against the growth of transplanted tumours and the formation of primary chemically induced and spontaneous tumours.

### 3.2 Immunoediting

Not only can the immune system prevent tumour formation but also promote or select tumour variants that have reduced immunogenicity, thus allowing developing tumours the ability to escape immunologic detection and elimination. In addition, the tumour itself may influence the immune system response. Taken together, these concepts have led to the development of the cancer immunoediting hypothesis, which is a modification to the cancer immunosurveillance theory. Cancer immunoediting encompasses three phases: Elimination, Equilibrium and Escape (Figure 3-2)

### 3.3 The elimination phase

In this stage tumour cells are destroyed by immune cells and cytokines from the innate and adaptive immune system and it represents the immunosurveillance of tumours (Figure 3-1)(Figure 3-2). Initially lymphocytes that participate in innate immunity (NKT, NK and gamma-delta T cells) recognize accumulated transformed cells and are stimulated to produce IFN-gamma leading to the induction of chemokines, with the ability to block neovascularization in the tumour and recruit NK cells, DC and other immune effector cells to the tumour site with cytocidal activity. IFN-gamma also has antiproliferative action on the developing tumor. Dead tumor cells or debris are

ingested by DC and transferred to the draining lymph node where $CD4^+$ and $CD8^+$ T cells that are specific for tumour antigens develop which when released can recognize and destroy tumour cells. Despite the central role that $CD8^+$ T cells have in tumour immunity, there is growing evidence supporting a similar important role for $CD4^+$ T cells (Th1 cells). Murine studies have clearly shown that Th1 cells mediate anti-tumour immunity through the activation of antigen-specific $CD8^+$ T cells and B cells. Moreover, they exert direct and indirect effects on tumour cells, even those not expressing MHC class II molecules. A number of clinical observations have provided evidence supporting the notion of tumour immune surveillance in humans. Immunosuppressed patients are associated with a high risk of malignany [186]. Some tumours have defects in DNA mismatch repair mechanisms which leads to the formation of areas termed microsatellite instability (MSI). In colorectal carcinoma the infiltration of tumours by $CD8^+$ T cells especially when it expresses high levels of MSI, is associated with a favorable prognosis [187-188]. Tumour infiltration by T cells, NK cells, or NKT cells has been associated with an improved prognosis for a number of different tumour types [189] and was observed in patients with melanoma [190]. The phenomenon of spontaneously regressing melanoma coupled with the clonal expansion of T cells offers the strongest evidence for the elimination phase of cancer immunoediting in humans [191].

### 3.4 Equilibrium Phase

Refers to the immune system reaching a balance with tumour cells and thus limits its progression. In this period tumour cells survive the elimination phase but yet remain dormant and not clinically detectable as malignant disease [192] [193]. The three probable end points to this phase include either the elimination of the tumour at a later stage or long term equilibrium between tumour cells and the immune system and finally the loss of balance in favour of the tumour reaching the escape phase and becoming apparent as a clinically existing malignancy (Figure 3-2). Clinical evidence supporting the existence of the equilibrium phase of is provided by a number of findings in haematological malignancies [194].

### 3.5 ESCAPE

In this phase tumour expands and evades immune control, by several mechanisms (Figure 3-1)(Figure 3-2). Tumours may not express neo-antigens that are immunogenic or they may fail to express co-stimulatory molecules required for the activation of T cells. In addition, certain tumours are known to lack or be poorly express MHC antigens. The amount of antigen may be too small to stimulate the immune system or due to the rapid proliferation of malignant cells the immune system is quickly overwhelmed. Some tumours may evade the immune system by secreting immunosuppressive molecules such as indoleamine 2,3-dioxygenase (IDO), produced by APC and tumour cells causing suppression of T cells , apoptosis and T-reg stimulation [195-196]. Tumours can activate the differentiation of circulating myeloid cells into suppressor cells, named myeloid-derived suppressor cells (MDSCs) [197], which inhibit $CD4^+$ and $CD8^+$ T cells in an MHC-independent manner [198]. MDSCs can also induce the development of T-regs [199]. Tumour-associated macrophages (TAMs), are another type of myeloid suppressor cells, recruited to tumours and promote tumor growth by enhancing inflammation and angiogenesis [200].

Also, T-regs play a role in preventing tumour immune surveillance [201]. CD4+ T-regs express CD25, cytotoxic T lymphocyte–associated antigen 4 (CTLA4), glucocorticoid-induced TNF receptor–related protein (GITR), and the transcription factor forkhead box P3 (Foxp3). They are generated in the thymus and the periphery [202] and exert their suppressive activity on multiple immune cells (T cells, NK cells, NKT cells, B cells, and APCs), through both contact-dependent and contact-independent mechanisms [203]. T-regs accumulation has been documented in various tumour tissues, in tumour-draining lymph nodes and in the peripheral circulation of patients with cancer. Tumour antigen–specific T-regs have also been described and an inverse correlation between the number of T-regs in the tumor and clinical outcome has been noted [204].

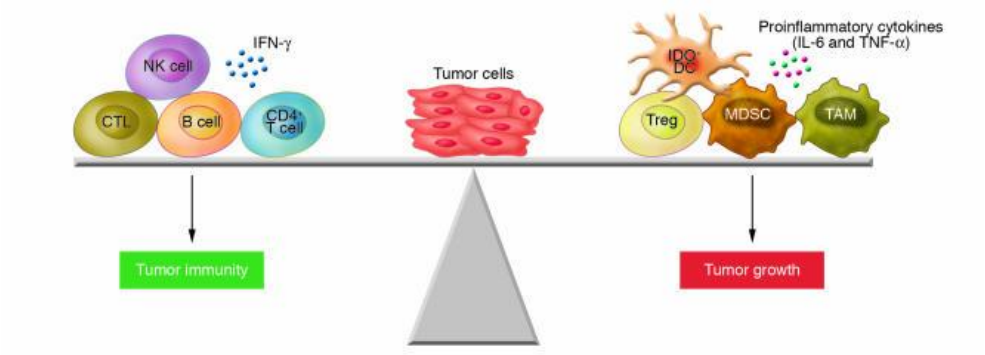



Figure 3-1: Cells of both the innate and adaptive arms of the immune system can mediate antitumor immunity, including CTLs, $CD4^+$ T cells, B cells, and NK cells. However, as tumors progress, they often develop ways in which to escape immune recognition. For example, they can induce the production of pro-inflammatory cytokines, the expression of IDO by APCs, and the differentiation of Tregs and various suppressor cells of myeloid origin [2]

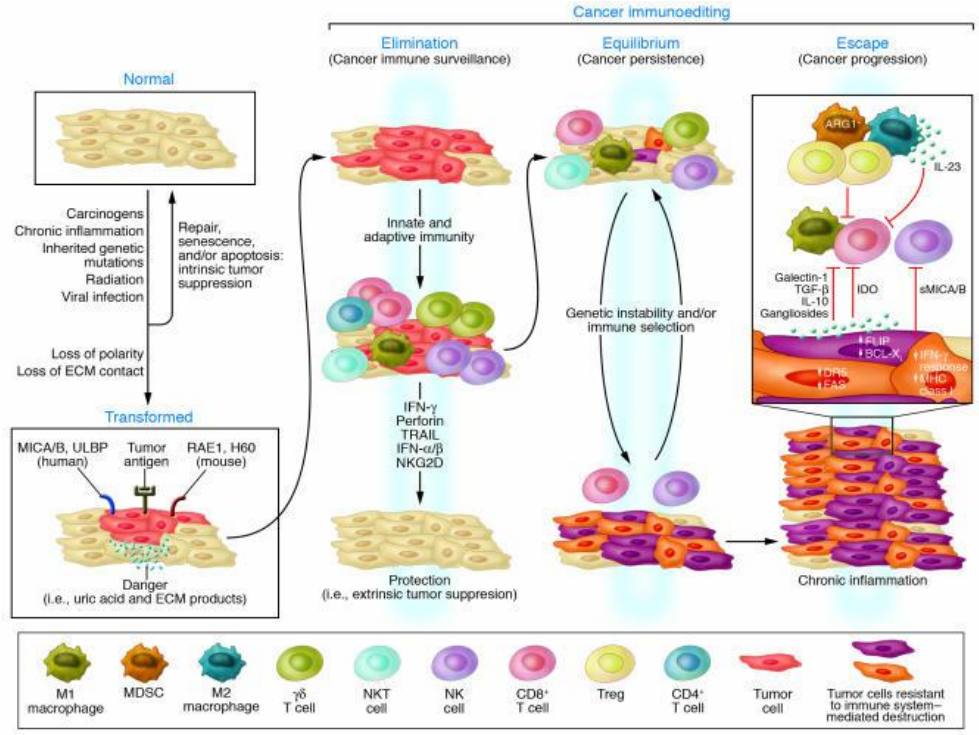



Figure 3-2: These phases have been termed the 3 Es of cancer immunoediting. DR5, death receptor 5; IDO, indoleamine 2,3-dioxygenase; MICA/B, MHC class I chain–related antigens A and B; RAE1, retinoic acid early transcript 1; sMICA/B, soluble MICA/B; ULBP, UL16-binding protein [3].

# CHAPTER 4: CANCER IMMUNOTHERAPY

Immunotherapy has been utilised as a new treatment modality to control cancer. Both active and passive immunotherapy techniques have been considered in stimulating the non-specific and specific immune systems.

## 4.1 Active Immunotherapy

The host actively participates in mounting an immune response following activation. Specific vaccines such as HBV vaccine and Human Papilloma virus vaccine or non-specific by activation with Bacillus Calmette-Guerin are examples. These methods activate macrophages to become tumouricidal.

## 4.2 Passive Immunotherapy

Passive immunotherapy is provided by the transfer of several factors to a host, such as preformed antibodies and immune cells. Monoclonal antibodies exert their effects by activating antibody dependent cellular cytotoxicity, triggering apoptosis, blockading growth factor receptors and complement activation. Hybridoma technology has allowed the mass production of specific antibodies against various tumours. Tumour antigens may be associated with class I MHC antigens and hence limits the efficiency of such antibodies. Tumours may change or alter their antigens or antigen-antibody complex, rendering such antibodies ineffectiveness. Some antibodies may be dysfunctional as they are not cytotoxic. Chimeric or humanized antibodies may retain a small murine component, which may itself be potentially immunogenic. Antibodies may bind to receptors of immune cells such as NK cells, B cells and macrophages thus blocking the binding sites to tumour cells. Anti CTLA-4 antibodies promote anti-tumour immunity by blocking the inhibitory effects of regulatory T cells [205]. In a murine model, it has been shown that CTLA-4 blockade with the addition of granulocyte macrophage colony stimulating factor (GM-CSF) immunotherapy was an effective treatment for melanoma [206].

Non-specific passive immunotherapy includes; (1) adoptive Transfer of lymphocytes, such as Lymphokine-activated killer (LAK) cells which are IL-2 activated T and NK cells or tumour infiltrating lymphocytes (TIL) (2) DCs pulsed with tumour antigens or tumour lysates may induce tumour specific T cell responses (3) cytokines such as IL2,

IFN-alpha, IFN-gamma and TNF-alpha (4) cytokine gene transfected tumor cells which activates LAK cell-mediated anti-tumor immunity.

### 4.3 T cell based immunotherapy

It is well established that both $CD4^+$ and $CD8^+$ T cells show anti-tumour activity. Antigens bind TCR and induce intracellular signaling which leads to proliferation of effector cytotoxic T cells and its stimulating cytokines. $CD8^+$ T cells recognize peptides presented by MHC class I molecules which is how the majority of tumor cells are presented. Loss of MHC class I expression is one method by which tumours evade recognition by $CD8^+$ T cells. $CD4^+$ cells also play an important role in fighting tumors [207] as antigens are presented to $CD4^+$ cells in the context of MHC II, resulting in the production of cytokines such as INF-gamma, which has inhibitory effect on tumour vasculature [208].

Antigen-specific T cells may be expanded *in vitro*, to avoid any immunosuppressive factors *in vivo* which may hamper the generation of an effector immune system. The removal of $CD4^+CD25^+$ regulatory cells resulted in the *in vivo* eradication of tumours in a murine model [209]. Depletion of lymphocytes *in vivo* resulted in the clearance of host MDSC or Tregs [210] and it is therefore vital to attempt to lymphodeplete patients before transferring the effector T cells in immunotherapy trials, which will allow transferred cells to persist *in vivo* [211].

# CHAPTER 5: NATURALLY OCCURRING CELL-MEDIATED IMMUNITY IN HCC

## 5.1 Tumour antigens expressed by HCC and their immunogenicity

Several tumour associated antigens (TAA) expressed by HCC have been identified, which could potentially be targeted in T cell-based immunotherapy and thus act as a tumour rejection antigens. AFP is a TAA known to be specific to HCC and testicular carcinoma. Depletion experiments and immunohistochemical analysis indicate that both CD8 and CD4 T cells facilitate the observed anti-tumour effect [212]. In animal models, AFP is shown to induce CD8 and CD4 T cell-mediated regression of AFP-expressing HCC by DNA-based immunization [213-214].

GPC3 which is also an oncofetal antigen over-expressed and released into the circulation in HCC and melanoma patients [215]. Glypicans belongs to a family of heparan sulphate (HS) proteoglycans that are linked to the cell membrane by a glycosyl-phosphatidylinositol anchor. Six types of glypicans have been identified in mammals (GPC1–GPC6). Glypicans are expressed predominantly during development and they are thought to play a role in morphogenesis [216]. Immunization of mice with DCs expressing GPC3 induces a protective antigen-specific T-cell response against GPC3-expressing tumour cells, demonstrating GPC3 immunogenic capabilities. In humans, HLA-A2 and A24 restricted peptide epitopes from glypicans have been identified and a cytotoxic T lymphocyte (CTL) response was detected to these peptides in healthy donors as well as HCC patients [217].

The NY-ESO-1 and MAGE super-family are members of the tumour-specific cancer-testis antigen and are expressed in many malignancies. HCC can express various cancer-testis antigens including NY-ESO-1, LAGE-1, members of the SSX family, members of the MAGE family, SCP-1 and CTP11 [218]. NY-ESO-1 and LAGE-1 are noted to be more immunogenic than other cancer-testis antigens and are considered target antigens for immunotherapy against different malignancies. Naturally occurring cell-mediated immunity to cancer-testis antigens is detected in patients with different types of cancer [219]. Humoral and CD8 T-cell responses to cancer-testis antigens were identified in HCC patients and this response was increased in late stage HCC [220]. Another observation was that T cells reacting to cancer-testis antigens are enriched in

tumour tissues [221-223]. Circulating cancer-testis-specific CD8 T cells are present in patients with HCC as well as patients with chronic liver disease secondary to HBV. However, cancer-testis-specific CD8 T cells from non-HCC patients were polyfunctional (IFN-$\gamma^+$, TNF-$\alpha^+$, CD107a$^+$) and those from HCC patients displayed an exhausted phenotype (IFN-$\gamma^+$, CD107a$^+$) [224].

BMI-1 and EZH2 are two classes of the polycomb group (PcG) protein that are expressed in HCC and, as other TAA, are considered as a prospective target for immunotherapy. They are important transcriptional repressors that regulate gene activity by the formation of multi-protein complexes [225]. They are associated with both humoral and T-cell responses and CD8 T-cell responses could be detected in both HCC patients and healthy donors [226].

HBV and HCV antigens are considered foreign antigens and therefore display more immunogenic capabilities than self-tumour antigens [224]. In animal models, the induction of cell-mediated immunity against hepatitis B surface and core antigens leads to the rejection of HCC [227]. Therefore, are also considered as target antigens for T-cell-based immunotherapy

**5.2 AFP-specific CD8 T cell responses**

The maintenance of peripheral tolerance to self proteins remains an area not fully understood in clinical immunology. However, in regards to AFP, many AFP-specific CD8 T cell clones are not deleted during ontogeny and AFP derived epitopes are recognized by both murine and human T cells later in life. It is established that CD8 T cells recognize AFP derived peptide epitopes in the context of MHC class I molecules and develop into cytotoxic T cells capable of recognizing and killing tumour cells. AFP-derived peptides with high binding to MHC class I (HLA-A2) were synthesized and tested *in vitro.* T cell clones which recognized several AFP-derived peptides were also identified. Earlier studies detected four peptides which were termed "immuno-dominant" based on their binding efficiency to MHC class I (HLA-A2) and ability to stimulate IFN-gamma production by CD8 T cells from healthy donors. These peptide epitopes were found to be both immunogenic and immunodominant in HLA-A2 transgenic mice [228]. Later studies discovered five other AFP-derived peptides containing HLA-A2402 binding motifs on hepatoma cells, recognized by CD8 T cells

which developed into cytotoxic T cells with the ability to produce IFN-gamma and allowed lysis of tumour cells [229]. We have reported that one of these epitopes ($AFP_{137–145}$) is recognised by both CTL and Th1 cells (12). Although several HLA-A2 and HLA-24 restricted CD8 T cell epitopes have been identified and classified as immuno-dominan/sub-dominant [229-230], this concept of dominance is no longer favorable as it has been shown that a high frequency of AFP-specific CD8 T cells (CTL) producing IFN-gamma are directed against different epitopes spreading across the entire AFP sequence [231] and our recent data also support these findings [232].

AFP-specific CD8 T cells can be detected in patients with HCC, non-HCC liver diseases as well as healthy donors [231, 233-234], however, it has been suggested that this response is stronger in HCC patients [231]. In addition, it was noted that CD8 T cells recognizing AFP, Glypican-3, NY-ESO-1 and MAGE-1 were detected in healthy donors [223, 231, 235-236]. In contrast, there is absence of anti-AFP CD4 T cell responses in healthy donors or patients with non-HCC liver diseases [232, 237-238]. A number of studies, including ours, supports the finding that AFP-specific CD8 T cells does not correlate with elevation of serum AFP, vascular invasion, liver function and the type of viral infection [231-233, 239]. No association was detected between the levels of serum AFP, Okuda staging in HCC patients and the presence of CD8 T cell responses to other HCC associated antigens such as NY-ESO-1 [224]. We have demonstrated an association between the stage of liver cirrhosis and the presence of anti-AFP CD8 T cell responses. Anti-AFP-CD8 T cell responses were more pronounced with advanced liver cirrhosis as the response was 46% in HCC patients with CP score of B or C whilst it was 17% in HCC patients with CP score of A [232]. The frequency of AFP-specific CD8 T cells in the liver of HCC patients was not higher than in the peripheral blood [231], contrary to the expectation of a high concentration of tumour specific CD8 T cells in the liver [223]. The liver infiltrating AFP-specific CD8 T cells may undergo apoptosis or stop responding to peptide stimulation due to exhaustion or expression of inhibitory molecules such as PD-1 which may explain this phenomenon. RFA or TACE treatment was noted to enhance the frequency of circulating AFP-specific CD8 T cells (*ex vivo*), but not viral-specific CD8 T cells in some HCC patients [229]. Therefore, it is envisaged that that tumour burden may suppress the expansion of anti-AFP CD8 T cell responses in HCC patients and tumour necrosis may also stimulates the expansion of anti-tumour immune responses.

### 5.3 AFP-specific CD4 T cell responses

CD4 T cells have a vital function in the control of tumour growth both in both animal and human cancer. It can provide induction for CD8 T cells responses which in turn capable of lysing tumour cells in a MHC class I, Fas ligand and perforin dependent manner. Th1 cells can also directly eliminate tumour cells without any involvement of CD8 T cells [240] as demonstrated by animal model experiments involving adaptive transfer of Th1 cells, but not CTLs, which provided protection against various transplanted or endogenous [241-242]. Similar results were reproduced in cancer patients, where adaptive transfer of a Th1 clone recognizing NY-ESO-1 antigen provided sustained clinical remission [243]. We have studied the role of circulating AFP-specific CD4+ T cell response in HCC patients in depth [232, 237-238, 244]. Several HLA-DR restricted CD4 T cell epitopes within the AFP sequence have been identified and assigned as immunodominant, in contrast to CD8 T cell responses. Th1 responses to the immuno-dominant epitopes were only observed in HCC patients and no response was detected in patients with non-HCC liver disease or healthy controls [232, 237-238] and more than 20% of HCC patients have a detectable response to this immunodominant epitope [238]. Anti-AFP CD4 T cell response are more likely to be present in patients with early stage disease (Okuda stage I) and low or moderately elevated serum AFP [238]. In our recent study it demonstrated that anti-AFP Th1 response is detectable in 58% of HCC patients with Okuda stage I tumours, 44% with a CP score of A, in contrast to 15.8% of patients with Okuda stage II or III tumours and15% with CP score of B or C [232]. This result is in keeping with our earlier reports showing that high concentrations of AFP suppress immune cell function *in vitro* [245] and CD4 T cells isolated from HCC patients with high concentrations of serum AFP are impaired [238]. This may indicate that Anti-AFP CD4 T cell response is impaired or exhausted in late stage HCC patients with high AFP as a result of different immune-regulatory mechanisms reported in patients with HCC [244-247]. The expansion of $CD4^{+}CD25^{+}$ regulatory T cells in the peripheral blood and tumour tissues of patients with HCC has been reported [246, 248-249] and this has an inverse correlation with the recurrence-free survival [250]. A number of preclinical murine studies suggested that the depletion of regulatory T cells augments the effects of immune-based therapies such as anti-tumour vaccines [251]. Low-dose cyclophosphamide treatment has been shown to deplete $CD4^{+}CD25^{+}$ regulatory T cells

in a murine tumour model [252]. In a recent report, the effect of a low-dose cyclophosphamide treatment in patients with advanced HCC, demonstrated a decreased frequency and suppressor function of the circulating $CD4^{+}CD25^{+}Foxp^{+}$ regulatory T cells in peripheral blood, thus unmasked anti-AFP T cell responses [253]. This indicates that cyclophosphamide may be used in combination with immunotherapeutic approaches in HCC. Also we reported that TGF-beta1 producing CD4 T cells could be induced by the AFP-derived peptide epitope and this may hamper anti-tumour immunity [244]. Our group has also shown that tumour necrotizing treatments such as TACE/TAE that reduce tumour burden may improve the function of AFP-specific CD4 T cells [237].

# CHAPTER 6: IMMUNOTHERAPY IN HCC

## 6.1 Strategies

Several different strategies have evolved to initiate anti-tumour immunity including making the tumour more immunogenic as an option. Also, eliminating factors in the tumour microenvironment which exert an immunosuppressive effect is another modality tested. Generalised as well as antigen specific immune activation has also been reported.

## 6.2 *In vitro* studies

To increase the immunogenicity of tumours, heat-shock or hyperthermia treatment of cells create stress signal and enhances the expression of heat shock proteins which have been shown to create an immunogenic delivery method for tumour-derived antigens. Tumour lysate from heat-shocked HepG2 cells has been shown to be more immunogenic when pulsed onto APC than lysate from non-heat shocked HepG2 cells [254].

T cells specific for HCC have been isolated and analysed *in vitro* to identify tumour antigen specific/non-specific cells with antitumour activity [255]. MAGE antigen-specific T cells have been isolated in TIL from HCC subjects [222] and AFP-specific T cells have been identified in patients with cirrhosis and HCC, with and without immunisation [234, 238, 256-257].

## 6.3 Human clinical trials

Immunotherapy trials for HCC to date fall into several categories:

**6.3.1** Cytokines

In a trial of patients with stage III–IV HCC, transarterial chemotherapy was combined with interferon gamma and interleukin (IL)-2 [258]. Fourteen of the twenty patients studied had some reduction in tumour size and serum AFP levels, indicating a clear positive biological effect.

A further trial of 15 patients with advanced inoperable HCC, subcutaneous injection of IFN-gamma and GM-CSF showed no clinical responses [259].

Another trial tested an adenovirus encoding IL-12 in intratumoural injections for a variety of digestive tumours. Of the 8 HCC patients studied, there were 6 who showed

disease stabilisations, 1 partial response and 1 progressive disease. The same group tested 8 patients with HCC by intratumoral injections of autologous DC transduced with an adenovirus encoding IL-12 and demonstrated trial safety and two patients had disease stabilisations [260].

### **6.3.2** Effector lymphocytes

The utilisation of different kinds of cytotoxic lymphocytes has been studied in various trials. In two trials, in which adriamycin was combined with LAK postresection of HCC, revealed a decrease in rate of recurrence in one trial whilst the second showed no overall benefit except for subjects with a negative margin of >1 cm post resection. One major set back with LAK cells is their lack of tumour antigen-specificity [261-262]. In contrast, TIL are derived from the tumour tissue, thus have been shown to contain tumour antigen-specific T cells. In a pilot study of $Indium^{111}$ labelled TIL, these cells delivered via intrahepatic artery infusion were shown to migrate to sites of disease preferentially. In a further study of 10 patients who received TIL activated by IL-2 and LAK cell supernatant, it demonstrated improved recurrence rates compared to controls [264]. In a large trial involved 150 patients randomised to receive either IL-2 and anti-CD3 activated peripheral blood Lymphocyets (PBL) or passive observation postcurative resection of HCC, there were significant improvements in the risk of recurrence, the time taken for recurrence and recurrence-free survival, but not overall survival [263].

### **6.3.3** Antigen presenting cells

Different modalities to improve uptake and presentation of TAA have been explored in various trials. For example, by adding tumour lysate or purified proteins to immature DCs as they are known to have an accelerated active uptake or by coating DCs with synthetic peptides. In one study two patients with metastatic HCC were treated with immature DCs, grown in GM-CSF and IL-4, and the results indicated a slowing down of tumour growth [265]. In another trial, a total of ten patients were vaccinated with autologous DCs which were generated *ex vivo* in the presence of GM-CSF and IL-4 then pulsed with tumour lysate, TNF-alpha and keyhole limpet hemocyanin. Immunization was well tolerated in all patients and in one patient there was a reduction in liver tumour size. In two patients, serum levels of tumour markers decreased after

vaccination [266]. In a further large trial thirty one patients with advanced HCC were enrolled and DCs were pulsed with autologous tumour lysates. Four patients exhibited partial response whilst seventeen patients had stable disease and ten patients had progressive disease. The overall 1-year survival rate of all 31 patients was improved significantly [267]. Recently, a phase 1 study was conducted in which 12 of the 14 patients recruited with metastatic stage HCC received two vaccinations of intratumoral injections with autologous immature DCs after a single fraction of conformal radiotherapy. This treatment induced tumour-specific and innate immunity responses as evident by two partial responses and four minor responses. A decrease in the AFP level of more than 50% was found in three patients. Six patients showed an increased NK cell cytotoxic activity after vaccination [268]. A phase II clinical trial was conducted where intravenous vaccination with mature autologous DCs pulsed *ex vivo* with a liver tumour cell line lysate (HepG2) in patients with advanced HCC not suitable for radical or loco-regional therapies. Of the 35 patients recruited 25 received at least three vaccine infusions and it illustrated the feasibility of generating autologous DCs despite advanced malignancy. These DCs were loaded *ex vivo* with multiple antigens from lysates of a liver cancer cell line and matured. This vaccine was safe when administered intravenously. Evidence of clinical response was noted with one patient achieving a radiological partial response associated with a significant decline in the serum AFP. Several other patients achieved disease stabilization and in three patients this was associated with significant reductions in serum AFP. Immune responses were assessed using an ELIspot assay of IFN-gamma release in 5 of 10 responder patients where there was induction of T cell responses to the vaccine and/or AFP following vaccination [226]. It is possible that tumour lysate-pulsed DCs can also activate the expansion of regulatory T cells which may diminish the effectiveness of this immunotherapy strategy [269].

