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**Phenotypic and Functional Evaluation of the Intra-hepatic Populations of  
Lymphocytes during Hepatocellular Carcinoma**

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## ABBREVIATIONS

AFB1:	aflatoxin B1
AFP:	alpha fetoprotein
AFU:	Alpha-l-Fucosidase
Ag:	antigen
ALT:	alanine amino transferase
ALT:	alanine transaminase
APC:	antigen presenting cell
CCL:	chemokine (C-C motif) ligand
CDK:	cyclin-dependent kinase
CLD:	chronic liver disease
CSIF:	cytokine synthesis inhibitory factor
CTA:	cancer testis antigen
CTA:	Cancer-testis antigen
CTL:	cytotoxic T lymphocytes
CX3CL:	chemokine (C-X3-C motif) ligand
CXCL:	chemokine (C-X-C motif) ligand
DC:	dendritic cell
DCP:	Des-gamma-CarboxyProthrombin
EASL:	European Association for the Study of the Liver
ERK:	extracellular signal-regulated protein kinase
GGTII:	Gamma-Glutamyl Transferase
GPC3:	Glypican-3
HBV:	hepatitis B virus
HCC	hepatocellular carcinoma
HCV:	hepatitis C virus
HGF:	hepatocyte growth factor
HIV:	human immunodeficiency virus
HLA:	human leukocyte antigen
HPRT:	Hypoxanthine Phosphoribosyl Transferase
HSC:	hepatic stellate cells
Hsp:	heat shock protein
hTERT:	Human Telomerase Reverse Transcriptase
ICAM:	intra-cellular adhesion molecule
IEL:	intraepithelial lymphocytes
IFN:	interferon
Ig:	immunoglobulin
IGF:	insulin-like growth factor
IHC:	ImmunoHistoChemistry
IL:	interleukin
IP:	inducible protein
IPP:	intraepithelial lymphocytes
IRES:	internal ribosome entry site
IRF:	Interferon Response Factor
LAK:	Lymphokine-activated killer cell
LDL:	Low Density Lipoproteins
LIL:	liver infiltrating lymphocytes
MCP:	monocyte chemotactic protein

MDC:	myeloid dendritic cell
MHC:	major histocompatibility complex
MIG:	monokine induced by gamma interferon
MTP:	microsomal triglyceride transfer protein
NK:	natural killer cell
NKT:	natural killer T cell
NS:	Non Structural proteins
NTR:	non-translated region
PBMC:	peripheral blood mononuclear cells
PDC:	plasmacytoid dendritic cell
PEG:	Poly Ethylen Glycol
PK:	protein kinases
PT:	prothrombin time
qRT- PCR:	quantitative real-time polymerase chain reaction
RANTES:	regulated upon activation normal T-cell expressed and secreted
SDF:	stromal cell-derived factor
SELDI:	Surface Enhanced Laser Desorption Ionization
SOCS:	Suppressor of Cytokine Signaling
TAA:	tumor associated antigen
TCR:	T cell receptor
TGF:	transforming growth factor
TGF:	Transforming Growth Factor
Th:	T helper lymphocyte
TIL:	tumor infiltrating lymphocytes
TNF:	tumor necrosis factor
TRAIL:	TNF-related apoptosis inducing ligand
Treg:	regulatory T lymphocytes
TSGF:	tumor specific growth factor
VAP:	vascular adhesion protein
VCAM:	vascular adhesion molecule
VEGF:	vascular endothelial growth factor
VLDL:	Very Low Density Lipoproteins
XCL:	chemokine (C motif) ligand



## Objectives of Work

Chronic hepatitis C is always accompanied by a massive lymphocyte infiltration in the liver which goes on increasing in the patients who develop HCC afterwards. These infiltrated lymphocytes compartmentalize principally in the portal/periportal tracts, lymphoid aggregates and in necro-inflammatory lesions. Among these lymphocytes, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes are more abundant than other types. Majority of the research work has been focused on characterization and functions of T lymphocytes. In the recent years, some work has also been done on NK cells and dendritic cells. However, the role of host immune response in the physiopathology of chronic HCV infections and HCC still remains to be defined. Generally, work on liver and tumor infiltrating cells is rare and fragmentary.

The main objective of our work was to generate a global phenotypic & functional picture of liver and tumor infiltrating lymphocytes and to assess the cytokines and chemokines profiles in the liver and tumor by using immunohistochemistry, flow cytometry and RT-PCR. Our second objective was to find out correlations among the immune cells, cytokines/chemokines profiles, presence and absence of HCV and presence and absence of tumor in these diseases.

This was completed by the following steps;

- i. Phenotypic characterization of liver and tumor infiltrating lymphocytes by quantitative immunohistochemistry and flow cytometry during the course of viral hepatitis and HCC.
- ii. Functional characterization of liver and tumor infiltrating lymphocytes, cytokines and chemokines status by RT-PCR during the course of disease.
- iii. Identification of new immune and tumor markers in the liver infiltrating lymphocytes and tumors of HCC patients by SELDI-TOF MS.

Certain clinical and histopathological features of chronic HCV and HCC, such as age, ALT, AFP, tumor type/stage, tumor size, tumor proliferation index and recurrence were also taken under consideration. These factors allow the clinicians to ensure the better management/treatment of the patients.

# **INTRODUCTION**

## **Introduction**

Hepatocellular carcinoma (HCC) is a common malignancy affecting approximately one million of people around the world every year and represents the fifth most common cancer worldwide with an incidence equal to the death rate (Marrero 2005; Motola-Kuba, Zamora-Valdes et al. 2006; Zerbini, Pilli et al. 2006). The incidence of HCC is low in the occidental world and high in Southeast Asia and sub-Saharan Africa. However, it has been rising in the last two decades in Europe, United States and Japan (Jemal, Murray et al. 2005; Motola-Kuba, Zamora-Valdes et al. 2006). Approximately 18,000 new cases of HCC are projected to occur in the United States in 2005 (Jemal, Murray et al. 2005; Jemal, Siegel et al. 2008), however, the incidence of HCC has doubled during the period from 1975 to 1995 and continued to rise through 1998 (El-Serag 2002; El-Serag, Davila et al. 2003). This trend is expected to continue because of the estimated 4,000,000 hepatitis C–seropositive individuals in the United States and the known latency of HCC development from the initial hepatitis C (HCV) infection, which may take two to three decades (El-Serag and Mason 1999). It is also known that non-alcoholic fatty liver disease–associated cirrhosis is increasing in the United States (Loguercio, De Simone et al. 2004; Ruhl and Everhart 2004). In France, the most striking increase in incidence was seen for primary liver cancer with an increase from 2000 incident cases in 1980 to nearly 6000 in 2000 (Lepage, Remontet et al. 2008). Reports from different countries suggest that the incidence of HCC is increasing, probably as a consequence of the increased prevalence of HCV infection, although increased alcohol consumption may be significant (El-Serag and Mason 1999). In 2001, around 4000-5000 deaths related to HCV and HBV infection occurred in France. Alcohol consumption and HIV infection were important co-factors. These data emphasize the need for ongoing, efficient public health programs that include screening, management and counseling for HCV and HBV infected individuals (Marcellin, Pequignot et al. 2008).

Although hepatic resection, transplantation, chemoembolization, ethanol injection and thermal ablation are available therapies for patients with HCC, the probability of residual tumor cells in the liver originating from intrahepatic metastasis results in a poor prognosis for patients after treatment (Geissler, Mohr et al. 2003). Hence, new treatment strategies such as immunotherapy for patients with HCC are urgently required.

Accordingly, the major clinical risk factor for the development of HCC is liver cirrhosis since 70–90% of HCCs develop into a cirrhotic liver. Most HCCs occur after many years of chronic hepatitis that provides the mitogenic and mutagenic environments to precipitate random genetic alterations resulting in the malignant transformation of hepatocytes and HCC development (Blum 2005). In most cases, HCC is asymptomatic and has a low life expectancy.

## **Hepatitis.**

Hepatitis implies injury to the liver characterized by the presence of inflammatory cells in the liver tissue. Hepatitis is “*acute*” when it lasts less than six months and “*chronic*” when it persists longer. A group of viruses known as the hepatitis viruses cause most cases of liver damage worldwide. Hepatitis can also be due to toxins (notably alcohol), other infections or from autoimmune.

### *Acute hepatitis:*

Clinically, the course of acute hepatitis varies widely from mild symptoms requiring no treatment to fulminant hepatic failure needing liver transplantation. Acute viral hepatitis is more likely to be asymptomatic in younger people. Symptomatic individuals may present after convalescent stage of 7 to 10 days, with the total illness lasting 2 to 6 weeks (Bain and M. Ma 2005). Initial features are of nonspecific flu-like symptoms, common to almost all acute viral infections and may include malaise, muscle and joint aches, fever, nausea or vomiting, diarrhoea and headache. More specific symptoms, which can be present in acute hepatitis from any cause, are: profound loss of appetite, aversion to smoking among smokers, dark urine, yellowing of eyes and skin (i.e., jaundice) and abdominal discomfort (Ryder and Beckingham 2001).

### *Chronic Hepatitis & Hepatitis C:*

Majority of patients will remain asymptomatic or mildly symptomatic, abnormal blood tests being the only manifestation. Features may be related to the extent of liver damage or the cause of hepatitis. Jaundice can be a late feature and may indicate extensive damage. Other features include abdominal fullness from enlarged liver or spleen, low grade fever and fluid retention (ascites). Extensive damage and scarring of liver (i.e., cirrhosis) lead to weight loss, easy bruising and bleeding tendencies.

Hepatitis C is a blood-borne infectious disease that is caused by the hepatitis C virus, affecting the liver. The infection is often asymptomatic, but once established, chronic infection can cause inflammation of the liver (chronic hepatitis). This condition can progress to scarring of the liver (fibrosis) and advanced scarring (cirrhosis). In some cases, those with cirrhosis will go on to develop liver failure or other complications of cirrhosis, including hepatocellular carcinoma.

#### *Grading and Staging of Hepatitis C:*

There are a number of systems in use for grading and staging of liver biopsies in chronic hepatitis, but the observations used for grading and staging are essentially the same no matter which system is employed. Periportal injury (“interface hepatitis” or “piecemeal necrosis”), lobular injury (apoptosis, “spotty necrosis”) and portal inflammation are all assessed as mild, moderate or marked and confluent (bridging or multilobular) necrosis is noted if present. In simple grading systems, an algorithm based on these observations is used to assign the grade, while in more complex systems, numbers are assigned for each feature and the numbers are added. Staging the degree of fibrosis requires a connective tissue stain for proper evaluation. There is progression in stage of disease as the fibrosis advances from none to fibrous portal expansion to bridging fibrosis to incomplete cirrhosis and finally to established cirrhosis. Intermediate stages, such as early bridging fibrosis, marked bridging fibrosis, early cirrhosis, and advanced cirrhosis can also be assigned and are used in some systems.

#### *French Metavir Scoring System:*

The Metavir scoring system was set up just for patients with hepatitis C. The scoring uses both a grade and a stage system. The *grade* tells us about the activity or amount of swelling and irritation (inflammation). The *stage* tells us the amount of fibrosis or scarring.

The grade is given a number based on the amount of inflammation. This is usually scored from “0-3.” A “0” is no activity, a “1” is mild activity, and “2” is moderate and “3” is severe activity. The amount of inflammation is important because it may lead to eventual scarring or damage. This is illustrated in the table below.

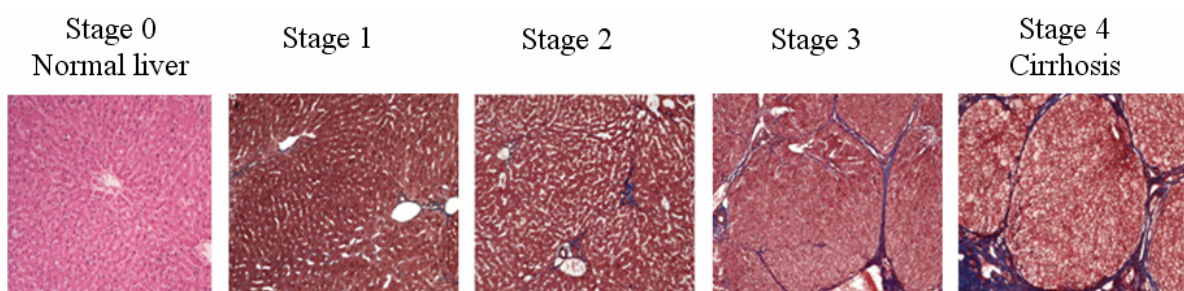
### *Metavir-Activity Scoring System*

Piecemeal necrosis 0	Lobular necrosis 0	Activity 0 (Inactive)
	Lobular necrosis 1	Activity 1 (mild)
	Lobular necrosis 2	Activity 2 (moderate)
Piecemeal necrosis 1	Lobular necrosis 0, 1	Activity 1 (mild)
	Lobular necrosis 2	Activity 2 (moderate)
Piecemeal necrosis 2	Lobular necrosis 0, 1	Activity 2 (moderate)
	Lobular necrosis 2	Activity 3 (marked)
Piecemeal necrosis 3	Lobular necrosis 0,1,2	Activity 3 (marked)

[http://www.xcellpath.com/healthcare/other/other\\_staging\\_liver.htm](http://www.xcellpath.com/healthcare/other/other_staging_liver.htm)

The stage is also given as number based on the amount of fibrosis or scarring. It is assigned a number from 0 to 4:

- 0 = No fibrosis or scarring
- 1 = Fibrosis expansion of some portal area with or without short fibrous septa.
- 2 = Fibrous expansion of most portal area with occasional portal to portal bridging.
- 3 = Fibrous expansion of portal area with marked bridging.
- 4 = Marked bridging with nodules (Cirrhosis stage).



**Fig. 1. Histological photograph of different stages of liver fibrosis.** (Photographs taken from the cellular pathology lab CHU Grenoble)

#### *Child-Pugh Score:*

The Child-Pugh score (sometimes the Child-Turcotte-Pugh score) is used to assess the prognosis of chronic liver disease, mainly cirrhosis. Although it was originally used to predict mortality during surgery, it is now used to determine the prognosis, as well as the required strength of treatment and the necessity of liver transplantation.

The score employs five clinical measures of liver disease. Each measure is scored 1-3, with 3 indicating the most severe derangement.

Measure	1 point	2 points	3 points	units
<i>Bilirubin (total)</i>	<34 (<2)	34-50 (2-3)	>50 (>3)	μmol/l (mg/dl)
<i>Serum albumin</i>	>35	28-35	<28	g/l
<i>INR</i>	<1.7	1.71-2.20	> 2.20	<i>no unit</i>
<i>Ascites</i>	None	Suppressed with medication	Refractory	<i>no unit</i>
<i>Hepatic encephalopathy</i>	None	Grade I-II (or suppressed with medication)	Grade III-IV (or refractory)	<i>no unit</i>

It should be noted that different textbooks and publications use different measures. Some older reference works substitute PT (Prothrombin Time) prolongation for INR (international normalized ratio).

Chronic liver disease is classified into Child-Pugh class A to C, employing the added score from above.

Points	Class	One year survival	Two year survival
5-6	A	100%	85%
7-9	B	81%	57%
10-15	C	45%	35%

### **Hepatocellular carcinoma (HCC).**

HCC is a primary malignancy of the liver. Most cases of HCC are secondary to either a viral hepatitis infection (hepatitis B or C) or cirrhosis (alcoholism being the most common cause of hepatic cirrhosis)(Kumar, Fausto et al. 2003). Treatment options of HCC and prognosis are dependent on many factors but especially on tumor size and staging. Tumor grade is also important. High-grade tumors will have a poor prognosis, while low-grade tumors may go unnoticed for many years. The usual outcome is poor because only 10 - 20% of hepatocellular carcinomas can be removed completely using surgery. If the cancer cannot be completely removed, the disease is usually deadly within 3 to 6 months.

### *Staging of HCC:*

In oncology, the prognosis of patients with solid tumors is solely related to tumor stage whereas other co-factors such as age or histological grade are only seldom considered. However, HCC patients constitute a particular case, as cirrhosis underlies the neoplasm in most individuals and thus, their outcome is related to these two entities, which simultaneously determine the applicability and efficacy of treatments. Accordingly, prognostic modeling in HCC patients is highly complex. The EASL (European Association for the Study of the Liver) panel of experts recommended the consideration of four related aspects: tumor stage, degree of liver function impairment, general condition of the patient, and treatment efficacy (Bruix, Sherman et al. 2001; Kitai, Kudo et al. 2008). Survival of early stage patients is modified by treatments and thus, prognostic prediction has to include treatment-related variables. Nowadays, experts in HCC management may choose among eight different staging systems (Table 1), none of them with universal acceptance (Chevret, Trinchet et al. 1999; Kitai, Kudo et al. 2008).

**Table. 1. Staging systems in hepatocellular carcinoma.**

<b>Classification</b>	<b>Type</b>	<b>Stages</b>	<b>Reference</b>
Okuda stage	System 3	Stage I, II, III	(Okuda, Ohtsuki et al. 1985)
French	Score 3	A: 0 points; B: 1–5 points; C: $\geq 6$ points	(Chevret, Trinchet et al. 1999)
CLIP	Score 7	0, 1, 2, 3, 4, 5, 6	(2000)
BCLC staging	Staging 5	0: Very early A: Early B: Intermediate C: Advanced D: End-stage	(Llovet, Bru et al. 1999)
CUPI	Score 3	Low risk: score $\leq 1$ Intermediate: score 2–7 High: score $\geq 8$	(Leung, Tang et al. 2002)
TNM staging	System 3	Stage I, II, III	(Vauthey, Lauwers et al. 2002)
JIS	Score 4	Stage I, II, III, IV	(Kudo, Chung et al. 2003)
ER	System 2	ER wild-type	(Villa, Colantoni et al. 2003)



Classification	Type	Stages	Reference
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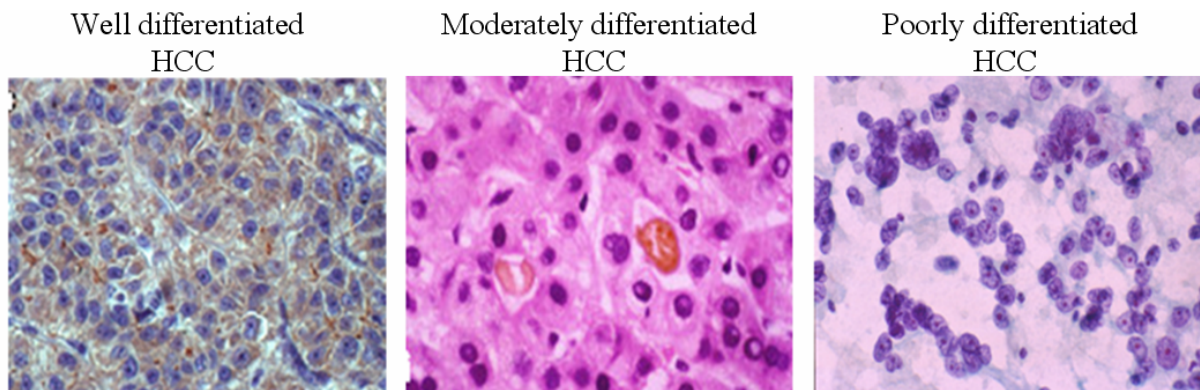
ER variant

HPB (Oxford). 2005; 7(1): 35–41.

doi: 10.1080/13651820410024058.(Kitai, Kudo et al. 2008).

*Grading of HCC:*

HCC can be classified as well, moderately and poorly differentiated. Well differentiated HCC consists of small liver like tumor cells arranged in thin trabeculae. Moderately differentiated HCC are larger with abnormal nuclei and eosinophilic cytoplasm; glandular structure may be seen. Poorly differentiated HCC have more numerous Giant tumor cells, with intensely hyperchromatic nuclei, little cytoplasm and loss of a trabecular pattern.