### **6.3.4** Autologous tumour

A randomised phase II trial was completed with forty one stage I-IIIa patients after curative resection. Nineteen received a vaccine consisting of formalin-fixed autologous tumour mixed with GM-CSF, IL-2 and BCG as a foreign immune stimulus. Early postoperative recurrence of HCC was reduced and overall survival improved. The best outcomes were observed in patients with low tumour burden [270].

In a follow up trial, 54 of 60 patients recruited with stage I/II HCC disease were immunised intradermally and randomised to observation or fixed autologous HCC vaccine, GM-CSF, IL-2 and tuberculin. The 3 year recurrence rate was 33% for immunised and 61% for controls, supporting a significant clinical benefit [271].

### **6.3.5** AFP-based T cell immunotherapy

After the identification of several HLA-A2 restricted CD8 T-cell epitopes *in vitro* [236, 272-273], two clinical trials targeting these epitopes have been conducted [256-257]. A pilot Phase I clinical trial in which HLA-A*0201 patients with AFP-positive HCC were immunized with three biweekly intradermal vaccinations of the four AFP peptides (100 - 500 μg each) emulsified in incomplete Freund's adjuvant. All of the 6 patients recruited generated T-cell responses to most or all of the peptides as measured by direct IFN-gamma production by the enzyme-linked immunospot (ELISPOT) and MHC class I tetramer assays suggesting that these peptide epitopes tested were immunogenic. No clinical responses were detected, nor did serum AFP levels decrease. The overall survival post-treatment ranged from 2 to 17 months. In a follow-up trial, 10 HCC patients with tumour stages III–IV were administered with autologous DCs pulsed with AFP-derived CD8 epitopes. Six of the vaccinated patients had a peptide-specific CD8 T-cell response, however, the immunization did not result in any clinical response. Novel vaccines are being tested in clinical trials and combinations of vaccines along side standard therapies are just beginning to be considered in clinical practice which may hold a promising future in the development immunotherapy for HCC.

## CHAPTER 7: IMMUNOSUPPRESSIVE FACTORS IN HCC

Despite the presence of tumour-specific immune responses, tumours progress in patients with HCC, implying the promotion of a number of immune suppressor factors. These factors include; production of T-regs, DCs dysfunction, induction of MDSC, impairement of NK cells, production of immunosuppressive factors such as Indoleamine 2,3-dioxygenase, tumour COX-2 and TGF-beta1. Clearly, these inhibitory factors ought to be eliminated prior to the design of any immunotherapy treatment.

### 7.1 CD4+CD25+ Foxp3 T-regs

Several studies have demonstrated that immunosuppression by T-regs leads to tumour immune evasion and could be the main hindrance for developing a successful tumour immunotherapy [274]. T-regs characterized by the expression of the transcription factor Foxp3 (CD4+CD25+ Foxp3 T-regs), are fundamental in suppressing the function of effector cells, such as CD8+ T cells, CD4+ T cells and NKT cells. They have been noted to be expressed at high frequencies in patients with haematological malignancies and several tumours [249, 275-276]. Studies have demonstrated a considerable increase in frequency of CD4+CD25+Foxp3+ T-regs in peripheral blood, tumour and ascites of HCC patients [248] as well as in studies of the tumour-infiltrating cells [277] or peritumour regions [246]. Various factors were implicated in the generation of T-regs. Amongst these factors, TGF-beta1 which convert peripheral CD4+CD25+ naive T cells to Foxp3- expressing T-regs [203]. Other factors include, the expression of VEGF in the tumour microenvironment of HCC [278] and high levels of IL-10 in the serum of HCC patients [279]. To eliminate the suppressive effects of T-regs on effector cells and to enhance T cell immune responses, T-regs were targeted either by monoclonal antibodies (mAbs) or immunosuppressive agents. In murine HCC, anti-CD25 mAb was found to enhance antitumour effects and tumour-specific CD4+ and CD8+ T cells [209, 280]. Administration of cyclophosphamide has been shown to enhance antitumour effects in preclinical models [252] and in HCC patients resulted in a marked decline in the frequency of T-regs [281].

### 7.2 Dendritic cell dysfunction

It is widely accepted that impairment of DC function contributes to the escape of the tumour from immune control [282]. DCs from cancer patients were Immature and did not stimulate T cells [283]. In HCC patients, DCs from peripheral blood and lymph nodes were reduced in number had an immature phenotype and an impaired function [284-285]. In the local hepatic lymph nodes, there were more immature plasmocytoid DC's than the mature myeloid DC's [286]. Tumour derived factors, such as VEGF [287], M-CSF, IL-6 [288] and IL-10 [289], were implicated in the aforementioned DC dysfunction. Our group has found that AFP expression in the serum of patients with HCC has also been identified as a possible inhibitor of DC function [245].

### 7.3 Myeloid-derived suppressor cells

MDSCs are a mixed population of myeloid derived suppressor cells regulating T cells function and include macrophages, granulocytes and myeloid cells at different stages of differentiation. In humans they are not well characterized but their immuno-suppressive function was noted in several malignancies and their phenotype was mainly CD34+, CD33+, CD15- and CD13+, CD14-/lin- [200, 290]. Recently, a new subset of MDSCs that expressed CD14, were noted to be increased in peripheral blood and tumour tissue of HCC patients. These cells when cultured with autologous T cells, induced CD4+CD25+Foxp3+ T-regs and suppressed the proliferation of CD4+ T cells [291]. Efforts to design immunotherapy for HCC should take into account and include targeting MDSCs as well CD4+CD25+Foxp3+ Tregs.

### 7.4 Impairment of NK cells

Natural killer cells have a vital role in the tumour microenvironment by initiating direct killing of tumour cells and releasing immunomodulatory cytokines which activate immunosuppressive leukocytes [292]. However, the frequency of peripheral blood and liver NK cells were reduced in HCC patients. In addition, they were found to have an impaired INF-gamma production and associated with increased CD4+CD25+ T-regs and MDSCs [293-294].

### 7.5 Suppressive factor overexpressed in HCC

**7.5.1** Indoleamine 2,3-dioxygenase (IDO) is an immunoregulatory enzyme which leads to arrest of T-cell proliferation and poor prognosis in patients with cancer [295]. It was found to be over expressed in HCC cell lines and tumor tissues leading to reduced recurrence-free survival [296].

**7.5.2** Tumour COX-2 is over-expressed in some cancers and HCC [297] and leads to the production of prostaglandins, which also cause immune suppression. Elevated tumour COX-2 levels correlated with an elevated VEGF level and increased invasiveness in HCC. Also, it was associated with lower CD8+ T-cells infiltrating HCC tumours and correlated with poor prognosis and disease-free survival rate [298].

**7.5.3** Transforming growth factor can also (TGF-beta1) dampen antitumour immunity [299]. TGF-beta1 is over-expressed in HCC tissue and the plasma TGF-beta1 was noted to be elevated in patients with HCC [300]. We have recently shown that AFP stimulated CD4 T cells with the production of TGF-β1 [244].

## CHAPTER 8: ALPHA-FETOPROTEIN IMPAIRS APC FUNCTION AND INDUCES THEIR APOPTOSIS

### 8.1 ABSTRACT

AFP is a TAA, and its serum level is elevated in patients with HCC. *In vitro*, AFP induces functional impairment of DCs. This was demonstrated by the down-regulation of CD40 and CD86 molecules and the impairment of allostimulatory function. Also, AFP was found to induce significant apoptosis of DCs, and AFP-treated DCs produced low levels of IL-12 and TNF-alpha, a cytokine pattern that could hamper an efficient antitumor immune response. *Ex vivo*, APCs of patients with HCC and high levels of AFP produced lower levels of TNF-alpha than that of healthy individuals. In conclusion, these results illustrate that AFP induces dysfunction and apoptosis of APCs, thereby offering a mechanism by which HCC escapes immunological control.

### 8.2 Introduction

Structurally related to human albumin, alpha-fetoprotein (AFP) is a well-characterized oncofetal Ag. It is normally expressed during embryogenesis and is present in only trace amounts in normal adults [83]. However, the expression of the AFP gene is reactivated in patients with testicular and HCC with high levels of AFP being found in the sera and tumor tissues. The determination of serum AFP aids in the diagnosis and the management of patients with HCC. A serum AFP level of >200 ng/ml is shown to have a specificity of 100% for HCC [301]. In a study of 68 Asian-American patients with HCC, serum AFP ranged from 0 to 636,000 ng/ml with the average being 5,200 ng/ml [302]. AFP, like serum albumin, shows relatively strong binding affinities for a variety of ligands. Various other specific physiological roles for AFP are being proposed such as its possible role in the regulation of immune cells adults [83]. A series of investigations has provided evidence that AFP causes selective down-regulation of MHC class II on monocytes [303-304] and the suppression of T responses [305-308] and B lymphocyte [309]. It has also been shown that AFP-mediated immunoregulation is an activity intrinsic to the molecule itself and cannot be attributed to either putative noncovalently bound moieties or to posttranslational modifications such as glycosylation and sialylation [310]. Stimulation of leukotriene synthesis by AFP in macrophages has been suggested as a possible mechanism for its immunoregulatory

effects [311]. In addition, microscopic autoradiography has exhibited binding of AFP almost exclusively on human peripheral monocytes but not on lymphocytes [88], suggesting that the regulatory effects of AFP may be via APCs.

Dendritic cells (DCs) are the most potent APCs of the immune system and are crucial in the initiation of the immune response against pathogens and tumors. DCs exist in two differing states of maturation: immature and mature. Immature DCs express low levels of surface molecules such as CD80, CD86, CD40, and MHC class II, and have low T cell-stimulatory capacity. Several stimulatory agents, such as proinflammatory cytokines and viral or bacterial products, can trigger DC activation and thus maturation. This is defined by the up-regulation of costimulatory molecules, an increase in the levels of TNF-alpha and IL-12 production, and improved capacity to stimulate T cells [312-313]. The activated DCs can rapidly activate other innate immune cells such as NK [314] and NKT cells [315]. Most knowledge about the biology of DCs has emerged from the ability to generate DCs *in vitro* from either CD34+ hemopoietic progenitors or peripheral blood monocytes.

In this study, we show that the treatment of monocyte-derived DCs with AFP induces DCs dysfunction as detected by the down-regulation of surface molecules and inhibition of their T cell-stimulatory capacity. In addition, AFP treatment reduces the ability of monocyte-derived DCs to produce TNF-alpha and IL-12 and induces apoptosis of DCs. Furthermore, we compare the ability of APCs from patients with HCC to produce TNF-alpha with that of control individuals. The data clearly show that HCC with high levels of serum AFP have reduced TNF-alpha production.

## 8.3 Materials and Methods

### 8.3.1 *Patients*

The patients involved in this study were all reviewed at the Liver Unit of the Cromwell Hospital (London, U.K.). The ethical committee's approval was granted, and informed consent was obtained. In total, there were 16 patients with an AFP value ranging from 8 to 1,141,205 ng/ml. The average serum AFP level in the HCC group was 80,409 ng/ml. There were seven patients with hepatitis C cirrhosis, two with cryptogenic cirrhosis, three with hepatitis B cirrhosis, two with hepatitis B and C cirrhosis, one with alcoholic liver cirrhosis, and one with alcoholic liver and hepatitis C-related cirrhosis. Five of them were Child's grade B and 11 were Child's grade A. Laboratory tests including aspartate aminotransferase, alanine aminotransferase, total bilirubin, platelet count,

prothrombin time, international normalized ratio, creatinine, hepatitis B surface Ag, anti-hepatitis C virus Ab, and hepatitis C virus RNA, were determined using standard, commercially available assays. All blood samples from patients with HCC were withdrawn before giving any therapy. The severity of cirrhosis was assessed by Child-Pugh score.

*8.3.2 AFP measurement*

Levels of serum AFP were measured using microparticle enzyme immunoassay (MEIA) kit obtained from Abbott Laboratories (Abbott Park, IL) and performed according to the manufacturer's instruction. In brief, anti-AFP microparticles were incubated with the blood specimen, and an aliquot of the reaction mixture was transferred to the matrix cell. The matrix cell was washed, removing unbound materials, and the anti-AFP conjugate was dispensed onto the matrix cell. The substrate was added to the matrix cell, and the fluorescent product is measured by the MEIA optical assembly.

*8.3.3 Cell culture*

RPMI 1640 medium, penicillin and streptomycin, and 10% heat-inactivated FCS were purchased from Invitrogen Life Technologies (Carlsbad, CA). Purified human cord blood AFP (purity, >95%; SDS-PAGE) and purified human albumin (purity, >97%; SDS-PAGE) were obtained from Calbiochem (San Diego, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Recombinant human GM-CSF and IL-4 were purchased from PeproTech (Rocky Hill, NJ).

*8.3.4 Monocyte purification and generation of monocyte-derived DCs*

Mononuclear cells were isolated from peripheral blood by centrifugation on Ficoll-Hypaque (Amersham Pharmacia, Uppsala, Sweden). Mononuclear cells from healthy individuals were incubated in 96-well plates in RPMI 1640 for 30 min, and nonadherent cells were removed by gentle wash. DCs were generated as described previously [316]. Briefly, adherent cells were cultured in DC medium (RPMI 1640 supplemented with 10% FCS) containing GM-CSF (500 IU/ml) and IL-4 (250 IU/ml) and in the presence or absence of AFP or human serum albumin (HSA). On days 3 and 5, the cells were fed with the DC medium and the above cytokines. Where indicated, AFP or HSA was added on day 5 to study the effect of AFP on different stages of DC

differentiation. DC maturation was induced by the addition of LPS (500 ng/ml; Sigma-Aldrich) to the culture on day 7 for 24 h.

*8.3.5 Analysis of DC surface markers and apoptosis*

DCs were stained with FITC- or PE-labeled mAbs (anti-human CD1a, CD11c, CD14, CD19, CD20, CD40, CD80, CD83, CD86, HLA-DR, or relevant isotype controls; BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Cells were gated according to their size (forward light scatter) and granularity (side light scatter) using a FACScan flow cytometer (BD Immunocytometry Systems, San Diego, CA). The DC surface marker expression was analyzed using the CellQuest program (BD Immunocytometry Systems). DC apoptosis was detected using Annexin VFITC, with dead cells identified by propidium iodide (PI) staining (BD Pharmingen).

*8.3.6 Mixed leukocyte reaction*

To avoid DC maturation, DCs were harvested following gamma irradiation. Gamma-irradiated allostimulatory DCs were incubated in round-bottom microtiter plates with 105 allogeneic T cells. Triplicate cultures were maintained for 5 days at 37°C in a 5% CO2 humidified atmosphere. T cell proliferation was measured by pulsing cells with 1 μCi of methylthymidine (Amersham Pharmacia) for 18 h.

*8.3.7 Intracellular cytokine assay*

PBMCs or DCs were cultured in a medium containing LPS (500 ng/ml) and brefeldin A for 3 h. The cells were stained with anti-HLA-DR Ab, permeabilized, fixed, and stained with anti-human IL-12, IL-10, TNF-alpha, or isotype control Abs. The expression of intracellular cytokines was analyzed using flow cytometry.

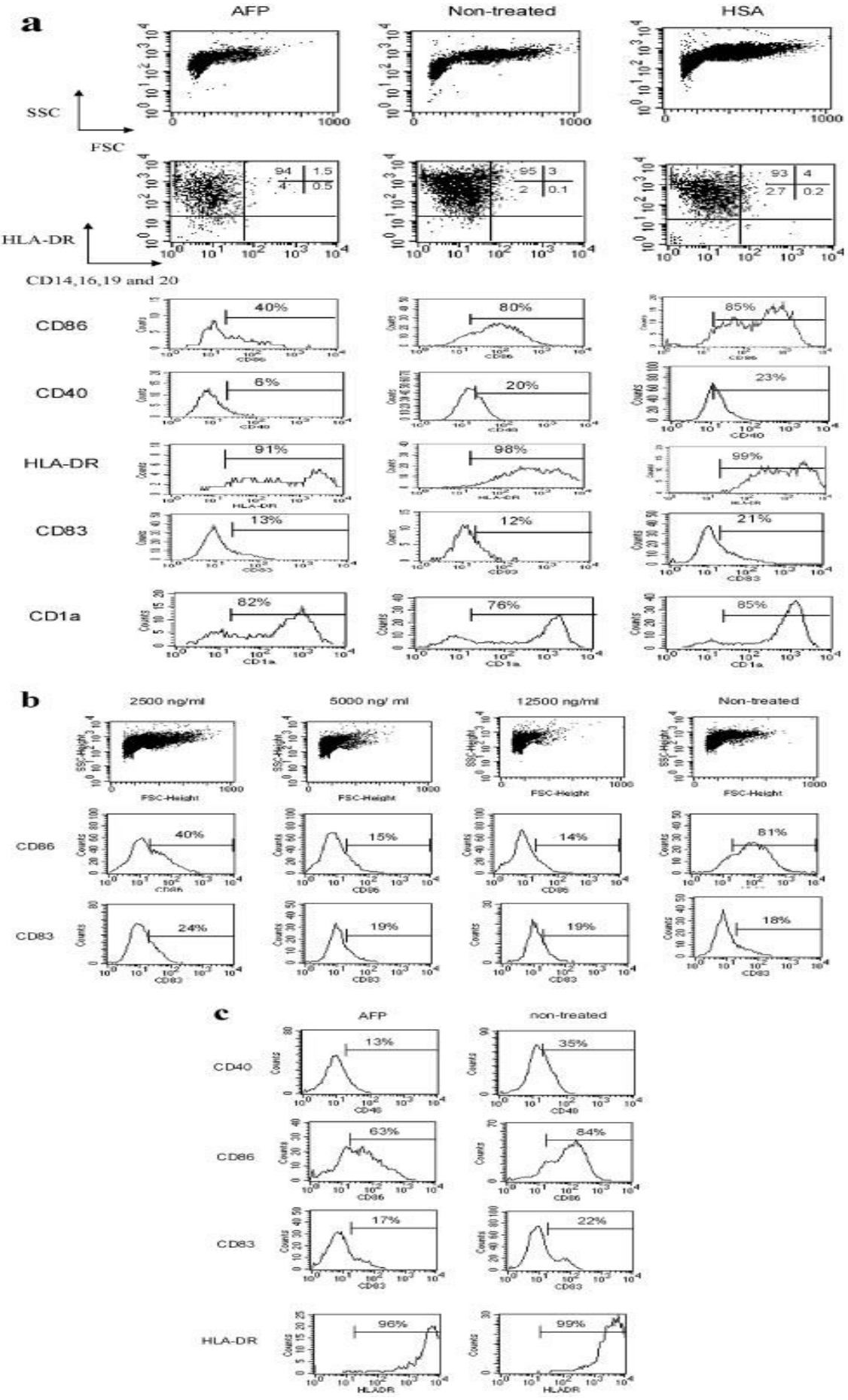
*8.3.8 ELISA*

Levels of biologically active IL-12 p70 and nonactive IL-12 p40 were measured using ELISA kit obtained from R&D Systems (Minneapolis, MN). ELISAs were performed in duplicate according to the manufacturer's instructions.

## 8.4 Results

### 8.4.1 AFP impairs DC function in vitro

In this study, we have examined the effects of AFP on the function of monocyte-derived DCs generated from healthy individuals. AFP was added at concentration similar to that of AFP level reported in the sera of patients with HCC. As assessed by flow cytometry, monocyte-derived DCs cultured for 7 days in the presence of GM-CSF and IL-4 developed into DCs characterized by the acquisition of CD1a and CD11c and loss of CD14 molecules. They expressed high levels of HLA-DR and CD1a molecules but did not express CD14, CD16, CD19, or CD20 (Figure 8-1a). Addition of AFP (as low as 2500 ng/ml) on day 0 of culture induced phenotypical alteration of DCs (Figure 8-1b). However, this was not demonstrated with the addition of HSA. CD86 expression was substantially down-regulated, and a slight reduction of CD40 median fluorescence was detected. The expressions of CD1a, HLA-DR, and CD83 did not alter (Figure 8-1a). Similar results were obtained when DCs were cultured in a medium containing 10% human serum (data not shown). When AFP was added on day 5 or 6 instead of day 0, a less pronounced decrease in CD86 expression was observed (data not shown). Addition of LPS on day 7 resulted in up-regulation of CD40, CD83, and CD86 molecules 24 h later (Figure 8-1c). However the expression levels of the surface molecules on the AFP-treated mature DCs were still significantly lower than that of the nontreated mature DCs (Figure 8-1c).


a
AFP
Non-treated
HSA
SSC
FSC
HLA-DR
CD14,16,19 and 20
CD86
CD40
HLA-DR
CD83
CD1a
40%
80%
85%
6%
20%
23%
91%
98%
99%
13%
12%
21%
82%
76%
85%
b
2500 ng/ml
5000 ng/ ml
12500 ng/ml
Non-treated
FSC-Height
SSC-Height
CD86
CD83
40%
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81%
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AFP
non-treated
CD40
CD86
CD83
HLA-DR
13%
35%
63%
84%
17%
22%
96%
99%

Figure 8-1: DCs treated with AFP express low levels of CD86 molecules. Monocytes were cultured with GM-CSF and IL-4, in the presence or absence of different concentrations (25,000 ng/ml in a and c; 12,500, 5,000, or 2,500 ng/ml in b) of AFP or 25,000 ng/ml HSA. Cells were harvested on day 7 of culture, stained with different mAbs, and analyzed using flow cytometry. Cells were stained with PE-labeled mAbs. A PE-labeled, isotype-matched control Ig was used (a–c). Cells were also stained with FITC-labeled anti-HLA-DR and PE-labeled anti-CD14, CD16, CD19, and CD20 (a). Numbers represent the percentage of cells expressing CD83, CD86, CD40, HLA-DR, and CD1a. Sideward light scatter (SSC) and forward light scatter (FSC) characteristics are shown (a and b). Results were similar in four different independent experiments. On day 7, cells were treated with LPS to induce DC maturation, and the expression of surface molecules was analyzed on day 8 (c). Results were similar in three different independent experiments.

### *8.4.2 AFP-treated DCs are poor stimulators of allogeneic T cell response in vitro*

AFP-treated DCs, but not HSA-treated or nontreated DCs, were shown to have low allostimulatory capacity. Graded numbers of viable DCs were cocultured with allogeneic T cells for 5 days in an MLR assay. The allostimulatory function of AFP-treated immature DCs was significantly reduced in a dose-dependent manner (Figure 8-2). HSA did not inhibit allostimulatory function of DCs. A significant inhibitory effect of AFP (25,000 ng/ml) was also detected when AFP was added on day 5 (data not shown).

AFP-treated immature DCs were stimulated with LPS for 24 h to induce DC maturation. The allostimulatory ability of AFP-treated mature DCs (10,000 cells/well) was analyzed. AFP-treated mature DCs had significantly lower T cell-stimulatory capacity than that of nontreated mature DCs (Figure 8-2c).

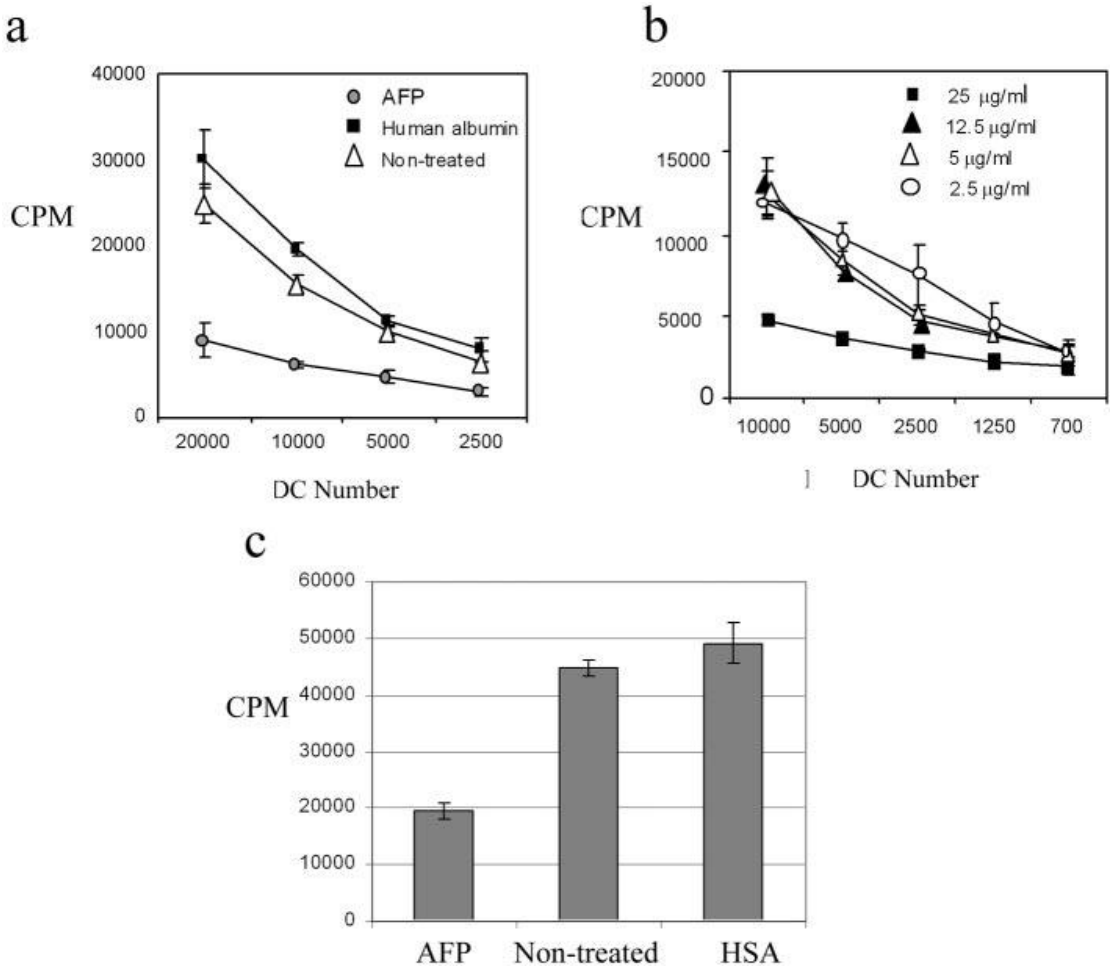



Figure 8-2: AFP reduces the DC allostimulatory function in a dose-dependent manner. Monocytes were cultured in the presence of GM-CSF and IL-4. AFP or HSA was added at different concentrations (25, 12.5, 5, or 2.5 µg/ml) (b) or 25 µg/ml (a and c) on day 0 of culture. Immature DCs (a and b) (day 7) or mature DCs (c) (day 8 after LPS stimulation for 24) were harvested, and graded numbers of viable cells were added to allogeneic T lymphocytes. After 5 days, T cell proliferation was assessed by the addition of thymidine for 18 h. The data shown are average counts per minute of three replicate determinations ± SD and are representative of three similar experiments.