**Fig. 2. Histological photograph of different grads of HCCs.** (Photographs taken from the cellular pathology lab CHU Grenoble)

## **HCC Biomarkers and Tumor-associated Antigens**

As for most types of cancer, hepatocarcinogenesis is a multi-state process involving different genetic alterations that ultimately lead to the malignant transformation of hepatocytes. HCCs are phenotypically (morphology and microscopy) and genetically heterogeneous tumors, possibly reflecting in part the heterogeneity of etiologic factors implicated in HCC development. Malignant transformation of hepatocytes may occur regardless of the etiologic agent through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration in a context of inflammation, immune response and oxidative DNA damage. This may result in genetic alterations that affect supra-molecular processes (Blum 2005).

The multi-state process of hepatocarcinogenesis is complex and heterogeneous and depends on a combination of many genetic, viral and environmental factors. Different genes have been implicated in hepatocarcinogenesis including genes regulating DNA damage responses, genes involved in cell cycle control, genes involved in growth inhibition and apoptosis and genes responsible for cell-cell interaction and signal transduction. The major molecular features of human HCC include aneuploidy and chromosomal aberrations, activation of proto-oncogenes and inactivation of tumor suppressor genes (Blum 2005; Cha and Dematteo 2005; Kim, Sills et al. 2005; Farazi and DePinho 2006).

### **HCC biomarkers.**

Four distinct families of HCC tumor markers have been proposed and currently applied in the clinical practice: (a) onco-fetal and glycoprotein antigens; (b) enzymes and iso-enzymes; (c) cytokines and (d) genes (Zhou, Liu et al. 2006). However, it is compulsory to underline that a truthful specific biomarker for early detection, diagnosis or clinical outcome/prognosis of HCC is still undiscovered.

#### ***Onco-fetal antigens and glycoprotein antigens.***

a) *Alpha FetoProtein (AFP)* is a fetal specific glycoprotein produced primarily by the fetal liver, yolk sac and gut, in the first trimester of pregnancy (Canick and MacRae 2005; Ding, Yang et al. 2005; Terentiev and Moldogazieva 2006). In normal conditions, its serum concentration falls rapidly after birth and its synthesis in adult life is repressed. Pathological elevation of AFP is seen in hepatocyte regeneration, hepatocarcinogenesis and embryonic carcinomas. AFP was first described as a marker for HCC by Abelev in the 1960s (Abelev

1968) and used as a serum marker for HCC in humans for many years (Daniele, Bencivenga et al. 2004). However, greater than 70% of HCC patients have a high serum concentration of AFP because of the tumor excretion. HCC patients with a high AFP concentration ( $\geq 400$  ng/mL) tend to have greater tumor size, bilobar involvement, massive or diffuse types, portal vein thrombosis and a lower median survival rate. Though the measurement of AFP serves as an important tool in screening HCC patients, some reports have indicated that it has limited utility in differentiating HCC from benign hepatic disorders for its high false-positive and false-negative rates and patients with acute exacerbation of viral hepatitis but no HCC may also have markedly increased AFP levels (Yuen and Lai 2005; Zhou, Liu et al. 2006).

b) *Glypican-3 (GPC3)* is one of the members of heparan sulfate proteoglycans. Although it is highly expressed during embryogenesis and is involved in organogenesis, its exact biological function remains unknown. It binds to the cell membrane through the glycosyl-phosphatidylinositol anchors. GPC3 is able to interact with various growth factors that either stimulates or inhibits the growth activity (Gebo, Chander et al. 2002; Capurro, Wanless et al. 2003; Yuen and Lai 2005; Zhou, Liu et al. 2006; Cheng, Tseng et al. 2008). GPC3 mRNA and protein are expressed in more than 80% of human HCC but not in normal tissues except for placenta and fetal liver (Filmus and Capurro 2004; Coston, Loera et al. 2008). It was found in the sera from 40–50% of HCC patients, although it was not detected in the sera from patients with liver cirrhosis or chronic hepatitis, or in sera from healthy individuals (Nakatsura and Nishimura 2005).

### ***Enzymes and iso-enzymes.***

a) *Gamma-Glutamyl Transferase (GGTII)* is a glycosylated membrane enzyme which catalyses the transfer of  $\gamma$ -glutamyl groups from peptide donors such as glutathione to a variety of acceptors including dipeptides, free amino acids or water. GGT activity is modulated in many physiological and pathological conditions, including differentiation and carcinogenesis (Daubeuf, Accaoui et al. 2001). In the liver GGT is mainly secreted in serum by macrophagic Kupffer cells and endothelial cells of bile ducts and its activity increases in tissues with HCC (Zhou, Liu et al. 2006). Sensitivities of GGTII have been reported to be 74.0% in detecting HCC and 43.8% in detecting small HCC (Cui, He et al. 2003). Recently, it has been shown that changes in GGTII occurring with changes in alcohol consumption were similar regardless of ethnicity.

b) *Alpha-l-Fucosidase (AFU)* is a glycosidase primarily found in lysosome and involved in the degradation of a variety of fucose-containing fucoglycoconjugates (Haydon and Hayes 1996). The alterations of AFU catalytic activity in human cells, tissues and body fluids have a diagnostic value for human tumors including primary HCC (Deugnier, David et al. 1984; Giardina, Matarazzo et al. 1992; Li, Qian et al. 2006; Zhou, Liu et al. 2006). The persistently elevated AFU level in the sera of patients with liver cirrhosis contributes to early detection of HCC (Haydon and Hayes 1996; Zhou, Liu et al. 2006).

c) *Des-gamma-CarboxyProthrombin (DCP)* is an abnormal prothrombin protein that is increased in the serum of patients with HCC. Generation of DCP is thought to be a result of an acquired defect in the post-translational carboxylation of the prothrombin precursor in malignant cells. The reduction of gamma-carboxylase activity had been determined to be due to defective gene expression in HCC. DCP is a well recognized tumor marker for HCC. It is an abnormal product from liver carboxylation disturbance during the formation of thrombogen and acts as an autologous mitogen for HCC cell lines (Suzuki, Shiraha et al. 2005; Zhou, Liu et al. 2006). DCP was more sensitive and specific than AFP for differentiating HCC from nonmalignant chronic liver disease (Semela and Dufour 2004).

### ***Cytokines.***

a) *Vascular Endothelial Growth Factor (VEGF)* is an endothelial cell mitogen that induces and promotes angiogenesis and endothelial cell proliferation, which plays an important role in regulating angiogenesis and which was initially identified as a vascular permeability factor. VEGF expression plays an important role in the development of HCC. Its degree of expression has been reported to be associated with tumor size and histological grade (Moon, Rhyu et al. 2003; Ariei 2004; Semela and Dufour 2004; Huang, Yang et al. 2005; Kanematsu, Osada et al. 2005; Zhou, Liu et al. 2006).

b) *Interleukin-8 (IL-8)* is a multifunctional CXC chemokine that affects human neutrophil functions, including chemotaxis, enzyme release and expression of surface adhesion molecules (Zhou, Liu et al. 2006). IL-8 also seems to have also direct effects on tumor and vascular endothelial cell proliferation, angiogenesis and tumor migration. It has been shown that the expression of IL-8 in human HCC has more relevance to metastatic potential, such as vessel invasion, than to angiogenesis or cell proliferation (Kubo, Ueno et al. 2005). Moreover, significant correlations of serum IL-8 levels with tumor size and tumor stage suggest that IL-8 may be directly or indirectly involved in the progression of HCC. These findings indicate that

serum IL-8 may be a useful biological marker of tumor invasiveness and an independent prognostic factor for patients with HCC (Takeda, Stoeltzing et al. 2002; Ren, Poon et al. 2003).

c) *Transforming Growth Factor-beta 1 (TGF- $\beta$ 1)* is a multifunctional cytokine involved in the regulation of growth and differentiation of both normal and transformed cells. TGF- $\beta$ 1 is a negative growth factor which correlates with cellular immunosuppression during the progression of HCC. It has been reported that TGF- $\beta$ 1 mRNA and its protein were over-expressed in HCC tissues and plasma TGF- $\beta$ 1 was elevated in patients with HCC. Its serum level in HCC patients has been shown to be elevated compared with those in healthy adults and patients with non-malignant hepatopathy (Song, Chung et al. 2002; Zhou, Liu et al. 2006). TGF- $\beta$ 1 mRNA was over-expressed in HCC compared with surrounding liver tissues, especially in small-sized and well-differentiated HCCs. In the liver, endogenous factors, such as TGF- $\beta$ 1 may be involved in induction of apoptosis. In HCC, birth and death rates of cells are several times higher than in surrounding liver. Consequently, tumor promoters may act as survival factors, *i.e.*, inhibit apoptosis preferentially in precancerous and even in malignant liver cells, thereby stimulating selective growth of precancerous lesions (Schulte-Hermann, Bursch et al. 1997).

d) *Tumor-Specific Growth Factor (TSGF)* is a product synthesized and released by malignant cells. It results in blood capillary amplification surrounding the tumor into peripheral blood during its growing period. Thus, the higher presence in serum of TSGF can be used as diagnostic marker in detecting HCC (Liang, Wan et al. 2002; Zhou, Liu et al. 2006). TSGF is the first tumor biomarker which obtained the approval by Chinese government for the commercial marketing (Liang, Wan et al. 2002).

It is essential to emphasize that the quantification of cytokines in serum/plasma, collected prior to the clinical recognition of HCC, may provide insights into the nature of a host immune system environment that contributes to cancer development and/or growth. Such studies need to be designed with knowledge of the biology of the cytokines to be measured and of the limitations inherent in the measurement of these molecules. Also, many cytokines are labile and/or are produced locally in immune system organs and not distributed systemically, so they cannot be detected in serum/plasma.

### **Genes.**

a) *Alpha-FetoProtein mRNA (AFP mRNA)* It has been demonstrated that AFP mRNA expression detected by reverse-transcription polymerase chain reaction (RT-PCR) in peripheral blood one week after surgery correlated with the recurrence of HCC and was a good predictor for tumor recurrence (Ding, Yang et al. 2005). In 1994, Matsumura *et al.* first reported that single HCC cell in circulation could be detected by means of RT-PCR, targeting AFP mRNA (Matsumura, Niwa et al. 1994). Subsequently, many investigators reported the value of AFP mRNA as a predictor for HCC recurrence, but the results are rather controversial. This may be due to the blood-borne dispersion of both tumor cells and normal liver cells during surgical manipulation and the mis-transcription of mRNA encoding AFP by peripheral mononuclear cells.

b) *Gamma-Glutamyl Transferase mRNA (GGT mRNA)* can be detected in the serum and liver tissues of healthy adults or patients with HCC, non-malignant hepatopathy, hepatic benign tumor and secondary tumor of the liver (Zhou, Liu et al. 2006). The human enzyme is encoded by a multi-gene family that includes at least seven genes but only one (gene I) appears to code for an active enzyme. These mRNA products are named after the organs or cells from which they were first characterized: mRNA firstly described in fetal liver (*type A*), the mRNA isolated from the human hepatoma cell line HepG2 cells (*type B*) and mRNA isolated from human placenta (*type C*) (Daubeuf, Accaoui et al. 2001). Type A is predominantly expressed in normal liver tissues or liver tissues without malignant hepatopathy, benign tumor or secondary liver cancer. On the contrary type B is commonly found in cancerous tissues of HCC. Sheen *et al.* have demonstrated that patients of HCC with type B gamma-GTP mRNA both in cancer and in non-cancerous tissue had a worse outcome, earlier recurrence and more post-recurrence death (Sheen, Jeng et al. 2003). The authors suggested that HepG2 mRNA might be of diagnostic value (Pettersen, Andersen et al. 2003).

c) *Human Telomerase Reverse Transcriptase mRNA (hTERT mRNA)* has been shown to be a rate-limiting determinant of the enzymatic activity of human telomerase. Human telomerase is a ribonucleoprotein that has three major components: human telomerase RNA component (hTERC); human telomerase-associated protein 1 (hTEP1); and human telomerase reverse transcriptase (hTERT). Of the three components, hTERT is the catalytic subunit responsible for telomerase activity. Telomerase is expressed in most human cancers and immortal cell lines but is inactive in normal somatic cell lines or tissues. Some reports support the concept

that activation of telomerase may be an important and obligate step in the development of most malignant tumors, including human HCC (Chen, Kyo et al. 2004). Telomerase activity is closely correlated with hTERT expression. A weak expression of hTERT and telomerase activity was observed in 30% of non-cancerous tissues, while strong hTERT expression and enzyme activity were observed only in HCC. The cellular origin of hTERT expression in non-cancerous liver is still controversial because infiltrating lymphocytes, precancerous liver parenchymal cells and harboring micro-metastases of cancer cells may be responsible. This ambiguity makes the value of hTERT transcripts in the diagnosis of HCC and the feasibility of the promoter in gene therapy uncertain (Onishi, Nouse et al. 2003; Fu, Yang et al. 2006; Oh, Kim et al. 2006).

d) *Insulin-like Growth Factor II-mRNA (IGF-II)* It has been shown that the abnormal expressions of IGF-II mRNA might be a useful tumor marker for HCC diagnosis, differentiation of extra-hepatic metastasis and monitoring postoperative recurrence. Moreover, serum IGF-II level seems to be an independent serologic marker or a complementary tumor marker to AFP for the diagnosis of small HCC (Himoto, Kuriyama et al. 2005).

It can be stressed that several other biomarkers have been proposed and preliminarily used in the early detection of HCC. AFP and DCP are, however, the most useful serum tumor markers for detection of HCC and the simultaneous determination of these markers might improve the accuracy, especially in differentiating HCC from precancerous lesions and nonmalignant hepatopathy.

### **Tumor-associated antigens.**

A family of *tumor-associated antigens* (TAAs) has recently been identified and their encoding genes have been extensively investigated. Among them are Cancer-testis antigens (CTA) whose name was given because of their restricted expression in the tumors and testis. They are also expressed in a limited number of normal tissues but mostly in the malignant tumors of unrelated histological origin and are being vigorously pursued as targets for new therapeutic strategies. They are also being evaluated because of their role in oncogenesis and the recapitulation of portions of the germ line gene-expression program, which might contribute characteristic features to the neoplastic phenotype such as immortality, invasiveness, immune evasion, hypomethylation and metastatic capacity.

## Cancer-Testis Antigens (CTA): a new class of biomarkers.

CTA represents a category of TAA normally expressed in male germ cells and placenta but not in adult somatic tissues (Scanlan, Gure et al. 2002). Concomitantly, the testis has a so-called blood-testis barrier, which limits the contact between testicular and immune cells in the seminiferous tubuli generated by the Sertoli cells (dos Santos, Torensma et al. 2000; Wang, Zhang et al. 2003). Due to the restricted CTA expression profile, the presence of multi-gene family and their X chromosomal location, CTA have the potential to provide specific antitumour immunity directly to malignant cells without harm to normal cells.

Actually, 44 distinct CT "gene" or "antigen" families have been reported in literature. Certain CT gene families contain multiple members, as well as splice variants and today a total of 89 distinct transcripts are known to be encoded by CT genes (Scanlan, Simpson et al. 2004). Nineteen of the 44 CT families encode proteins associated with an immune response in humans and can be considered *bona fide* CT antigens. They can either be elicit cell mediated or humoral immune response against these antigens (Table 2) (Van den Eynde, Lethé et al. 1991; Stockert, Jager et al. 1998; Jager, Nagata et al. 2000; Ayyoub, Stevanovic et al. 2002).

Antigen	Immune Response
MAGEA/CT1	cellular and humoral
BAGE/CT2	cellular
MAGEB/CT3	humoral
GAGE/CT4	cellular
SSX/CT5	cellular and humoral
NY-ESO-1/CT6	cellular and humoral
MAGEC/CT7	humoral
SYCP1/CT8	humoral
SPANXB1/CT11.2	humoral
NA88/CT18	cellular
CTAGE/CT21	humoral
SPA17/CT22	humoral
OY-TES-1/CT23	humoral
CAGE/CT26	humoral
HOM-TES-85/CT28	humoral
HCA661/CT30	humoral
NY-SAR-35/CT37	humoral
FATE/CT43	humoral
TPTE/CT44	humoral

**Table 2. Different Cancer-testis antigens and their immune response (Wang, Han et al. 2002)**

## CT gene families encoding immunogenic gene products (CTA).

The tumor types can be differentiated in three distinct groups on the basis of the number of CT genes expressed and their expression frequency:



- a) *high CT expressors*: tumors which express more than 50% of the CT-antigens;
- b) *moderate CT expressors*: tumors which express between 30–50% of the CT transcripts;
- c) *low CT expressors*: tumors which express less than 30% of the CT transcripts.

This data is taken from mRNA expression frequencies, which do not necessarily correlate directly with protein expression frequencies. The expression of these antigens may correlate with advanced stage and grade of tumors.

### **Cancer-Testis Antigens expression in HCC.**

A number of CTA have been found expressed with a high percentage and specificity in HCC and their products are promising targets for antigen-specific immunotherapy of this tumor. Moreover, the high frequent co-expression of multiple members of CTA in HCC provides possibility of polyvalent vaccinations for HCC.

MAGE1, MAGE-A3, MAGE-B2, MAGE-C1, MAGE-C2 (HCA587), LAGE-1, SSX-1, SSX-2, SSX4, SSX-5, NY-ESO-1, CTp11, SCP-1, TSPY and transgelin-2 mRNA expressions were detectable in HCC samples (Wang, Han et al. 2002; Zhao, Mou et al. 2004; Peng, Chen et al. 2005; Shi, Wang et al. 2005; Yin, Li et al. 2005; Zhang, Xiao et al. 2005; Zhang, Xiao et al. 2005; Wu, Lu et al. 2006; Grizzi, Franceschini et al. 2007; Chiriva-Internati, Grizzi et al. 2008). Conversely, non or only limited expression of SSX-1, SSX-2, SSX5 and NY-ESO-1 was detected in the adjacent non-HCC tissues or peripheral blood (Zhao, Mou et al. 2004; Grizzi, Franceschini et al. 2007; Chiriva-Internati, Grizzi et al. 2008). Specific expression of CTA was observed in AFP-negative HCC, suggesting the application of their mRNA as tumor markers to detect circulating HCC cells as adjuvant diagnostic tool, and as indicators of recurrence and prognosis (Wu, Lu et al. 2006). It was found that the expression of MAGE-A3, SSX-1, SSX-2, SSX-4, MAGE-B2, MAGE-C1 and MAGE-C2 correlated significantly with older age. Moreover, the expressions of MAGE-A4 and SCP-1 were related to AFP abnormality and the expression of NY-ESO-1 was related to early tumor stage (Peng, Chen et al. 2005).

It appears that about more than 80% of the patients with HCC would be eligible for specific immunotherapeutic approaches with at least one CT antigen in ways similar to those currently being evaluated in malignant melanomas, which express CT antigens at a similar frequency as

HCC (Luo, Huang et al. 2002). HCC-specific CD8<sup>+</sup> T cells response, specific for MAGE-1 and MAGE-3 has been demonstrated in the tumor-infiltrating cells (Zerbini, Pilli et al. 2004), in the tumor and peripheral blood of HCC patients. These results were also confirmed by the identification of CD8<sup>+</sup> T cells specific for some additional CT-antigens: MAGE-10, MAGE-n, NY-ESO-1b and SSX-2 (Dong, Sui et al. 2004; Shang, Chen et al. 2004; Bricard, Bouzourene et al. 2005). Specific CD4<sup>+</sup> T-cell responses was also detected by *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) with the recombinant HCA587 protein (Li, He et al. 2004). Recently, three new HLA-A-restricted CD8<sup>+</sup> T cell epitopes in the CT antigen HCA587 have been identified, which may serve as targets for peptide-based immunotherapy for HCC patients (Xing, Pang et al. 2008).