*8.4.3 AFP induces apoptosis of DCs in vitro*

The actual DC and PBMC recovery was determined after 7-day culture in the presence or absence of AFP. The cell recovery in the AFP-treated DCs, but not AFP-treated PBMCs, was significantly reduced. In the DC cultures, the number of cells recovered was 113,000 ± 10,000 for the AFP-treated group, and 236,000 ± 10,000 for the nontreated group. In PBMC cultures, the number of cells recovered was 143,000 ± 12,000 for the AFP-treated group, and 123,000 ± 4,000 for the nontreated group. The reduction of cell viability in the DC cultures after exposure to AFP may be due to induction of apoptosis. To investigate this possibility, cells were treated with AFP on day 0 of culture and analyzed on day 7 for the presence of apoptotic cells. AFP induced a significant increase in the number of apoptotic DCs as assessed by Annexin VFITC/PI staining (Figure 8-3a). To test whether AFP exerts apoptotic effects on lymphocytes, PBMCs were treated with AFP for 7 days and annexin V binding was assessed on CD3+ cells. AFP did not induce apoptosis of CD3+ cells (Figure 8-3a).

AFP inhibitory effects on DCs could be due to the induction of DC apoptosis or the suppression of DC function or both. We characterized the phenotype (Figure 8-3b) and the function (Figure 8-3c) of nonapoptotic DCs. The cells were gated on annexin V-negative, PI-negative cells (nonapoptotic), and the expression levels of CD86 molecule were analyzed. The nonapoptotic DCs exposed to AFP expressed slightly lower levels of CD86 than that on nonapoptotic, nontreated DCs (Figure 8-3b). To test the functional ability of nonapoptotic DCs, we first determined the percentage of apoptotic cells in the cultures (annexin V-positive, PI-negative cells). The ability of nonapoptotic cells to stimulate T cell proliferation was determined in an MLR assay. The number of nonapoptotic cells in unsorted cells was estimated, and AFP-treated or nontreated nonapoptotic DCs, i.e., annexin V-negative, PI-negative cells, were added (20,000 cells/well) to allogeneic T lymphocytes. After 5 days, T cell proliferation was assessed by the addition of thymidine for 18 h. The nonapoptotic cells from AFP-treated group had a low ability to stimulate T cell proliferation (Figure 8-3c).

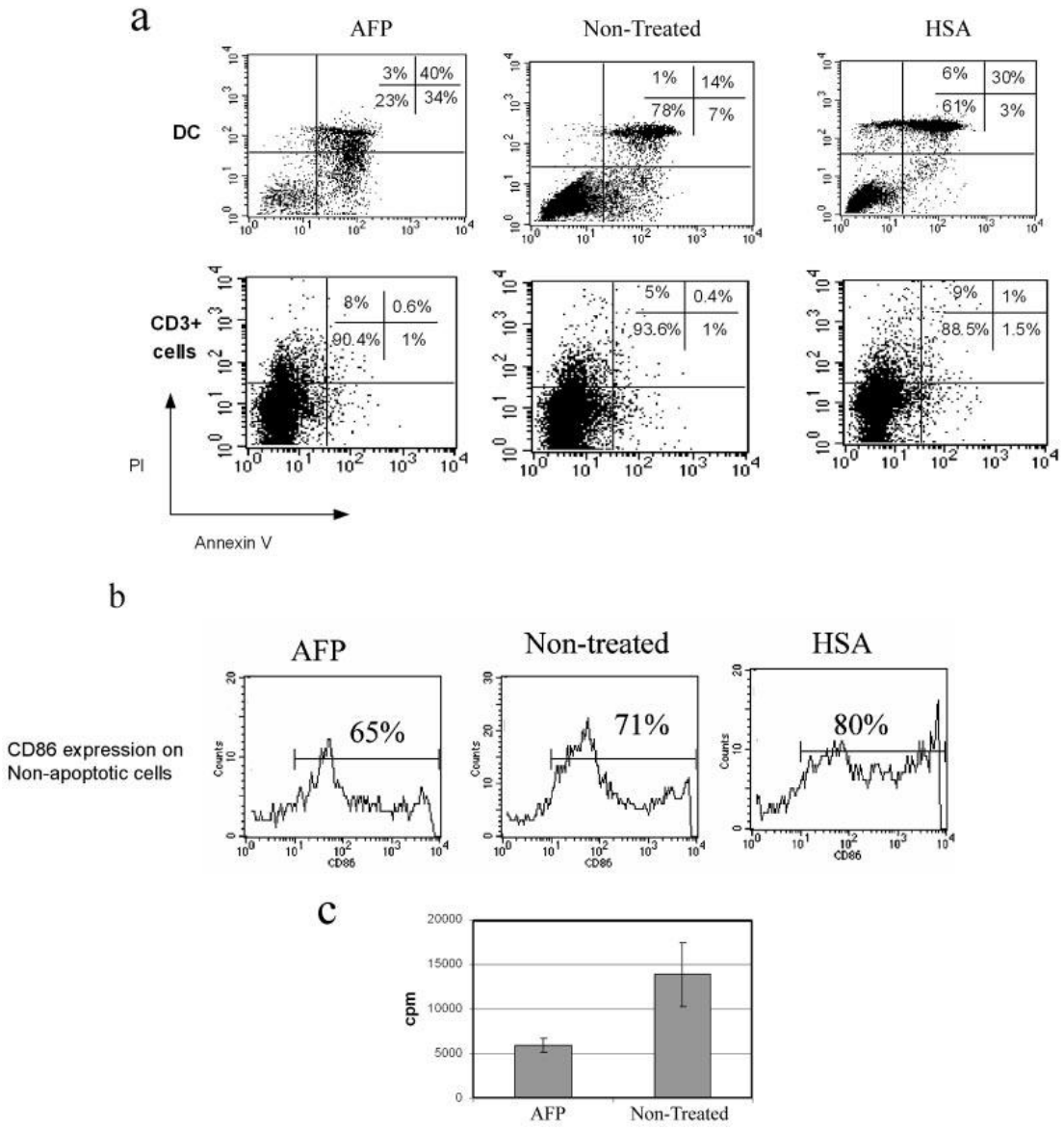



Figure 8-3: AFP induces apoptosis of monocyte-derived DCs but not in CD3+ cells. Monocytes were cultured in the presence of GM-CSF and IL-4, and AFP or HSA (25,000 ng/ml) was added on day 0 of culture. PBMC were also cultured in the presence of AFP or HSA (25,000 ng/ml) for 7 days. A two- or three-color staining with annexin V and PI with (c) or without (a) anti-CD86 Ab was conducted on day 7 of DC culture. A three-color staining was performed on PBMC culture (annexin V, PI, and anti-CD3 Ab). Numbers represent the percentage of cells in each quadrant. The cells in PBMC culture were gated on CD3+ cells, and the expression of annexin V-positive, PI-negative cells (apoptotic cells) was analyzed (a). The cells in DC culture were gated on nonapoptotic cells (annexin V-negative, PI-negative), and the expression levels of CD86 molecules were analyzed (b). The counted cells for the AFP-treated group were 340,000 cells, and for nontreated and HSA-treated group was 10,000 cells. AFP-treated or nontreated nonapoptotic DCs, i.e., annexin V-negative, PI-negative cells, were added (20,000 cells/well) to allogeneic T lymphocytes. After 5 days, T cell proliferation was assessed by the addition of thymidine for 18 h (c). The data shown are average counts per minute of three replicate determinations ± SD. The data are representative of three similar experiments.

*8.4.4 AFP-treated DCs produce lower levels of TNF-alpha and IL-12 in vitro*

DCs were stimulated with LPS for 3 h, and the production of intracellular TNF-alpha, IL-12, and IL-10 was analyzed. DCs that were differentiated in the presence of AFP had an impaired ability to produce IL-12 and TNF-alpha. The lowest level of AFP used in vitro with inhibitory effects on IL-12 production was 12,500 ng/ml. HSA treatment did not significantly reduce the ability of DCs to produce TNF-alpha and IL-12. When AFP was added on day 5 instead of day 0 of culture, a significant decrease in IL-12 production occurred. However, a greater decrease was seen when AFP was added on day 0 (Figure 8-4). The serum AFP levels in patients with HCC (n = 16) are shown in (Figure 8-5). The lowest amount of AFP used in vitro to induce CD86 down-regulation on DC was 2,500 ng/ml and for inhibition of IL-12 production was 12,500 ng/ml. There was an undetectable level of IL-10-producing cells in all three experimental groups (data not shown).

The level of IL-12 (p70) and IL-12 (p40) in supernatant of AFP-treated DCs (1 x 105/well) was measured using ELISA after 48-h stimulation with LPS. AFP-treated DCs produced lower levels of IL-12 (p40) and bioactive IL-12 (p70) than that of nontreated DCs. No IL-12 (p40) or IL-12 (p70) was detected in the supernatant of AFP-treated DCs. The levels of IL-12 (p40) and IL-12 (p70), produced by nontreated DCs, were 2200 ± 230 and 508 ± 127 pg/ml, respectively.

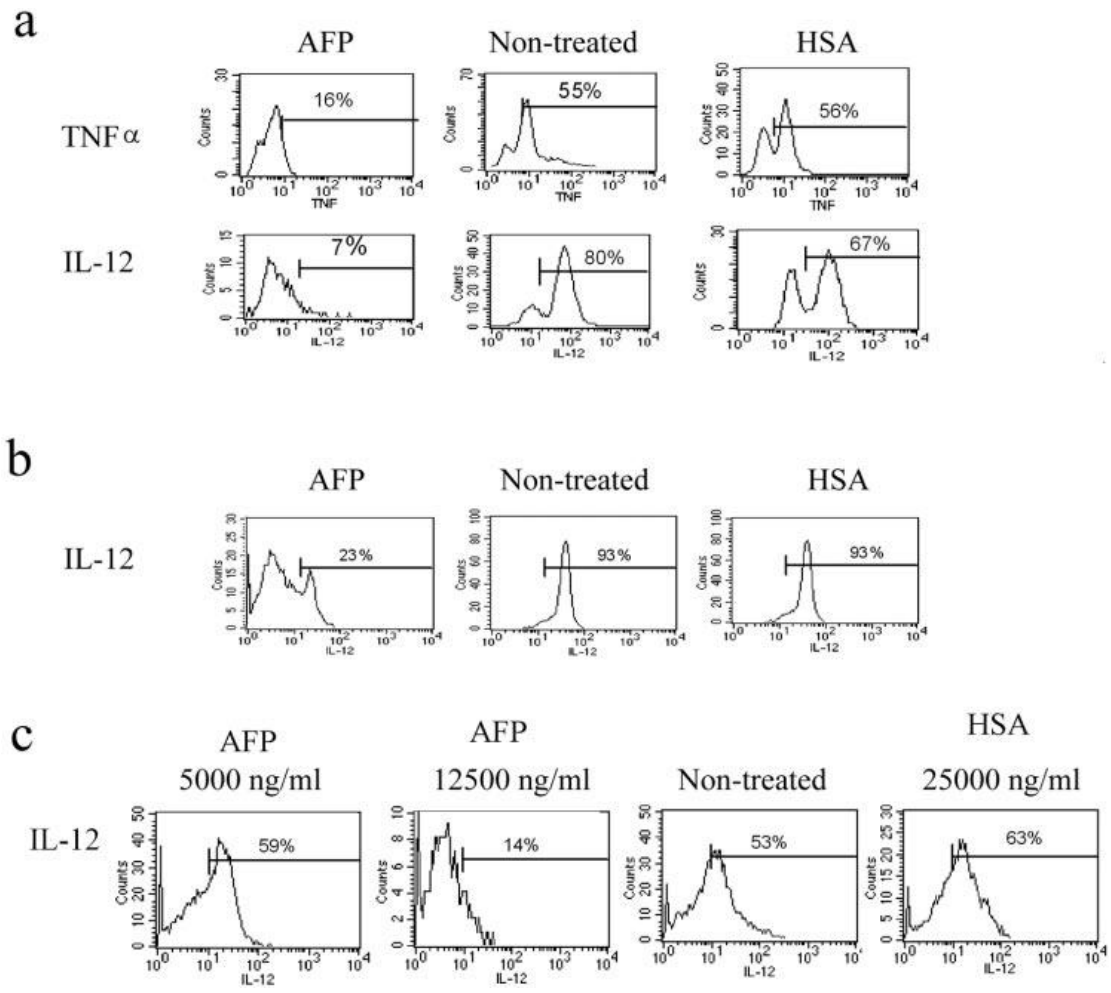



Figure 8-4: AFP treatment impairs IL-12 and TNF-$\alpha$ production from monocyte-derived DCs. Monocytes were cultured in the presence of GM-CSF, IL-4, and different concentrations of AFP (25,000 ng/ml in a and b; 12,500 or 5,000 ng/ml in c) or HSA (25,000 ng/ml) were added on day 0 (a and c) or 5 (b) of culture. Cells were harvested on day 7 of culture and stimulated in vitro with LPS. Cells were stained with PE-conjugated mAb for intracellular IL-12 or TNF-alpha, and analyzed by flow cytometry. A PE-labeled isotype-matched mAb was also used. Numbers represent the percentage of cells expressing the intracellular cytokines. The results were similar in three independent experiments.

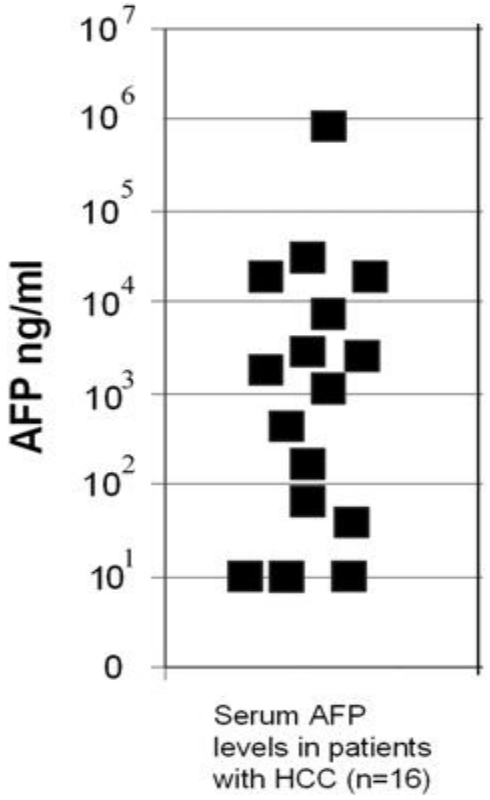



Figure 8-5: AFP induces functional impairment of DCs at concentrations close to those detected in the sera from patients with HCC. The level of serum AFP in patients with HCC was measured using MEIA. The dots represent results from patients. The lowest levels of AFP used in culture that had inhibitory effects (down-regulation of CD86 molecule and reduced IL-12 production) are shown.

*8.4.5 APCs of patients with HCC produce low levels of TNF-alpha ex vivo*

Flow cytometry was used to examine TNF-alpha secretion profiles of PBMCs in patients with HCC (n = 16) and healthy individuals (n = 7), in response to stimulation with LPS. The cells were gated on HLA-DR-positive or CD14-positive cells, and the expression of intracellular TNF-alpha was determined. In both healthy individuals and HCC patients, 70–98% of TNF-alpha-producing cells expressed high levels of HLA-DR molecule and 71–93% expressed CD14 molecule. The percentage of TNF-alpha-producing cells was lower in HCC patients with high levels of serum AFP than that of the healthy group (Figure 8-6). To examine whether the reduction of TNF-alpha production could be attributed to the loss of HLA-DR-positive cells, the percentage and mean fluorescence intensity of cells expressing HLA-DR molecules were analyzed. No significant loss of HLA-DR-positive cells was observed (Figure 8-6b&c) When the ability of HLA-DR-positive cells to produce IL-10 was analyzed, there was no significant difference between patients with HCC and healthy individuals (data not shown).

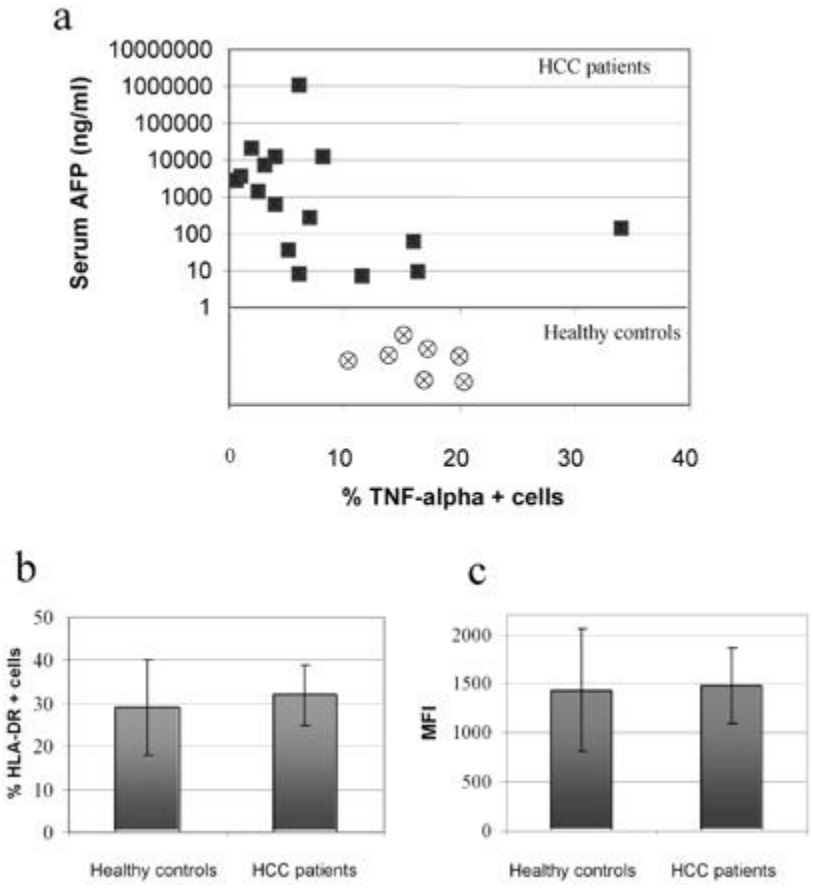



Figure 8-6: PBMCs of HCC patients with high levels of serum AFP produced lower TNF-alpha ex vivo. PBMCs were stimulated with LPS for 3 h, harvested, and stained with FITC-labeled anti-HLA-DR mAb for surface molecules and PE-labeled TNF-alpha for intracellular cytokine. Cells were analyzed using flow cytometry, and the percentages of HLA-DR+TNF-alpha+ cells were determined. The closed dots (■) represent results obtained from patients with HCC (n = 16), and open dots (⊗) represent results obtained from healthy controls (n = 7), with the y-axis representing percentage of HLA-DR+TNF-alpha+ cells and the x-axis representing the levels of serum AFP (a). The percentage (b) and mean fluorescent intensity (c) of cells expressing HLA-DR molecules in PBMC of patients with HCC and healthy individuals are shown.

## 8.5 Discussion

Inadequate presentation of tumour Ags by the host professional APCs is one potential mechanism by which tumors evade the host's immune response. Several clinical observations suggest that DC infiltration of solid tumors correlates with better prognosis [317]. Conversely, impairment of the DCs is associated with increased immunosuppression and tumour progression [288, 318] [319] [320] [321]. It has been shown that both circulating DCs ex vivo [311] and monocyte-derived DCs [285] are functionally impaired in patients with HCC. The impairment of DC function has also been reported in other tumors, a process mostly related to tumor-derived soluble factors, several of which have been identified [288, 318] [322]. A large variety of murine and human tumor cell lines produce as-yet-unidentified soluble factors causing increased apoptosis of DCs.

In this study, the effect of AFP on the functions and viability of DCs was analyzed *in vitro*. The data clearly show that DCs treated with AFP (as low as 2,500 ng/ml) become dysfunctional. These cells express low levels of costimulatory molecules and produce low levels of IL-12 and TNF-α, which correlates with their low allostimulatory capacity. Furthermore, the results suggest that the impairment of DCs is not only due to apoptosis. Further studies are required to determine the inhibitory effects of AFP on DC differentiation *in vivo*. We used a concentration of AFP (2,500–25,000 ng/ml) that is in a range similar to that detected in the sera of patients with HCC (ranging from 8 to 1,141,205 ng/ml).

Many patients with HCC have a serum AFP level of <12,500 ng/ml, and this may imply that the level of serum AFP in most patients is not high enough to exert immunoregulatory effects. However, one can hypothesize that higher concentrations of AFP can be found in the vicinity of the tumors where tissue-specific or infiltrating APCs may be exposed to high levels of AFP and therefore become dysfunctional. Our *ex vivo* results support this notion and show that the ability of monocytes, isolated from patients with elevated levels of serum AFP (>1,000 ng/ml), to produce TNF-alpha is impaired. This was not observed for monocytes isolated from PBMCs of patients with low serum AFP (<200 ng/ml). In this study, we decided to analyze monocytes rather than DCs as the majority of TNF-α-producing cells in peripheral blood are monocytes (10% of PBMCs) and DC subsets comprise only a very small percentage. In addition, to analyze DC subsets, we would have needed large amounts of blood from patients

who had already severe pancytopenia. Further studies are required to determine the functional ability of DC subsets in patients with HCC. The impairment of TNF-alpha production by monocytes may have important clinical implications and prognostic values. However, one cannot assume that the dysfunction of the monocytes (i.e., impairment of TNF-alpha production) is solely attributable to high levels of AFP. Other regulatory soluble factors released by the tumors may contribute to the impairment of APCs. For example, an elevation of TGF-beta1 has been reported in the serum of patients with HCC [323]. TGF-beta1 is known to have the ability to suppress APCs [324]. In addition to soluble factors, regulatory T cells can also suppress DC function [193] and several studies have shown that the frequency of the regulatory T cells is significantly increased in some patients with cancer [325-326], including HCC (our unpublished data). However, the mechanisms by which regulatory T cells suppress APCs in patients with HCC remains poorly understood.

In conclusion, this study demonstrates for the first time that AFP severely impairs the function of DCs and induces their apoptosis. In addition, the ability of APCs, of patients with HCC and high levels of serum AFP, to produce proinflammatory cytokines is reduced. This provides new insights into understanding the mechanisms underlying the suppression of immune recognition of tumour in patients with HCC.

# CHPATER 9: ANALYSIS OF CD4+ T-CELL RESPONSE TO A NOVEL ALPHA-FETOPROTEIN–DERIVED EPITOPE IN HEPATOCELLULAR CARCINOMA PATIENTS

## 9.1 Abstract

**Purpose**: AFP is a TAA in HCC and is a target for the development of a cancer vaccine. Four immunodominant AFP-derived HLA-A*0201-restricted peptides have been identified and the administration of these peptides with an adjuvant has stimulated AFP-specific CTL responses in HCC patients. However, no AFP-derived CD4 T-cell epitope has yet been reported and the status of AFP-specific CD4+ T-cell responses in HCC patients is not fully understood. The aim of this study was to analyze naturally occurring CD4+ T-cell responses toAFP. **Experimental Design**: We analyzed the ability of CD4+ T cells to recognize an HLA-DR-restricted AFP-derived epitope in 41 HCC patients and 24 non HCC control patients using intracellular cytokine assays for IFN-gamma. Results: Here, for the first time, we report the identification of an AFP-derived CD4+ T-cell epitope that is recognized by circulating lymphocytes from HCC patients in association with HLA-DR. The absence of detectable responses in healthy donors and patients with chronic liver disease suggests that AFP-specific CD4+ T-cells in the responder patients had been previously expanded in vivo in response to the tumor. The anti-AFP CD4+ T-cell response was only detected in HCC patients with normal or mildly elevated serum AFP levels who were in the early stage of disease.
**Conclusion**: Our data will be instrumental in the development of cancer vaccine using AFP-derived immunogens.

## 9.2 Introduction

Several studies in patients with malignant tumors have shown the existence of immune responses to antigens expressed by both malignant and normal cells. However, this naturally occurring antitumor immune response fails to induce regression of established tumors in some patients. It has been suggested that the rationale behind an epitope-based therapeutic vaccine is to enhance preexisting CD4+ and CD8+ T-cell responses thus controlling tumor growth. The identification and characterization of T-cell

epitopes on tumour-associated antigens is a crucial prerequisite in the design of epitope-based therapeutic vaccines [327].

AFP is a tumour-associated antigen in HCC and testicular carcinoma and is a target for immunotherapy [213, 272, 328-329]. In patients with HCC, the AFP gene is reactivated and the level of serum AFP is increased. The measurement of serum AFP plays an important role in the diagnosis of HCC and in monitoring the response to various treatment modalities [213, 272, 328-329]. One of the biological properties of AFP is its regulatory effects on immune response [245, 307, 309, 330]. We have shown that APCs of HCC patients with high levels of AFP, not those with normal or mildly elevated AFP levels, are dysfunctional and *in vitro* AFP impairs dendritic cell function and induce their apoptosis [245]. Naturally occurring immune response against AFP can be mounted in patients with HCC [234, 331-332], but there is limited information about its association with HCC staging and serum AFP levels. Furthermore, no AFP-derived CD4+ T-cell epitope has yet been identified [213-214, 236, 272, 328-329, 333] and the effects of AFP expression levels on antigen-specific T-cell response are unknown.

In this study, we identified the first AFP-derived T-cell epitope recognized by circulating CD4+ T cells from HCC patients in association with HLA-DR. The response was only detected in HCC patients with normal or mildly elevated AFP levels who were in the early stage of disease. However, the lack of response in patients with high serum AFP maybe due to a nonspecific CD4+ T-cell suppression and hence a reduction in IFN-gamma levels. Our data indicate that the 364-373 sequence plays a role in the induction of CD4+ T-cell responses to AFP, and the identification and detection of this response might be important in the development and application of AFP-based vaccines.

## 9.3 Materials and Methods

### 9.3.1 *Patients*

All patients were reviewed at the Liver Unit of Cromwell Hospital in London with the ethical committee's approval. Peripheral blood was collected from the patients with their informed consent and peripheral blood mononuclear cells (PBMC) were isolated. The clinical staging of patients with hepatocellular carcinoma was determined using Okuda classifications [334].

*9.3.2 HLA typing*

Following centrifugation, buffy coats were separated from the red cell pellet and the white cells frozen in freezing mix (FCS 20%, Terasaki Park Medium 70%, and DMSO 10%). DNA was extracted by using a modified salting-out procedure. HLA-A, HLA-B, and HLA-DR typing was done by molecular techniques in line with recent recommendations [335]. HLA analysis was done on DNA extracted from a ~5-mL sample of blood by a modified salting out technique [336]. The typing systems used defined all HLA-A, HLA-B, and HLA-DR specificities [337] with the exception of A203 and A210 (included in A2), A2403 (included in A24), B703 (included in B7), B2708 (included in B27), B3901 and B3902 (included in B39), B5101 and B5103 (included in B51), B64 and B65 (included in B14), and DR1403 and DR1404 (included in DR14), and additionally HLA-B82 was defined.

*9.3.3 Synthetic peptides*

Peptides corresponding to the sequence of AFP were purchased from Mimotopes Pty, Ltd. (Clayton, Victoria, Australia). ProPed MHC class II binding prediction server (http://www.imtech.res.in/raghava/propred) was used to predict HLA-DR13-binding region of AFP.

*9.3.4 Cells and cell lines*

RPMI 1640, MEM, penicillin and streptomycin, and 10% heat-inactivated FCS were purchased from Life Technologies (Grand Island, NY). Purified human cord blood AFP (purity, >95% SDS-PAGE) and purified bovine serum albumin (purity >97%) were obtained from Calbiochem (La Jolla, CA) and Sigma-Aldrich (St. Louis, MO), respectively. EBV-immortalized B cells were generated from PBMCs by culturing 5 x 106 cells with 1 mL of EBV B95/8 supernatant and 0.4 µg/mL cyclosporine A (Sigma-Aldrich).