Another proteomic approach is mass spectrometry, which can detect proteins and present them as charged ions. The mass spectrometric technique termed *Surface Enhanced Laser Desorption Ionization* (SELDI), releases proteins in a sample from a capturing surface that can specifically bind groups of proteins which share common features (i.e. hydrophobic, negatively charged) and the expression of thousands of proteins can be monitored simultaneously (El-Aneed and Banoub 2006). Interestingly, using SELDI, an elevated level of complement C3a has been found in the patients with chronic hepatitis C and HCV-related HCC (Lee, Chen et al. 2006).

Thus, high frequency and specificity of CTA in HCC indicates that their products may be new potential promising targets for antigen-specific immunotherapy of HCC. Moreover, the high frequent co-expression of genes in HCC provides a possibility of polyvalent vaccinations for this neoplasia.

## **Hepatocarcinogenesis and HCV**

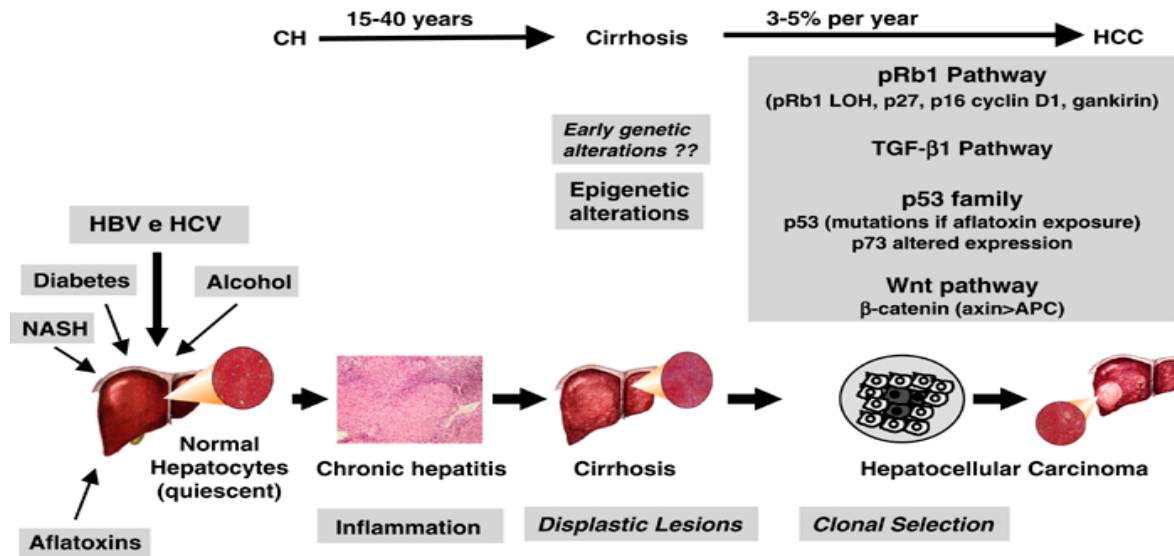
Chronic hepatitis B, C and associated liver cirrhosis represent major risk factors for HCC development, being implicated in more than 70% of HCC cases worldwide. The rise in the incidence of mortality from HCC recently observed in most industrialized countries likely reflects the increased prevalence of HCV infection (Deuffic, Buffat et al. 1999; El-Serag, Davila et al. 2003). A large analysis of HCC in Europe, based on both serology and molecular tests, has demonstrated the major impact of hepatitis B virus (HBV) and hepatitis C virus (HCV). Only 29% of HCC cases were found negative for these viruses. The hepatitis B surface antigen (HBsAg) and anti-HCV antibodies were detected in 19 and 40.1% of the patients respectively, with HCV 1b being the most prevalent genotype (Brecht, Jaffredo et al. 1998).

### **Hepatocarcinogenesis.**

As for most types of cancer, hepatocarcinogenesis is a multistep process involving different genetic alterations that ultimately lead to malignant transformation of the hepatocyte. Although significant progress has been achieved in recognizing the sequence of events involved in other forms of cancer, notably colorectal cancer and certain hematopoietic malignancies, we still lack a coherent understanding of the mechanisms of HCC development.

Malignant transformation of hepatocytes is believed to occur, regardless of the etiological agent, through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration, in a context of inflammation and oxidative DNA damage. This microenvironment facilitates the occurrence of genetic and epigenetic alterations. Chronic viral hepatitis, alcohol, metabolic liver diseases such as hemochromatosis and  $\alpha$ -1-antitrypsin deficiency, as well as non-alcoholic fatty liver disease may act predominantly through this pathway of chronic liver injury, regeneration and cirrhosis. Accordingly, the major clinical risk factor for HCC development is liver cirrhosis and 70–90% of all HCCs develop in a cirrhotic liver. The risk of HCC in patients with liver cirrhosis depends on the activity, duration and the etiology of the underlying liver disease. The co-existence of multiple etiologies, for example, HCV infection with overt or occult HBV, aflatoxin B1 (AFB1) and

HBV infection, HCV infection and alcohol or HCV infection and liver steatosis, increases the relative risk of HCC development (Fig.3).



**Fig. 3. Pathogenesis of human hepatocellular carcinoma (HCC).** Chronic hepatitis B and C and associated liver cirrhosis represent major risk factors for HCC development, being implicated in more than 70% of HCC cases worldwide. Additional etiological factors, which often represent co-factors of an underlying HBV- or HCV-related chronic liver disease, include toxins and drugs (e.g., alcohol, aflatoxins, microcystin, anabolic steroids), metabolic liver diseases (e.g., hereditary hemochromatosis, 1-antitrypsin deficiency), steatosis, non-alcoholic fatty liver diseases and diabetes. Hepatocarcinogenesis is a multistep process that may last for decades and involves the progressive accumulation of different genetic alterations ultimately leading to malignant transformation. Regardless of the etiological agent, malignant transformation of hepatocytes is believed to occur through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration, in a context of inflammation and oxidative DNA damage. Dysplastic nodules and macroregenerative nodules are considered as pre-neoplastic lesions. The detailed analysis of HCC development in experimental animals and the comparison of the results with HCC in humans has identified a variety of genomic and molecular alterations in fully developed HCC and to a lesser extent in morphologically defined pre-neoplastic precursor lesions. At least four pathways that regulate either cell proliferation or cell death (i.e., the phospho-retinoblastoma (pRb), p53, transforming growth factor- (TGF-) and -catenin pathways) are affected in HCCs (Levrero 2006).

#### *Cirrhotic nodules as a pre-neoplastic lesion:*

Dysplastic nodules and macroregenerative nodules are considered pre-neoplastic lesions (Furuya, Nakamura et al. 1988; Terada, Ueda et al. 1993). Several observations support this hypothesis. Firstly, direct outgrowth of HCC from cirrhotic nodules has been described as the nodule-in-nodule appearance. Secondly, 50–60% of the cirrhotic macronodules have a monoclonal origin when examined for the X-chromosome methylation pattern. Thirdly, chromosome aberrations and allelic losses are found in half of cirrhotic nodules and in small cell dysplasia, thus indicating that they represent true pre-malignant lesions of HCC. Only in less than 10% of the cases, HCCs are observed in non-cirrhotic livers and even without inflammatory lesions (Fig.3).

### ***Molecular pathways in human hepatocarcinogenesis.***

At least four pathways that regulate either cell proliferation or cell death i.e. phospho-retinoblastoma (pRb), p53, transforming growth factor- $\beta$  (TGF- $\beta$ ) and  $\beta$ -catenin pathways are affected in HCCs (Ozturk 1999; Moradpour and Blum 2005).

#### *Phospho-Retinoblastoma (pRb) pathway & HCC:*

The retinoblastoma protein (pRb) is a tumor suppressive protein that is dysfunctional in many types of cancer (Murphree and Benedict 1984). pRb prevents the cell from replicating damaged DNA by preventing its progression along the cell cycle through G1 (first gap phase) into S (synthesis phase) (Das, Hashimoto et al. 2005). The pRb tumor suppressor pathway is altered in more than 50% of human HCCs owing to genetic and epigenetic changes (Ozturk 1999) The Rb protein and its regulators p16INK4A and cyclin D are involved in the G1/S progression of the cell cycle. Loss of heterozygosity at the RB1 locus is quite frequent and RB1 gene mutations are found in about 15% of human HCCs. Both mutations of the p16INK4A gene and its inactivation by methylation have been described (Liew, Li et al. 1999; Tommasi, Pinto et al. 2007). In addition, overexpression of gankyrin, a six ankyrin repeat protein, that mediates pRb degradation by the 26S proteasome, has also been described (Higashitsuji, Itoh et al. 2000; Umemura, Itoh et al. 2008), thus providing an additional liver-specific mechanism of pRb inactivation (Fig.3).

#### *P53 & HCC:*

p53 (also known as protein 53 or tumor protein 53), is a transcription factor encoded by the *TP53* gene. p53 regulates the cell cycle and functions as a tumor suppressor that is involved in preventing cancer. p53 has been described as "the guardian of the genome", "the guardian angel gene", and the "master watchman", referring to its role in conserving stability by preventing genome mutation. A G-to-T mutation at the third base position of codon 249 of the p53 tumor suppressor gene is found in up to 50–70% of HCCs (Bressac, Kew et al. 1991). The p53-related tumor suppressor p73 is also deregulated in HCC. Mutations in the p73 gene have not been described (Levrero, De Laurenzi et al. 2000), but the amino-terminal-deleted isoform DNp73, which acts as a dominant *trans*-repressor of both p53 and proapoptotic TAp73 proteins (Vossio, Palescandolo et al. 2002), accumulates progressively in chronic

hepatitis, cirrhosis and HCC and confers to HCC cells a chemoresistant phenotype (Muller, Schilling et al. 2005; Buhlmann, Racek et al. 2008). A recent study showed the oncogenic capability of DNp73 to drive hepatocarcinogenesis *in vivo* (Tannapfel, John et al. 2008).

#### *β-catenin pathway & HCC:*

Accumulation of nuclear β-catenin-containing complexes leads to the unrestricted transcription of several cell-cycle control genes. Deregulated expression of β-catenin, resulting from Adenomatous Polyposis of the Colon (APC) defects, β-catenin gene mutations and/or Wnt signaling pathway alterations, appears to play a role in more than 50% of HCCs (Ozturk 1999). Moreover, mutations in β-catenin along with Frizzled receptor and Axin have been reported in HCC (Merle, de la Monte et al. 2004; Merle, Kim et al. 2005; Kim, Lee et al. 2008; Yang, Yan et al. 2008) Interestingly, β-catenin mutations and/or deregulation in HCC correlate(s) with a low rate of loss of heterozygosity (LOH), suggesting that β-catenin pathway activation over-rides the need for multiple genetic/epigenetic events and loss of tumor suppressor genes in the multistep process of hepatocarcinogenesis and may lead more directly to the liver malignant phenotype (Legoix, Bluteau et al. 1999).

#### *Transforming growth factor-β and other growth factors in HCC:*

The upregulation of growth factors expression and the activation of components of their signaling pathways also play an important role in hepatocarcinogenesis. Insulin-like growth factor (IGF) II, insulin receptor substrate 1, hepatocyte growth factor (HGF), overexpression of the angiogenic factors vascular endothelial growth factor and TGF-α and β have been involved in the development of HCC (Kitisin, Ganesan et al. 2007; Nussbaum, Samarin et al. 2007; Nussbaum, Samarin et al. 2008; Pang, Joh et al. 2008). Unexpectedly, HGF, a mitogen for normal hepatocytes, inhibits the growth of hepatoma cell lines *in vitro* and the development of HCC in transgenic mouse models of hepatocarcinogenesis (Santoni-Rugiu, Preisegger et al. 1996; Nussbaum, Samarin et al. 2008).

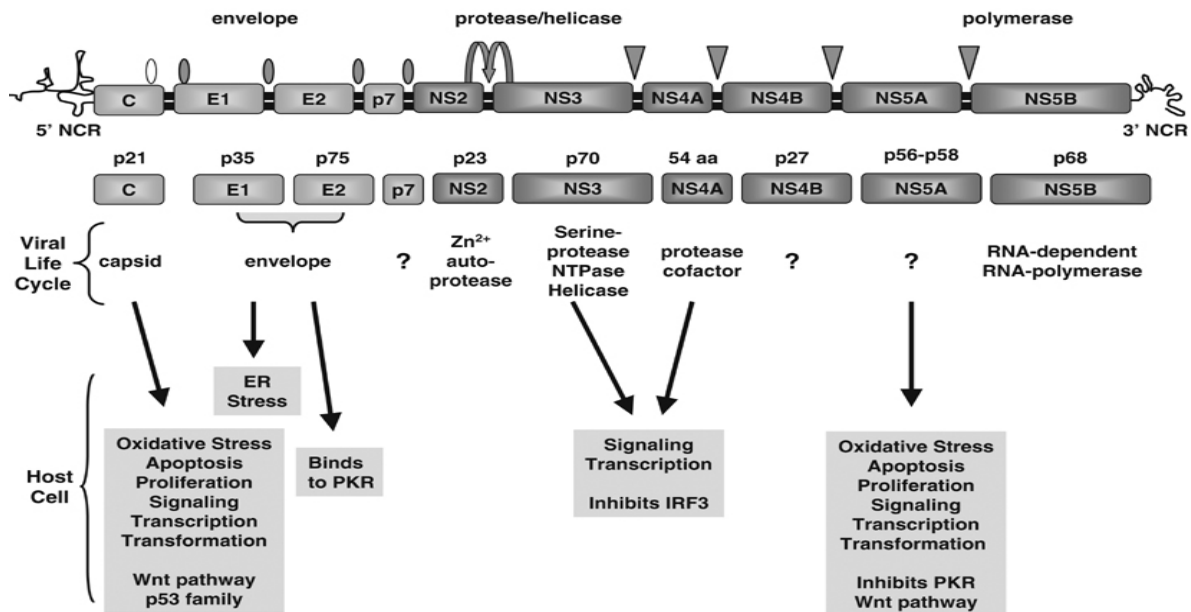
The overall emerging picture is that of HCCs as genetically very heterogeneous tumors. This is not completely unexpected, given the heterogeneity of etiological factors implicated in the development of HCC, the complexity of hepatocyte functions and the late stage at which HCCs are usually detected and analyzed.

## **Hepatitis C virus and hepatocellular carcinoma.**

The notion of HCV, a completely cytoplasmic-replicating virus that induces oncogenic transformation, has challenged the current conventional biological models for viral oncogenesis. As HCC associated with HCV infection evolves after many years of chronic infection and is generally preceded by the development of cirrhosis, the role of chronic liver injury followed by regeneration, cirrhosis and the development of HCC has been the leading hypothesis. Increasing experimental evidences, however, raise the possibility that HCV might also contribute through more direct pathways in promoting malignant transformation of hepatocytes. Different viral proteins, notably core, NS3 and NS5A have been implicated in transformation and HCC development (Yoshida, Hanada et al. 2002; Kasprzak and Adamek 2008; Tanaka, Moriya et al. 2008).

### **The hepatitis C virus.**

Hepatitis C virus is a member of the Flaviviridae family of enveloped, positive-strand RNA viruses and is the only member of the genus *Hepacivirus* (Tellinghuisen and Rice 2002). The HCV genome consists of an RNA molecule, of approximately 9.6 kb, that contains a large open-reading frame flanked by structured 5' and 3' non-translated regions (NTRs). Viral proteins are translated as a polyprotein precursor from an internal ribosome entry site (IRES) located in the 5' NTR. The polyprotein undergoes a complex series of co- and post-translational cleavage events catalyzed by both host and viral proteinases to yield the individual HCV proteins (Fig. 4). The structural proteins include the core protein and the envelope glycoproteins E1 and E2. The non-structural proteins include the P7 polypeptide, NS2-3 autoprotease and NS3 serine protease, an RNA helicase located in the C-terminal region of NS3, the NS4A polypeptide, NS4B and NS5A proteins and NS5B RNA-dependent RNA polymerase (Tellinghuisen and Rice 2002). An additional HCV protein, F (for frameshift protein) or ARFP (for Alternate Reading Frame Protein), generated by an overlapping reading frame in the core (C) protein coding sequence, has been proposed (Walewski, Keller et al. 2001; Xu, Choi et al. 2001; Varaklioti, Vassilaki et al. 2002).



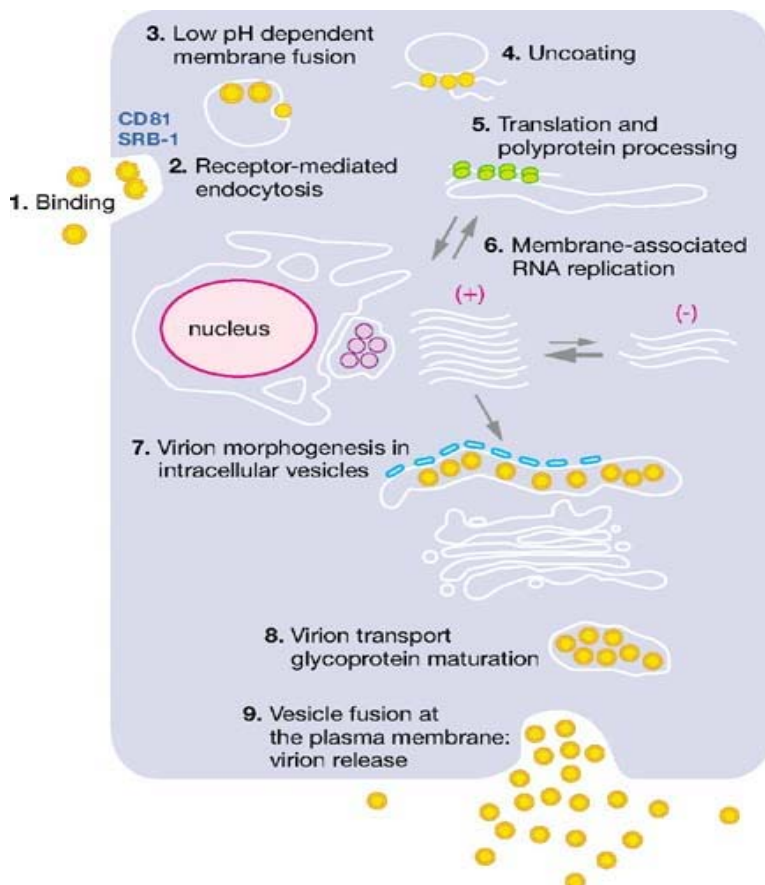
**Fig. 4. Hepatitis C virus (HCV) proteins. Genetic organization and polyprotein processing of HCV.** The 9.6 kb positive-strand RNA genome is composed of a 5' non-coding region (NCR), a long open-reading frame encoding a polyprotein precursor of about 3000 amino acids and a 3' NCR. The polyprotein precursor is processed into structural and non-structural proteins by cellular and viral proteases. Solid rods denote cleavage sites of the endoplasmic reticulum signal peptidase. The open rod indicates the additional C-terminal processing of the core protein by signal peptide peptidase. Arrow heads indicate cleavages by HCV NS2-3 and NS3 proteases. Besides their roles in the viral life cycle, HCV proteins interact with many host-cell factors and impact on a wide range of cellular activities, including cell signaling, transcriptional modulation, transformation, apoptosis, membrane rearrangements, vesicular trafficking and translational regulation (Levrero 2006).

The identification of the receptors recognized by the HCV, which allows its internalization, is not definitively known. Nevertheless, some candidate molecules have been proposed (Cocquerel, Voisset et al. 2006; Dubuisson, Helle et al. 2008). Low density lipoproteins (LDL) and very low density lipoproteins (VLDL) could also be function like receptors (Agnello, Abel et al. 1999; Wunschmann, Muller et al. 2006) owing to the fact that HCV interacts physically with the particles of LDL and VLDL (Thomssen, Bonk et al. 1992; Andre, Komurian-Pradel et al. 2002). E2 protein is able to bind *in vitro* to CD81 which is present on the cell membrane of the hepatocytes like many other types cells (Pileri, Uematsu et al. 1998; Wunschmann, Medh et al. 2000). HCV interacts with other molecules through E2 on hepatocytes such as human “scavenger” receptors class B type I (SR-BI) (Scarselli, Ansuini et al. 2002; Helle and Dubuisson 2008), L-SIGN (liver and lymph node-specific intercellular adhesion molecule-3-grabbing integrin) and DC-SIGN (dendritic concealment-specific intercellular adhesion molecule-3-grabbing nonintegrin) (Gardner, Durso et al. 2003; Lozach, Lortat-Jacob et al. 2003; Khoo, Chan et al. 2008).

After entry in the cell, the viral genome is released in the cytoplasm, where the synthesis of the polyprotein takes place. The structural proteins, namely C, E1 and E2 are cleaved from the



polyprotein by the endoplasmic reticulum (ER) signal peptidases and, after maturation in the ER, are assembled into the progeny virions in internal membrane compartments. E2 is also responsible for binding the putative host-cell receptor(s), including the CD81 tetraspannin. The function of the small hydrophobic p7 protein, located in the polyprotein at the junction of the structural and non-structural proteins, is unknown. The non-structural proteins form the viral replicase complex. NS2 protein, together with the amino-terminal region of the NS3 protein, constitutes the NS2–NS3 proteinase, which catalyses a single autocatalytic cleavage between NS2 and NS3. Once released from NS2, the amino-terminal domains of the NS3 proteins serve as serine proteinases for the release of the remaining non-structural proteins from the polyprotein. The carboxy-terminal region of the NS3 protein has RNA helicase activity. The NS4A protein acts as a co-factor/enhancer for the activities of NS3. The function of the hydrophobic integral membrane protein NS4B is unknown, but it interacts with the viral replicase. NS5A is a hydrophilic membrane-associated protein and exists in multiple phosphorylation states. The NS5B protein is the RNA-dependent RNA polymerase (Fig.4). Viral RNA replication is thought to occur in the perinuclear membrane. The replication complex contains the viral polymerase, helicase as well as a number of host-cell factors (Levrero 2006; Suzuki, Ishii et al. 2007).



**Fig. 5. Life Cycle of HCV**

1. Binding to receptor
2. Endocytosis of virus
3. & 4. Release in the cytoplasm
5. Translation & polyprotein synthesis.
6. RNA replication.
7. Virions assembly
8. Maturation.
9. Release of virions (Guidotti and Chisari 2006)

Little is known about the assembly of HCV or its virion structure since efficient production of authentic HCV particles has only recently been achieved. As with related viruses, the mature HCV virion likely consists of a nucleocapsid and outer envelope composed of a lipid membrane and envelope proteins. The virus could be released as nucleocapsids would overlap with an envelope inside the endoplasmic reticulum by budding and that thereafter, the viral particles might be released outside the cell by exocytosis (Fig. 5) (Guidotti and Chisari 2006; Suzuki, Ishii et al. 2007).

Owing to the enormous amount of information that has been generated regarding the properties of HCV structural and non-structural proteins and their interactions with cellular proteins and functions, only the findings that are potentially relevant for malignant transformation are summarized below.

#### *Hepatitis C virus core:*

Hepatitis C virus core is involved in binding viral RNA, regulating HCV RNA translation, making homotypic interactions for particle assembly and interacting with the glycoproteins to generate a complete virion. In addition to these more predictable functions, the core gene product has been proposed to be also involved in cell signaling, transcriptional activation, apoptosis, lipid metabolism and transformation. Hepatitis C virus core binds to p53 (Ray, Steele et al. 1997; Lu, Lo et al. 1999), p73 (Alisi, Giambartolomei et al. 2003) and pRb (Cho, Baek et al. 2001) tumor suppressor proteins but the functional consequences of these interactions have not fully been elucidated. p73/core interaction results in the nuclear translocation of HCV core protein in the presence of either p73 $\alpha$  or p73 $\beta$  tumor suppressor proteins. Hepatitis C virus core protein also modulates the expression of the cyclin-dependent kinase (CDK) inhibitor p21/Waf which is a transcriptional target of p53 and regulates the activities of cyclin/CDK complexes involved in cell-cycle control and tumor formation (Wang, Yoshida et al. 2000).

The tumor necrosis factor receptor (Zhu, Khoshnan et al. 1998) and the lymphotoxin- $\beta$  receptor (Matsumoto, Hsieh et al. 1997) have been shown to interact with core and play a role in inhibiting apoptosis (Ruggieri, Harada et al. 1997; Balachandran, Kim et al. 1998; Saito, Meyer et al. 2006). Hepatitis C virus core has also been proposed to have immunosuppressive activities through inhibition of activated T cell proliferation (Yao, Eisen-Vandervelde et al. 2003), induction of apoptosis by Fas-FasL in T cells (Ruggieri, Murdolo et al. 2003) and by

interaction with the complement receptor C1qR on T cells (Kittlesen, Chianese-Bullock et al. 2000), thus contributing to chronic infection.

TGF- $\beta$  signaling not only controls cell proliferation, differentiation and apoptosis but also plays an important role in liver repair processes and fibrogenesis through its action on the extracellular matrix. HCV core variants isolated from liver tumor but not from adjacent non-tumor tissue interact with Smad3 and inhibit the TGF- $\beta$  pathway (Pavio, Battaglia et al. 2005), thus block antiproliferative effects of TGF- $\beta$  in the tumor. In addition, HCV core upregulates TGF- $\beta$  expression which induce fibrogenesis by hepatic stellate cells (Bataller, Paik et al. 2004; Gressner, Rizk et al. 2008). HCV core protein associates with cellular membranes (Barba, Harper et al. 1997; Hope, Murphy et al. 2002; Roingeard, Hourieux et al. 2008) and lipid vesicles (Moriya, Yotsuyanagi et al. 1997), binds to apolipoprotein II (Sabile, Perlemuter et al. 1999; Perlemuter, Sabile et al. 2002; Petit, Jooste et al. 2007) and reduces microsomal triglyceride transfer protein (MTP) activity, leading to defects in the assembly and secretion of very-low-density lipoproteins (Perlemuter, Sabile et al. 2002) and steatosis. The relevance *in vivo* of this interaction is supported by the development of steatosis (Perlemuter, Sabile et al. 2002; Roingeard and Hourieux 2008) and liver cancer (Lerat, Honda et al. 2002; Tanaka, Moriya et al. 2008).

#### *E2 protein:*

The impact of other structural proteins on malignant transformation is more indirect. The E2 glycoprotein has been shown to interfere with interferon actions *in vitro* by inhibition of protein kinase R (PKR), an important intermediate of interferon effects (Crotta, Stilla et al. 2002; Tseng and Klimpel 2002). In addition, a soluble form of E2 (with the transmembrane domain truncated) interacts specifically with the cell surface marker CD81 and exerts inhibitory effect on the activation of T and natural killer cells *in vitro* (Crotta, Stilla et al. 2002; Tseng and Klimpel 2002). E2 interaction with CD81 as well as with the LDLR on the cell surface activates the MAPK/extracellular signal-regulated protein kinase (ERK) pathway, including the downstream transcription factor ATF-2 and promotes cell proliferation and cell survival (Zhao, Wang et al. 2005; Zhao, Zhang et al. 2006).

#### *NS3:*

The NS3 serine protease domain alone can transform mammalian cells by interaction with the tumor suppressor p53, although the link between this interaction and HCC is not clear (Sakamuro, Furukawa et al. 1995; Ishido, Muramatsu et al. 1997; Ishido and Hotta 1998).

NS3 also contains a histone-binding site and binds histones H2B and H4 (Borowski, Kuhl et al. 1999). NS3 interacts with protein kinase A (PKA) and C (PKC) and inhibits their signaling (Borowski, Kuhl et al. 1999). NS3 has also been shown to inhibit Interferon Response Factor (IRF)-3-mediated induction of type I interferon in response to viral infection, which may be important in the ability of HCV to escape immune surveillance (Foy, Li et al. 2003).

#### *NS5A:*

NS5A has been implicated in diverse cellular functions, including apoptosis, signal transduction, transcriptional activation and cellular transformation. NS5A also induces interleukin-8, leading to the inhibition of the antiviral effects of IFN (Polyak, Khabar et al. 2001; Khabar and Polyak 2002). NS5A has been implicated both in the modulation of cytoplasmic signaling pathways and in the regulation of the cellular transcriptional machinery (Sato, Hirota et al. 2000; Brass, Bieck et al. 2002; Qadri, Iwahashi et al. 2002). NS5A may effects Wnt/ $\beta$ -catenin pathway which is a common target for HCV (independently from axin/ $\beta$ -catenin gene mutations) in human HCCs (Ding, Xia et al. 2005). Moreover, a recent data shows that NS5A may play an important role in the development of HCV-related liver diseases and HCC (De Mitri, Cassini et al. 2007; Hung, Chen et al. 2008).

In addition, HCV F protein is a newly identified protein encoded by an alternative open reading frame has a regulatory properties in cellular oncogene c-myc and anti-oncogene p53, which may be implicated in the formation of hepatocellular carcinoma (Wu, Shao et al. 2007).

#### *Hepatitis C virus, endoplasmic reticulum stress and oxidative stress:*

Hepatitis C virus has been shown to induce endoplasmic reticulum (ER) stress. (Jordan, Wang et al. 2002; Waris, Tardif et al. 2002; Tardif, Waris et al. 2005; Christen, Treves et al. 2007). ER stress is a homeostatic mechanism that regulates cellular metabolism and protein synthesis in response to perturbations in protein folding and biosynthesis (Ma and Hendershot 2004). Mild ER stress modulates protein synthesis initiation and causes a reduction in cell growth, whereas extreme or prolonged ER stress leads to apoptosis and may cause liver injury (Kaufman 1999; Kaplowitz, Than et al. 2007). Markers of acute intracellular oxidative stress are elevated in patients with chronic HCV (Sumida, Nakashima et al. 2000). HCV core and NS5A proteins can induce oxidative stress that correlate with HCC development (Moriya, Nakagawa et al. 2001; Waris, Tardif et al. 2002; Farinati, Cardin et al. 2007).

## Immune Cells and HCC

One of the important roles of the immune system is to identify and eliminate the tumors. The *transformed cells* of tumors express antigens that are not found on normal cells. These antigens appear foreign and their presence causes immune cells to attack the transformed tumor cells. The main response of the immune system to tumors is to destroy the abnormal cells using killer T cells, sometimes with the assistance of helper T cells (Gerloni and Zanetti 2005; Romero, Cerottini et al. 2006). Clearly, some tumors evade the immune system and go on to become cancers (Seliger 2005). Tumor cells often have a reduced number of MHC class I molecules on their surface, thus avoiding detection by killer T cells (Seliger, Ritz et al. 2006).

An average human liver contains  $\sim 1 \times 10^{10}$  lymphocytes, made up of ‘conventional’  $\alpha\beta$  T cells with a diverse T-Cell Receptor (TCR) repertoire and the surface markers similar to T cells in the peripheral blood, as well as ‘unconventional’ T cells that belong to subsets rare in blood, such as  $\gamma\delta$  T cells, Natural Killer T (NKT) cells, or T cells displaying unusual marker combinations. In addition, intrahepatic lymphocytes comprise a large fraction of NK cells and some B cells (below). Compared with blood, the liver contains higher frequencies of effector/memory cells among the conventional  $CD4^+$  T cells (Nascimbeni and Rehermann 2003; Klugewitz, Adams et al. 2004).

<u>Lymphocyte subpopulation in the liver</u>	<u>Frequency [%]</u>
→ NK cells	11.6 – 51.3
→ NKT cells	13.5 – 54.7
→ B cells	1.0 – 7.0
→ T cells	25.0 – 93.0
→ $\alpha\beta$ -T cells	66.0 – 93.7
→ $\gamma\delta$ -T cells	6.8 – 34.0
→ CD4 <sup>+</sup> CD8 <sup>-</sup> T cells	2.7 – 29.0
→ CD4 <sup>+</sup> CD8 <sup>+</sup> T cells	3.0 – 11.6
→ CD4 <sup>+</sup> T cells	7.0 – 31.0
- Local / resident	10.0 – 22.0
- Liver-Infiltrating	3.0 – 21.0
- HCV epitope -specific	<b>0.3– 1.3</b>
→ CD8 <sup>+</sup> T cells	57.0 – 89.0
- Local / resident	60.0 – 75.0
- Liver-Infiltrating	3.0 – 14.0
- HCV epitope-specific	<b>0.1– 2.8</b>

Relative proportion of lymphocyte subpopulations in the human liver (Nascimbeni and Rehermann 2003).

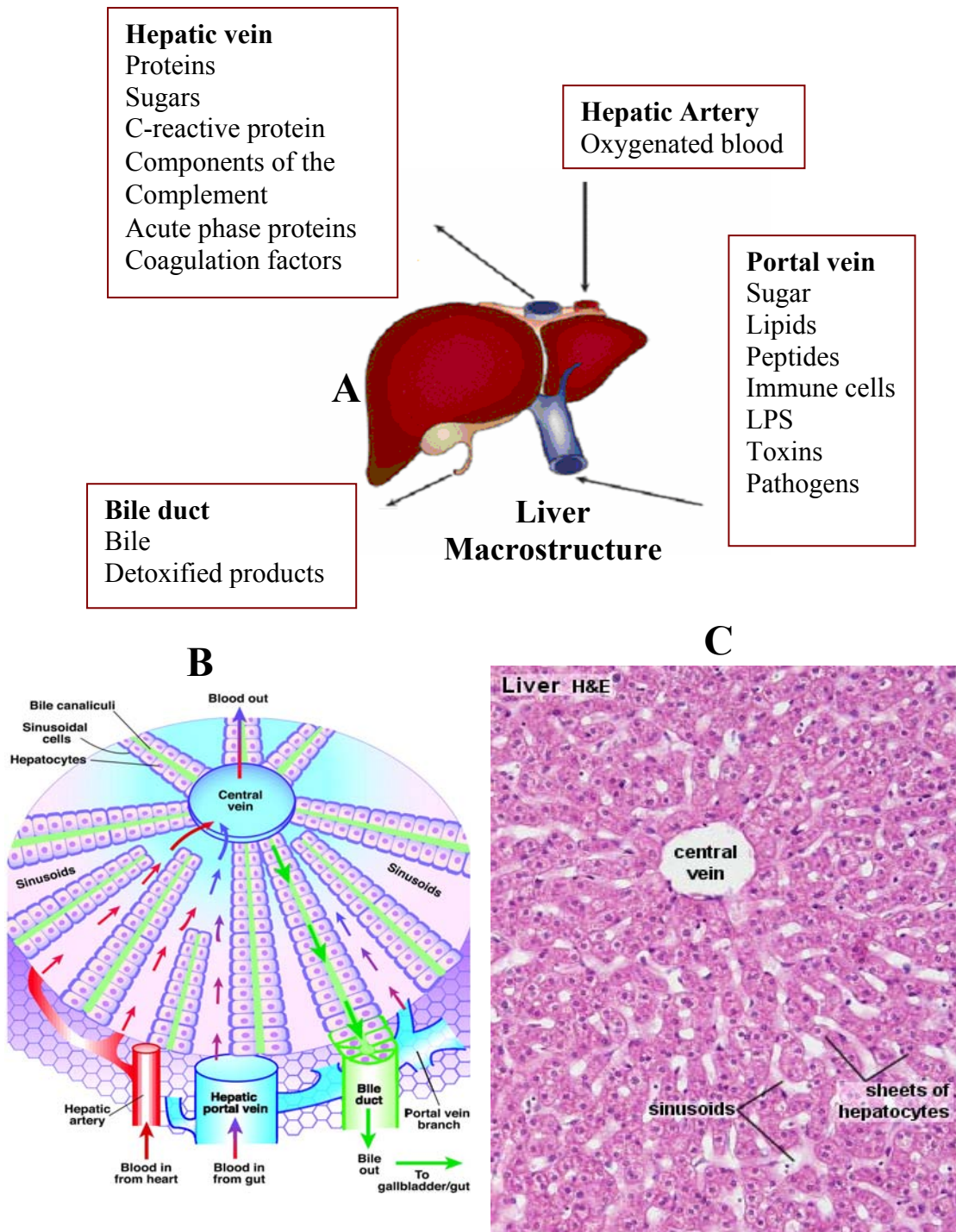
Most HCC occur in chronic liver disease, such as chronic hepatitis and liver cirrhosis. In chronic hepatitis C, the resident intrahepatic lymphocytes (IHL) are markedly increased but most of them are apoptotic (Nutti, Rosa et al. 1998; Bertolino, Klimpel et al. 2000). They include helper and suppressor T lymphocytes, natural killer cells, B lymphocytes, and macrophages (Balch, Riley et al. 1990). It has been observed that when the tumor tissues are associated with the Tumor-Infiltrating Lymphocytes (TIL) at a high density, good post-operative survival rates for cancer have been reported in various organs (Underwood 1974; Shimokawara, Imamura et al. 1982; Itoh, Platsoucas et al. 1988; Kawata, Une et al. 1992; Wada, Nakashima et al. 1998). Others have suggested that TIL are neither cytotoxic nor influential in biological defence immediately after isolation (Moy, Holmes et al. 1985; Miescher, Whiteside et al. 1986).

### **The microanatomy of the liver.**

The functional requirements of the liver have resulted in it developing a unique dual blood supply, with oxygenated blood entering through the hepatic artery and blood from the gut, which is rich in nutrients and bacterial endotoxin, entering through the hepatic portal vein. Blood from both of these vessels percolates through the sinusoids, which provide a large vascular bed that maximises exchange of materials prior to exit of blood via the hepatic vein. The portal vein provides a route through which infectious organisms can enter the liver and mechanisms have evolved to allow rapid and selective immune responses within this tissue.

The liver is composed of different cell populations with distinct functions (Fig. 6). Hepatocytes constitute the major cell type in the parenchyma and are arranged into cell plates separated by narrow sinusoids lined with endothelial cells. The lumen of these channels is narrow and is penetrated by resident macrophages called Kupffer cells and liver-associated natural killer lymphocytes or Pit cells that sit on the luminal surface of the sinusoidal endothelial cells. The sinusoids allow oxygenated, nutrient-rich blood arriving from the portal tract (which is composed of a branch of the hepatic artery, a portal vessel and a bile duct) to percolate slowly past the hepatocyte cell plates, allowing maximal exchange of materials before the blood leaves the liver through the central vein branches that run to the hepatic vein (Fig. 7). Thus, leukocytes entering the liver can arrive through vascular endothelial cells

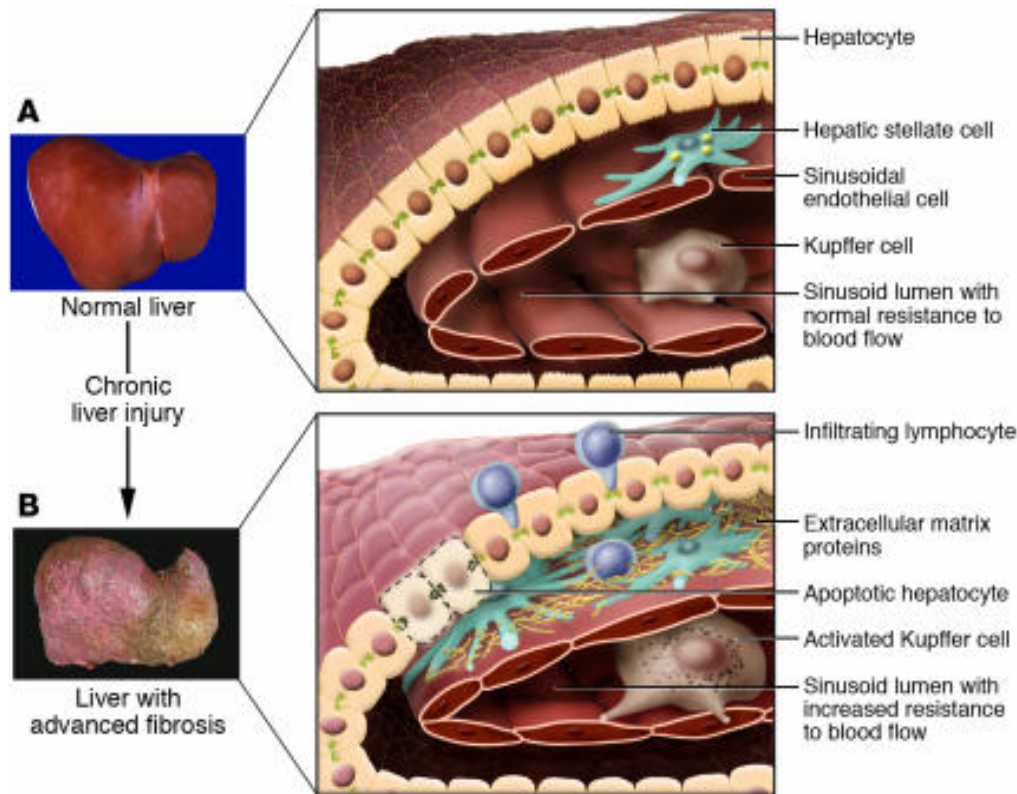
lining the portal vein in the portal tract, or through the microvascular endothelial cells that line the sinusoids or through the terminal hepatic veins (Lalor and Adams 2002).