*9.3.5 Generation of T-cell lines*

T-cell lines were generated as described previously [338]. In brief, PBMCs were resuspended at a concentration of 1.5 x 106/mL in MEM, 10% FCS (Life Technologies). PBMCs were stimulated with individual peptides (1 µmol/L) or peptide pools in 96-well plates. Recombinant interleukin-2 (rIL-2, 25 IU/mL) was added on day 3 of culture and cells were analyzed after a total of 10 to 12 days of culture.

*9.3.6 Intracellular cytokine staining*

AFP-specific T-cell lines were incubated for 5 hours at 37°C at 1 x 106 cells/mL in the cell culture medium with the AFP-derived peptides (1 µmol/L) and in the presence of Brefeldin A (10 µg/mL, Sigma-Aldrich). Cells were surface stained with Cychrome-conjugated anti-CD4 or anti-CD8 antibodies (BD PharMingen, Cowley, United Kingdom). The cells were then permeabilized and fixed using Cytofix/Cytoperm (BD PharMingen). Afterwards, the cells were stained for intracellular cytokines with FITC-conjugated anti IFN-γ. FITC-conjugated anti-IL-2 PE-conjugated anti-tumor necrosis factor-α, PE-conjugated anti-IL-5, or isotype controls (R&D Systems, Abingdon, United Kingdom); washed twice; and analyzed by

*9.3.7 Inhibition of T-cell responses with anti-MHC class I and II antibodies*

To determine the HLA molecules responsible for presenting AFP-derived epitopes to CD4 T cells, antibody-blocking assays were done. The murine monoclonal antibodies HL-39 and SPVL-3 which block peptide presentation to CD4+ T cells by HLA-DR and HLA-DQ, respectively, were added at 5 µg/mL to two separate wells containing T-cell lines followed by 1 µmol/L peptide 90 minutes later. Two controls were used, a positive control using only the peptide and a negative control that did not contain any peptide or antibody. All samples were tested in duplicates and peptide-specific intracellular IFN-gamma production was analyzed using flow cytometry. The murine monoclonal antibody w6/32, which blocks peptide presentation by HLA class I to CD8+ T cells, was used as described above.

*9.3.8 CD4+ T-cell proliferation assay*

AFP364-373-specific T-cell lines were labeled with carboxyfluorescein diacetate succinimidyl ester (1 µmol/L) and the cells were washed twice in PBS and resuspended in medium supplemented with 10% human serum (Sigma-Aldrich). Carboxyfluorescein diacetate succinimidyl ester–labeled cells (3 x 106 cells/mL) were incubated in 96-well plates (Nunc, Naperville, IL) in the presence of 1 µmol/L AFP364-373 peptide or an irrelevant peptide. Phytohemagglutinin (1 µg/mL) and rIL-2 (20 units/mL) were used as positive controls. After 5 days of in vitro incubation, cells were stained with PE-Cy5-labeled anti-CD4- and peptide-specific CD4+ T-cell proliferation on gated CD4+ T cells was analyzed using flow cytometry.

*9.3.9 Alpha-Fetoprotein measurement*

Levels of serum AFP were measured using microparticle enzyme immunoassay kit obtained from Abbott Laboratories (Abbott Park, IL) and done according to the manufacturer's instruction. In brief, anti-AFP microparticles were incubated with the blood specimen, and an aliquot of the reaction mixture was transferred to the matrix cell. The matrix cell was washed, removing unbound materials, and the anti-AFP conjugate was dispensed onto the matrix cell. The substrate was added to the matrix cell and the fluorescent product is measured by the microparticle enzyme immunoassay optical assembly.

*9.3.10 Statistical analysis*

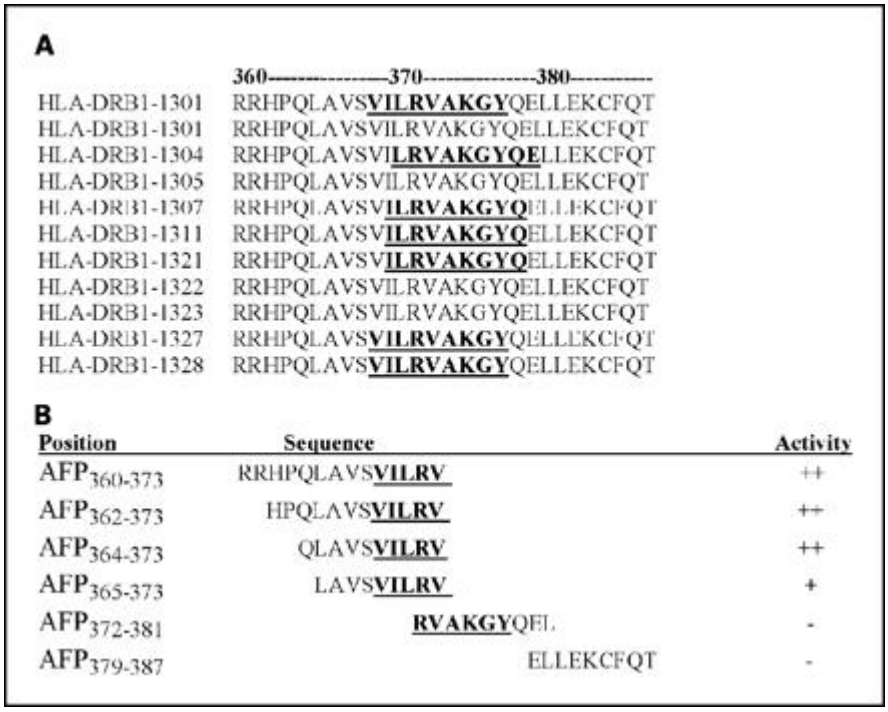
The Mann-Whitney U test was used to compare the levels of serum AFP in hepatocellular carcinoma patients with or without CD4+ T-cell response to the identified epitope (responder and nonresponder). The $\chi 2$ was used to evaluate the significant difference in the prevalence of Okuda grade 2 and 3 in responders and nonresponders. The Spearman rank coefficient was used to evaluate correlations between serum AFP levels and the percentages of CD4+ IFN-gamma-producing cells. Statistical significance was defined at $P \leq 0.05$ for all analyses done.

## 9.4 Results

9.4.1 *Isolation of alpha-fetoprotein-specific CD4+ T cells from circulating lymphocytes of a hepatocellular carcinoma patient*

The region of 369 to 379 within AFP sequence was predicted to bind HLA-DR13 molecules using ProPed MHC class II binding prediction server. Nine to 14 amino-acid-long peptides were synthesized to cover the region (Figure 9-1). PBMCs were isolated from an HLA-DR13+ hepatocellular carcinoma patient (HCC041) and stimulated with the AFP-derived peptides. Two weeks later, cultures were tested using a pool containing AFP-derived peptides (AFP364-373, AFP372-381, and AFP379-387) or individual peptides. The presence of specific CD4+ T cells was analyzed by intracellular staining with IFN-gamma and CD4-specific monoclonal antibodies (Figure 9-2). The peptide pool and AFP364-373 peptide stimulated significant levels of IFN-gamma-producing CD4+ T cells as compared with samples with no peptide or

irrelevant peptides (AFP379-387 and AFP372-381) (Figure 9-2). AFP364-373-specific CD4+ T cells recognized AFP365-373 (9 amino acid long), AFP 362-373 (12 amino acid long), and AFP 360-373 (14 amino acid long) peptides and produced peptide-specific IFN-gamma but did not recognize irrelevant peptides (AFP379-387 and AFP372-381; Fig. 1B). T-cell lines expanded in the presence of shorter (9 amino acid long) or longer peptides (12 and 14 amino acid long) recognized AFP364-373 peptide (data not shown). The frequency of IFN-gamma-producing cells among CD4+ T cells was lower in cells stimulated with AFP365-373 peptide, but there was no difference in the ability of other peptides (i.e., AFP364-373, AFP 362-373, and AFP 360-373)(Figure 9-1)



**A**

| | 360----------370----------380---------- |
|---|---|
| HLA-DRB1-1301 | RRHPQLAVS**VILRVAKGY**QELLEKCFQT |
| HLA-DRB1-1301 | RRHPQLAVSVILRVAKGYQELLEKCFQT |
| HLA-DRB1-1304 | RRHPQLAVSVI**LRVAKGYQE**LLEKCFQT |
| HLA-DRB1-1305 | RRHPQLAVSVILRVAKGYQELLEKCFQT |
| HLA-DRB1-1307 | RRHPQLAVSV**ILRVAKGYQ**ELLEKCFQT |
| HLA-DRB1-1311 | RRHPQLAVSV**ILRVAKGYQ**ELLEKCFQT |
| HLA-DRB1-1321 | RRHPQLAVSV**ILRVAKGYQ**ELLEKCFQT |
| HLA-DRB1-1322 | RRHPQLAVSVILRVAKGYQELLEKCFQT |
| HLA-DRB1-1323 | RRHPQLAVSVILRVAKGYQELLEKCFQT |
| HLA-DRB1-1327 | RRHPQLAVS**VILRVAKGY**QELLEKCFQT |
| HLA-DRB1-1328 | RRHPQLAVS**VILRVAKGY**QELLEKCFQT |

**B**

| Position | Sequence | Activity |
|---|---|---|
| $AFP_{360-373}$ | RRHPQLAVS**VILRV** | ++ |
| $AFP_{362-373}$ | HPQLAVS**VILRV** | ++ |
| $AFP_{364-373}$ | QLAVS**VILRV** | ++ |
| $AFP_{365-373}$ | LAVS**VILRV** | + |
| $AFP_{372-381}$ | **RVAKGY**QEL | - |
| $AFP_{379-387}$ | ELLEKCFQT | - |

Figure 9-1: A, amino acid sequence of 360-387 region of AFP and predicted epitope (bold and underlined) within this region for different HLA-DR13 alleles (ProPed class II binding prediction server was used, http://www.imtech.res.in/raghava/propred). B, positions and sequences of AFP-derived peptides analyzed in this study for their activity. No response (–), peptide-specific response (+), and potent peptide-specific response (++).

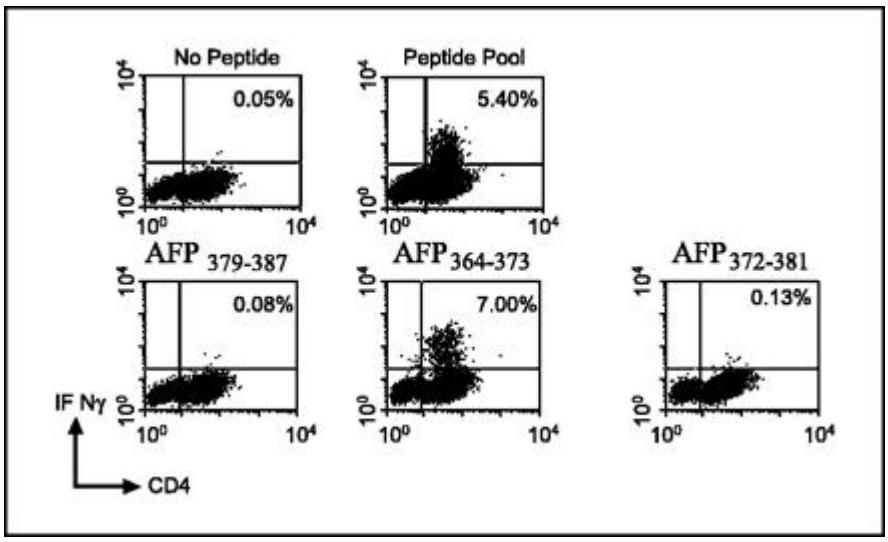



Figure 9-2: Detection of AFP364-373-specific CD4+ T cells among circulating T lymphocytes from patient HCC041 after stimulation with a pool containing AFP-derived peptides. The presence of peptide-specific CD4+ T cells in the culture was assessed by staining with anti-IFN-gamma monoclonal antibody after incubation in the absence of added peptide or after stimulation with a pool containing AFP-derived peptides or individual peptides. An example of two individual peptides with no activity. Numbers in the top right quadrants are the percentage of cytokine-producing cells among CD4+ T cells.

The AFP364-373-specific CD4+ T cells were expanded in vitro to further define the epitope. The obtained CD4+ T cells recognized the AFP364-373 peptide in a dose-dependent manner and produced IFN-gamma but did not recognize an irrelevant peptide (Figure 9-3a). Furthermore, AFP364-373-specific CD4+ T cells recognized AFP364-373 peptide and produced peptide-specific tumor necrosis factor-α and IL-2 (Th1-type cytokine) and expanded in vitro but did not recognize an irrelevant peptide (Figure 9-3b&c). The AFP364-373-specific CD4+ T cells did not produce IL-5 (Figure 9-3b).

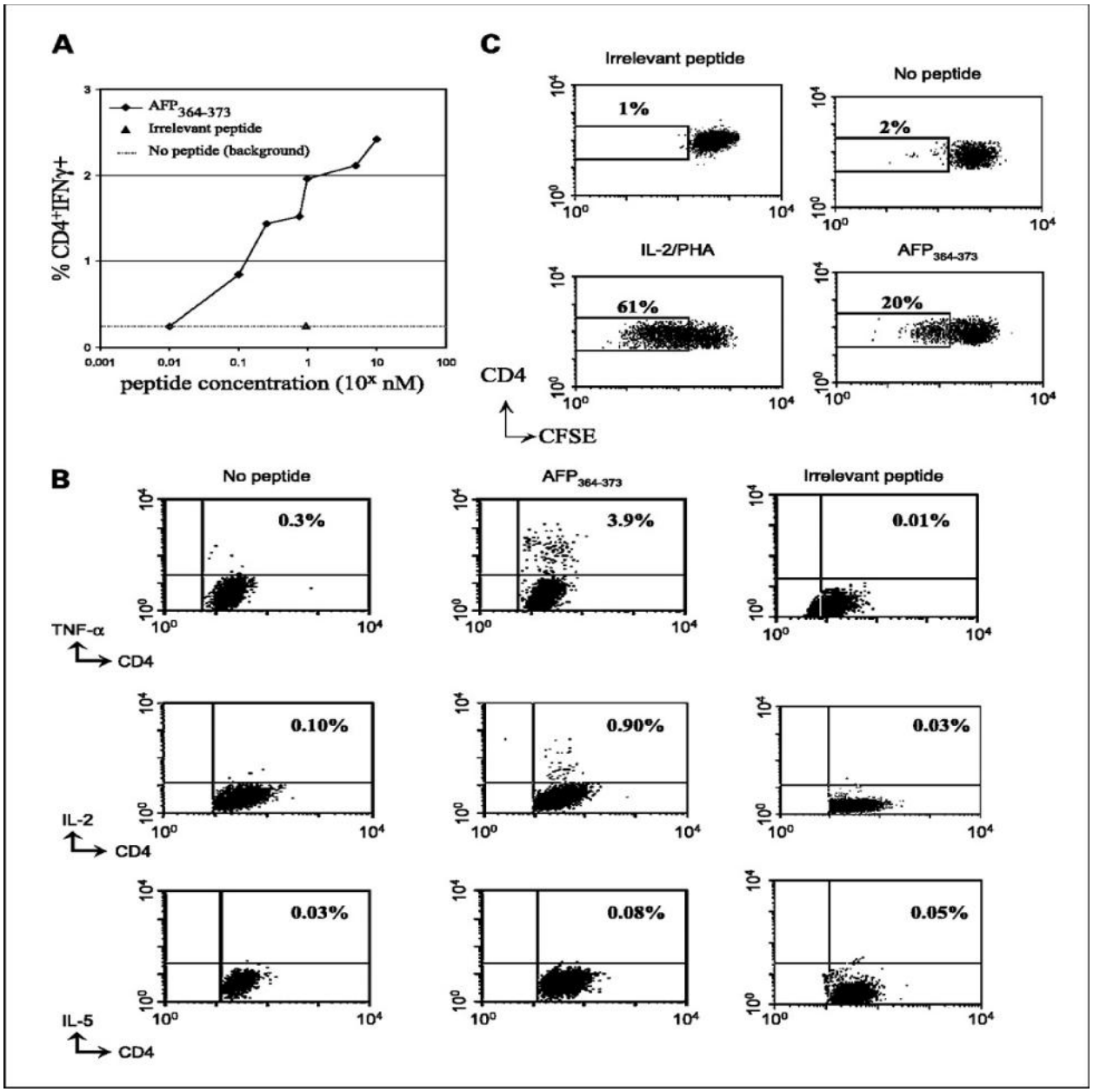



Figure 9-3: A, AFP364-373 peptide activity is shown in peptide titration experiment. AFP364-373-specific CD4+ T-cell line (isolated from PBMCs of patient HCC041) was stimulated with different concentration of AFP364-373 peptide or an irrelevant peptide and the production of peptide-specific IFN-gamma by CD4+ T cells was analyzed using intracellular cytokine assay. B, production of tumor necrosis factor-alpha (TNF-alpha), IL-2 (Th1-type cytokines), and IL-5 (Th2-type cytokine) by AFP364-373-specific CD4+ T cells was assessed by staining with anti-tumor necrosis factor-alpha, anti-IL-2 or anti-IL-5 monoclonal antibodies after incubation in the absence of peptide or after stimulation with AFP364-373 or an irrelevant peptide. Numbers in the top right quadrants are the percentage of cytokine-producing cells among CD4+ T cells. C, carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled T-cell lines were incubated in vitro with AFP364-373 peptide, an irrelevant peptide or stimulated with Phytohemagglutinin and rIL-2 as positive control. After 5 days of incubation, cells were stained with PE-Cy5-labeled- anti-CD4 antibody and the peptide-specific CD4+ T-cell proliferation was analyzed using flow cytometry. The numbers indicated in each quadrant are the percentage of CD4+ CFSElow T cells. Representative of two different experiments.

*9.4.2 AFP364-373 is recognized by peptide-specific CD4+ T cells from patient HCC041 in the context of HLA-DR13*

To determine the HLA molecules responsible for presenting the identified epitope to CD4+ T cells, an antibody-blocking assay was done. The T-cell line was incubated with the relevant peptides in the presence of anti-HLA-DR, anti-HLA-DQ, or anti-HLA class I antibodies. T-cell reactivity was measured using an intracellular cytokine assay for IFN-gamma. The addition of anti-HLA-DR antibody blocked recognition of peptide AFP364-373 by a peptide-specific CD4+ T cells. This response was not blocked by antibodies to HLA-DQ or HLA class I. This result indicates that CD4+ T cells recognize AFP364-373 in a HLA-DR-restricted manner (Figure 9-4a).

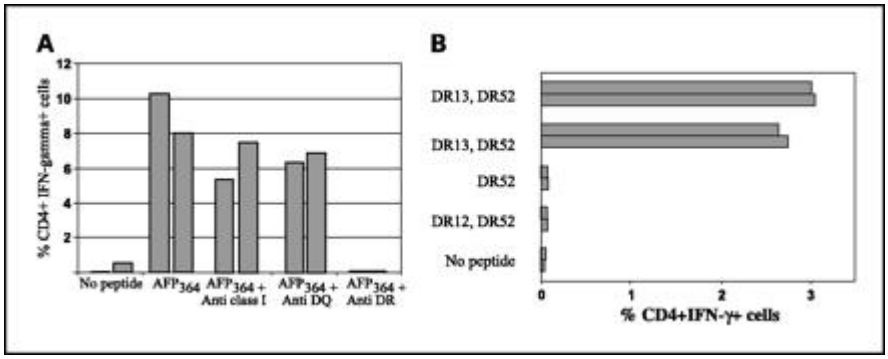



Figure 9-4: HLA class II restriction of AFP364-373-specific CD4+ T cells. A, peptide recognition was assessed either in the absence or in the presence of anti-HLA-DR, DQ, or anti-MHC class I antibodies. B, AFP364-373-specific CD4+ T-cell line generated from PBMCs from patient HCC041 (HLA-DR12, HLA-DR13, and HLA-DR52) was incubated with partially histocompatible EBV-B cells pulsed with the relevant or control peptide. HLA-DR class II alleles of the target cells matched with patient HCC041 are indicated. Percentage of peptide-specific IFN-gamma-producing cells among CD4+ T cells in duplicate. Representative of two individual experiments.

To establish the presenting allele(s), we first determined the HLA-DR alleles of the patient. Patient HCC041 expressed HLA-DR12, HLA-DR13, and HLA-DR52. Then, we assessed presentation by partially matched APCs from other individuals. Partially histocompatible EBV-B cells pulsed with peptide AFP364-373 or a control peptide were used to find matching HLA-DR class II alleles. In the case of two EBV-B cells expressing HLA-DR13 and HLA-DR52, we obtained efficient presentation of the peptide to the CD4+ T cells from patient HCC041, whereas in the case of two EBV-B cells expressing HLA-DR12 and/or HLA-DR52 but not HLA-DR13, no presentation was observed. This suggest that peptide AFP364-373 was recognized by an HLA-DR13-restricted CD4+ T cells in the case of patient HCC041 (Figure 9-4b). However, we cannot rule out that other DRB gene products can also present the identified epitope

to specific CD4+ T cells. This was confirmed when some HLA-DR13-negative hepatocellular carcinoma patients (HCC157, HCC159, HCC028, and HCC018) exhibited CD4 T-cell responses to the identified epitope (Table 9-1).

| Patient ID | %CD4+ IFNγ+ | AFP (ng/mL)* | Gender | Age (y) | Aetiology | HLA-DR13 |
|---|---|---|---|---|---|---|
| HCC010 | 0 | 180 | M | 62 | HCV/HBV | + |
| HCC014 | 0 | 200 | M | 60 | HBV/HCV | ND |
| HCC017 | 0 | 170 | M | 63 | HCV | – |
| HCC030 | 0 | 13,077 | M | 63 | HCV/ALD | + |
| HCC032 | 0 | 23,463 | M | 35 | HBV/HCV | + |
| HCC034 | 0 | 41,930 | M | 59 | ALD | – |
| HCC035 | 0 | 3,677 | M | U | HBV | + |
| HCC039 | 0 | 8 | M | 32 | Cryptogenic | + |
| HCC040 | 0 | 813,500 | F | 71 | Cryptogenic | + |
| HCC050 | 0 | 5 | M | 61 | ALD | – |
| HCC062 | 0 | 12,432 | F | 67 | HBV | – |
| HCC081 | 0 | 10 | F | 46 | HCV | + |
| HCC088 | 0 | 157 | M | 53 | HCV | ND |
| HCC104 | 0 | 2,997 | M | 78 | HCV | – |
| HCC133 | 0 | 46,160 | M | 75 | HCV | + |
| HCC160 | 0 | 8 | M | 83 | ALD | – |
| HCC161 | 0 | 105 | M | 62 | HBV | – |
| HCC100 | 0 | 3,791 | M | 60 | HCV | + |
| HCC056 | 0 | 1 | M | U | HCV/HBV | – |
| HCC048 | 0 | 1,234 | M | 61 | HCV/ALD | – |
| HCC053 | 0 | 1,501 | M | 62 | HBV/HCV/ALD | – |
| HCC054 | 0 | 188 | M | 67 | ALD | ND |
| HCC116 | 0 | 9 | F | 69 | HCV | – |
| HCC130 | 0 | 44,450 | M | 49 | Cryptogenic | + |
| HCC140 | 0 | 14 | M | 73 | HBV | – |
| HCC016 | 0 | 36 | M | 53 | HCV | – |
| HCC087 | 0 | 14 | M | 54 | HCV | + |
| HCC095 | 0 | 108 | M | 58 | HCV | – |
| HCC163 | 0 | 9 | M | 85 | HCV | + |
| HCC172 | 0 | 17,350 | F | 61 | HCV | + |
| HCC013 | 2.2 | 8 | M | 62 | HCV | + |
| HCC018 | 1 | 32 | M | 66 | HCV | – |
| HCC019 | 15 | 312 | M | 66 | HCV | + |
| HCC028 | 2.1 | 60 | M | 55 | Cryptogenic | – |
| HCC036 | 2.9 | 14 | F | 58 | HBV/HCV | + |
| HCC111 | 9.3 | 969 | M | 47 | HCV | + |
| HCC114 | 1.9 | 4 | M | 58 | HBV | + |
| HCC138 | 2.9 | 10 | M | 35 | HBV | + |
| HCC157 | 11.5 | 25 | M | 63 | HCV | – |
| HCC041 | 7 | 70 | M | 79 | HCV | + |
| HCC159 | 0.2 | 4 | M | 60 | HBV | – |

Table 9-1: List of patients with hepatocellular cancer tested in this study for the presence of circulating AFP364-373-specific CD4+ T-cell response

-NOTE: The ability of short-term T-cell lines generated from PBMCs of patients with hepatocellular cancer to recognize $AFP_{364-373}$ peptide was analyzed based on their ability to produce peptide-specific IFN-γ using an intracellular cytokine assay.

Abbreviations: HCV, hepatitis C infection; HBV, hepatitis B infection; ALD, alcohol liver disease; U, unknown. ND, not determined. * Serum AFP level.

*9.4.3 Recognition of purified AFP by AFP365-373-specific CD4 T cells*

PBMCs isolated from patient HCC041 were stimulated with purified AFP (13 µg/mL) and rIL-2. After 2 weeks, the resulting cells were restimulated with AFP364-373 or an irrelevant peptide (AFP379-387) or cultured in medium only (no peptide) and the presence of AFP-reactive CD4+ T cells were analyzed using intracellular cytokine assay for IFN-gamma. The AFP-specific CD4+ T cells recognized AFP364-373 peptide but not the irrelevant peptide and produced IFN-gamma (Figure 9-5).

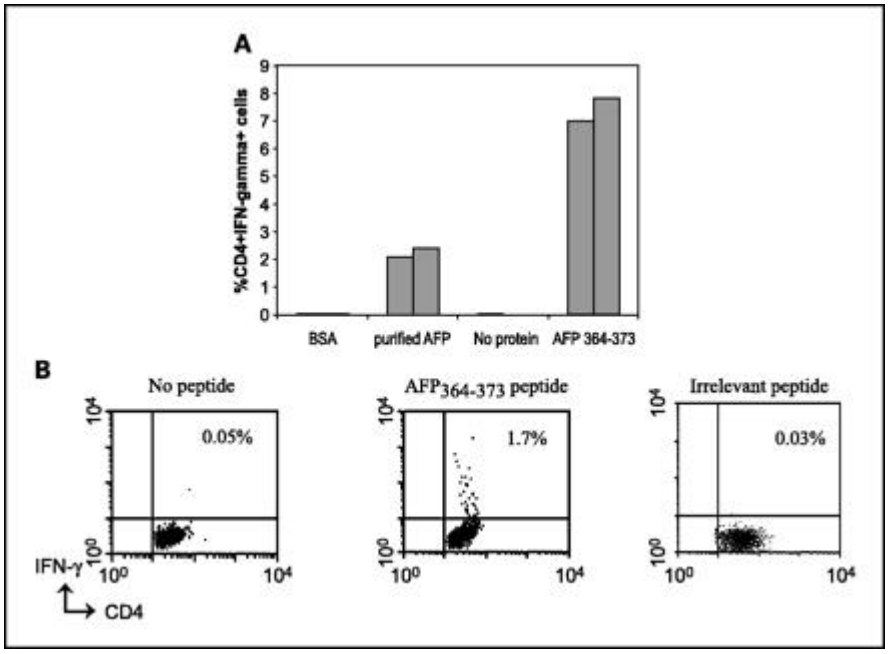



Figure 9-5: A, the recognition of purified AFP by AFP364-373-specific CD4+ T cells. PBMCs were stimulated with AFP364-373 peptide and 2 weeks later, the culture was restimulated with purified AFP, control protein (bovine serum albumin), or AFP364-373 peptide. The production of antigen-specific IFN-gamma by CD4+ T cells was assessed using intracellular cytokine assay. Percentage of antigen-specific CD4+ IFN-gamma+ cells. Representative of two individual experiments. B, detection of AFP-specific CD4+ T cells among circulating lymphocytes isolated from patient HCC041 after stimulation with purified AFP antigen. The presence of AFP364-373-specific CD4+ T cells was assessed using intracellular cytokine assay for IFN-gamma after incubation of the culture in the absence of added peptide, irrelevant peptide, or after stimulation with AFP364-373 peptide, as indicated. Numbers in the top right quadrants are the percentage of cytokine-producing cells among CD4+ T cells.