**Fig. 6. Hepatic Architecture.**

(A) Representation of liver pathways of nutrients and other components. (B) Schematic representation of liver anatomy showing the hepatocytes are organized in lines bordered by the sinusoids (pink). Blood arrives by the hepatic artery coming from the heart and by the hepatic portal vein coming from the intestine, drains fabric and sets out again in the middle by the central veins which join the lower vena cava. The hepatocytes are in contact with plasma, which allows the exchange of nutrients and metabolites (According to Strain, Science, 2002). (C) Microscopic image of normal liver after coloring E/H. (www.anatomy.dal.ca)(Crispe 2003).





**Fig. 7. Changes in the hepatic architecture during chronic liver injury.** (A) associated with advanced hepatic fibrosis (B). Following chronic liver injury, inflammatory lymphocytes infiltrate the hepatic parenchyma. Some hepatocytes undergo apoptosis, and Kupffer cells activate, releasing fibrogenic mediators. HSCs proliferate and undergo a dramatic phenotypical activation, secreting large amounts of extracellular matrix proteins. Sinusoidal endothelial cells lose their fenestrations, and the tonic contraction of HSCs causes increased resistance to blood flow in the hepatic sinusoid (Bataller and Brenner 2005).

### Cytotoxic and helper T cells.

T cells are a group of lymphocytes which play a central role in cell-mediated immunity. They are characterized by having a special receptor on their cell surface called T cell receptor (TCR). Several different subsets of T cells have been described, each with a distinct function.

CD4<sup>+</sup> T cells, also called T helper cells, accumulate in the hepatic microenvironment in the patients with chronic HCV by almost twofold compared with blood (Artini, Nisini et al. 1995). They express an antigen experienced (CD45RO<sup>+</sup>) and activated (CD69<sup>+</sup>) phenotype (Fiore, Angarano et al. 1997). Several groups have shown that peripheral and intrahepatic HCV-specific CD4<sup>+</sup> T-cell responses are associated with viral clearance in acutely infected humans and chimpanzees (Diepolder, Zachoval et al. 1995; Missale, Bertoni et al. 1996; Thimme, Spangenberg et al. 2002; Fontenot, Gharavi et al. 2003). In addition, depletion study of CD4<sup>+</sup> cells in chimpanzees has clearly demonstrated that CD4<sup>+</sup> cells are required for effective antiviral CD8<sup>+</sup> T-cell immunity (Grakoui, Shoukry et al. 2003). Typically, HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells are compartmentalized to the liver and not the periphery (He,



Rehermann et al. 1999; Grabowska, Lechner et al. 2001). CD4<sup>+</sup> HCC specific cytotoxic T-lymphocytes have also been reported in patients with HCC. These cells were identified from both peripheral blood and from TILs and may play a role in immunity against HCC (Nakao, Sata et al. 1997). The diversificity of TCR increases in correlation with the number of liver T lymphocytes in HCV infected patients arguing for the establishment of a multi-specific immune responses which are unable to resolve the infection (Vigan, Jouvin-Marche et al. 2003). In the patients with hepatocellular carcinoma, most of the infiltrating lymphocytes were identified as T lymphocytes and CD8<sup>+</sup> T lymphocyte was more predominant than CD4<sup>+</sup> T lymphocytes (Wada, Nakashima et al. 1998), but in another study CD4<sup>+</sup> cells are predominant than CD8<sup>+</sup> cells in the tumor of HCC patients (Yoong, McNab et al. 1998). The presence of tumor Ag-specific CD8<sup>+</sup> T cells (specific for MAGE-A1 and MAGE-A3) has been demonstrated in the tumor of HCC patients (Zerbini, Pilli et al. 2004). In some cases (melanoma and a fibrosarcoma), both CD4<sup>+</sup> and CD8<sup>+</sup> T cells or only CD8<sup>+</sup> T cells (murine myeloma ) were found to be required for tumor rejection (Onizuka, Tawara et al. 1999; Jones, Dahm-Vicker et al. 2002). It is possible that the role of CD4<sup>+</sup> T cells is simply to behave as helper cells for the activation of CD8<sup>+</sup> CTLs, particularly in the case where the tumor cells are poorly immunogenic.

Despite the presence of a lymphocytic infiltration within and surrounding the tumors, a continued tumor growth has been observed in various kinds of tumors, suggesting a failure of the immune control. Many mechanisms have been proposed for an attenuated immune response to the tumors; these include partial antigen masking, failure of antigen processing, inadequate costimulation and direct suppression of effector cells (Chouaib, Asselin-Paturel et al. 1997). It has been suggested that the chronic inflammatory response can cause hepatic damage leading to liver cirrhosis and cancer (Liaw, Lee et al. 1995; Jin, Fuller et al. 1997). Down-regulation of immune response in HCV infected host may play a crucial role in the mechanism of HCV persistence and contribute to the malignant transformation of hepatocytes and outgrowth of HCC. These results implied that not only T cells in HCC but also T cells in non-cancerous liver showed dysfunction in HCV infected patients. Defects in antigen-specific CD4<sup>+</sup> T cell function have been described in the patients with chronic HCV infections and HCC. In contrast, patients exposed to HCV who resolve viraemia have circulating CD4<sup>+</sup> T cells that react to a wide variety of HCV antigens (Ferrari, Penna et al. 1990; Jung, Spengler et al. 1991; Penna, Del Prete et al. 1997; Maini, Boni et al. 1999). TGF- $\beta$  has been found in high concentration in chronic HCV and HCC. It may inhibit FasL expression on CD8<sup>+</sup> and

CD4<sup>+</sup> T cells (Bodmer, Strommer et al. 1989; Smyth, Strobl et al. 1991; Wang, Holmes et al. 2006). Furthermore, TGF- $\beta$ 1 induces the expression of CD95/NKG2, a major histocompatibility complex (MHC) class I receptor that inhibits T cell mediated cell function in T cells (Bertone, Schiavetti et al. 1999), suggesting that there is another mechanism of tumor escape from CTL-mediated control (Mingari, Moretta et al. 1998). Another study demonstrated that hepatitis C virus (HCV) core protein might promote the apoptosis of T cells during HCV infection via the Fas signaling pathway (Hahn, Cho et al. 2000). Moreover, abnormalities in signal-transducing molecules of the tumor infiltrating T cells and intrahepatic T cells from HCC bearing or HCV infected hosts have also been reported (Maki, Matsuda et al. 2003). Defects in CTL cytolytic molecules have also been observed. In one report, only 10% MAGE-specific lymphocytes infiltrating HCC expressed perforin (Zerbini, Pilli et al. 2004). Other studies confirm that perforin and Granzyme B expression were deficient in the majority of CD8<sup>+</sup> T-cells infiltrating in HCC and chronic HCV (Unitt, Rushbrook et al. 2005; Wang, Holmes et al. 2006). Taken together, cytotoxic and helper T cells are abundantly present in the tumors as well as in the surroundings of the tumors but they, somehow, are not able to perform their functions completely or correctly to eradicate tumors.

### **Th17 cells.**

Recently, a novel lineage of CD4<sup>+</sup> T cells have been described in various kinds of cancer (Ciree, Michel et al. 2004; Harrington, Mangan et al. 2006; Miyahara, Odunsi et al. 2008; Zhang, Rong et al. 2008). Th17 cells are characterized by the ability to secrete IL-17, IL-21, IL-22 and IL-6 and act as pro-inflammatory cells (Ye, Rodriguez et al. 2001; Wei, Laurence et al. 2007; Wilson, Boniface et al. 2007). Kryczek et al reported the presence of naturally occurring Th17 cells in the tumor microenvironment and tumor-draining lymph nodes in both human and mice tumors (Kryczek, Wei et al. 2007). Interestingly, it is the only pro-inflammatory cell known in human till now which do not suppressed by CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T (Evans, Suddason et al. 2007), suggesting that Th17 cells may be regulated by the other mechanism. In a very recent study, Ag specific Th17 cells in HCV infected patients have been induced and they can be suppressed by TGF- $\beta$  and IL-10 (Rowan, Fletcher et al. 2008). Anti-tumor functions of Th17 cells have been demonstrated recently in melanoma and they can become a candidate for the immunotherapy of HCC (Muranski, Boni et al. 2008).

## **Regulatory T cells (T<sub>reg</sub>).**

T<sub>reg</sub> are the major regulators of concomitant tumor immunity as they efficiently suppressed CTL mediated concomitant immunity against a rechallenge with the same tumor (Turk, Guevara-Patino et al. 2004). T<sub>reg</sub> cells abrogated CD8<sup>+</sup> T cell mediated tumor rejection in a transgenic murine colon carcinoma model by specifically suppressing cytotoxicity of CTLs (Chen, Pittet et al. 2005). In a murine fibrosarcoma model, the majority of tumor-infiltrating lymphocytes (TILs) at late stage of tumor progression were T<sub>reg</sub> cells and their depletion successfully enhanced antitumor immunity (Linehan and Goedegebuure 2005).

T<sub>reg</sub> cells can inhibit TAA-specific CD8<sup>+</sup> T-cell cytotoxicity and contributed to tumor growth *in vivo* in a human and chimeric model (Curiel, Coukos et al. 2004). An accumulation of T<sub>reg</sub> cells in the tumor predicted a striking reduction of patient survival (Curiel, Coukos et al. 2004). T<sub>reg</sub> that were isolated from peripheral blood, ascites or solid tumors of patients can suppress T-cell activation *in vitro* (Curiel, Coukos et al. 2004). An increased numbers of T<sub>reg</sub> cells in patients with non-small-cell lung cancer and ovarian cancer were observed as compared to normal donors and they also secrete TGF- $\beta$  (Woo, Chu et al. 2001). Furthermore, a higher frequency of T<sub>reg</sub> cells in peripheral blood and tumors was reported in patients with various cancers (Woo, Chu et al. 2001; Liyanage, Moore et al. 2002; Somasundaram, Jacob et al. 2002; Gray, Arosio et al. 2003; Ichihara, Kono et al. 2003; Sasada, Kimura et al. 2003; Wolf, Wolf et al. 2003; Karube, Ohshima et al. 2004; Marshall, Christie et al. 2004; Viguier, Lemaitre et al. 2004; Ormandy, Hillemann et al. 2005). Prevalence of T<sub>reg</sub> cells increased again in patients having a relapse after tumor resection (Kono, Kawaida et al. 2006).

It has been suggested that the accumulation of intrahepatic T<sub>reg</sub> increases the risk of malignancy by inhibiting anti-tumor responses and their numbers are high in the tumors of HCC patients (Ormandy, Hillemann et al. 2005; Hoofnagle and Seeff 2006). T<sub>reg</sub> isolated from HCC suppressed proliferation of autologous circulating CD4<sup>+</sup>CD25<sup>-</sup> cells, perforin expression and proliferation of autologous CD8<sup>+</sup> T cells. The proportion of circulating T<sub>reg</sub> cells in patients with HCC was similar to that of healthy controls but the proportion of circulating T<sub>reg</sub> cells that were also TGF- $\beta$ <sup>+</sup> was elevated in HCC as compared to controls (Unitt, Rushbrook et al. 2005).

T<sub>reg</sub> are increased in peripheral blood mononuclear cells (PBMCs) from hepatocellular carcinoma (HCC) patients (with HCV) and positively correlate with the tumor burden and

inhibit CD4<sup>+</sup>CD25<sup>-</sup> T-cell proliferation (Cao, Cabrera et al. 2007). There were also high numbers of T<sub>reg</sub> in the tumor-infiltrating lymphocytes of HCC patients comparable with the increase in their peripheral blood (Ormandy, Hillemann et al. 2005). T<sub>reg</sub> cells represent approximately 10% of TILs and *in vitro* study indicates that they are effective at these ratios and that tumor T<sub>reg</sub> cells suppress immune responses of the circulating lymphocytes (Unitt, Rushbrook et al. 2005). The suppressive effect of T<sub>reg</sub> in HCC patients was not cytokine mediated but rather cell-contact mediated as shown by Transwell analysis (Ormandy, Hillemann et al. 2005). In addition, T<sub>reg</sub> also suppress the cytotoxicity of NK cells (Wolf, Wolf et al. 2003), suggesting that T<sub>reg</sub> suppress the cytotoxicity of effector cells and reduce their potential to control the tumor growth.

In addition, Intrahepatic virus-specific *IL-10-producing CD8 T cells* prevent liver damage during chronic hepatitis C virus infection. These cells are not cytotoxic and are unable to control viral replication. They can attenuate hepatocellular necrosis, liver fibrosis and inflammation mediated by bystander T cells and may thus represent antigen-induced regulatory CD8 T cells (Abel, Sene et al. 2006). The presence of similar kind of cells in the liver with HCC cannot be rolled out because most of HCC develop in the liver with chronic HCV.

### **γδT cells.**

γδ T cells represent a small subset of T cells that possess a γδ instead of αβ TCR on their surface. This group of T cells is usually much less common than αβ T cells but are found in their highest abundance in the gut mucosa, within a population of lymphocytes known as intraepithelial lymphocytes (IELs) (Beetz, Wesch et al. 2008). Certain tumor cells might produce metabolites such as IPP (isopentenyl pyrophosphate) at increased levels, which then can be recognized by γδ T cells (Gober, Kistowska et al. 2003; Uchida, Ashihara et al. 2007). In both instances, γδT cells respond immediately with release of proinflammatory cytokines and/or lysis of infected cells or tumor cells.

The livers of patients with chronic viral hepatitis contain elevated numbers of T cells that express γδ form of T-cell receptor for antigen (Nuti, Rosa et al. 1998). γδ T cells have also been shown to have cytotoxic activity and to produce both Th1 and/or Th2 cytokines (Ferrick, Schrenzel et al. 1995; Chien, Jores et al. 1996). Additionally, many studies have shown that elevated numbers of γδT cells exist at inflammatory sites in the viral infections including HCV (Carding, Allan et al. 1990; Ferrick, Schrenzel et al. 1995; Chien, Jores et al. 1996;

Salerno and Dieli 1998; Tseng, Miskovsky et al. 2001). The fact that  $\gamma\delta$  T cells can produce Th1 cytokines and recognize and kill not only virus-infected cells (Bukowski, Morita et al. 1994; Wallace, Malkovsky et al. 1995) but also cells expressing stress proteins (Groh, Steinle et al. 1998) or heat shock proteins (Hsps) (Doherty, Allan et al. 1991; Kaufmann 1996) suggests that these cells may have a role in the liver pathology that develops in HCV-infected individuals including HCC.  $\gamma\delta$ T cell deficient mice show increased susceptibility to solid tumors (Girardi, Oppenheim et al. 2001). Tumor-infiltrating  $\gamma\delta$ T lymphocytes are correlated with a brief disease free interval in advanced ovarian cancer (Raspollini, Castiglione et al. 2005). In HCC,  $\gamma\delta$ T cells acquire the effector memory phenotype CD45RA<sup>-</sup>CD45RO<sup>high</sup>CD27<sup>-</sup> and display a strong lytic activity toward a broad panel of tumor cell lines (Bouet-Toussaint, Cabillic et al. 2008). Immunohistochemical study of  $\gamma\delta$  T cell in hepatocellular carcinoma showed that they are significantly increased in the tumor capsules compared to amounts in the fibrous septa in non-cancerous cirrhotic areas of the same liver, and as compared to amounts in the liver of patients with cirrhosis. Phenotypic characterization by the two-color double-staining technique showed that CD8/ $\gamma\delta$  cells were significantly increased in the tumor capsule, and that more than one-third of  $\gamma\delta$  also expressed CD56 positive cells (Chin, Morise et al. 1995).

### **NKT cells.**

The innate immune system represents a critical first line of host response to infectious, injurious and inflammatory insults. NKT cells (Natural Killer T-cells) are an important, but relatively poorly understood, component of the innate immune response. Moreover, NKT cells are enriched within the liver, suggesting that within the hepatic compartment NKT cells probably fulfill important roles in the modulation of the immune response to infection or injury. NKT cells are characterized by their rapid activation and secretion of large amounts of numerous types of cytokines, including those within Th1, Th2 and Th17 groups, which in turn can interact with a multitude of other cell types within the liver. In addition, NKT cells are capable of participating in a wide array of effector functions via NKT cell-surface-molecule expression [e.g. FASL (FAS ligand) and CD40L (CD40 ligand)] and the release of mediators (e.g. perforin and granzyme) contained in cellular granules, which in turn can activate or destroy other cells (i.e. immune or parenchymal cells) within the liver. Given the huge scope of potential actions that can be mediated by NKT cells, it has become increasingly apparent that NKT cells may fulfill both beneficial (e.g. clearance of virally infected cells) and harmful

(e.g. induction of autoimmunity) roles in the setting of liver disease (Swain 2008). Human NKT cells express a restricted TCR repertoire, usually containing the invariant  $V\alpha 24J\alpha Q$  chain, and recognize antigens in the context of the nonclassical major histocompatibility complex molecule CD1 (Nascimbeni and Rehermann 2003; Klugewitz, Adams et al. 2004). Although, NKT are frequent in the normal liver, variable percentages of this population has been detected in the chronic HCV liver (Pernollet, Jouvin-Marche et al. 2002).  $CD56^+CD3^-$  NK cells and  $CD56^+CD3^+$  NKT cells are involved in immunosurveillance and antitumor responses (Schmidt-Wolf, Negrin et al. 1997; Yokoyama 1998; Tortorella, Gewurz et al. 2000). NKT cells exhibit both cytotoxic and anti-tumor activities (Carnaud, Lee et al. 1999; Doherty, Norris et al. 1999), and an increase in NKT and  $CD8^+$  T cells has been associated with an antitumour effect in HCC (Shibolet, Alper et al. 2003). Stimulated NKT cells have a potential to reduce tumor burden and even eradicate HCC in mice (Shibolet, Alper et al. 2003; Margalit, Shibolet et al. 2005). In patients infected by HCV, NKT cells expressing NKG2A, or KIR\_ receptors are impaired. Furthermore, NKG2A NKT cells, in the liver and in a lesser extent in the peripheral blood, were positively correlated to histological activity in HCV-infected patients (Bonorino, Leroy et al. 2007). In conclusion, NKT cells are one of the most important immune cells and can be a potential candidate in the immunotherapy against HCC.