To establish the physiologic relevance of the identified epitope, AFP364-373-specific CD4+ T-cell line was incubated with purified AFP (13 µg/mL) and the production of antigen-specific IFN-gamma production was assessed using intracellular cytokine assay. AFP-specific CD4+ T cells recognized purified AFP but not control protein (bovine serum albumin) and produced IFN-gamma (Figure 9-5b). To stimulate AFP364-373-specific CD4+ T-cell line, we used purified AFP at concentrations ranging from 1 to 20 µg/mL. The T-cell lines restimulated with purified AFP at concentration <8 µg/mL did not produce detectable IFN-gamma and 13 µg/mL was the optimal concentration in our system. The activation of PBMCs or control T-cell lines with purified AFP (13 µg/mL) did not induce nonspecific IFN-gamma production.

*9.4.4 Assessment of AFP364-37-specific CD4+ T cells in HCC patients and control groups*

To further analyze the spontaneous immunogenicity of the identified epitope, we assessed the responsiveness to AFP364-373 using PBMCs from 40 additional hepatocellular carcinoma patients as well as seven healthy donors, 13 patients with liver cirrhosis, and four patients with non-hepatocellular carcinoma secondary liver cancer (Tables 9-1&2). Short-term T-cell lines were tested against AFP364-373 peptide and two irrelevant peptides (AFP372-381 and AFP379-387). AFP364-373-specific CD4+ T cells were detected in 11 of 41 patients and seven responder patients expressed HLA-DR13 molecules (Table 9-1). AFP364-373-specific CD4+ T cells recognized AFP364-373 peptide but did not recognize AFP372-381 and AFP379-387 peptides (data not shown). No CD4+ T-cell response to the tested AFP-derived peptides (AFP364-373, AFP372-381, and AFP379-387) was detected in the control groups. AFP364-37-specific IFN-gamma production by CD4+ T cells isolated from six hepatocellular carcinoma patients is shown (Figure 9-6). The results obtained from antibody blocking assay show that CD4+ T cells isolated from the responders recognize AFP364-373 in association with HLA-DR molecules (data not shown).

| Patient ID | AFP (ng/mL)* | Gender | Age (y) | Aetiology |
|---|---|---|---|---|
| LC021 | ND | M | U | HBV/ALD |
| LC043 | 11 | M | 34 | HBV/HCV/ALD |
| LC057 | 10 | M | 54 | ALD |
| LC089 | 4 | M | 54 | ALD |
| LC102 | ND | M | 49 | HBV/ALD |
| LC139 | ND | M | 61 | ALD |
| LC052 | ND | F | U | Cryptogenic LC |
| LC092 | 10 | F | 59 | Cryptogenic LC |
| LC113 | 10 | M | 51 | Cryptogenic LC |
| LC086 | 5 | F | 75 | HCV |
| LC091 | 9 | F | 58 | HCV |
| LC101 | 5 | M | 49 | HBV/ALD |
| LC131 | 105 | M | 77 | ALD |
| NC065 | ND | M | 35 | Normal control |
| NC066 | ND | M | 38 | Normal control |
| NC069 | ND | M | 42 | Normal control |
| NC078 | ND | M | 42 | Normal control |
| NC083 | ND | M | 35 | Normal control |
| NC090 | ND | F | 23 | Normal control |
| NC156 | ND | M | 35 | Normal control |
| TC044 | ND | F | U | Colonic cancer |
| TC045 | ND | F | 40 | Breast cancer |
| TC046 | ND | F | U | Ovarian cancer |
| TC055 | 44 | M | 71 | Adenocarcinoma |

Table 9-2: List of control groups (liver cirrhosis patients, healthy controls, and patients with secondary liver non-hepatocellular cancer tumors) analyzed in this study

-NOTE: Short-term T-cell lines were generated from PBMCs and the presence of $AFP_{364-373}$-specific $CD4^+$ T cells were analyzed using intracellular cytokine assay for IFN-gamma.

Abbreviations: LC, liver cirrhosis; ND, not determined; U, Unknown; HBV, hepatitis B infection; ALD, alcohol liver disease; NC, normal control; TC, tumor control; HCV, hepatitis C infection.

-* Serum AFP level.

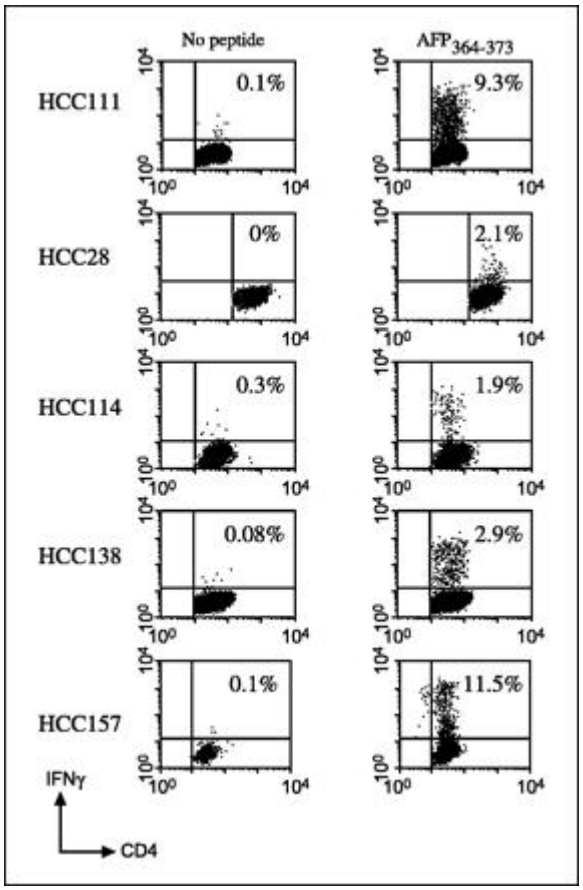



Figure 9-6: Detection of AFP364-373-specific CD4+ T cells in circulating lymphocytes of different patients with hepatocellular cancer. PBMCs were stimulated with AFP364-373 peptide and the presence of AFP364-373-specific CD4+ T cells was assessed 2 weeks later after incubation of the culture with AFP364-373 peptide. Numbers in the top right quadrants are the percentage of IFN-gamma-producing cells among CD4+ T cells.

*9.4.5 Association between the presence of a detectable alpha-fetoprotein-specific CD4+ T-cell response and low serum AFP level*

In this study, we have shown that the AFP364-373-specific CD4+ T-cell response was only detected in hepatocellular carcinoma patients with normal or mildly elevated serum AFP. In addition, median serum AFP levels were 7.2-fold higher in nonresponders than that in responders (P = 0.0044, Mann-Whitney U test)(Figure 9-7a). There is a significant difference in the prevalence of Okuda stage II and III in responders and nonresponders ($\chi^2$, P = 0.002; 95% confidence intervals)(Figure 9-7b). This indicates that patients in stage II or III are significantly less likely to have CD4+ T-cell response to the identified epitope. However, it was unknown whether the lack of AFP364-373-specific CD4+ T-cell response in hepatocellular carcinoma patients with high levels of serum AFP was due to a general immunosuppression of CD4+ T cells or anergy of AFP-specific CD4+ T cells. To address this question, eight hepatocellular carcinoma patients with different levels of serum AFP were chosen and PBMCs isolated from these patients were stimulated with phorbol 12-myristate 13-acetate and ionomycin ex vivo and the frequency of CD4+ T cells producing nonspecific IFN-gamma was analyzed. High levels of serum AFP and IFN-gamma production by CD4+ T cells are inversely correlated (p = –0.738; P = 0.037, Spearman rank-order test). As shown in (Figure 9-7c), HCC patients with high levels of serum AFP produced significantly lower levels of IFN-gamma, suggesting that there is a general CD4+ T-cell dysfunction in terms of IFN-gamma production in this group of patients. CD4+ T cells isolated from this group of patients did not proliferated in the presence of rIL-2 (data not shown), suggesting that their CD4+ T cells are dysfunctional. Although, it has been shown that AFP has immunoregulatory properties [245, 307], this does not prove that the impairment of CD4+ T cells in HCC patients with high levels of AFP is solely induced by AFP. Other regulatory factors released by the tumor (such as TGF-beta) may be involved. We are currently studying the role of AFP, transforming growth factor-ß and tumor-specific regulatory T cells in the induction of CD4+ T-cell dysfunction.

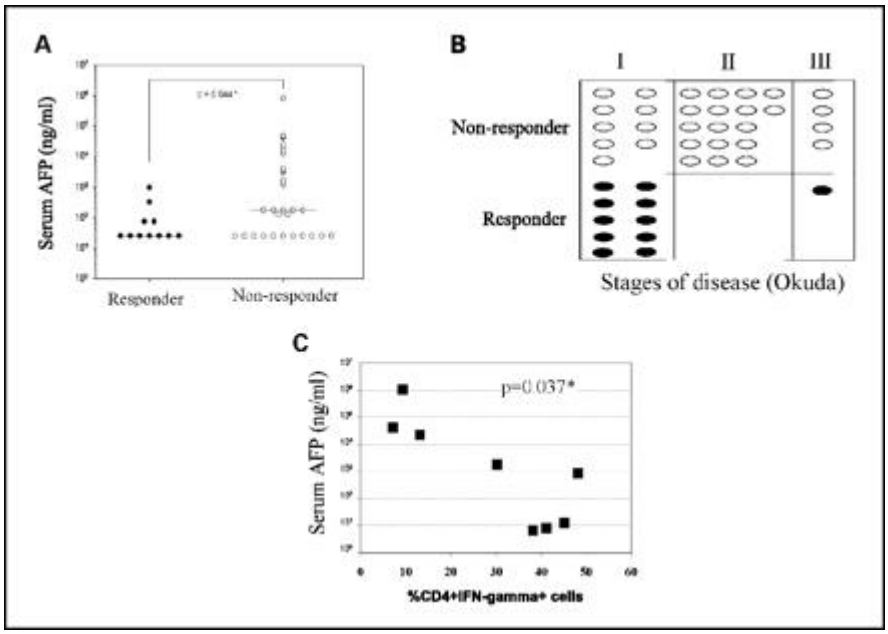



Figure 9-7: Serum AFP levels, disease stage, and the presence of AFP-specific CD4 T-cell response in hepatocellular cancer patients. A, AFP364-373-specific CD4+ T cells were only detected in hepatocellular cancer patients with normal or mildly elevated serum AFP. Levels of serum AFP in patients with and without AFP364-373-specific CD4+ T-cell response. Median of serum AFP levels for the responders and nonresponders (P = 0.044). Closed dots are responders and open dots are nonresponders. B, the majority of hepatocellular cancer patients with AFP364-373-specific CD4+ T-cell response were in their early disease stage. Disease stage was determined using Okuda classification (stages I, II, and III). Closed dots are responders (n = 11) and open dots are nonresponders (n = 30). $\chi^2$ was used to evaluate the significant difference in the prevalence of grade 2 and 3 in responders and nonresponders (P = 0.002; difference in proportions, –0.6; 95% confidence interval, –0.26 to –0.76). C, CD4+ T cells isolated from patients with hepatocellular cancer and high levels of serum AFP produced significantly lower IFN-gamma. PBMCs were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 5 hours, harvested, and stained with Cychrome-conjugated anti-CD4 monoclonal antibody for surface molecules and FITC-conjugated IFN-gamma for intracellular cytokine. Cells were analyzed using flow cytometry. Percentages of CD4+ T cells that express IFN-gamma were analyzed. Results obtained from HCC patients (■, n = 8), with the y-axis representing percentage of IFN-gamma-producing cells among CD4+ T cells and the x-axis representing the levels of serum AFP. The correlation coefficients were calculated by using the Spearman rank-order ($\rho$ = –0.738; P = 0.037 for serum AFP levels compared with percentage of CD4+ IFN-gamma-producing cells) *, statistically significant.

## 9.5 Discussion

The AFP-derived peptide described here, QLAVSVILRV (AFP364-373), was recognized by CD4+ T cells isolated from 11 different patients with hepatocellular carcinoma. The CD4+ T-cell lines also recognized AFP360-373, AFP 362-373, and AFP365-373. We initially concentrated our analysis on patient HCC041, who expressed HLA-DR13, who had an elevated serum AFP. After a single *in vitro* stimulation, we assessed the presence of specific CD4+ T cells among circulating lymphocytes from this patient using 9 to 14 amino-acid-long peptides from the 362 to 387 region. As revealed by the analysis of AFP-derived peptides, the peptide corresponding to the epitope recognized by AFP-specific CD4+ T cells is located between AFP residues 64 and 73, a region that partially overlaps the predicted epitope for HLA-DR13 molecules. In support of the physiologic relevance of the identified epitope, we showed that APCs cultured with purified AFP can stimulate IFN-gamma production by AFP364-373-specific CD4+ T cells. This indicates that processing and presentation of tumor-derived AFP antigen by APCs, through the exogenous pathway, was the mechanism through which spontaneous responses to AFP occurred *in vivo*. In addition, we detected AFP364-373-specific CD4+ T cells from PBMCs isolated from patient HCC041 that had been cultured in the presence of purified AFP. This shows that APCs can take up purified AFP, process, and present the epitope to specific CD4+ T cells. AFP364-373 epitope also seemed recognized by CD4+ T cells from 11 of 41 hepatocellular carcinoma patients analyzed. However, not all the responders expressed HLA-DR13 molecules suggesting that other HLA-DR molecules may be able to present the identified epitope. This is not surprising as many CD4+ T-cell epitopes can be presented by different HLA class II molecules. Six of 11 hepatocellular carcinoma patients with the circulating AFP-specific CD4+ T cells had a mildly elevated serum AFP and five patients showed a normal serum AFP level (normal serum AFP level is between 10 and 20 ng/mL). Various frequencies of AFP-specific CD4+ T cells were recorded in the responder group, which could not be explained by the difference in their clinical data (age, gender, serum AFP level, and the presence of different viral hepatitis) or their T-cell ability to expand in vitro. It is noteworthy that AFP staining can be shown in hepatocellular carcinoma tissues of hepatocellular carcinoma patients with serum AFP level of <20 ng/mL [339]. The CD4+ T-cell response was not detected

in patients with high levels of serum AFP (>1,000 ng/mL) and the majority of patients with a response were in the early stage of disease. CD4+ T cells from hepatocellular carcinoma patients with high levels of serum AFP did not exhibited anti-AFP CD4+ T cells responses (cytokine production and antigen-specific T-cell proliferation). Furthermore, CD4+ T cells isolated from this group of patients did not respond to activation by rIL-2 as measured by T-cell proliferation (data not shown) or nonspecific cytokine production, suggesting that CD4+ T cells from this group of patients are dysfunctional. It should be emphasized that the identified AFP-derived epitope did not exhibit any regulatory property and the regulatory ability of AFP should be attributed to other sections of AFP sequence. The lack of anti-AFP CD4+ T-cell response in patients with high levels of AFP could be due to immuno-regulatory effects of AFP in high levels [245, 307], T-cell exhaustion or anergy caused by high levels of antigen [340], other regulatory cytokines released by tumor (such as TGF-ß), and/or tumor specific Treg cells. Further studies using peptide-specific MHC class II tetramers are required to determine whether AFP364-373-specific CD4+ T cells are dysfunctional or deleted in HCC patients with high levels of serum AFP. Before one can proceed with this experiment, peptide-specific class II tetramers must be constructed and fully characterized, which is beyond the scope of the present study. Whatever mechanism, the results obtained from this study imply that only HCC patients with normal or mildly elevated serum AFP (up to 1,000 ng/mL) who are in the early stage of disease exhibit CD4+ T-cell response to the identified epitope and thus might benefit from an anti-AFP immunotherapy vaccination. A recent report by Ritter et al. [233] has also shown the presence of AFP-specific CD4+ and CD8+ T cells directly *ex vivo* in the peripheral blood of HCC patients. CD4 T-cell responses to AFP (the whole protein) were detected in higher percentages of HCC patients (59%) than that in our study (about 20% responded to the identified epitope), suggesting that CD4 T cells from patients with HCC may recognize, not yet unidentified, CD4 epitopes. We are currently examining this possibility and will determine whether CD8 T-cell responses are also dysfunctional in HCC patients with high levels of AFP (>1,000 ng/mL). The control groups (n = 24) tested in this study did not exhibit any response to the AFP-derived peptides and no AFP-specific CD4+ T-cell response was detected in this group suggesting that AFP-specific CD4+ T cells in the responder patients had been previously expanded *in vivo* in response to the autologous tumor. Other authors have succeeded in showing a specific B- and T-cell response to AFP both in patients with HCC and patients with liver

cirrhosis [234, 331]. One explanation for this discrepancy could be that the CD4+ T cells of cirrhotic patients may recognize yet unidentified AFP-derived epitopes. Identification of a larger number of AFP-derived CD4+ T-cell epitopes and analysis of the CD4+ T-cell response to these epitopes in cirrhotic patients and hepatocellular carcinoma patients may cast a light on the role of the AFP-specific CD4+ T-cell response in disease progression and may be used as a prognostic/diagnostic marker. In conclusion, we report a dominant role of 364-373 region of AFP antigen in the induction of specific CD4+ T-cell responses in hepatocellular carcinoma patients. The identified epitope is presented to specific CD4+ T cells by HLA-DR molecules. This finding is likely to contribute significantly to the development of AFP-based vaccines.

# CHAPTER 10: HUMAN CD4+ T CELLS RECOGNIZE AN EPITOPE WITHIN ALPHA-FETOPROTEIN SEQUENCE AND DEVELOP INTO TGF-PRODUCING CD4+ T CELLS

## 10.1 Abstract

There is limited information on the influence of tumor growth on the expansion of tumor-specific Th3 cells in man and yet no Th3 epitope has been identified. Alpha-fetoprotein (AFP) is an oncofetal antigen and has intrinsic immunoregulatory properties. Here, we report the identification and characterization of subsets of $CD4^+$ T cells that recognize an epitope within AFP sequence ($AFP_{46-55}$) and develop into TGF-β producing CD4+ T cells. In a peptide specific and dose dependent manner, $AFP_{46-55}$ $CD4^+$ T cells produce TGFβ, GM-CSF and IL-2 but not Th1, Th2, Th17 or Tr1 type cytokines. These cells express CTLA-4 and GITR and inhibit T cell proliferation in a contact dependent and TGF-β independent manner. Here we show that the frequency of $AFP_{46-55}$ $CD4^+$ T cells is significantly higher (p=001) in patients with Hepatocellular Carcinoma (HCC) than in healthy donors, suggesting that these cells are expanded in response to tumor antigen. In contrast, tumor necrosis-inducing treatments that are shown to improve survival rate, can shift Th1/Th3 balance in favor of Th1 responses. Our data demonstrate that tumor antigens may contain epitopes which activate the expansion of inducible Treg cells, leading to evasion of tumor control.

## 10.2 Introduction

There are two types of Treg; natural and inducible. Natural CD4+CD25+ Treg cells are derived in thymus and express CD25 and Foxp3. Inducible Treg cells are generated in the periphery in response to pathogen or self antigens and produce IL-10 (Tr1 cells) or TGF-β (Th3 cells). Natural and inducible Treg cells can be beneficial to the host by regulating anti-self response in autoimmune patients or during infection by preventing pathogen-induced immunopathology. However, the induction or activation of Treg cells by tumors or pathogens may suppress protective immunity [341].

It has been suggested that some tumor may activate the expansion of inducible Treg cells [251]. The majority of tumor associated antigens are self antigens with the ability

to stimulate inducible Treg cells that can inhibit the development of an effective anti-tumor immunity [342]. To avoid unwanted expansion of inducible Treg cells by vaccines targeting tumor antigens, it is crucial to identify $CD4^+$ Treg cell epitopes within tumor-associated antigen sequences. On the other hand, the expansion of Treg cells in auto-immune diseases could suppress anti-self immune responses [343]. Therefore, MHC class II-restricted T cell epitopes with the ability to induce the expansion of Treg cells *in vivo* could be used in the treatment of auto-immune diseases. Moreover, it is clear that desirable peptides for therapeutic vaccines should be promiscuous T cell epitopes, which could be recognized by $CD4^+$ T cells with different alleles, allowing broad population coverage.

Alpha-fetoprotein (AFP) is an oncofetal antigen with intrinsic immunoregulatory properties [245, 307] and is also a tumor rejection antigen in Hepatocellular Carcinoma (HCC) [228]. Several immuno-dominant AFP-derived Th1 and Tc1 epitopes have been recently identified [230, 237-238]. However, there is little information on the ability of AFP to stimulate the expansion of inducible Treg and as yet no AFP-derived Treg epitope has been identified. Here, we report the identification of the first self antigen-derived Th3 epitope in man and demonstrate that over-expression of AFP stimulate the expansion of AFP-specific Th3 cells in patients with HCC.

## 10.3 MATERIAL AND METHODS

### *10.3.1 Synthetic peptides and cell lines*

In total 94 peptides spanning the AFP sequence were synthesized by Mimotopes. Sixty-two were soluble in DMSO and were tested in this study (Table 10-1).

| Amino Acid Start | Sequence | Amino Acid Start | Sequence |
|---|---|---|---|
| 1 | MKWVESIFL | 350 | FLASFVHEY |
| 8 | FLIFLLNFT | 365 | QLAVSVILRV |
| 11 | FLLNFTESRT | 372 | RVAKGYQEL |
| 20 | TLHRNEYGI | 379 | ELLEKCFQT |
| 30 | SILDSYQCTA | 385 | FQTENPLEC |
| 35 | YQCTAEISL | 410 | ALAKRSCGL |
| 37 | CTAEISLADL | 419 | FQKLGEYYL |
| 40 | EISLADLATI | 427 | LQNAFLVAYT |
| 46 | LATIFFAQFV | 431 | FLVAYTKKA |
| 54 | FVQEATYKEV | 441 | QLTSSELMAI |
| 65 | KMVKDALTAI | 449 | AITRKMAAT |
| 70 | ALTAIEKPT | 453 | KMAATAATCC |
| 86 | CLENQLPAFL | 462 | CQLSEDKLL |
| 89 | NQLPAFLEEL | 468 | KLLACGEGA |
| 125 | FLAHKKPTPA | 475 | GAADIIIGHL |
| 137 | PLFQVPEPV | 485 | CIRHEMTPV |
| 140 | QVPEPVTSC | 489 | EMTPVNPGV |
| 158 | FMNKFIYEI | 492 | PVNPGVGQC |
| 164 | YEIARRHPFL | 498 | GQCCTSSYA |
| 172 | FLYAPTILL | 507 | NRRPCFSSLV |
| 179 | LLWAARYDKI | 514 | SLVVDETYV |
| 187 | KIIPSCCKA | 531 | FIFHKDLCQA |
| 217 | SLLNQHACAV | 536 | DLCQAQGVAL |
| 235 | FQAITVTKL | 542 | GVALQTMKQ |
| 249 | KVNFTEIQKL | 545 | LQTMKQEFLI |
| 277 | CLQDGEKIM | 548 | MKQEFLINL |
| 298 | KITECCKLTT | 555 | NLVKQKPQI |
| 306 | TTLERGQCII | 562 | QITEEQLEAV |
| 325 | GLSPNLNRFL | 570 | AVIADFSGL |
| 343 | SSGEKNIFL | 576 | SGLLEKCCQ |
| 347 | KNIFLASFV | 598 | KLISKTRAAL |

Table 10-1: AFP-derived peptides

*10.3.2 Patient recruitment*

The study was approved by ethical committees and all patients gave written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of patients with HCC or healthy donors.

*10.3.3 Generation of short-term and long-term T cell lines*

Short-term T cell lines were generated as described previously [238]. In brief, PBMCs were resuspended in AIM-V medium (Invitrogen Life Technologies) and cultured with individual peptides (1μM). rIL-2 (25 IU/ml) was added on days 2 and 3 of culture and the cells were analyzed after a total of 10–12 days of culture. The experiments presented in this study (excluding inhibition assay and ex vivo data) were performed on short-term T cell lines.

To generate long-term T cell lines (for inhibition assay), PBMCs were resuspended in AIM-V medium (Invitrogen Life Technologies) and cultured with individual peptides (1 μM). rIL-2 (25 IU/ml) was added on days 2 and 3 of culture. After 10–12 days of culture, CD4+ T cells were isolated from short-term T cell lines using Dynabeads and the cells (1 cell/well) were cultured with gamma-irradiated 5 x $10^4$ allogeneic PBMCs as feeder cells, and rIL-2 (30 IU/ml) culture medium was changed once a week with fresh medium, rIL-2, and feeder cells. On day 21, T cells were tested for GMCSF production using ELISA. CD4+ T cell lines that produced peptidespecific GM-CSF were selected and expanded.

*10.3.4 Flow cytometry and intracellular cytokine assay*

AFP-specific T cells were incubated for 5 h at 37°C with AFP-derived peptides (1 μM) or peptide-pulsed or protein pulsed APCs and brefeldin A. Cells were surface stained with Abs to CD3, CD4, CD8, CD25, TCR-alpha-beta, HLA-DR, CD62L, CD45, and GITR (BD Pharmingen). The cells were then permeabilized, fixed, and stained for intracellular molecules (GMCSF,TGF-beta, IL-2, IFN-gamma, IL-10, TNF-beta, IL-5, IL-13, IL-17, and CTLA-4) or isotype controls (R&D Systems), washed twice, and the frequency of peptide-specific T cell responses was quantified by flow cytometry. Anti-TGF-beta antibodies(Abs) for intracellular staining were obtained from R&D Systems and IQ Products. Cells were stained with Abs to Foxp3 (eBioscience) as described by the manufacturer's instructions. An immunological responder was defined as a 2-fold increase in frequency of cytokine-producing cells

above control peptides or proteins. AFP46–55-spcific T cell lines or control T cell lines (AFP364–373) were washed and cultured in serum-free medium in the presence of relevant or irrelevant peptides for 48 h, and the amount of total TGF-beta and GM-CSF were measured in culture supernatants by ELISA (R&D Systems).

*10.3.5 ELISPOT assay*

TGF-beta-releasing cells were detected upon specific peptide stimulation using an ELISPOT assay ex vivo. Nitrocellulose-backed plates (96-well, MAHA S45; Millipore) were coated with mouse anti-human latent TGF-beta capture Ab overnight at 4°C. The wells were washed five times with PBS and blocked using blocking buffer (1% BSA and 5% sucrose PBS) for 2 h. PBMCs and the peptides were then added into the wells and incubated for 18 h at 37°C in 5% CO2. The wells were washed with wash buffer (0.05% Tween 20 in PBS), then 1 μg/ml secondary biotin-conjugated anti-human latent TGF-beta Ab (R&D Systems) was added and incubated at 4°C overnight. The color development was done using ELISPOT blue color module
(R&D systems). After 30 min, the wells were washed with tap water, dried and the spots counted.