### **NK cells.**

Natural Killer (NK) cells are the major component of innate immunity (Kawasaki, Shinkai et al. 1992). According to the surface expression level of CD56, NK cells can be divided into two subsets with important functional distinctions. The vast majority of NK cells displays a moderate level of CD56 ( $CD56^{dim}$ ) with a marked cytotoxicity potential, while a small proportion harbors a high CD56 expression level ( $CD56^{bright}$ ) having a greater capacity to secrete cytokines (Cooper, Fehniger et al. 2001; Jacobs, Hintzen et al. 2001).

The various strategies that many viruses have evolved to evade NK cell effector functions illustrate the central role for these cells in early host defense against viral infections (Orange, Fassett et al. 2002; Lodoen and Lanier 2005). It is known that, both HCV and HBV, impair human and murine NK cell activity (Crotta, Stilla et al. 2002; Tseng and Klimpel 2002). Multiple studies were conducted regarding the functional potential of NK cells in the persistent HCV infections and HCC (Duan, Wang et al. 2004; Lin, Gonzalez et al. 2004; Chen, Wei et al. 2005; Meier, Owen et al. 2005; Morishima, Paschal et al. 2006) but the evidence of an NK cell defect and their role in these chronic infections remains to debate. It

has been established that NK cell frequencies in HCV infected patients and in HCV related HCC are lower than that observed in the uninfected population (Yuen and Norris 2001; Meier, Owen et al. 2005; Morishima, Paschal et al. 2006; Golden-Mason, Madrigal-Estebas et al. 2008). Concerning with dim and bright populations of NK cells, it was reported that the frequency of peripheral blood cytolytic CD56<sup>dim</sup> NK cells is decreased in HCV infected patients (Morishima, Paschal et al. 2006).

NK cells predominantly reside in the liver, in contrast to a relatively small percentage in the peripheral blood (Vermijlen, Seynaeve et al. 2004; Bai, Wang et al. 2006; Subleski, Hall et al. 2006). Several studies have shown that liver NK cells mediate higher cytotoxic activity against tumor cells than spleen or peripheral blood NK cells in rodents (Vermijlen, Seynaeve et al. 2004; Bai, Wang et al. 2006; Subleski, Hall et al. 2006). In patients with a variety of tumors, peripheral NK cell cytotoxicity was found to be significantly decreased compared with non-cancer-bearing controls (Cao, Xi et al. 2007; Jarahian, Watzl et al. 2007; Kano, Soda et al. 2007). This decrease in NK cell cytotoxicity was more marked in patients with advanced disease stages (Steinhauer, Doyle et al. 1982; Pross and Lotzova 1993; Jovic, Konjevic et al. 2001). In particular, NK cells from the liver of HCC patients displayed a reduced cytotoxicity against HCC cell line after *in vitro* IL-2 stimulation, compared with peripheral NK cells and healthy NK cells from donor liver (Ishiyama, Ohdan et al. 2006). This evidence suggests that functional defects in NK cells might be responsible for the failure of anti-tumor immune responses (Sung, Johnson et al. 1996). It is unclear what mechanisms are responsible for impaired NK cytotoxicity, particularly in HCC patients. Recently, it has been found that HCC patients display a dramatic reduction in peripheral CD56<sup>dim</sup> NK subsets as compared to the healthy subjects. A significant reduction of CD56<sup>dim</sup> NK subsets was also found in the tumor regions as compared to non-tumor regions in the livers of these HCC patients. Both these peripheral and tumor-infiltrating NK cells exhibited poor capacity to produce IFN- $\gamma$  and cytotoxicity (Cai, Zhang et al. 2008). In summary, along with much reduced NK cell population, they are also functionally impaired in the liver of HCC patients, suggesting that NK cells might not be able to control the tumor growth in this malignancy.

### **B cells.**

B cells play a large role in the humoral immune response against diseases. The principal functions of B cells are to make antibodies against antigens, perform the role of Antigen Presenting Cells (APCs) and eventually develop into memory B cells after activation by

antigen interaction. HCV-specific antibodies have been detected in the patients with chronic HCV and HCC patients (Chen, Sallberg et al. 1999; Logvinoff, Major et al. 2004; Chen, Fu et al. 2008). Sometimes antibodies are generated against tumor cells allowing for their destruction by the complement system (Guevara-Patino, Turk et al. 2003). Humoral immune response has been detected in HCC patients to a known tumor-specific antigen, NY-ESO-1 protein (Korangy, Ormandy et al. 2004). It had been observed that CD20<sup>+</sup> cells are mainly concentrated in the lymphoid follicles/aggregates outside of the tumor, where they are localized in the centre and are surrounded by CD3<sup>+</sup> cells zone (Murakami, Shimizu et al. 1999). B cells in the liver of HCC patients were found significantly higher than normal control and liver cirrhosis patients. Moreover, their numbers in the cancerous tissues of stage I and II was significantly higher than that of stages III and IV, suggesting that the number B cells decrease in the patients with the progression of HCC (Chen, Luo et al. 2006). A population innate B cells, expressing CD5, with an increased expression of CD81 was found expanded in the livers and peripheral blood of chronic HCV-infected patients, suggesting a role in viral specific activation and clonal proliferation in chronic HCV infection (Curry, Golden-Mason et al. 2003). Recently, antibodies against certain tumor antigen have been detected in HCC patients, which indicates that disease-specific humoral response is induced in HCC patients and the corresponding antibodies may be used as tumor markers for HCC (Chen, Fu et al. 2008).

### **Dendritic Cells.**

Dendritic Cells (DCs) are the immune cells which process antigen and present it on the surface to other cells of the immune system, thus functioning as Antigen Presenting Cells (APCs). They can be found in an immature state in the blood. Once activated, they migrate to the lymphoid tissues where they interact with T cells and B cells to initiate and shape the adaptive immune response. They can be divided as myeloid dendritic cells (MDC) and plasmacytoid dendritic cells (PDC). MDC are most similar to monocytes and their main function is antigen presentation. PDCs look like plasma cells but have certain characteristics similar to myeloid dendritic cells. They can produce high amounts of IFN- $\alpha$  and were known as IPC (interferon-producing cells) before their dendritic cell nature was revealed.

Tumor environmental factors, such as vascular endothelial growth factor (VEGF), IL-10 and TGF- $\beta$  suppress DC differentiation and function, resulting in immature and/or partially differentiated DCs (Gabrilovich 2004; Zou, Barnett et al. 2004). Plasmacytoid DCs associated



with ovarian cancer induced CD8<sup>+</sup>FOXP3<sup>-</sup>IL-10<sup>+</sup> regulatory T cells (Zou, Machelon et al. 2001; Wei, Kryczek et al. 2005), and dysfunctional myeloid DCs also induced IL-10<sup>+</sup> regulatory T cells *in vitro* (Jonuleit, Schmitt et al. 2000) and *in vivo* in the patients with cancer (Dhodapkar, Steinman et al. 2001; Chakraborty, Chattopadhyay et al. 2004). Tumors convert DCs into TGF- $\beta$ -expressing immature myeloid DCs that are capable of promoting T<sub>reg</sub> proliferation (Ghiringhelli, Puig et al. 2005), suggesting that tumor microenvironments promote the immature form of DCs which intern suppress the tumor immunity perhaps via regulatory T cells. Reduced numbers of myeloid DCs with impaired allo-stimulatory capacity and a reduced IL-12 secretion have been observed in the patients with HCC (Ormandy, Farber et al. 2006). Moreover, hepatic lymph nodes of the patients with HBV or HCV infection complicated by HCC showed a reduction in numbers of mature MDCs and an increase in numbers of PDCs in their T-cell areas as compared to viral hepatitis only (Tang, Vukosavljevic et al. 2006). In addition, HCC ablation induces a functional transient activation of MDC but not of PDC (Ali, Grimm et al. 2005), indicating that HCC alter the distribution and function of DCs in favor of tumor progression.

Dendritic cells are professional antigen-presenting cells able to initiate a T-cell-mediated immune response against tumor-associated antigens (TAA) (Banchereau and Steinman 1998). We and others have shown previously that TAA-loaded DCs can stimulate cytotoxic lymphocytes significantly to lyse HCC cells *in vitro* (Gonzalez-Carmona, Marten et al. 2006). DC vaccination induced protective immunity toward the development of HCC. However, DC-based approaches have not been substantially effective in inducing tumor regression of established HCC (Vollmer, Eilber et al. 1999; Lee, Wang et al. 2001). In clinical trials, TAA-loaded DCs evoked immunological responses in patients with HCC but therapeutic effects were limited (Chi, Liu et al. 2005; Lee, Wang et al. 2005). Despite this, DC-based immunotherapy is considered promising for cancer treatment. However, new strategies to improve its efficacy are necessary.

## Cytokines and HCC

A component of the immune system that modulates the host response to foreign pathogens is cytokines, which are secreted or membrane-bound proteins that regulate the growth, differentiation and activation of immune cells. Cytokines are released in response to a diverse range of cellular stresses including infection, inflammation and carcinogen-induced injury. In fact, many cytokines have pleiotropic functions and some can act in a synergistic manner. Several cytokines, particularly those produced by CD4<sup>+</sup> Th cells, are defined as Th1 or Th2 and are comprised mainly of ILs. Th1 cytokines (e.g., IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-12p35, IL-12p40, IL-15, and non-ILs, e.g., TNF- $\alpha$  and IFN- $\gamma$ ) are generally referred as proinflammatory. On the other hand, Th2 cytokines (e.g., IL-4, IL-8, IL-10 and IL-5) induce anti-inflammatory responses.

The liver hosts many cell types that are susceptible to the actions of cytokines. *Hepatocytes* bear a variety of cytokine receptors such as IL-1, TNF- $\alpha$  and IL-6. Non-parenchymatous cells, such as the resident liver macrophages (*Kupffer cells*), not only synthesize many cytokines, but the actions of these immune cells can also be affected by the cytokine environment. Liver *sinusoidal endothelial cells* are also targets and producers of various cytokines. *NKT cells* are generally increased in chronically infected livers and produce profibrotic cytokines (IL-4 and IL-13) (Wiltrout 2000; de Lalla, Galli et al. 2004). Cytokines have therefore been implicated in liver development and regeneration but may also contribute to the pathogenesis of liver-related diseases such as cirrhosis, fibrosis and cancer.

The effect of cytokine function on tumorigenesis and progression is complex as a result of the pleiotropy and apparent redundancy of cytokine action. Despite this, many experiments have begun to elucidate the complex associations between cytokines and tumors. The continued expression of cytokines and recruitment of activated lymphomononuclear cells to the liver affects many cellular pathways and ultimately results in fibrosis, cirrhosis and/or HCC (Budhu and Wang 2006). Intrahepatic production of cytokines and chemokines by HCV infection promotes the recruitment of nonspecific lymphocytes. In the absence of viral clearance, this pathway boosts itself, leading to necroinflammatory and fibrotic liver disease. In HCV transgenic mice, a significant lymphocyte infiltration was observed in the portal tracts of livers and was associated with increasing serum alanine transaminase (ALT) levels

(Wakita, Katsume et al. 2000). These changes in liver function are associated with the development and progression of HCC. In the liver, an organ enriched with immune cells, cell damage and regeneration, mediated by viral hepatitis-induced immune responses, may lead to liver cancer by promoting cell proliferation and death (Nakamoto, Guidotti et al. 1998; Nakamura and Imawari 2000; Rehermann 2000). It has been proposed that necrosis of hepatocytes, as a result of chronic inflammation and consequent regeneration, enhances mutagenesis in host cells, which can accumulate and culminate in HCC (Freeman, Marinos et al. 2001; Rapicetta, Ferrari et al. 2002; Rehermann 2003).

Mounting evidence indicates the involvement of cytokines in hepatocarcinogenesis. Thus, various avenues have been taken to elucidate changes in cytokine expression levels in patients with HCC. To determine whether cytokine expression correlates with disease progression, many comparisons have been made, including HCC tumor and nontumor samples, chronic liver disease (CLD) patients and normal healthy individuals. As cytokines are secreted and can be measured in the serum and plasma, several groups have analyzed certain cytokines for their predictive capacity.

### **Cytokine expression levels in HCC.**

The mRNA and protein expression of cytokines in HCC has been demonstrated in the blood, tumors and comparative normal liver samples by immunohistochemistry (IHC), quantitative real-time PCR (qRT-PCR) and ELISA.

#### *Anti-inflammatory cytokines in HCC:*

IL-10 was originally isolated from mouse T<sub>H</sub>2 cells as a cytokine synthesis inhibitory factor (CSIF) and was thought to be specifically produced by these cells to inhibit T<sub>H</sub>1-cell responses (Fiorentino, Bond et al. 1989; Moore, de Waal Malefyt et al. 2001). However, it soon became clear that this cytokine was also produced by many other cell types, including B cells (O'Garra and Vieira 1992; Moore, de Waal Malefyt et al. 2001; O'Garra, Vieira et al. 2004), mast cells, eosinophils (Moore, de Waal Malefyt et al. 2001), macrophages, DCs (Moore, de Waal Malefyt et al. 2001; Edwards, Manickasingham et al. 2002; Dillon, Agrawal et al. 2004; Boonstra, Rajsbaum et al. 2006) and many subsets of T cells, such as CD8<sup>+</sup> T cells and T<sub>Reg</sub> cells (Kamanaka, Kim et al. 2006; Uhlig, Coombes et al. 2006). Furthermore, T<sub>R</sub>1 cells induced by IL-10 and IFN- $\alpha$  secrete enhanced levels of both IL-10 and IFN- $\gamma$

(Levings, Sangregorio et al. 2001). In one study, CD4<sup>+</sup> T cells that express T-bet and secrete large amounts of IFN- $\gamma$  were found to be the source of IL-10 that regulates the immune response against *T. gondii* (Jankovic, Kullberg et al. 2007). The principal routine function of IL-10 appears to be to limit and ultimately terminate inflammatory responses. The suppressive function of IL-10 involves the inactivation of macrophages and DCs, with a consequent inhibition of pro-inflammatory cytokine secretion (e.g. TNF- $\alpha$ ) and inhibition of the expression of MHC class II and co-stimulatory molecules (de Waal Malefyt, Abrams et al. 1991; Fiorentino, Zlotnik et al. 1991; Ding, Linsley et al. 1993; Moore, de Waal Malefyt et al. 2001). It also inhibits the activation and effector function of T cells, monocytes and macrophages. In addition to these activities, IL-10 regulates growth and/or differentiation of B cells, NK cells, cytotoxic and helper T cells, T regulatory cell, mast cells, granulocytes, dendritic cells, keratinocytes and endothelial cells (Moore, de Waal Malefyt et al. 2001). In contrast to its major suppressive functions, IL-10 can also have a positive effect on certain immune responses, which include the direct enhancement of IL-10 production by CD4<sup>+</sup> regulatory T cells (Barrat, Cua et al. 2002).

Of the many anti-inflammatory cytokines, the most studied with regard to HCC expression is IL-10. Serum IL-10 levels in patients with HCC resections were tested and found significantly higher in HCC than in healthy individuals. Patients with high IL-10 had a worse disease-free survival and a multivariate analysis implied that IL-10 may be a predictor of the postresection outcome of HCC patients (Chau, Wu et al. 2000; Zekri, Ashour et al. 2005). Several groups have shown that IL-10 is highly expressed in HCC tumors versus nontumorous-surrounding tissue (Kakumu, Okumura et al. 1997; Chia, Ban et al. 2002; Kitaoka, Shiota et al. 2003; Beckebaum, Zhang et al. 2004; Zekri, Ashour et al. 2005). These studies suggest that increases in IL-10 and perhaps other Th2 cytokines correlate with progression.

Th2 lymphocytes also produce **IL-4**, which leads to fibrogenesis in liver injury to a greater extent than Th1 lymphocytes. The increased abundance of IL-4 message identified in stages F2 to F4 of fibrosis is likely to contribute to the overall fibrogenic process (Lau, Luxon et al. 2005).

#### *Pro-inflammatory cytokines in HCC:*

Pro-inflammatory cytokine **IL-1 $\beta$** , mainly produced by mononuclear cells, plays a central role in the communication between cells of the immune system (Neta and Oppenheim 1988). It is a co-mitogen for thymocyte proliferation, induces  $\kappa$ -light chain upregulation in pre B-cells,

promotes B cell proliferation and in a network with other cytokines induces proliferation of haematopoietic progenitor cells (Fibbe and Falkenburg 1990). It causes a rapid induction of transcriptional regulator genes such as AP-1 and nuclear factor (NF)- $\kappa$ B (Bankers-Fulbright, Kalli et al. 1996; Funakoshi, Sonoda et al. 2001). IL-1 $\beta$  mRNA was lower in the tumors versus the surrounding tissue (Bortolami, Venturi et al. 2002). Two members of the common  $\gamma$  chain family of cytokines, IL-7 and IL-15, are central to homeostatic proliferation and survival of mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Boyman, Purton et al. 2007). In one study, the serum level of **IL-15** was higher in HCC and reflected the degree of inflammation in the liver (Kakumu, Okumura et al. 1997). The expression of **IL-18** was shown to be slightly higher in normal surrounding tissue versus the tumor (Chia, Ban et al. 2002) IL-18 is up-regulated in the tumors of HCV-positive HCC patients (Asakawa, Kono et al. 2006).

**TNF- $\alpha$**  is produced in response to tissue injury and is associated with an increase in cell-cycle progression and oxidative stress through the formation of 8-oxo-deoxyguanosine, an established marker of DNA damage associated with chronic hepatitis in human livers (Wheelhouse, Chan et al. 2003). TNF- $\alpha$  takes part in immune stimulation enhancing the expression of human leukocyte antigen (HLA) class II, affecting the generation of cytotoxic T cells), inflammation, acute phase response and tissue remodeling (Tracey and Cerami 1994; Wallach 1997). TNF- $\alpha$  is involved in the initiation of liver regeneration inducing the activation of transcriptional factors (NF- $\kappa$ B and STAT3) which make hepatocytes capable of proliferating and responding to growth factors (HGF and TGF) (Kirillova, Chaisson et al. 1999; Wheeler, Yamashina et al. 2001). On the other hand, TNF- $\alpha$ , originally known as a tumoricidal protein, induces apoptosis of different cell types (Elzey, Griffith et al. 2001). Serum TNF- $\alpha$  expression and its receptors (TNF- $\alpha$ Rs, TNF $\alpha$ RI and TNF $\alpha$ RII) were elevated in chronic HCV with cirrhosis and HCC patients, especially those with recurrence (Nakazaki 1992; Sato, Asanuma et al. 1996; Kakumu, Okumura et al. 1997; Huang, Hwang et al. 1999). However, in other studies, TNF- $\alpha$  levels were lower in HCC tumor tissue versus the tissue surrounding the tumor and in HCC patients versus healthy individuals (McGuinness, Painter et al. 2000; Bortolami, Venturi et al. 2002; Zekri, Ashour et al. 2005). Cytokine stimulation of TNF- $\alpha$ , IL-1 $\beta$  or IL-18 has also been shown to induce expression of TRAIL in HCC cell lines (HepG2, Hep3B, Huh7) (Shiraki, Yamanaka et al. 2005). The expression of TRAIL on the HCC cell surface might contribute to tumor cell immune evasion by inducing apoptosis of activated human lymphocytes. Kupffer cells and monocyte-infiltrating cells have been considered to be the major source of IL-1  $\beta$  and TNF-  $\alpha$ , which have diverse effects on

different target cells (Whicher and Evans 1990; Dinarello 2000). Moreover, the mean levels of IL-1  $\beta$  and TNF- $\alpha$  mRNA in HCC patients were significantly higher in the pericancerous tissues than in the related cancer tissues (Bortolami, Venturi et al. 2002).