*10.3.6 Proliferation assays*

CD4_CD25_ T cells (2 x $10^5$) isolated from PBMCs by Ab-coated beads were cultured for 5 days in 96-well plates containing 5 x $10^4$ CD3-depletedAPCs, 0.5 μg/ml anti-CD3 mAb, and different numbers of regulatory (AFP46–55) or effector (AFP364–373 peptide) CD4+ T cells in medium containing 10% human serum. The proliferation of responder T cells was assessed by the incorporation of [3H]thymidine for the last 18 h of culture. Cells were harvested and radioactivity was counted in a scintillation counter. All experiments were performed in triplicates. For some experiments, Ab against TGF-beta (R&D Systems) was added in the assay at a final
concentration of 5 μg/ml.

Transwell experiments were performed in 24-well plates with a 0.4 μm pore size (Corning Glass). Purified naive CD4+ T cells (1 x $10^5$) were cultured in the outer wells in medium containing 0.5 μg/ml anti-CD3 Ab and 2 x $10^5$ APCs. Equal numbers of AFP46–55 CD4_ T cells or AFP364–373 CD4_ T cells were added into the inner wells in the same medium containing anti-CD3 and 2 x $10^5$ APCs. The cells in the inner and

outer wells were harvested separately and transferred into 96-well plates after 3 days of culture. [3H]Thymidine was added, and the cells were cultured
for another 18 h before being harvested for counting the radioactivity with a liquid scintillation counter.

*10.3.7 Statistical analysis*

The Mann-Whitney *U* test (two tailed) was used to compare the frequencies of AFP46–55 -specific GM-CSF- producing CD4+ T cells in healthy and cancer patients. The statistical significance was defined at $p < 0.05$.

## 10.4 RESULTS

*10.4.1 Alpha-fetoprotein stimulates GM-CSF and TGF- production by CD4+ T cells*

GM-CSF is a cytokine that is produced by different T cell populations including inducible Tregs [204] and TGF-β is an immuno-regulatory cytokine produced by Th3 cells. Short term T cell lines were generated in medium containing rIL-2 with or without purified AFP (5μg/ml). Cells were washed, counted and cultured in serum free medium in the presence or absence of purified AFP (5μg/ml). The amounts of GM-CSF and total TGF-β were measured in cell culture supernatant using ELISA assay. T cell lines stimulated with AFP produced TGFβ and GM-CSF (Figure 10-1a&b). The depletion of $CD4^+$ cells but not CD8 T cells prior to re-stimulation reduced AFP specific GM-CSF and TGFβ production by T cell lines (Figure 10-1a&b), suggesting that CD4+ T cells are the source of GM-CSF and TGF-beta.

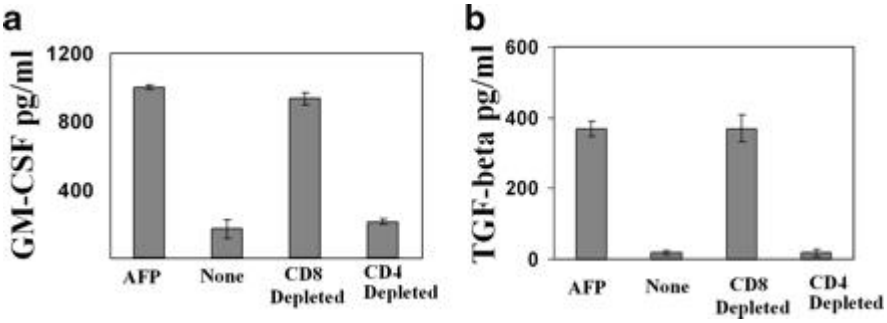



Figure 10-1**:** AFP stimulate GM-CS F and TGF-β production by $CD4^+$ T cells. The amount of GM-CSF (*a*) and total TGF-beta (*b*) were measured in cell culture supernatants of T cell lines stimulated with AFP (5 μg/ml). $CD4^+$ or $CD8^+$ T cells were depleted from T cell lines before restimulation to determine the source of cytokine production.

*10.4.2 $CD4^+$ T cells recognize an epitope within AFP sequence and produce GM-CSF*

To identify AFP derived epitopes that can stimulate GM-CSF production by CD4+ T cells, short term T cell lines were generated in the presence of the 62 different peptides spanning the AFP sequence (Table 10-1). Ten days later, the reactivity was analyzed using intracellular cytokine staining for GM-CSF. Among 62 peptides, $AFP_{46-55}$ peptide (LATIFFAQFV) stimulated GM-CSF production by CD3+CD4+ T cells (Figure 10-2a&b) in a dose dependent manner (Figure 10-2c). Similar results were observed in T cell lines generated from 3 other individuals. GM-CSF production by $AFP_{46-55}$ specific CD4 T cells is peptide specific as determined using ELISA assay (Figure 10-2e). To identify the optimal length of peptide sequence, $AFP_{46-55}$ $CD4^+$ T cells were stimulated with $AFP_{47-55}$ (9 amino acid long), $AFP_{46-55}$, $AFP_{44-57}$ (14 amino acid long), $AFP_{42-55}$ (14 amino acid long) and the frequency of peptide-specific GM-CSF producing $CD4^+$ T cells was analyzed. $AFP_{46-55}$ $CD4^+$ T cells recognized $AFP_{47-55}$, $AFP_{46-55}$, $AFP_{44-57}$, $AFP_{42-55}$ but not an irrelevant peptide ($AFP_{364-373}$) and produced peptide-specific GM-CSF. The frequency of GM-CSF-producing cells among $CD4^+$ T cells was highest in cells stimulated with $AFP_{46-55}$ (Figure 10-2d).

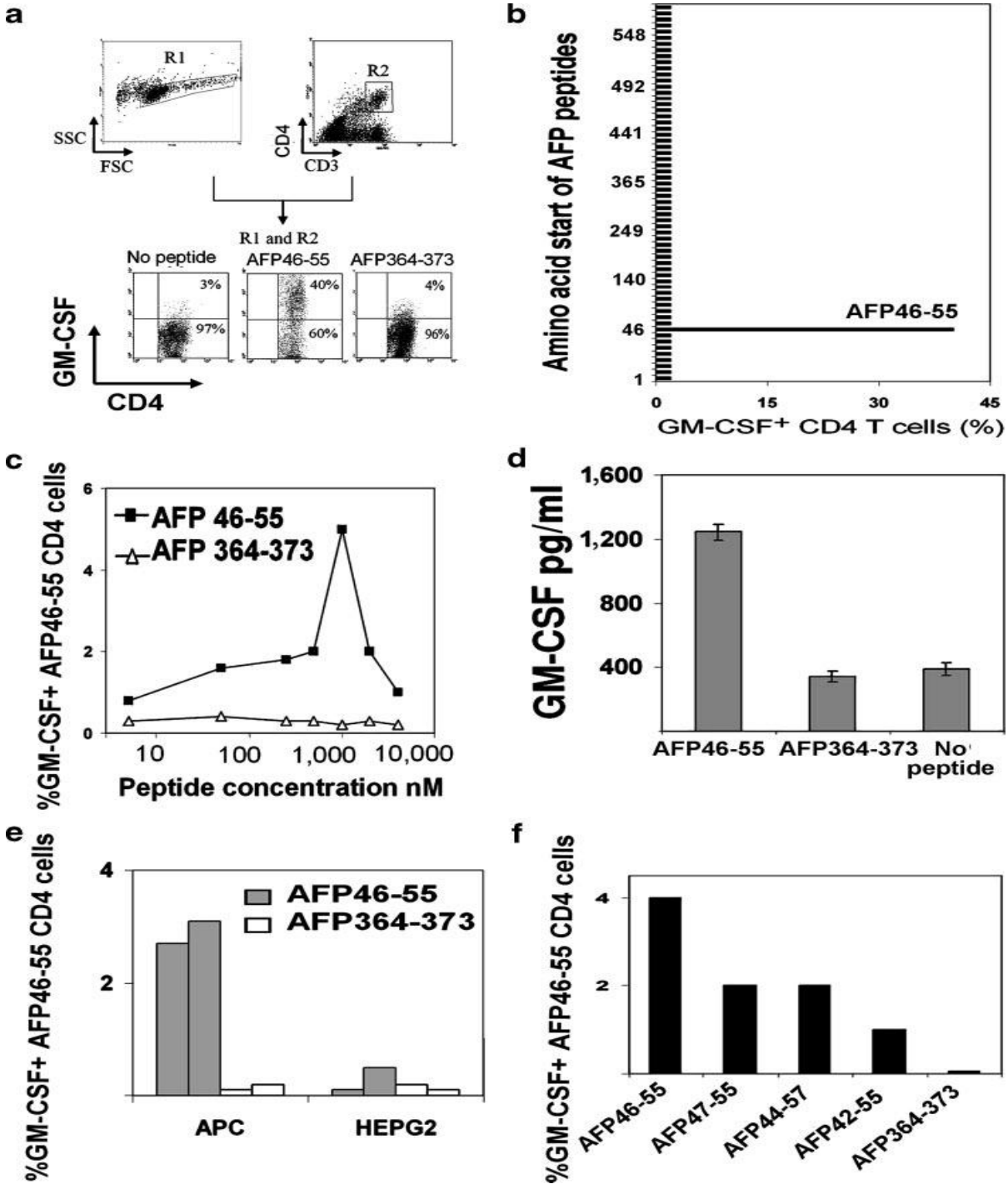



Figure 10-2: Identification of AFP-derived peptide epitope that stimulates GM-CSF production. *a* and *b*, Short-term T cell lines were generated from PBMCs isolated from HCC patients in the presence of 62 different peptides spanning the AFP sequence. After 10 days, cells were restimulated with relevant or irrelevant peptides (*c*) at different concentrations. Numbers indicate percentages of GM-CSF-producing cells within $CD3^+CD4^+$ T cells. *d*, The production of peptide-specific GM-CSF was detected in the supernatant of $AFP_{46–55}$ $CD4^+$ T cells using ELISA (in triplicate wells ± SD). *e*, Peptide-pulsed EBV B cells or MHC class II- deficient cells (HepG2 cells) were cultured with $AFP_{46–55}$ $CD4^+$ T cells, and peptide recognition was analyzed an using intracellular cytokine assay for GM-CSF. *f*, To determine optimal peptide length required for T cell recognition, $AFP_{46–55}$ $CD4^+$ T cells were restimulated with $AFP_{46–55}$, $AFP_{47–55}$, $AFP_{44–57}$, $AFP_{42–55}$, or $AFP_{364–373}$ peptide and the peptide recognition by $CD4^+$ T cells was determined using an intracellular cytokine assay for GM-CSF. Two independent experiments were performed and the results are confirmed in three other individuals.

To study the role of IL-2, IL-7 and IL-15 on the generation and expansion of $AFP_{46-55}$-specific $CD4^+$ T cells, PBMCs were cultured in the presence or absence of different combinations of these cytokines. $AFP_{46-55}$ $CD4^+$ T cells were not expanded in the absence of exogenous IL-2, suggesting that IL-2 is essential for the expansion of these cells. The highest percentage of $AFP_{46-55}$ $CD4^+$ T cells was detected in cultures expanded in the presence of IL-2 (25 IU/ml), IL-7 (20 ng/ml) and IL-15 (20 ng/ml) (data not shown).

*10.4.3 $AFP_{46-55}$ $CD4^+$ T cells produce TGF in a peptide specific and dose dependent manner*

To test the ability of T cell lines to produce antigen specific TGFβ, $AFP_{46-55}$ or $AFP_{364-373}$ T cell lines (Th1 cells) [238] were washed and stimulated ($2 \times 10^5$ cells/well) with increasing concentrations of $AFP_{46-55}$ or $AFP_{364-373}$ peptides in serum free medium for 48 hrs. The amounts of total TGF-β were measured in the culture supernatant using an ELISA for TGF-β. $AFP_{46-55}$ T cell lines stimulated with $AFP_{46-55}$ produced TGFβ in a dose dependent manner (Figure 10-3a). The depletion of $CD4^+$ cells or $CD2^+$ cells prior to peptide re-stimulation but not the depletion of CD8+ T cells reduced peptide specific TGFβ production by $AFP_{46-55}$ T cell lines (Figure 10-3b), suggesting that $CD4^+$ T cells are the source of TGFβ. To test the recognition of purified AFP by $AFP_{46-55}$ $CD4^+$ T cells, $AFP_{46-55}$ CD4+T cell lines or a control $AFP_{364-373}$ T cell line were cultured with APCs-pulsed with purified AFP (5 μg/ml) or a control protein (human serum albumin) for 48h. The amount of total TGF-β was measured in the culture supernatant using an ELISA for TGF-β. $AFP_{46-55}$ $CD4^+$ T cell lines stimulated with purified AFP but not with control protein produced large quantities of TGFβ (Figure 10-3c). The production of TGF-β by $AFP_{46-55}$ CD4+ T cells was confirmed using intracellular cytokine assay (Figure 10-3d). Both anti-TGF-β antibodies from R & D systems and IQ product stained similar percentages of peptide specific TGF-β producing CD4+ T cells (data not shown).

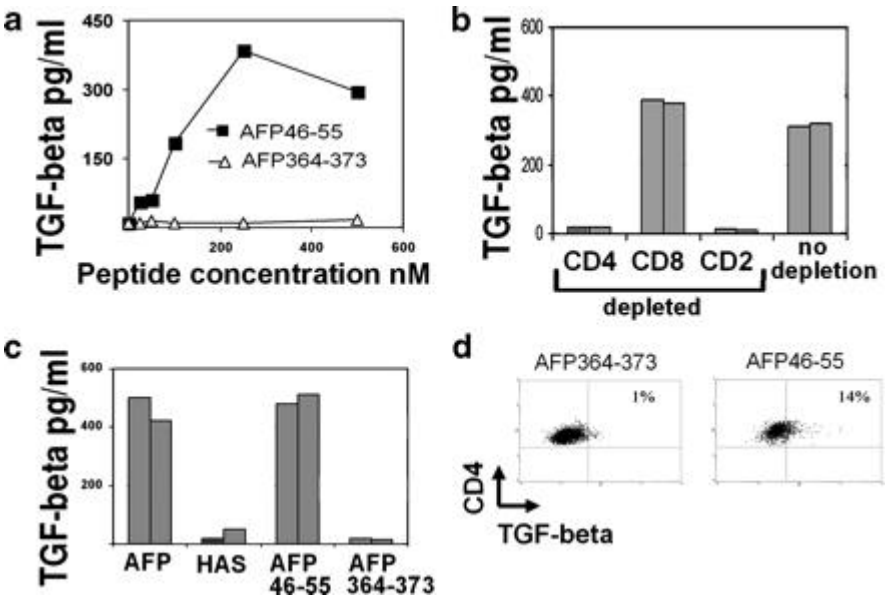



Figure 10-3: $AFP_{46–55}$ $CD4^+$ T cells produce TGF-β in a dose-dependent manner. The amounts of total TGF-β were measured in the culture supernatant of $AFP_{46–55}$ $CD4^+$ T cells restimulated with different concentrations of the relevant or irrelevant peptides (*a*). *b*, $CD4^+$, $CD2^+$, or $CD8^+$ cells were depleted from PBMCs and cultured in medium containing $AFP_{46–55}$ peptide and rIL-2 for 10 days. The levels of total TGF-β were measured in the supernatant of the T cell lines. *c*, The amounts of TGF-beta produced by $AFP_{46–55}$ $CD4^+$ T cells upon restimulation with purified AFP, human serum albumin, $AFP_{46–55}$ peptide, or $AFP_{364–373}$ peptide are shown. *d*, The percentage of intracellular TGF-β produced by $CD4^+$ T cells is shown. Two independent experiments were performed and the results were confirmed in five other individuals.

### *10.4.4 $AFP_{46-55}$ $CD4^+$ T cells do not produce Th1, Th2, Th17, Tr1 type cytokines*

Antigen-specific CD4+ T cells can be classified as Th1, Th2, Th17 or Tr1 based on their ability to produce different cytokine profile. To classify $AFP_{46-55}$ CD4+ T cells, we analyzed their ability to produce different cytokines upon peptide stimulation. $AFP_{46-55}$ -specific CD4+ T cells were generated from HCC patients and their ability to produce cytokines was evaluated using intracellular cytokine assays. $AFP_{46-55}$ $CD4^+$ T cells did not produce Th1 type (IFN-γ, TNF-α), Th2 (IL-5, IL-13), Tr1 (IL-10) or Th17 (IL-17) type cytokines. $AFP_{46-55}$ -specific $CD4^+$ T cells recognized the relevant peptide and produced TGFβ, GM-CSF and IL-2 (Figure 10-4). As determined using six-color flow cytometry, IL-2, GM-CSF and TGF-β are produced by the same $AFP_{46-55}$ -specific $CD4^+$ T cells. $AFP_{46-55}$ T cell lines generated from 5 other individuals produced similar pattern of cytokine production (data not shown).

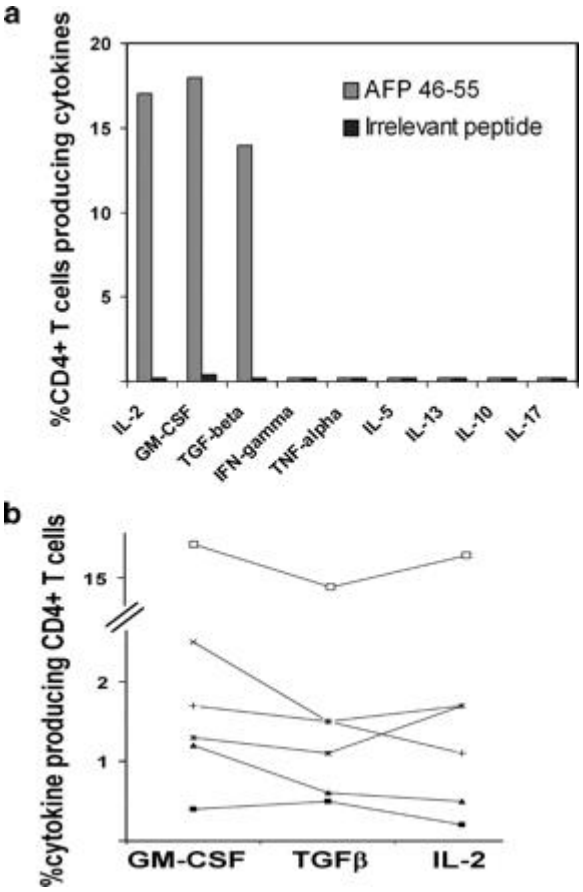



Figure 10-4: $AFP_{46–55}$ $CD4^+$ T cells do not produce Th1-, Th2-, Th17-, or Tr1-type cytokines. Cell lines were restimulated with $AFP_{46–55}$ peptide or an irrelevant peptide ($AFP_{364–373}$), and the production of IFN-gamma, TNF-alpha, IL-5, IL-13, IL-17, IL-2, GM-CSF, TGF-beta, and IL-10 by $CD4^+$ T cells was analyzed using an intracellular cytokine assay. *a*, The percentages of peptide-specific cytokine-producing $CD4^+$ T cells are shown. Two independent experiments were performed. *b*, A summary of cytokines produced by CD4 T cells from six different individuals is shown. Each symbol represents percent cytokine-producing $CD4^+$ T cells from an individual.

### *10.4.5 $AFP_{46-55}$ CD4+ T cells express CTLA-4 and GITR but do not express Foxp3*

We then analyzed cell surface phenotype of $AFP_{46-55}$ $CD4^+$ T cells in short term T cell lines. The expression of surface and intracellular molecules (CD4, CD45RO, CD62L, CTLA-4 and GITR) were analyzed. The majority of $AFP_{46-55}$ $CD4^+$ GM-CSF producing T cells expressed surface CD45RO, and intracellular CTLA-4 but not CD62L. Non-responder $CD4^+$ T cells (cells not producing GM-CSF) did not express GITR or intracellular CTLA-4 (Fig 5a). $AFP_{46-55}$ $CD4^+$ T cells expressed CD3, TCRαβ, CD25 but not CD8, CD14 or CD16 (data not shown).

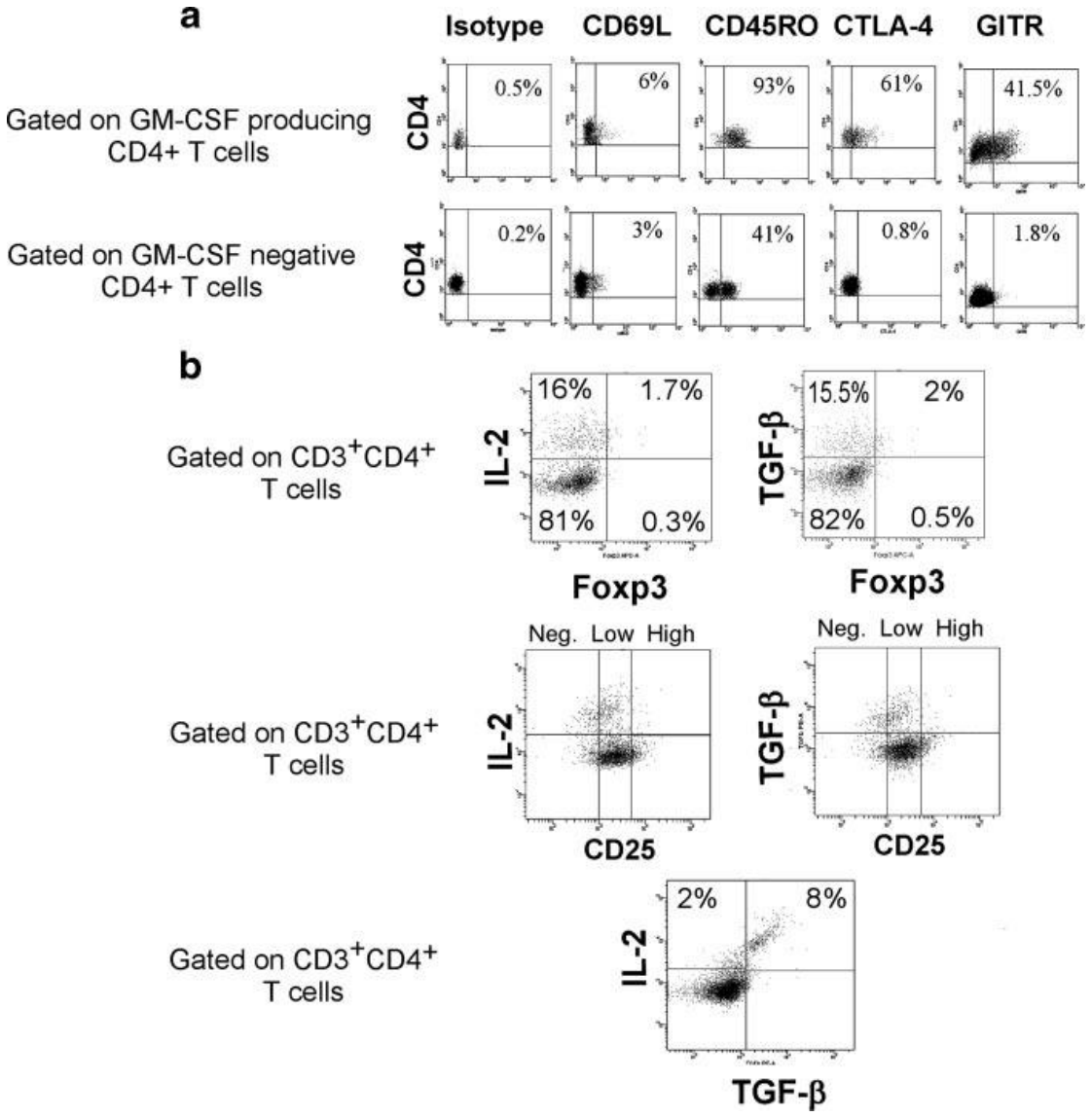



Figure 10-5: Phenotypic characterization of peptide-specific $CD4^+$ T cells. $AFP_{46–55}$ $CD4^+$ T cell line was restimulated with $AFP_{46–55}$ peptide and the cells were stained with mAbs to detect surface CD4, CD69L, CD45RO, and GITR molecules, intracellular CTLA-4 and GM-CSF, and intranucleus Foxp3. Isotype control Abs served as negative control staining. *a*, $CD4^+$ GM-CSF-producing T cells or $CD4^+$ GM-CSF-negative cells were gated and the expression level of surface and intracellular molecules is shown. *b*, The expression levels of Foxp3 and CD25 in IL-2- and TGF-beta-producing $AFP_{46–55}$ $CD4^+$ T cells are shown. Two independent experiments are performed in T cell lines generated from five other individuals.

Foxp3 is expressed by CD4+CD25+ T cells but its expression in inducible Treg is controversial. We analyzed the expression of Foxp3 in $AFP_{46-55}$ $CD4^+$ T cells using flow cytometry. The majority of $AFP_{46-55}$ peptide specific IL-2 and TGFβ producing CD3+ CD4+ did not express Foxp3 and only expressed low levels of CD25 (Figure 5b). In some T cell lines, small percentages of $AFP_{46-55}$ $CD4^+$ T cells were Foxp3 positive (data not shown).

*10.4.6 $AFP_{46-55}$ -specific $CD4^+$ T cells have inhibitory effect on T cell proliferation in vitro*

It has been shown that recombinant TGF-β induces certain $CD4^+$ T cells in human peripheral blood to develop contact-dependent suppressive activity that is not antagonized by anti-TGFβ. This suppressive activity was only partially abrogated when recombinant IL-2 was added to the culture [344]. Here, we examined the suppressive effects of $AFP_{46-55}$ $CD4^+$ T cells that are shown to produce both TGF-β and IL-2. Four CD4+ T cell clones (clones 1, 2, 3 & 4) were generated from PBMCs of a healthy donor using limited dilution methods as described in material and methods. Antigen-specificity and phenotypic characterization of $AFP_{46-55}$ $CD4^+$ T cell clones were analyzed after re-stimulation with peptide-pulsed APCs (adherent cells) and detection of intracellular GM-CSF. The majority of CD4+ T cells from these clones recognized the peptide and produced GM-CSF, suggesting that these cells are antigen specific. Next we tested the ability of these T cell clones to inhibit anti CD3-induced T cell proliferation. The proliferation rate of responding cells without T cell clones was considered as 100% proliferation. Clone 1 moderately inhibited T cell proliferation, suggesting that these cells may have some inhibitory function. $AFP_{46-55}$ $CD4^+$ T cell clone 3 did not inhibit T cell proliferation (Figure 10-6a). The addition of anti TGFβ antibody did not reverse or alter the inhibitory effects of the inhibitory T cells (Figure 10-6b).

CD3+CD4+ T cell clone 1 (with moderate inhibitory effect) and clone 3 (with no inhibitory effect) were selected for further phenotypic studies using flow cytometry. There was no significant difference in the percentages of GM-CSF producing cells between these two clones (Figure 10-6b). Eighty four percentages of T cells from clone 1 produced TGFβ in a peptide specific manner but only 32% of T cells from clone 3 produced TGFβ. Small percentages of TGFβ or IL-2 producing CD4+ T cells expressed Foxp3 (Figure 10-6c).