**IFN- $\alpha$**  is secreted by many cell types including lymphocytes (NK cells, B-cells and T-cells), macrophages, fibroblasts, endothelial cells, osteoblasts and others. They stimulate both macrophages and NK cells to elicit an anti-viral response and are also active against tumors. In HCC patients, tumor and non-tumor sections were more positive for IFN- $\alpha$  mRNA than histologically normal liver, however, deficient IFN- $\alpha$  expression in HCC was shown at the protein level (d'Arville, Nouri-Aria et al. 1993). Higher levels of IFN- $\alpha$  in cHCV and HCC patients can up-regulated DC production of IL-15, which is capable of activating NK and CTLs. Therefore, IFN- $\alpha$  may function to control tumorigenicity (Yamaji, Nabeshima et al. 2006). IFN- $\alpha$  treatment induced monocyte production of TNF- $\alpha$ , IL-6 and IL-12 (Szabo, Catalano et al. 2001). Moreover, IFN- $\alpha$  has also been shown to act directly on CD8<sup>+</sup> cells to improve expansion of antigen-specific CD8<sup>+</sup> cells *in vivo* (Kolumam, Thomas et al. 2005; Le Bon, Durand et al. 2006).

**IFN- $\gamma$**  is secreted by Th1 cells, Tc cells, DCs and NK cells and has antiviral, immunoregulatory and anti-tumor properties (Schroder, Hertzog et al. 2004). The expression of IFN- $\gamma$  was slightly higher in the adjacent normal tissue than tumor in HCC liver (Chia, Ban et al. 2002). It has been shown that normal hepatocytes express weak levels of IFN- $\gamma$ R, but the expression of IFN- $\gamma$ R is up-regulated in acute and chronic liver diseases. Further examination revealed that tumor size, ALT, metastasis and proliferation were higher in IFN- $\gamma$ R-negative liver tissue cases. Therefore, the loss of IFN- $\gamma$ R might contribute to immune escape (Nagao, Nakajima et al. 2000). Increased hepatic IFN- $\gamma$  levels have been found in acute as well as chronic liver diseases (Napoli, Bishop et al. 1996; Dumoulin, Wennrich et al. 2001). In addition, IFN- $\gamma$  can stimulate CD8<sup>+</sup> T cells to mediate their expansion during viral infection (Whitmire, Benning et al. 2005). On the other hand, IFN- $\gamma$  have a potent indirect influence on memory CD8<sup>+</sup> cell homeostasis as they can induce production of IL-15 and IL-15R $\alpha$  in antigen-presenting cells (APCs) (Whitmire, Benning et al. 2005). IFN- $\gamma$  can also dampen the extent of T cell activation and limit T cell-mediated tissue damage (Eriksson, Kurrer et al. 2001; Pewe, Haring et al. 2002). Chronic viral hepatitis is associated with impaired effector functions of HCV-specific CD8<sup>+</sup> T cells that probably contribute significantly to viral persistence (Boettler, Spangenberg et al. 2005).

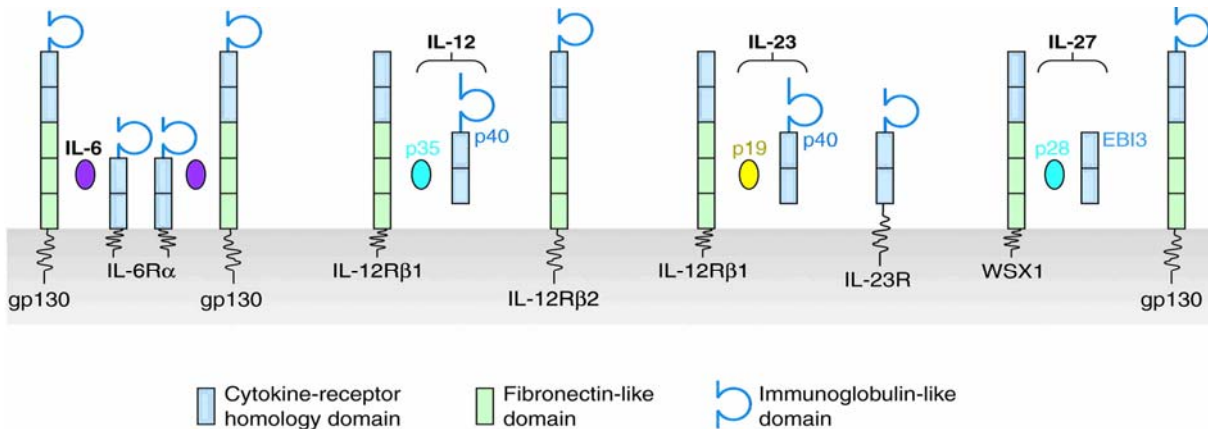
**IL-2** is a cytokine which plays role in differentiation, proliferation and maturation of T cells and NK cells. It was measured and compared with the density of CD8<sup>+</sup> tumor-infiltrating lymphocytes (TILs) in HCC resection patients. A decrease in CD8<sup>+</sup> cells was seen in tumor versus noncancerous tissue and an increase in CD8<sup>+</sup> cells was observed in the tumors with IL-2 expression. As patients with IL-2<sup>+</sup> tumors have a favorable prognosis, thus concluded that IL-2 activates CD8<sup>+</sup> T cells in TILs and may be a good prognostic marker (Ikeguchi and Hirooka 2005), however, signals mediated by IL-2–IL-2R have also been linked to the development, maintenance, survival and expansion of regulatory T cells populations (Malek and Bayer 2004). The proinflammatory cytokines IL-2 and IL-12 are also increased in serum (Kitaoka, Shiota et al. 2003) and in the surrounding tissues (Ikeguchi and Hirooka 2005) in HCC patients.

**IL-12** is naturally produced by DCs, macrophages and B-cells in response to antigenic stimulation. It is also required as ‘third’ signal other than antigen and costimulatory receptors for naïve CD8<sup>+</sup> T cells for their optimal proliferation and effector functions (Mescher, Curtsinger et al. 2006). The molecular events that control this third signal are not clear to date but recent evidence suggested that, whereas antigen and costimulatory signals were sufficient for upregulation of the anti-apoptotic proteins Bcl-2 and Bcl-x<sub>L</sub>, IL-12 was needed for Bcl-3 upregulation (Valenzuela, Hammerbeck et al. 2005; Li, Eppolito et al. 2006). IL-12 can act directly on CD8<sup>+</sup> cells and is reported to be involved in the regulation of T-bet (Intlekofer, Takemoto et al. 2007). Previous reports demonstrated potent antitumor effects associated with IL-12 gene therapy (Tahara, Zitvogel et al. 1995) and DCs (Tatsumi, Takehara et al. 2007) in murine tumor models. In cancer patients, DCs are often found in an immature and hypostimulatory state (Grohmann, Belladonna et al. 1998; Cerundolo, Hermans et al. 2004) demonstrated that IL-12 directly activates DC by signals mediated by IL-12 receptors expressed on DCs. Thus, IL-12 transfection into DCs isolated from cancer patients might be expected to lead to recovery of their impaired function via a positive feedback loop. DCs secreting abundant IL-12 would be expected to activate both T cells and NK cells in the liver that could prove therapeutic. IL-12 transfection of DCs restores the impaired phenotype/function of endogenous DCs isolated from tumor-bearing animals and intratumoral injection of IL-12 gene-transduced DCs activates both innate and acquired immunity in a liver tumor model (Tatsumi, Takehara et al. 2007).

**IL-23** has pro-inflammatory activity, inducing **IL-17** secretion from activated CD4<sup>+</sup> T cells and stimulating the proliferation of memory CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells that specifically produce IL-17 are now called T helper 17 (T<sub>H</sub>17) cells. T<sub>H</sub>17-cell differentiation is initiated by IL-6 in the presence of TGF-β. IL-23 is now believed to be necessary for expansion and/or maintenance of T<sub>H</sub>17 cells (Harrington, Hatton et al. 2005; Yoshimura, Naka et al. 2007). Th17 differentiation is strongly inhibited by IL-12 or IFNγ (Bettelli, Carrier et al. 2006; Ivanov, McKenzie et al. 2006; Veldhoen and Stockinger 2006). IL-23/IL-17 pathway is associated with local tissue inflammation that produces swelling, heat and pain, and sets up an environment with heightened immune responses. In this sense, the IL-12/IFN-γ and IL-23/IL-17 immune responses may work together to control microbial infections. As IL-17 is the signature Th17 effector cytokine, it is important to consider its biological activities. It was initially discovered as a potent proinflammatory cytokine produced by activated CD4<sup>+</sup> memory T cells. It is a locally produced cytokine that acts on stromal, epithelial and endothelial cells and a subset of monocytes to induce secretion of proinflammatory mediators such as IL-8, CXCL1, TNF and G-CSF that promote rapid neutrophil recruitment and are important for the control of acute infection (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006; Veldhoen and Stockinger 2006). **IL-23** is significantly upregulated in the overwhelming majority of human carcinoma samples (Langowski, Zhang et al. 2006).

**IL-27** is a new IL-6/IL-12 cytokine family member that not only has a role in the early regulation of T<sub>H</sub>1-cell differentiation but also exerts an inhibitory effect on immune responses, including T<sub>H</sub>17-cell differentiation. IL-27 can suppress the effect of IL-23 and reduce T<sub>H</sub>17-cell differentiation. In a tumor model with poorly immunogenic tumor cells were transduced to express IL-27 revealed that they suppressed tumor-induced neovascularization, and this factor could act directly on endothelial cells to induce the production of the antiangiogenic chemokines IP-10 and MIG (Shimizu, Shimamura et al. 2006). Indeed, the IL-27R is also expressed on other lymphocytes including NK cells, NKT cells, T regulatory cells, B cells macrophages, mast cell and certain memory populations (Pflanz, Timans et al. 2002; Hamano, Himeno et al. 2003; Artis, Villarino et al. 2004; Yamanaka, Hamano et al. 2004; Holscher, Holscher et al. 2005; Larousserie, Charlot et al. 2006). Together, this data suggests that IL-27 can suppress effector functions of a range of immune cell types involved in innate and adaptive immunity.





**Fig. 8.** IL-6, IL-12, IL-23 & IL-27 share some common receptor peptides and domains (Kastelein, Hunter et al. 2007).

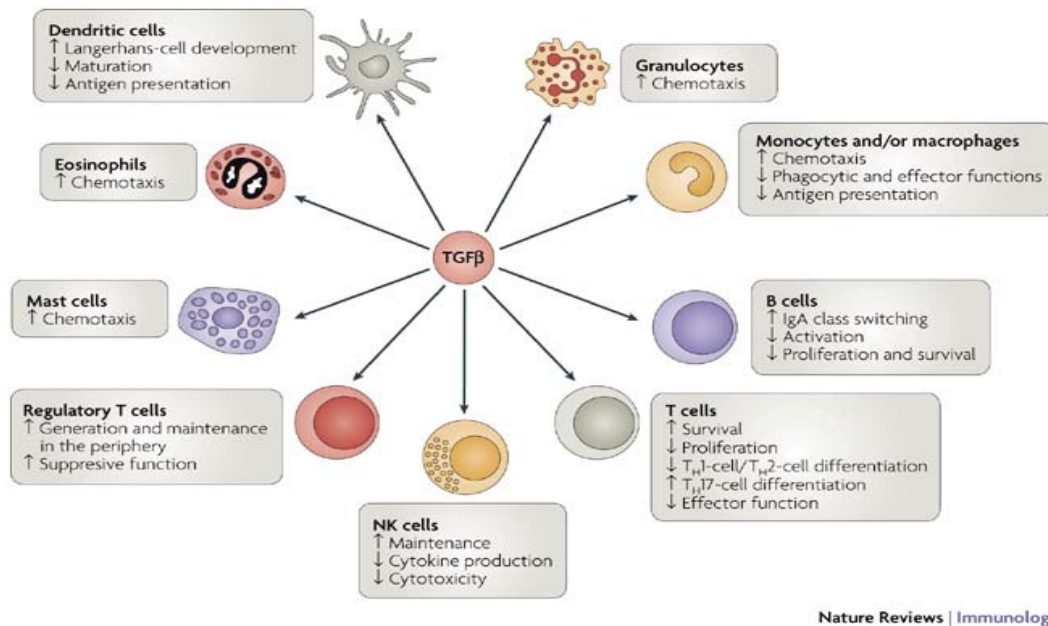
*Cytokines neither Th1 nor Th2 and HCC:*

Several cytokines, which do not function specifically in a Th1 or Th2-restricted group or do not fall into IL family, have also been correlated with HCC. **IL-6**, a cytokine categorized as pro- and anti-inflammatory with a multifunctional role in host defense, was present at higher levels in the serum of HCC and chronic HCV patients compared with healthy individuals (Hu, Lee et al. 1999; Huang, Hwang et al. 1999). However, IL-6 has also been shown to be down-regulated in chronic hepatitis and cirrhotic tissue versus normal liver tissue (McGuinness, Painter et al. 2000). In a separate study, IL-6, IL-6R, and downstream pathway members were measured in liver samples from HCC rat model versus normal surrounding tissue. HCC has decreased IL-6R as compared with normal surrounding tissue. IL-6 was also shown to inhibit serum-stimulated DNA synthesis and cell mitogenesis in HCC cells *in vitro* (Moran, Mayes et al. 2005). Therefore, IL-6 and other non-Th1/Th2 cytokines may contribute to the pathogenesis of HCC at the stages of transformation and metastasis.

The transforming growth factor- $\beta$  (**TGF- $\beta$** ) family of proteins is a large group of secreted pleiotropic cytokines that have a wide range of functional properties which inhibits cytolytic and T helper 1 (T<sub>H</sub>1)-cell differentiation, proliferation and their effector functions and also required for the maintenance of the peripheral CD4<sup>+</sup> regulatory T (T<sub>Reg</sub>)-cell subset (Kehrl, Wakefield et al. 1986; Marie, Letterio et al. 2005; Li, Wan et al. 2006; Marie, Liggitt et al. 2006). TGF- $\beta$ 1 can act in both an autocrine and paracrine manner. Macrophages, T cells, dendritic cells (DCs) and other immune-cell types can serve as both its source and its

physiological targets (Letterio and Roberts 1998; Li, Wan et al. 2006). Furthermore, stromal cells in the primary, secondary and tertiary lymphoid organs, including epithelial cells and fibroblasts, also secrete TGF- $\beta$ 1 in substantial amounts (Lagneaux, Delforge et al. 1993; Filer, Pitzalis et al. 2006). TGF- $\beta$  was demonstrated to have lower or similar concentrations in HCC and in the surrounding tissue (Jaskiewicz and Chasen 1995; Grasl-Kraupp, Rossmannith et al. 1998). However, in other studies, serum levels of TGF- $\beta$  were higher in HCC patients versus healthy individuals (Yuen, Norris et al. 2002; Kim, Lee et al. 2003). Similarly, TGF- $\beta$  serum level was higher in patients with alcohol-related cirrhosis and chronic HCV patients versus healthy individuals (Yuen, Norris et al. 2002; Kim, Lee et al. 2003). In addition, Activin A, a member of the TGF- $\beta$  family, and its receptors are expressed in HCC and have been shown to stimulate VEGF transcription, DNA binding, and the transactivation potential of specificity protein 1 (Wagner, Peters et al. 2004).

Naive T cells can also differentiate into ‘induced’ regulatory T cells (iTreg) or inflammatory T cells that secrete IL-17. TGF- $\beta$  regulates Foxp3 expression and iTreg differentiation in cell culture (Zheng, Gray et al. 2002; Chen, Jin et al. 2003; Fantini, Becker et al. 2004; Weber, Harbertson et al. 2006). TGF- $\beta$  also controls differentiation of the novel Th17 subset of effector T cells, characterized by production of the inflammatory cytokine IL-17 (Dong 2006; Weaver, Harrington et al. 2006). TGF- $\beta$  and IL-6 cooperatively instruct expression of the orphan nuclear receptor ROR $\gamma$ t, which in turn drives naïve T-cell differentiation into Th17 effector T-cell subset that produces IL-17, but not IL-4 or IFN $\gamma$  (Ivanov, McKenzie et al. 2006). Both TGF- $\beta$  and IL-6 are absolutely required for the development of Th17 cells *in vivo*, as evidenced by the paucity of Th17 cells in the periphery of mice that lack IL-6 (Ivanov, McKenzie et al. 2006). However, IL-6 and STAT3 oppose Foxp3 expression and iTreg differentiation in cultured T cells in a cell-intrinsic manner, through a mechanism that does not appear to involve STAT3 binding to the *Foxp3* promoter (Yang, Panopoulos et al. 2007; Yao, Kanno et al. 2007).



**Fig. 9.** Positive or negative effects of TGF-β signaling on the indicated aspects of leukocyte biology are marked by arrows pointing up or down. Only cellular processes with a well-documented role for TGF-β are indicated in this figure. NK, natural killer; T<sub>H</sub>, T helper (Rubtsov and Rudensky 2007).

### SOCS, cytokines and HCC.

Suppressor of Cytokine Signaling (SOCS) proteins comprise a family of intracellular proteins, several of which have been shown to regulate the responses of immune cells to cytokines (Ilangumaran, Ramanathan et al. 2004; Davey, Starr et al. 2005; Fletcher and Starr 2005; Naka, Fujimoto et al. 2005). Most SOCS proteins are induced by cytokines and therefore act in a classical negative-feedback loop to inhibit cytokine signal transduction (Yoshimura, Naka et al. 2007).

SOCS1 interferes with IFN signaling even at low levels (Qing, Costa-Pereira et al. 2005; Fenner, Starr et al. 2006), moreover, it also interferes with the maturation of DCs and cytotoxic T lymphocytes (Ilangumaran and Rottapel 2003; Bartz, Avalos et al. 2006) and with T<sub>H</sub>17 cells development (Harrington, Hatton et al. 2005). Most human hepatocellular carcinomas (HCCs) are a consequence of hepatitis C virus (HCV) infection. The expression of SOCS1 is often silenced in these tumors by hypermethylation of CpG islands of the SOCS1 promoter (Yoshikawa, Matsubara et al. 2001; Yoshimura 2006) and the deletion of SOCS1 in tumor cells might enhance IL-6-mediated cell proliferation. Therefore, SOCS1 is considered to be an anti-oncogene because it is a suppressor of signals induced by a growth factor (IL-6). Consistently, *Socs1*<sup>+/-</sup> mice are shown to be hypersensitive to dimethylnitrosamine- induced hepatocarcinogenesis (Yoshida, Ogata et al. 2004). However,

the story may not be so simple. We found that silencing of SOCS1 was frequently observed even in pre-malignant HCV-infected patients (Yoshida, Ogata et al. 2004). Liver injury is associated with hyperactivation of STAT1 and reduced activation of STAT3 (Ogata, Chinen et al. 2006). Therefore, reduced expression of SOCS1 might enhance tissue injury and inflammation by hyperactivation of STAT1, promoting the turnover of epithelial cells and enhancing their susceptibility to oncogenesis. The negative role of SOCS1 in inflammation-associated tumor development is supported by the study which shows that *Socs1*<sup>-/-</sup>-transgenic mice, in which *Socs1* expression is deleted in all types of cells except T and B cells, developed chronic colitis and colon tumors (Hanada, Kobayashi et al. 2006). This study strongly indicates that chronic activation of the IFN $\gamma$ -STAT1 pathway that occurs in the absence of *Socs1* causes colitis-induced colon tumors. Therefore, *Socs1* is a unique anti-oncogene that prevents carcinogenesis by suppressing chronic inflammation.