An $AFP_{46-55}$ CD4+ T cell clone was generated from PBMCs of a HCC patient. CD3+ CD4+ $Foxp3^{neg}$ T cells produced TGF-β, IL-2 and GM-CSF in a peptide specific manner (data not shown). In an inhibitory assay, T cells from the HCC patient suppressed anti CD3-induced T cell proliferation. $AFP_{364-373}$ specific Th1 cells [238] generated from the same patient did not inhibit T cell proliferation in vitro (Figure 10-6d).

Transwell experiments were performed to test whether cell-cell contact is required for $AFP_{46-55}$ $CD4^+$ T cells to exert their suppressive activity. $AFP_{46-55}$ $CD4^+$ T cells when cultured in the inner well containing medium with anti-CD3 and the purified APCs, did not inhibit the proliferative activity of $CD4^+$ T cells cultured in the outer well containing medium, anti-CD3 and APCs (Figure 10-6e). Taken together, these results indicate that $AFP_{46-55}$ $CD4^+$ T cells exert its T cell inhibitory properties in a contact dependent manner.

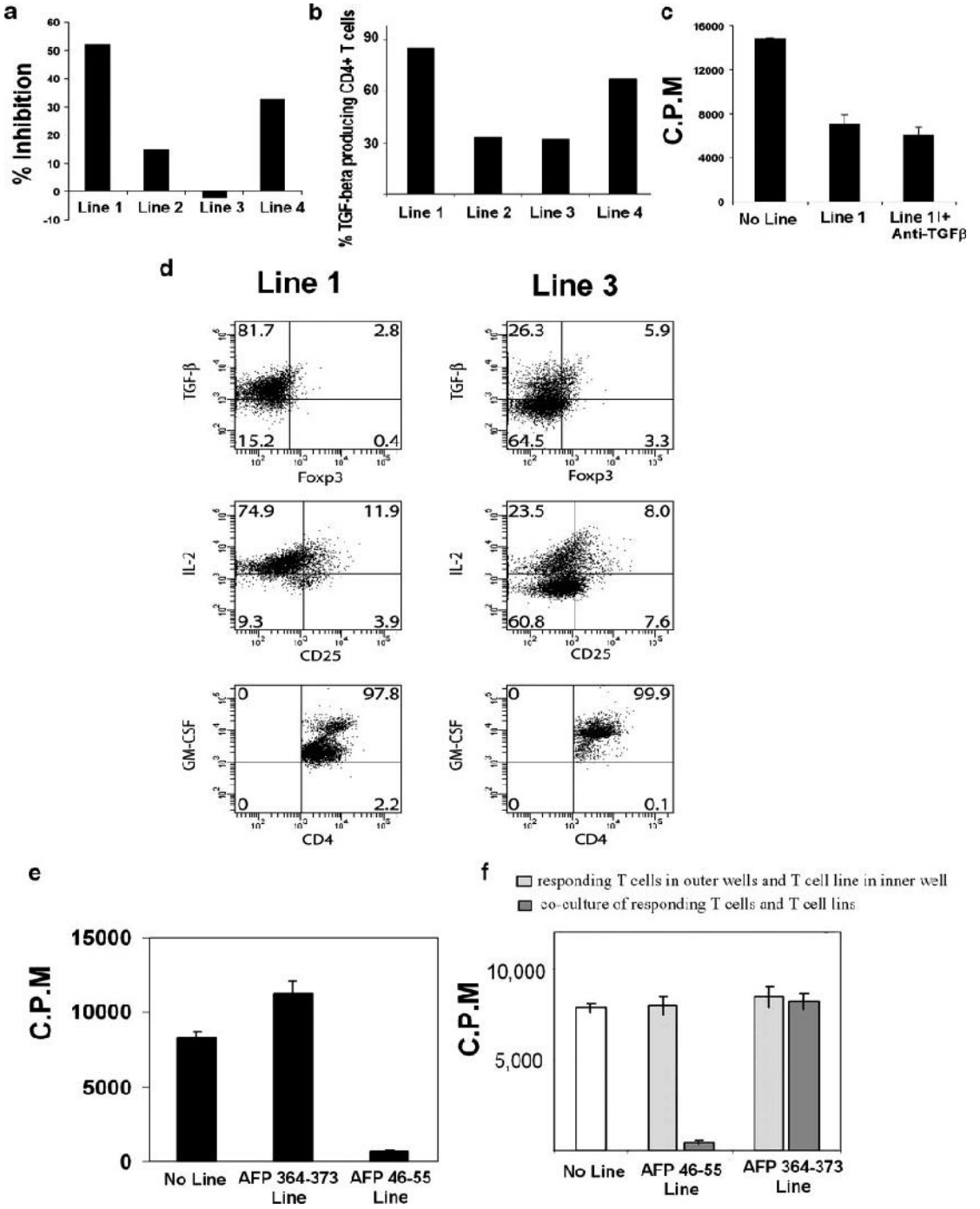



Figure 10-6: $AFP_{46–55}$ $CD4^+$ T cells suppress T cell proliferation to anti-CD3 Ab stimulation in a cell-cell contact manner. *a*, In an inhibition assay, responding T cells were cultured with or without $AFP_{46–55}$ $CD4^+$ T cells (lines 1–4) generated from a healthy donor at a 1:1 ratio and anti-CD3 Ab-induced T cell proliferation was measured. The percentages of inhibition recorded in wells containing $AFP_{46–55}$ $CD4^+$ T cells are shown. *b*, The percentages of TGF-β-producing $AFP_{46–55}$ $CD4^+$ cells are shown for each long-term T cell line. *c*, Anti-TGF-β mAb did not abrogate the inhibitory effects of $AFP_{46–55}$ $CD4^+$ T cells. *d*, Cytokine profile in $AFP_{46–55}$ $CD3^+CD4^+$ T cells (lines 1 and 3). The percentages of cytokine-producing $CD4^+$ T cells are shown. *e*, The suppressive effects of $AFP_{46–55}$ and $AFP_{364–373}$ $CD4^+$ T cells (Th1) from a HCC patient were evaluated in an anti-CD3-induced T cell proliferation assay. *f*, In a Transwell system, $AFP_{46–55}$ or $AFP_{364–373}$ $CD4^+$ T cells were cocultured or cultured separately (in inner wells) with responding $CD4^+$ T cells and stimulated with anti-CD3 Ab. Mean $[^3H]$thymidine incorporation indicated as cpm (±SD) in triplicate wells. Two independent experiments were performed.

*10.4.7 Expansion of $AFP_{46-55}$ $CD4^+$ T cells in patients with HCC*

Short term T cell lines were generated from PBMCs isolated from 10 healthy donors (6 males and 4 females) and 15 HCC patients (12 males and 3 females) and the frequency of GM-CSF producing $AFP_{46-55}$ T cells was analyzed using an intracellular cytokine assay. $AFP_{46-55}$ -specific CD4+ T cells were detected in all healthy donors and HCC patients (Figure 10-7a). A significantly higher frequency of $AFP_{46-55}$ $CD4^+$ T cells was detected in HCC patients than healthy controls (p=0.01) (Figure 10-7a), suggesting that these cells are expanded *in vivo* in response to the tumor antigen. Anti-human TGF-β mAb for an intracellular assay was not available to us when we were performing these experiments and there are no data on TGF-β production by $AFP_{46–55}$ T cells in this group of patients.

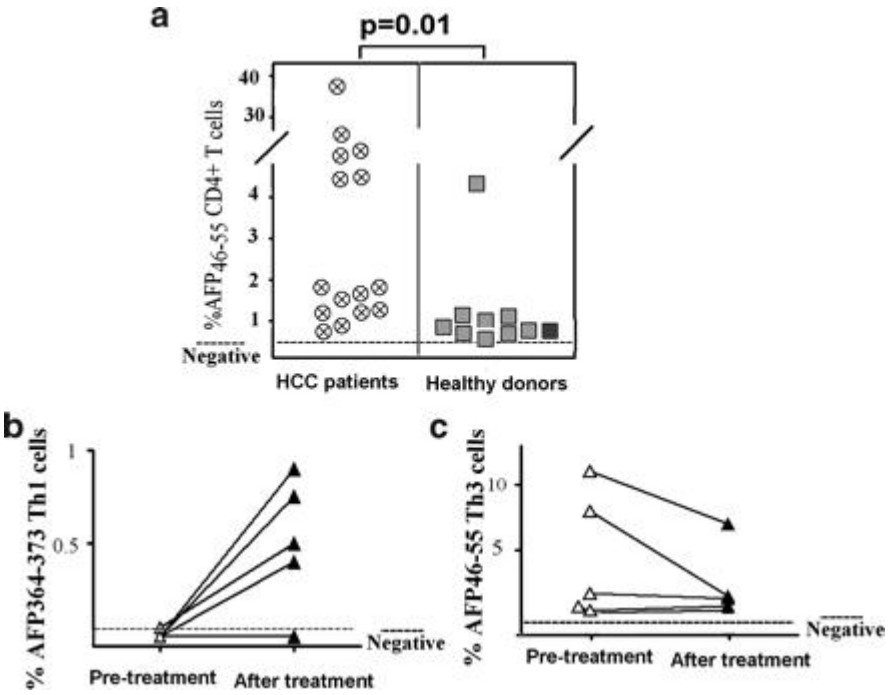



Figure 10-7: $AFP_{46–55}$ $CD4^+$ T cells are expanded in peripheral blood of HCC patients. *a*, The frequency of $AFP_{46–55}$ $CD4^+$ T cells was determined in HCC patients ($n$ = 15) and healthy donors ($n$ = 10) using an intracellular cytokine assay for GM-CSF. The Mann-Whitney *U* test (two tailed) reveals a significant difference ($p$ = 0.01) between patients and healthy donors. Frequencies of IFN-gamma-producing $AFP_{364–373}$ $CD4^+$ T cells (*b*) and GM-CSF-producing $AFP_{46–55}$ $CD4^+$ T cells (*c*) were determined before and 3 mo after transarterial chemoembolization/TAE treatment in five HCC patients. The percentages of cytokine-producing cells are shown.

We have previously shown that induction of tumor necrosis improved survival of HCC patients and expanded AFP-specific Th1 cells *in vivo* [237]. We analyzed the effects of embolisation on the frequency of circulating Th1 ($AFP_{137-145}$, $AFP_{249-258}$, $AFP_{364-373}$) and $AFP_{46-55}$ $CD4^+$ T cell responses before and 3 months after the treatment in 5 consecutive patients. Th1 responses to at least one of AFP-derived epitopes were expanded after treatment in all 5 patients after embolisation. The frequency of IFN-gamma producing $AFP_{364-373}$ $CD4^+$ T cells is shown (Figure 10-7b). $AFP_{46-55}$ $CD4^+$ T cells were detected before and after treatment in all 5 patients. The responses ranged from 0.2% to 11% of $CD4^+$ T cells producing peptide specific GM-CSF (Figure 10-7c). In HCC03, the frequency of $AFP_{46-55}$ $CD4^+$ T cells was reduced from 8% of CD4+ T cells before TAE (serum AFP=2625 ng/ ml) to 1.5% after TAE (serum AFP=1640 ng/ml). In HCC04, the response was reduced from 11% of $CD4^+$ T cells before TACE (serum AFP=7 ng/ml) to 7% after the treatment (serum AFP=10 ng/ml) (Figure 10-7b). Due to a limited number of cells available from this group of patients, intracellular cytokine assay for TGFβ or IL-2 was not performed.

### *10.4.8 Ex vivo detection of $AFP_{46–55}$-specific TGF-β-producing cells*

The frequency of peptide-specific TGF-β-releasing cells was analyzed using ELISPOT assays for TGF-β. PBMCs isolated from four healthy donors (HD-1, HD-2, HD-3, and HD-4) were stimulated with $AFP_{46–55}$ or $AFP_{364–373}$ for 18 h, and the frequency of peptide-specific TGF-β-producing cells was analyzed ex vivo. PBMCs from three of four patients (HD-11, HD-2, and HD-3) responded to $AFP_{46–55}$ and released TGF-β (Figure 10-8). $AFP_{364–373}$ peptide did not stimulate TGF-β production above the background (cells cultured in medium only) (data not shown).

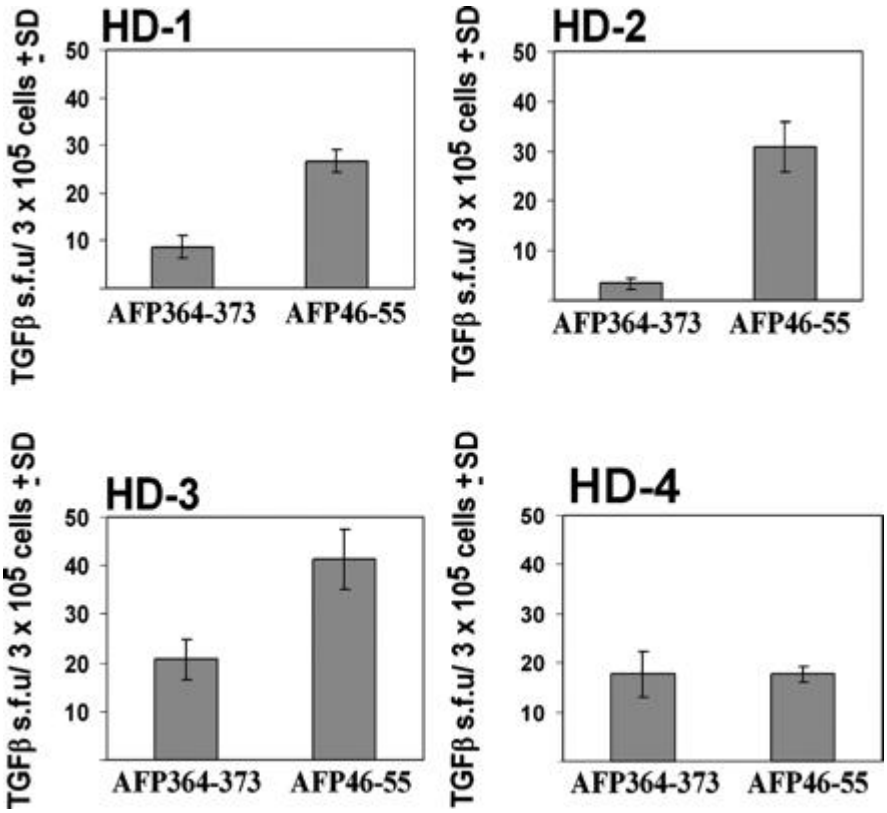



Figure 10-8: Detection of $AFP_{46–55}$-specific TGF-β-releasing cells ex vivo. The frequency of $AFP_{46–55}$-specific-TGF-β-releasing T cells was analyzed in PBMCs of four healthy donors (HD-1, HD-2, HD-3, and HD-4) ex vivo using an ELISPOT assay for TGF-beta. The results are presented as spot-forming units (s.f.u) per 3 x $10^5$ cells and average spots with SD are shown. The experiments were performed in triplicate and the results are representative of two experiments performed on different days.

## DISCUSSION

Here, we show that AFP stimulates the expansion of a subset of CD4+ T cells that produce TGF-β, GM-CSF and IL-2. These cells express CD3, CD4, CTLA-4 and GITR and exert inhibitory effects on T cell proliferation in a contact dependent and TGF-β independent manner. An epitope within AFP sequence ($AFP_{46-55}$) that stimulates these CD4+ T cells are identified and T cells recognizing $AFP_{46-55}$ produce a unique cytokine profile, suggesting that these cells differ from Th1, Th2, Th17 or typical antigen-induced Treg cells that produce IL-10 [345]. In independent studies, clones derived from mice that have been orally tolerized with low antigen dose primarily produce TGF-β, and these cells have been termed Th3 cells. Treg cells that exclusively produce TGFβ have not been observed in other models [345]**.** To our knowledge, this is the first report describing peptide epitope-specific TGFβ producing Treg cells in man.

TGF-beta is an immuno-regulatory cytokine that can act on different populations of leukocytes including T cells. The inhibitory effects of TGF-beta on T cell proliferation is thought to be through the induction of Treg cells in vitro [344]. In this study, we have shown that TGF-beta producing $AFP_{46-55}$ CD4+ T cells inhibit T cell proliferation in a contact dependent manner and this inhibition was not abrogated with anti TGF-beta antibody. However, TGF-beta may play an important role in the generation of inhibitory $AFP_{46-55}$ CD4+ T cells. This notion is supported by the fact that T cells that produced high levels of TGF-beta also exhibited inhibitory function in vitro. The regulatory effects of TGF-beta could be altered by the presence of other known or unknown stimulatory or regulatory cytokines [346]. It is known that the function of some immune cells from HCC patients is impaired and this may influence the inhibitory effects of TGF-beta producing CD4+ T cells in this group of patients. We are currently studying these factors and will analyze their effects on the function of $AFP_{46-55}$ -specific TGF-beta producing CD4+ T cells.

GM-CSF and IL-2 are also produced by $AFP_{46-55}$ $CD4^+$ T cells. It has been shown that GM-CSF enhances protection against tumors and infections, but GM-CSF-deficient mice develop inflammatory disease [347-348]. Many tumors constitutively secrete low

levels of GM-CSF, which may be linked with disease progression [349]. Moreover, it has been shown that the administration of GM-CSF expands regulatory $CD4^+CD25^+$ T cells and suppresses autoimmune diseases in animal models. This suppression is believed to be through activation and generation of regulatory antigen presenting cells [198, 350-354].

IL-2 is not produced by natural Treg cells or Tr1 cells but it has been shown that in combination with TGF-β induces the expansion of Treg cells in vitro and in vivo [344, 355]. In contrast to its in vivo function, IL-2 can reduce the inhibitory function of Tregs on T cell proliferation in vitro [344]. It is possible, but not proven, that IL-2 produced by $AFP_{46\text{-}55}$ $CD4^+$ T cells partially abrogates the inhibitory effect of TGF-beta on T cell proliferation in vitro.

AFP is an oncofetal antigen and has intrinsic immunoregulatory properties [245, 307, 309, 356-357] and recombinant AFP is being considered for treatment of autoimmune diseases. The administration of the intact antigen would avoid the selection of specific epitopes to suit MHC-disparate individuals. This is not the case for the AFP-derived epitope identified, as the response to this epitope can be detected in all individuals tested. In this study, donors and patients (30 in total) were not selected based on their HLA haploytpes and determination of HLA class II haplotypes from some patients showed that $AFP_{46\text{-}55}$ T cell responses are detectable in patients with completely different HLA class II haplotypes (data not shown), suggesting that $AFP_{46\text{-}55}$ is a promiscuous epitope and its recognition is not restricted to one HLA class II haplotype. We believe that the processing and presentation of this epitope by antigen-presenting cells can take place via the exogenous pathway, rather than by direct recognition of tumor, as HCCs do not express MHC class II molecules on the cell surface [358] and these cells are unable to present the peptide to $AFP_{46\text{-}55}$ $CD4^+$ T cells (data not shown). Although $AFP_{46\text{-}55}$ $CD4^+$ T cells were detected after short term T cell culture in both HCC patients and healthy donors, the frequency of these cells in HCC patients was significantly higher than that in healthy donors. This suggests that these cells are expanded *in vivo* in response to the HCC antigen. It has recently been shown that the expansion of circulating Treg is directly associated with poor survival in HCC patients

[249]. Further studies are required to establish any association between AFP-specific Th3 cell prevalence with HCC progression and patient survival.

In support of this notion, we have shown that the reduction of tumor mass reduced the frequency of $AFP_{46-55}$ $CD4^+$ T cells in patient with expanded $AFP_{46-55}$ T cells. In contrast, this treatment results in the activation and expansion of IFN-γ producing Th1-specific CD4+ T cells in the same group of patients. Reduction in tumor burden/regulatory factors by embolisation may explain in part the observed concomitant expansion of AFP-specific Th1 and reduction of $AFP_{46-55}$ $CD4^+$ T cells.

In conclusion, we have identified and characterized self-antigen specific $CD4^+$ T cells from HCC patients and healthy donors. Their cytokine profile, phenotype and functional characteristics suggest that these cells are antigen-specific Th3 cells and recognize an AFP peptide as a natural ligand. Here we show that tumor may stimulate the expansion of AFP-specific T cells and the removal of antigen source reduces the frequency of cells *in vivo*. These results will be instrumental in the development of peptide-based immunotherapy for treatment of cancer as well as autoimmune disease.

# CHAPTER 11: EXPANSION OF ANTI-AFP Th1 AND Tc1 RESPONSES IN HEPATOCELLULAR CARCINOMA OCCUR IN DIFFERENT STAGES OF DISEASE

## 11.1 Abstract

AFP is a tumour-associated antigen in HCC and is a target for immunotherapy. However, there is little information on the pattern of CD4 (Th1) and CD8 (Tc1) T-cell response to AFP in patients with HCC and their association with the clinical characteristics of patients. We therefore analysed CD4 and CD8 T-cell responses to a panel of AFP-derived peptides in a total of 31 HCC patients and 14 controls, using an intracellular cytokine assay for IFN-gamma. Anti-AFP Tc1 responses were detected in 28.5% of controls, as well as in 25% of HCC patients with Okuda I (early tumour stage) and in 31.6% of HCC patients with stage II or III (late tumour stages). An anti-AFP Th1 response was detected only in HCC patients (58.3% with Okuda stage I tumours and 15.8% with Okuda stage II or III tumours). Anti-AFP Th1 response was mainly detected in HCC patients who had normal or mildly elevated serum AFP concentrations ($P$=0.00188), whereas there was no significant difference between serum AFP concentrations in these patients and the presence of an anti-AFP Tc1 response. A Th1 response was detected in 44% of HCC patients with a Child–Pugh A score (early stage of cirrhosis), whereas this was detected in only 15% with a B or C score (late-stage cirrhosis). In contrast, a Tc1 response was detected in 17% of HCC patients with a Child–Pugh A score and in 46% with a B or C score. These results suggest that anti-AFP Th1 responses are more likely to be present in patients who are in an early stage of disease (for both tumour stage and liver cirrhosis), whereas anti-AFP Tc1 responses are more likely to be present in patients with late-stage liver cirrhosis. Therefore, these data provide valuable information for the design of vaccination strategies against HCC.

## 11.2 Introduction

HCC overexpresses several tumour-associated antigens [359]. Some of these antigens, such as MAGE, Glypican-3 and NY-ESO-1, are also expressed by many other types of cancer cells [215, 220], whereas $\alpha$-fetoprotein (AFP) is specific to HCC and testicular carcinoma. The synthesis of AFP decreases dramatically after birth and only trace amounts are expressed in the adult liver. During liver regeneration and hepatocarcinogenesis, expression of the *AFP* gene is reactivated in adults [360-361], with the majority of HCC patients showing an increase in serum AFP levels. The induction of an anti-AFP cell-mediated immune response can control tumour growth in animal models [213]. In humans, it has been shown that B and T cells can recognise peptide epitopes within the AFP sequence and can develop into effector and/or regulatory lymphocytes. Several AFP-derived HLA class I (HLA-A2 and HLA-A24) and class II (HLA-DR) restricted CD4 (Th1) and CD8 (Tc1) epitopes have been identified [236, 272-273, 331] [238, 244] [229] [230] [237]. The results from several studies show that the detection of AFP-specific CD8 T cells is restricted to HCC patients, but does not correlate with elevated serum AFP concentrations, vascular invasion or type of viral infection (Butterfield et al., 2007; Ritter et al., 2004; Thimme et al., 2008). In contrast, we have shown that CD4 T-cell responses to an immunodominant AFP-derived peptide are only detectable in HCC patients, and this response is mainly detected in patients with low serum AFP concentrations and early-stage disease [238]. To address these conflicting results, we analysed Th1 and Tc1 responses in parallel in the same group of patients/controls. Our findings demonstrate that a Th1 response is only detectable in HCC patients, whereas a Tc1 response is detectable in both HCC and controls. Moreover, these results suggest that anti-AFP Th1 responses are more likely to be present in an early tumour stage or liver cirrhosis, whereas anti-AFP Tc1 responses are more likely to be present in patients with more severe liver cirrhosis.

## 11.3 Materials and methods

### *11.3.1 Synthetic peptides*

Peptides corresponding to the sequence of AFP were purchased from Mimotopes Pty Ltd. (Clayton Victoria, Australia)

*11.3.2 Patients*

This study was approved by ethical committees and all patients gave written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of patients with HCC or from healthy donors.

*11.3.3 Expansion of T cells in vitro*

The RPMI 1640 medium, MEM medium, penicillin and streptomycin and 10% heat-inactivated FCS were purchased from Life Technologies (Grand Island, NY, USA). PBMCs were re-suspended at a concentration of 1.5 × 106 per ml in MEM, 10% FCS (Life Technologies), and stimulated with individual peptides (1 μM) or peptide pools in 96-well plates. Recombinant IL-2 (50 IU ml−1) was added on day 3 of culture, and cells were analysed after a total of 10–12 days of culture.

*11.3.4 Intracellular IFN-gamma staining*

Expanded T cells were incubated for 5h at 37°C at $1 \times 10^6$ cells per ml in MEM, 10% FCS with AFP-derived peptides (1$\mu$M) and in the presence of Brefeldin A (10$\mu$g/ml$^{-1}$ Sigma-Aldrich, St Louis, MO, USA). Cells were surface stained with Cy-chrome-conjugated anti-CD4 or anti-CD8 antibodies (BD PharMingen, Cowley, UK). The cells were then fixed and permeabilised using Cytofix/Cytoperm (BD PharMingen), and stained for intracellular cytokines with FITC-conjugated anti-IFN$\gamma$ or isotype control (R&D Systems, Abingdon, UK), washed twice and analysed by flow cytometry. The expansion of T cells did not induce non-specific (no peptide) IFN-*gamma*-producing T cells and all background levels (no peptide or irrelevant peptide) were between 0.1 and 0.5%. An immunological response/responder was defined as a five-fold increase in the frequency of cytokine-producing cells above control peptides/no peptide.

*11.3.5 AFP measurement*

Concentrations of serum AFP were measured using a microparticle enzyme immunoassay kit obtained from Abbott Laboratories (Abbott Park, IL, USA) and carried out according to the manufacturer's instructions. In brief, anti-AFP microparticles were incubated with the blood specimen, and an aliquot of the reaction mixture was transferred to the matrix cell. The matrix cell was washed, and the anti-

AFP conjugate was added to it. The substrate was then added to the matrix cell and the fluorescent product was measured by the microparticle enzyme immunoassay optical assembly.

*11.3.6 Statistical analysis*

The Mann–Whitney *U*-test was used to compare the concentration of serum AFP in HCC patients and the ability of T cells to respond to the identified epitope (responder *vs* non-responder). The $\chi^2$ test was used to compare whether the percentage of CD4 T-cell responders was predominantly in patients with Okuda tumour stage I or II+III. Statistical significance was defined as $P \leq 0.05$.

## 11.4 Results

*11.4.1 An anti-AFP Tc1 response was detected in controls and HCC patients, whereas an anti-AFP Th1 response was detected only in HCC patients*

Peripheral blood mononuclear cells were isolated from a total of 31 patients with HCC (HCC01-HCC31), from 8 patients with cirrhosis (LC01-08) and from 6 healthy individuals (NC01-06) (Tables 11-1&2). T cells were expanded in culture in the presence of AFP-derived peptide pools (Table 11-3). The reactivity of T-cell lines to relevant and irrelevant peptide pools was analysed using an intracellular cytokine assay for IFN-gamma after 10–12 days of culture (Figure 11-1a). An anti-AFP CD4 T-cell response was detected in 32% of HCC patients (10 out of 31), whereas no CD4 T-cell response was detected in controls. In contrast, an anti-AFP CD8 T-cell response was detected in 29% of HCC patients (9 out of 31) and in 29% of the control group (4 out of 14) (Figure 11-1b). The presence of both a CD4 and a CD8 T-cell response to AFP peptides was detected in three HCC patients (Figure 11-1c).

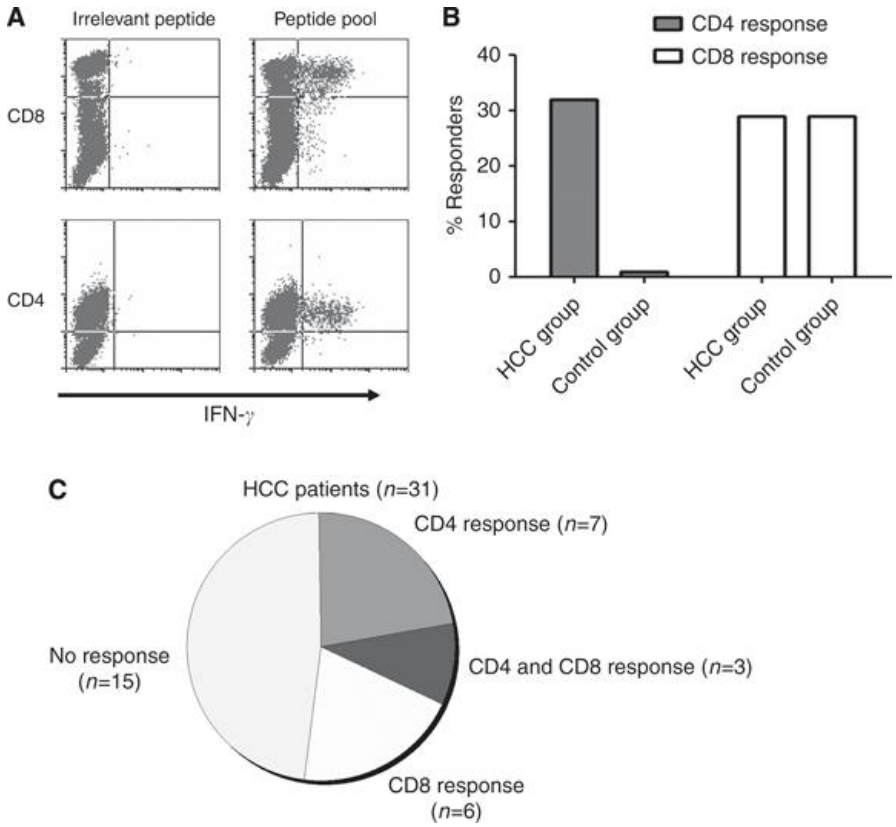



Figure 11-1: Anti-AFP CD4 T-cell responses are only detected in HCC patients. (**A**) Peptide-specific intracellular IFN-γ production by CD4 and CD8 T cells was determined using flow cytometry. Representatives of CD4 and CD8 T-cell responses against AFP-derived peptides are shown. (**B**) The percentages of HCC patients or controls with CD4 or/and CD8 T cells reacting to AFP-derived peptides are shown. (**C**) The number of HCC patients with or without anti-AFP CD4, CD8 and CD4 and CD8 T-cell responses is presented.

| Sample ID | Gender[a] | Age | Cirrhosis | Tumour | Hepatitis | Okuda[b] | AFP[c] (ng/ml) |
|---|---|---|---|---|---|---|---|
| HCC01 | M | 62 | + | Multifocal | HCV | III | 180 |
| HCC02 | M | 62 | + | Single | HCV | I | 8 |
| HCC03 | M | 60 | + | Single | HBV/HCV[d] | II | 200 |
| HCC04 | M | 53 | + | Single | HBV/HCV | II | 37 |
| HCC05 | M | 63 | + | Multifocal | HCV | I | 64 |
| HCC06 | M | 66 | + | Multifocal | HCV | I | 32 |
| HCC07 | M | 66 | + | Multifocal | HCV | I | 10 |
| HCC08 | M | 55 | + | Multifocal | - | II | 145 |
| HCC09 | M | 66 | + | Multifocal | HBV | I | 8 |
| HCC10 | M | 52 | + | Single | HBV | II | 48 |
| HCC11 | M | 55 | - | Multifocal | - | I | 60 |
| HCC12 | M | 63 | + | Diffuse | - | I | 13077 |
| HCC13 | M | 35 | + | Multifocal | HBV/HCV | III | 96730 |
| HCC14 | M | 59 | + | Single | - | I | 41930 |
| HCC15 | F | 32 | + | Multifocal | HBV | II | 31 |
| HCC16 | F | 58 | + | Multifocal | HBV/HCV | I | 14 |
| HCC17 | M | 67 | + | Multifocal | HBV | II | 10 |
| HCC18 | M | 32 | + | Multifocal | - | I | 8 |
| HCC19 | F | 71 | + | Multifocal | HBV | II | 813500 |
| HCC20 | M | 79 | + | Multifocal | HCV | III | 70 |
| HCC21 | M | 61 | - | Diffuse | HCV | II | 1234 |
| HCC22 | M | 61 | + | Multifocal | - | III | 5 |
| HCC23 | M | 62 | + | Multifocal | HBV/HCV | II | 1501 |
| HCC24 | M | 67 | + | Diffuse | HBV | II | 188 |
| HCC25 | M | 84 | + | Single | HCV/HBV | I | 1 |
| HCC26 | F | 67 | + | Diffuse | HBV | II | 12432 |
| HCC27 | M | 62 | + | Single | HBV/HCV | II | 165 |
| HCC28 | F | 46 | + | Single | HBV/HCV | II | 10 |
| HCC29 | M | 54 | + | Single | HCV | II | 14 |
| HCC30 | M | 53 | + | Multifocal | HCV | II | 157 |
| HCC31 | M | 58 | + | Multifocal | HCV | I | 108 |

Table 11-1: Hepatocellular carcinoma (HCC) patients evaluated in this study.

[a] F, Female, M, Male
[b] Okuda is clinical staging systems for hepatocellular carcinoma (HCC)
[c] AFP: Alpha-fetoprotein
[d] HBV and HCV: Hepatitis B and C virus

| Variables | All Controls ($n$ = 14) |
|---|---|
| Patients with liver cirrhosis $n$ =8 | |
| Median Age (years) | 56.5 (34-75) |
| Patients with HCV or HBV/ others | 4/4 |
| Median AFP (ng/ ml) | 9.5 (4-11) |
| Healthy donors $n$ =6 | |
| Median Age (years) | 35.8 (23-42) |
| AFP (ng/ ml) | Unknown |

Table 11-2: Clinical characteristics of the controls.

| Amino Acid Start | Sequence | Amino Acid Start | Sequence |
|---|---|---|---|
| 1 | MKWVESIFL | 350 | FLASFVHEY |
| 8 | FLIFLLNFT | 365 | QLAVSVILRV |
| 11 | FLLNFTESRT | 372 | RVAKGYQEL |
| 20 | TLHRNEYGI | 379 | ELLEKCFQT |
| 30 | SILDSYQCTA | 385 | FQTENPLEC |
| 35 | YQCTAEISL | 410 | ALAKRSCGL |
| 37 | CTAEISLADL | 419 | FQKLGEYYL |
| 40 | EISLADLATI | 427 | LQNAFLVAYT |
| 46 | LATIFFAQFV | 431 | FLVAYTKKA |
| 54 | FVQEATYKEV | 441 | QLTSSELMAI |
| 65 | KMVKDALTAI | 449 | AITRKMAAT |
| 70 | ALTAIEKPT | 453 | KMAATAATCC |
| 86 | CLENQLPAFL | 462 | CQLSEDKLL |
| 89 | NQLPAFLEEL | 468 | KLLACGEGA |
| 125 | FLAHKKPTPA | 475 | GAADIIIGHL |
| 137 | PLFQVPEPV | 485 | CIRHEMTPV |
| 140 | QVPEPVTSC | 489 | EMTPVNPGV |
| 158 | FMNKFIYEI | 492 | PVNPGVGQC |
| 164 | YEIARRHPFL | 498 | GQCCTSSYA |
| 172 | FLYAPTILL | 507 | NRRPCFSSLV |
| 179 | LLWAARYDKI | 514 | SLVVDETYV |
| 187 | KIIPSCCKA | 531 | FIFHKDLCQA |
| 217 | SLLNQHACAV | 536 | DLCQAQGVAL |
| 235 | FQAITVTKL | 542 | GVALQTMKQ |
| 249 | KVNFTEIQKL | 545 | LQTMKQEFLI |
| 277 | CLQDGEKIM | 548 | MKQEFLINL |
| 298 | KITECCKLTT | 555 | NLVKQKPQI |
| 306 | TTLERGQCII | 562 | QITEEQLEAV |
| 325 | GLSPNLNRFL | 570 | AVIADFSGL |
| 343 | SSGEKNIFL | 576 | SGLLEKCCQ |
| 347 | KNIFLASFV | 598 | KLISKTRAAL |

Table 11-3: AFP-derived peptides

To determine the reacting peptide(s) within the peptide pools, expanded T cells were treated with individual peptides of the reacting pools, and peptide-specific intracellular IFN-γ production by CD4 and CD8 T cells was analysed using flow cytometry (Figure 11-2). Among the responders, PBMCs were available from LC01, LC03, NC02, HCC06, HCC20 and HCC25 for further studies. Dot plots of anti-AFP-specific IFN-gamma-producing CD4 or CD8 T cells from these individuals are shown. These results suggest that Th1 and Tc1 responses are multi-specific.

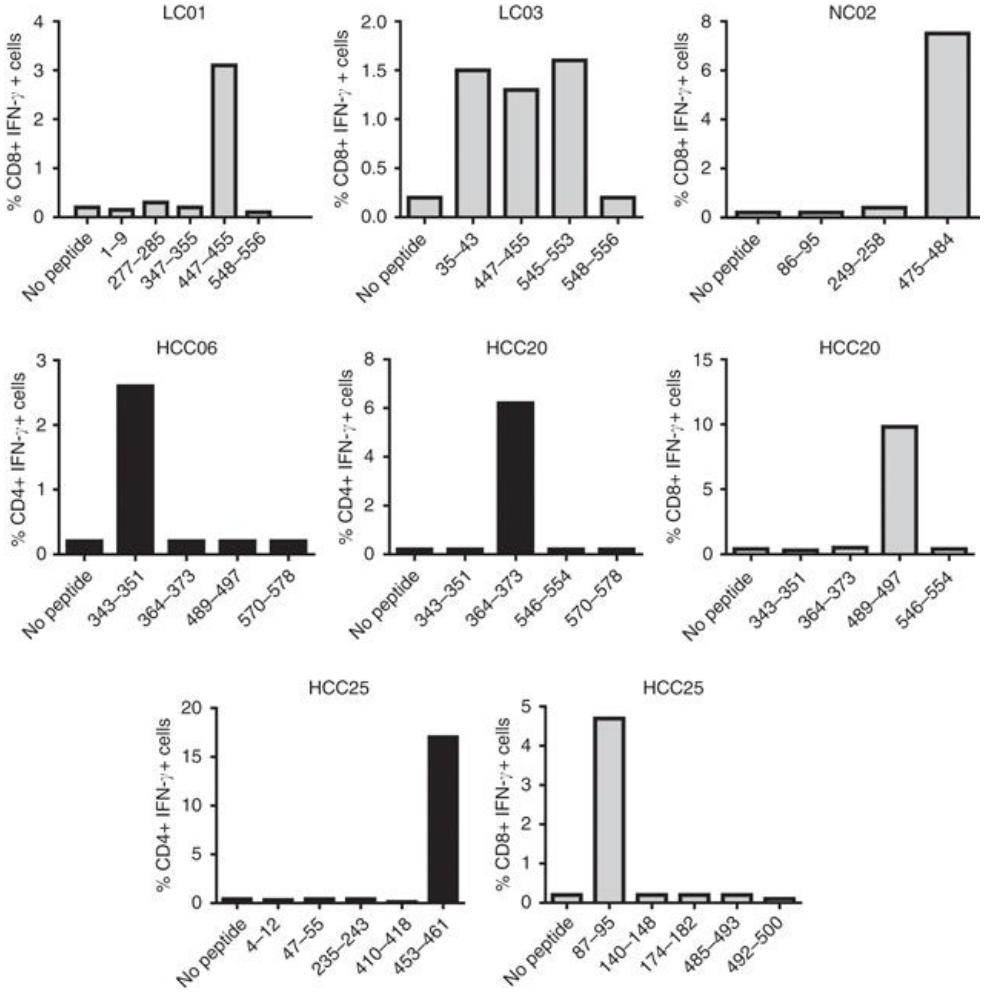



Figure 11-2: Multi-specific CD4 and CD8 T-cell responses in HCC patients and controls. Expanded T cells were incubated with indicated AFP peptides and peptide-specific intracellular IFNγ was analysed using flow cytometry. Naturally occurring CD4 and CD8 T-cell responses against individual AFP-derived peptides were analysed in three patients with HCC and in three controls. The percentages of peptide-specific CD4 (black) or CD8 (grey) T-cell responses to individual peptides are shown.

*11.4.2 Anti-AFP Th1 responses are mainly detected in an early tumour stage*

An AFP-specific CD4 T-cell response was mainly detected in HCC patients with normal or mildly elevated serum AFP ($P$=0.0188, Mann–Whitney $U$-test). In contrast, there was no significant difference in AFP concentrations between CD8 T-cell responders and non-responders (Figure 11-3a&c). Median serum AFP concentrations were 7.5-fold higher in HCC patients who lacked a CD4 T-cell response (non-responders) than in those who had a CD4 T-cell response (responders) (Figure 11-3b). There was also a significant difference in the number of patients with Okuda tumour stage I *vs* those with stage II or III in CD4 T-cell responders ($\chi^2$, $P$=0.04; 95% confidence intervals), whereas there was no difference in the number of patients with Okuda tumour stage I *vs* stage II or III in CD8 or total T-cell responders. This indicates that patients in stage II or III are significantly less likely to have an anti-AFP Th1 response, whereas anti-AFP Tc1 responses remain unchanged.

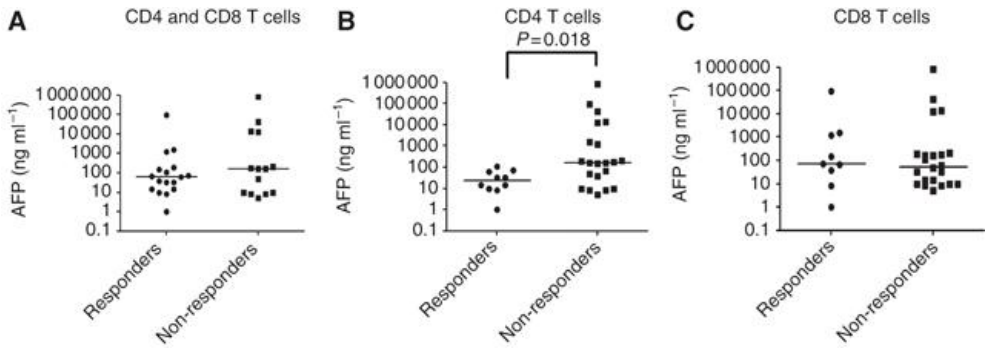



Figure 11-3: HCC patients with anti-AFP CD4 T-cells responses have significantly lower levels of serum AFP. Serum AFP levels in HCC patients with or without (A) anti-AFP total T-cells responses (both CD4 and CD8), (B) CD4 T-cell responses or (C) CD8 T-cell responses are shown. Anti-AFP CD4 T-cell response was detected in patients with low or moderately elevated serum AFP (n¼0.018). Median of serum AFP levels for responders and non-respondersare presented and each dot represents a patient with HCC.

HCC patients with anti-AFP CD4 T-cells responses have significantly lower levels of serum AFP. Serum AFP levels in HCC patients with or without (**A**) anti-AFP total T-cells responses (both CD4 and CD8), (**B**) CD4 T-cell responses or (**C**) CD8 T-cell responses are shown. Anti-AFP CD4 T-cell response was detected in patients with low or moderately elevated serum AFP ($n$=0.018). Median of serum AFP levels for responders and non-responders are presented and each dot represents a patient with HCC.

Overall, the results demonstrate that CD8 T-cell responses to AFP are detectable in control groups (28.5%), as well as in Okuda tumour stage I HCC patients (25%) and Okuda tumour stage II+III HCC patients (31.6%). In contrast, AFP-specific CD4 T-cell responses are only detected in HCC patients and response was mainly detected in Okuda tumour stage I HCC patients (58.3%) and to a lesser extent in Okuda tumour stage II or III HCC patients (15.8%) (Figure 11-4b).

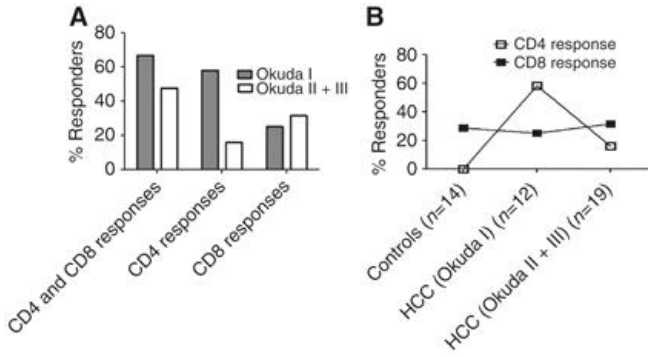



Figure 11-4: Anti-AFP CD4 T-cell response is more likely to be detected in HCC patients with early stage of disease. (A) The percentages of anti-AFP T-cell response (both CD4 and CD8), CD4 T-cell response or CD8 T-cell response are shown in HCC patients with different stage of disease (Okuda I *vs* Okuda II+III). (B) The percentages of anti-AFP CD4 or CD8 T-cell responses in controls, HCC patients with Okuda I and HCC patients with Okuda II+III are shown**.**

### *11.4.3 The balance between anti-AFP Th1 and Tc1 responses switches to an anti-AFP Tc1 response as liver cirrhosis progresses*

Child–Pugh grading (CP)is the most commonly used method to evaluate liver cirrhosis and liver function in HCC patients, and is used to assess prognosis, evaluate the required strength of treatment required and determine the necessity for surgical intervention. Of the 31 HCC patients assessed, 18 were classified as having a Child–Pugh A score and 13 patients were classified as having a Child–Pugh B or C score. We analysed the presence of AFP-specific CD4 and CD8 T-cell responses in HCC patients with these different Child–Pugh scores. Just over 44% of HCC patients with a Child–Pugh A score had a CD4 T-cell response (8 out of 18), whereas only 15% of HCC patients with a Child–Pugh B or C score had as CD4 T-cell response (2 out of 13), suggesting that an AFP-specific Th1 response is mainly detected in HCC patients with a Child–Pugh A score (Figure 11-5). A CD8 T-cell response was detected in 17% of HCC patients with a Child–Pugh A score (3 out of 18), and in 46% of HCC patients

with a Child–Pugh B or C score (6 out of 13)(Figure 11-5). These results suggest that, in contrast to a Th1 response, a Tc1 response is mainly detected in HCC patients with a Child–Pugh B or C score. However, total T-cell response (CD4 and CD8) was detected in 50 (9 out of 18) and 53% (7 out of 13) of HCC patients with a Child–Pugh A score and a Child–Pugh B+C score, respectively. Taken together, these results suggest that the balance between anti-AFP Th1 and Tc1 responses switches to an anti-AFP Tc1 response as liver cirrhosis progresses.

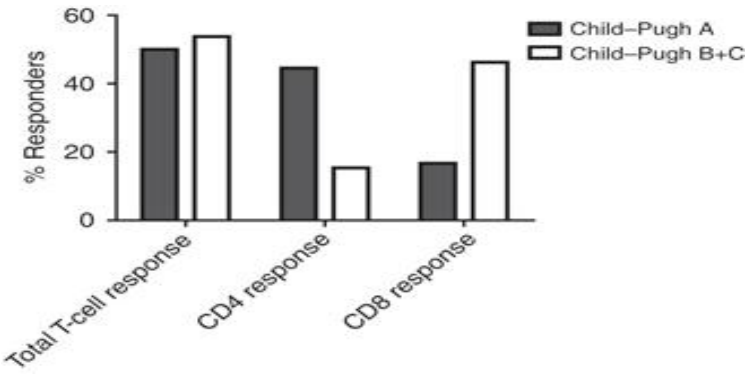



Figure 11-5: Th1 response is mainly detected in HCC patients with Child–Pugh A grading. The percentages of anti-AFP T-cell response (both CD4 and CD8), CD4 T-cell response or CD8 T-cell response are shown in HCC patients with different Child–Pugh scores (A or B+C).

### 11.5 Discussion

There are contrasting reports on the presence of anti-AFP T-cell responses and their association with clinical characteristics of patients, such as serum AFP concentration and the stage of disease in HCC patients [233, 238] [231, 237, 239, 362]. We hypothesised that these contrasting reports may be due to a difference in the presence of anti-AFP CD4 and CD8 T-cell responses over the course of disease. A concomitant analysis of anti-AFP CD4 and CD8 T-cell responses in the same group of patients showed that anti-AFP CD4 T-cell responses are present in HCC patients but not in controls, whereas anti-AFP CD8 T-cell responses are present in both HCC patients and controls. CD4 T cells from HCC patients reacted to a wide range of peptide pools, suggesting that the anti-AFP CD4 T-cell response is multi-specific (data not shown). However, owing to the limited number of cells isolated from HCC patients, we were unable to analyse the responses to individual peptides in all patients with a T-cell response. For the same reason, we were also unable to analyse the *ex vivo* responses in

these groups of patients. However, we have previously reported that AFP-specific CD4 T-cell responses are not detectable *ex vivo* [237].

In this study, we used a panel of 9–10-mer peptides that are normally expected to bind to MHC class I molecules. However, we showed that 10 mer AFP-derived peptides can also be presented by MHC class II molecules, and that CD4 T cells could respond equally well to 14- or 10-mer peptides and produce IFN-gamma [238]. AFP-specific Th1 cells may produce IFN-gamma, IL-2 and TNF-alpha [238]. Interleukin-2 is often associated with a memory response, and it can be produced by both antigen-specific CD4 and CD8 T cells. In our experience, not all AFP-specific IFN-γ producing T cells produced detectable intracellular IL-2. Thus, we decided to analyse the expansion of antigen-specific Th1 cells that are characterised by IFN-gamma production.

It has been suggested that healthy donors have a weak response to the whole AFP protein and this response is only detectable when the antigen is presented by DCs [362]. The presentation of peptide epitopes to T cells by DCs has been shown to amplify the underlying response and improve the sensitivity of assays [363]. This indicates that a weak anti-AFP Th1 response is present in healthy donors and that the detection of this response would require professional antigen-presenting cells. Accordingly, we were able to detect IFN-gamma-producing AFP-specific CD8 T cells in healthy donors. In contrast to our finding, [229] were unable to detect this response in healthy donors. This discrepancy could be explained by the methods used in different studies, with *ex vivo* assays detecting effector and expanded T cells detecting memory populations. Furthermore, a comprehensive and careful analysis of anti-AFP CD8 T-cell responses by [231] demonstrated that anti-AFP CD8 T-cell responses are detectable in controls and in HCC patients [231].

The results from several studies support the fact that the presence of AFP-specific CD8 T cells does not correlate with clinical features of disease such as elevated serum AFP concentrations [239] [231, 233]. Moreover, no association has been observed between the concentration of serum AFP in HCC patients and the presence of CD8 T-cell responses to non-AFP tumour-associated antigens, such as NY-ESO-1 [224]. Our results support these findings and demonstrate that anti-AFP Tc1 responses are detected in all groups of patients. In contrast, anti-AFP Th1 responses are more likely to be detected in HCC patients in an early stage of disease and in cases in which the

concentration of serum AFP is low. This suggests that there is a difference in the activation of anti-AFP CD4 *vs* CD8 T cells in HCC patients, with a CD4 T-cell response expanding in early stages of disease, which is usually associated with low concentrations of serum AFP, and with exhaustion of this response in later stages of disease in which there is a high concentration of serum AFP. This is in accordance with our earlier reports showing that high concentrations of AFP suppress immune cell function *in vitro* [245], and CD4 T cells isolated from HCC patients with high concentrations of serum AFP are impaired [238]. Our results demonstrate that Th1 response was mainly detected in HCC patients with a Child–Pugh A score and Tc1 response was largely detected in HCC patients with a Child–Pugh B or C score. To our knowledge, this is the first report suggesting that the balance between anti-AFP Th1 and Tc1 responses switches to an anti-AFP Tc1 response as liver cirrhosis progresses. However, it is not clear why Tc1 response is preferentially expanded in patients with severe liver cirrhosis.

In conclusion, these results suggest that anti-AFP Th1 responses are more likely to be present in an early disease stage, whereas anti-AFP Tc1 responses are more likely to be present when liver cirrhosis has developed. We believe that the results presented in this paper will advance our understanding of anti-tumour immune response in HCC patients and will therefore have important implications for the development of vaccines or vaccination strategies.

## CHAPTER 12: CONCLUSION

The rising incidence of HCC world wide has necessitated a better understanding of the risk factors and molecular pathogenesis implicated in the development of HCC. The rise in HCV, ALD and NASH related HCC will undoubtedly further increase the disease burden and require better early disease prevention and treatment.

The introduction of surveillance programmes for at risk populations allows for early diagnosis of HCC. Improvement in HCC staging and linking disease stage to treatment modality facilitates improved prediction of prognosis.

Although LR and LT offer the best opportunity of cure in patients with early HCC, recent evidence suggests that RFA may offer comparable survival results. For patients who cannot be offered curative options, the implementation of embolisation therapy and sorafenib may improve survival. There remains a pressing need for increasing research into newer directed therapies for HCC, along with the potential use of combination therapy and immunotherapy.

As the immune system plays a crucial role in the control of tumour growth in animal models, it has been suggested that the expansion of anti-tumour effector responses and/or removal of regulatory cells may enhance the effectiveness of anti-tumour immunity. This notion is supported by the fact that tumour cells suppress the function of the immune system cells and this may dampen the effectiveness of immunotherapy vaccines [245].

We revealed that the treatment of monocyte-derived DCs with AFP led to DCs dysfunction and induced their apoptosis. This indicated that AFP has a negative effect on anti tumour immunoprotective cells. Our identification of an AFP-derived T cell epitope which was recognized by circulating CD4+ T cells from patients with HCC was a significant step in supporting the immunoprotective role of CD4+ T cells. It is noted that the induction T-regs by tumours may lead to tumour escape from immunological protective response. We have demonstrated that AFP contained an epitope which activated the expansion of inducible T-regs. We observed that CD4 T-cell response expanded in patients with HCC, at an early stage of disease with Child–Pugh A score and with low concentrations of serum AFP. Furthermore, CD8 T-cell response was largely detected in HCC patients with Child–Pugh B or C score. It is noted that necrosis

of tumour cells can activate both innate and adaptive antitumor immunity. HCC embolisation therapy increases the frequency of anti-tumour T cells [237] and retracts the number of tumour-specific regulatory T cells in HCC patients [244]. We have therefore proposed to combine embolisation therapy and T cell-based immunotherapy targeting both CD4 and CD8 T cell responses [228] to improve survival in patients with HCC [237-238].

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## PUBLICATIONS ARISING FROM THIS THESIS