SOCS3 binds to gp130-related cytokine receptors (IL-6 & IL-27), including the phosphorylated tyrosine 757 (Tyr757) residue of gp130 and Tyr800 of IL-12 receptor  $\beta$ 2 and suppress their effects (Yamamoto, Yamaguchi et al. 2003). SOCS3 expression levels were high in infected non-tumor areas of the patients infected with HCV (Ogata, Chinen et al. 2006). Huang et al. also reported that the levels of SOCS3 are elevated in patients infected with HCV, as well as in chimpanzee models (Huang, Feld et al. 2007). This group suggested that the activation of SOCS3 contributes to the defective hepatic response to IFN- $\gamma$  in HCV-infected liver. Indeed, the levels of SOCS3 were inversely correlated with STAT3 activation in regions of human livers with and without HCC (Ogata, Chinen et al. 2006). The mechanism behind this observation is more easily explicable than that of SOCS1 because numerous studies have shown that hyperactivation of STAT3 can contribute to tumorigenesis by inducing multiple tumor-promoting genes (Yoshikawa, Matsubara et al. 2001; Yoshimura 2006).

Although a multitude of studies have presented evidence for changes in cytokine expression in HCC tumors, the direction of the change is not always consistent. In addition, the cytokine levels found in the local regions, such as near or within the tumor, do not necessarily correspond to their level in serum. In conclusion, an imbalance between Th1 and Th2 cytokines has been observed in the serum, outside and inside the tumor in HCC patients.

### **HCC cytokine expression levels and metastasis/recurrence.**

Some studies have correlated changes in cytokine expression with HCC metastasis and/or recurrence. It has been demonstrated that serum IL-6 level in rat HCC model and in the breast cancer patients is positively correlated with the metastatic potential (Reichner, Mulligan et al. 1998; Coskun, Bukan et al. 2004). The proinflammatory cytokines TNF- $\alpha$ , IL-1 $\alpha$ , and IL-1 $\beta$  were measured in HCC and in metastatic liver carcinoma patients before and after operation and were found high in HCC and metastases patients before operation and increased postoperatively (Nakazaki 1992; Bortolami, Venturi et al. 2002). High expression of IL-8, an anti-inflammatory cytokine with angiogenic action, in cancerous liver tissue was associated with a higher frequency of portal vein, venous, and bile duct invasion in HCC patients with surgical resection and may therefore be important in invasion and metastasis (Akiba, Yano et al. 2001). Moreover, high peritumoral M-CSF is associated with HCC progression, disease recurrence, and poor survival after hepatectomy, highlighting the importance of peritumoral tissue in the recurrence and metastasis of HCC (Budhu, Forgues et al. 2006; Zhu, Zhang et al. 2008). Another study shows that IFN- $\alpha$  inhibits metastasis and angiogenesis by down-regulating vascular endothelial growth factor (VEGF) but has no effect on fibroblast growth factor (Wang, Wu et al. 2003). Thus, it appears that some cytokines may contribute to the metastatic process, and several play a role in the prometastatic angiogenic phenotype.

### **HCC cytokine expression levels and prognosis**

High level of anti-inflammatory cytokine IL-10 in patients with HCC have a worse disease-free survival and IL-10 may be a predictor of the postresection outcome in these patients (Chau, Wu et al. 2000). In addition, patients with high serum IL-18 levels have a poor survival than those with low serum IL-18 levels in HCC (Asakawa, Kono et al. 2006; Tangkijvanich, Thong-Ngam et al. 2007). HCC patients with high TGF- $\beta$  concentration have a shorter survival period than those with concentrations below that of healthy individuals. Therefore, the concentration of TGF- $\beta$  was shown to be a predictor of outcome of patients with unresectable HCC (Okumoto, Hattori et al. 2004). In contrast, HCC patients with IL-2<sup>+</sup> tumors, a pro-inflammatory cytokine, have a favorable prognosis (Ikeguchi and Hirooka 2005). In conclusion, these studies show that the expression level of several cytokines is associated with disease outcome, and they may therefore serve as HCC prognosticators.

### **Cytokine-based HCC therapy.**

Antiviral strategies have been used to treat patients with HCC related to chronic HCV infection. Current evidence is sufficient to recommend IFN- $\alpha$  treatment for all patients with acute hepatitis. With the advent of a combined IFN- $\alpha$  and ribavirin therapy, the sustained virological response rate has been improved greatly. Whether the long-term effects of IFN- $\alpha$  plus ribavirin therapy can reduce the incidence of HCC needs to be established. The current gold standard of efficacy for treatment of patients with chronic HCV is the combination of pegylated IFN- $\alpha$  (PEG- IFN- $\alpha$ ) and ribavirin, which is more effective in improving liver histology. Pooling data from the literature suggests a slight preventive effect of IFN- $\alpha$  on HCC development in patients with HCV-related cirrhosis. Despite the low magnitude of this effect, some patients have benefited from such regimens.

Antitumour strategies for patients with HCC include various methodologies to boost or decrease the level of certain cytokines, particularly in the liver, or harness and expand reactive immune cells, which can be re-infused, and release their therapeutic cytokines. Lymphokine-activated killer cells (LAK) have been used as a therapeutic approach for HCC. LAKs produce IL-2, IFN- $\alpha$ , and IL-12 and thereby induce cytolytic activity (Kountouras, Boura et al. 2002). DC pulsed with heat-shock protein 70 peptide have been shown to stimulate high T cell proliferation, cytokine secretion, and induction of CTLs, which specifically killed HCC cells by a MHC Class I-restricted mechanism (Wang, Qin et al. 2005). NKT cells have been proposed to mediate the effects of IL-12 and immune-modulated NKT cells lead to suppression of HCC (Andrews, Ribas et al. 2000; Margalit, Shibolet et al. 2005). Gene therapies for different cytokines have been performed and found effective in different tumor models. IL-12 gene therapy has been tested which inhibit angiogenesis and induce tumor regression in HCC mouse model (Andrews, Ribas et al. 2000; Putzer, Stiewe et al. 2001; Peron, Couderc et al. 2004). Moreover, IL-2 and combination of IL-12 and GM-CSF gene therapies also have promising results in HCC models (Junbo, Li et al. 1999; He, Tang et al. 2000; Wang, Qiu et al. 2001; Qiu, Ye et al. 2002). In addition, *Glossogyne tenuifolia*, a traditional antipyretic and hepatoprotective herb, has been shown to inhibit the release of IFN- $\gamma$  and IL-6 in activated PBMC from HCC patients, thus producing anti-inflammatory effects (Wu, Weng et al. 2005).

## Chemokines and HCC

Chemokines (**chemotactic cytokines**) are small heparin-binding proteins that direct the migration of circulating leukocytes to sites of inflammation or injury. All chemokines are small proteins, with a molecular mass of between 8 and 10 kDa. They share a common structure that is stabilised by disulfide bonds between conserved cysteine residues. The major role of chemokines is to act as a chemoattractant to guide the migration of cells. Some chemokines have roles in development, promote angiogenesis and homeostasis. Chemokines are further involved in immune cell activation, proliferation, differentiation, survival and apoptosis. In addition, chemokines are critically involved in embryogenesis, angiogenesis and lymphoid organogenesis. Furthermore, chemokines play a critical role in tumor development and metastasis, and they also contribute to the process of wound healing and tissue organization/regeneration following damage (Gerard and Rollins 2001; Fernandez and Lolis 2002; Rot and von Andrian 2004; Charo and Ransohoff 2006).

Chemokines exert their cellular effects by activating seven- transmembrane-domain G-protein-coupled receptors. Whether a leukocyte responds to a particular chemokine is determined by its complement of chemokine receptors. Chemokine binding activates a signal transduction cascade that activates phosphatidylinositol-3 kinase, increases levels of inositol trisphosphate and intracellular calcium, activates Rho and mitogen-activated protein kinases, and eventually leads to actin re-arrangement, shape change, and cell movement. Although not yet fully understood, the signaling pathways that lead to chemotaxis rely on G $\alpha$ i as the initial link to the activated receptor and appear to be dependent on the activation of one or more isoforms of phosphatidylinositol 3-kinase (Weiner, Neilsen et al. 2002; Xu, Wang et al. 2003).

There are  $\approx$ 50 human chemokines, which are divided into four major families based on differences in their structure (on the bases of spacing of their first two cysteine residues) and function.

### *CC chemokines:*

The CC chemokines ( $\beta$ -chemokines) have two adjacent cysteines near their amino terminus. There have been at least 27 distinct members of this subgroup reported for mammals, called CC chemokine ligands (CCL)-1 to -28; CCL10 is the same as CCL9 (Laing and Secombes

2004). They induce the migration of monocytes/macrophages and other cell types such as NK cells, T cells, dendritic cells, basophiles, eosinophiles, bone marrow cells and thymocytes. CC chemokines induce cellular migration by binding to and activating CC chemokine receptors, ten of which have been discovered to date and called CCR1-10. These receptors are expressed on the surface of different cell types allowing their specific attraction by the chemokines (Laing and Secombes 2004). High expression of CCL2, CCL3, CCL5, CCL20 and CCL21 chemokines and their receptors in the liver of chronic HCV and HCC patients have been reported (Table 3) (Kim, Kunkel et al. 2001; Wald, Weiss et al. 2007; Zeremski, Petrovic et al. 2007).

#### *CXC chemokines:*

The two N-terminal cysteines of CXC chemokines ( $\alpha$ -chemokines) are separated by one amino acid, represented in this name with an "X". There have been 17 different CXC chemokines described in mammals called CXC chemokine ligands (CXCL)-1 to -17. They bind to CXC chemokine receptors, of which seven have been discovered to date designated as CXCR1-7. They induce the migration of neutrophils, activated T cells, B cells, NK cells and fibroblasts (Table 3) (Laing and Secombes 2004). Some of chemokines of this group like CXCL6, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL16 and their receptors have a role in chronic HCV infections and HCC (Kim, Kunkel et al. 2001; Wald, Weiss et al. 2007; Zeremski, Petrovic et al. 2007).

#### *C chemokines:*

The third group of chemokines is known as the C chemokines ( $\gamma$  chemokines), and is unlike all other chemokines in that it has only two cysteines; one N-terminal cysteine and one cysteine downstream. Two chemokines have been described for this subgroup and are called XCL1 and XCL2. These chemokines attract T cell precursors to the thymus (Table 3) (Laing and Secombes 2004). They also induce T cell proliferation and strong CTL reaction against allogenic HCC cells (Sheng and Zhang 2007).

#### *CX<sub>3</sub>C chemokines:*

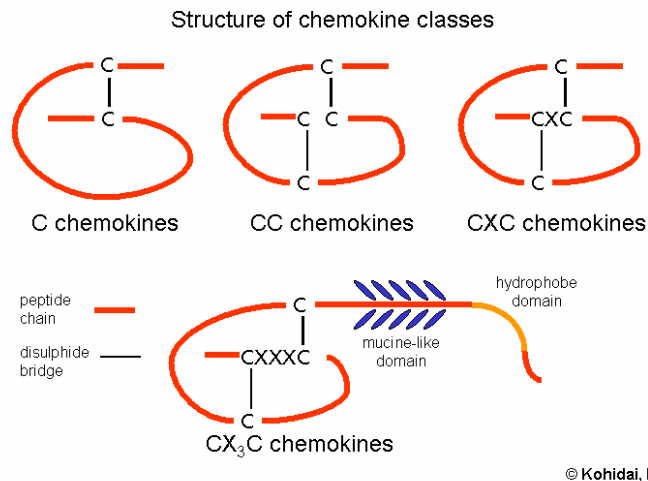
A fourth group has also been discovered and its members have three amino acids between the two cysteines. They are termed as CX<sub>3</sub>C chemokine ( $\delta$ -chemokines). The only CX<sub>3</sub>C chemokine discovered to date is called fractalkine (CX<sub>3</sub>CL1). It is both secreted and tethered



to the surface of the cell that expresses it, thereby serving as both a chemoattractant and as an adhesion molecule. It attracts T cells and monocytes (Table 3) (Laing and Secombes 2004). High level of CX<sub>3</sub>CL1 has been reported in chronic liver injuries and HCC (Efsen, Grappone et al. 2002; Matsubara, Ono et al. 2007).

**Table 3.** Showing names of chemokines, their receptors and the target cells of the immune system.

<b>Chemokines</b>			
<b>CC chemokines</b>			
<b>Name</b>	<b>Other name(s)</b>	<b>Receptor</b>	<b>Target cells</b>
CCL1	I-309, TCA-3	CCR8	Neutrophil (TCA-3 only), T cell
CCL2	MCP-1	CCR2, CCR2	T cell, monocyte, basophil
CCL3	MIP-1 $\alpha$	CCR1	Monocyte/macrophage, T cell (T <sub>H</sub> 1 > T <sub>H</sub> 2), NK cell, basophil, immature dendritic cell, bone marrow cell
CCL4	MIP-1 $\beta$	CCR1, CCR5	Monocyte/macrophage, T cell (T <sub>H</sub> 1 > T <sub>H</sub> 2), NK cell, basophil, immature dendritic cell, bone marrow cell
CCL5	RANTES	CCR5	Monocyte/macrophage, T cell (memory T cell > T cell; T <sub>H</sub> 1 > T <sub>H</sub> 2), NK cell, basophil, eosinophil, dendritic cell
CCL6	C10, MRP-2	CCR1	
CCL7	MARC, MCP-3	CCR2	T cell, monocyte, eosinophil, basophil, dendritic cell
CCL8	MCP-2	CCR1, CCR2B, CCR5	T cell, monocyte, eosinophil, basophil
CCL9/CCL10	MRP-2, CCF18, MIP-1 $\gamma$	CCR1	
CCL11	Eotaxin	CCR2, CCR3, CCR5	Eosinophil
CCL12	MCP-5		Eosinophil, monocyte, T cell
CCL13	MCP-4, NCC-1, Ck $\beta$ 10	CCR2, CCR3, CCR5	T cell, monocyte, eosinophil, basophil, dendritic cell
CCL14	HCC-1, MCIF, Ck $\beta$ 1, NCC-2, CCL	CCR1	Monocyte
CCL15	Leukotactin-1, MIP-5, HCC-2, NCC-3	CCR1, CCR3	T cell, monocyte, neutrophil (?), dendritic cell
CCL16	LEC, NCC-4, LMC, Ck $\beta$ 12	CCR1, CCR2, CCR5, CCR8	Monocyte
CCL17	TARC, dendrokinine, ABCD-2	CCR4	T-cell (T <sub>H</sub> 2 > T <sub>H</sub> 1), immature dendritic cell, IA NK cell, T cell (T <sub>H</sub> 2 > T <sub>H</sub> 1), thymocyte
CCL18	PARC, DC-CK1, AMAC-1, Ck $\beta$ 7, MIP-4		Naive T cell > T cell
CCL19	ELC, Exodus-3, Ck $\beta$ 11	CCR7	Naive T cell, mature dendritic cell, B cell
CCL20	LARC, Exodus-1, Ck $\beta$ 4, MIP3 $\alpha$	CCR6	T cell (memory T cell > T cell), peripheral blood mononuclear cell, bone marrow cell-dendritic cell
CCL21	SLC, 6Ckine, Exodus-2, Ck $\beta$ 9, TCA-4	CCR7	Naive T cell, B cell, mesangial cells (?)
CCL22	MDC, DC $\beta$ -CK	CCR4	Immature dendritic cell, IA NK cell, T cell (T <sub>H</sub> 2 > T <sub>H</sub> 1), thymocyte
CCL23	MPIF-1, Ck $\beta$ 8, MIP-3, MPIF-1	CCR1	Monocyte, T cell (resting), neutrophil (?)
CCL24	Eotaxin-2, MPIF-2, Ck $\beta$ 6	CCR3	T cell (?), eosinophil, basophil
CCL25	TECK, Ck $\beta$ 15	CCR9	Macrophage, thymocytes, dendritic cell
CCL26	Eotaxin-3, MIP-4 $\alpha$ , IMAC, TSC-1	CCR3	
CCL27	CTACK, LLC, Eskine, PESKY, skinkine	CCR10	T cell
CCL28	MEC	CCR3, CCR10	T cell, eosinophil
<b>CXC chemokines</b>			
<b>Name</b>	<b>Other name(s)</b>	<b>Receptor</b>	<b>Target cells</b>
CXCL1	Gro- $\alpha$ , GRO1, NAP-3	CXCR2	Neutrophil
CXCL2	Gro- $\beta$ , GRO2, MIP-2 $\alpha$	CXCR2	Neutrophil
CXCL3	Gro- $\gamma$ , GRO3, MIP-2 $\beta$	CXCR2	Neutrophil
CXCL4	PF-4	CXCR3B	Fibroblast
CXCL5	ENA-78	CXCR2	Neutrophil
CXCL6	GCP-2	CXCR1, CXCR2	Neutrophil
CXCL7	NAP-2, CTAPIII, $\beta$ -Ta, PEP		Fibroblast, neutrophil
CXCL8	IL-8, NAP-1, MDNCF, GCP-1	CXCR1, CXCR2	Neutrophil, basophil, T cell
CXCL9	MIG, CRG-10	CXCR3	Activated T cell
CXCL10	IP-10, CRG-2	CXCR3	Activated T cell (T <sub>H</sub> 1 > T <sub>H</sub> 2)
CXCL11	I-TAC, $\beta$ -R1, IP-9	CXCR3	Activated T cell
CXCL12	SDF-1, PBSF	CXCR4	CD34 <sup>+</sup> bone marrow cell, T cell, dendritic cell, B cell, naive B cell, activated CD4 T cell
CXCL13	BCA-1, BLC	CXCR5	Naive B cells, activated CD4 T cells
CXCL14	BRAK, bolekinine		
CXCL15	Lungkine, WEICHE		
CXCL16	SRPSOX	CXCR6	T cell, NK T cell
CXCL17	DMC, VCC-1		
<b>C chemokines</b>			
<b>Name</b>	<b>Other name(s)</b>	<b>Receptor</b>	<b>Target cells</b>
XCL1	Lymphotactin $\alpha$ , SCM-1 $\alpha$ , ATAC	XCR1	T cell, NK cell
XCL2	Lymphotactin $\beta$ , SCM-1 $\beta$	XCR1	
<b>CX3C chemokines</b>			
<b>Name</b>	<b>Other name(s)</b>	<b>Receptor</b>	<b>Target cells</b>
CX3CL1	Fractalkine, Neurotactin, ABCD-3	CX3CR1	T cell, monocyte, neutrophil (?)



**Fig. 10.** Structures of four classes of chemokines (Kohidai 1999)

### Lymphocyte recruitment in the normal and cHCV infected liver.

The liver contains a large population of lymphocytes, including CD4<sup>+</sup> and CD8<sup>+</sup> T cells, NK cells and NKT cells (Doherty, Norris et al. 1999; Doherty and O'Farrelly 2000). These populations can be rapidly expanded during inflammatory liver disease or in response to viral infection (Shields, Morland et al. 1999). It has been suggested that most lymphocytes in the normal liver are activated, terminally differentiated T cells that are removed from the circulation by the liver where they are destined to die by apoptosis (Crispe, Dao et al. 2000; Hamann, Klugewitz et al. 2000). However, although this is partly true, the liver also contains multiple lymphocyte cells that provide protection against pathogens and tumor cells, and many of these cells migrate through the liver as part of the process of continuing immune surveillance (Salmi, Adams et al. 1998).

#### *Initial capture or adhesion:*

The hepatic sinusoids are lined by specialized endothelium that supports lymphocyte adhesion and recruitment in a unique low-shear (i.e. low blood flow velocity) environment (Lalor and Adams 1999; Knolle and Gerken 2000; Lalor, Shields et al. 2002). Hepatic endothelium also has a distinct phenotype compared with endothelium from other vascular beds. Most strikingly, sinusoidal endothelial cells *in vivo* fail to express selectins, even in the presence of inflammation (Steinhoff, Behrend et al. 1993; Adams, Hubscher et al. 1996) and selectins play a minimal role in leukocyte recruitment by the sinusoids in liver (Wong, Johnston et al. 1997). The lack of expression of these classical selectin 'tethering' or rolling receptors in the sinusoids, coupled with the low-shear environment might indicate that primary capture

receptors play only a minimal role at this site. Thus, a lymphocyte moving slowly through the narrow, irregular sinusoids might be able to interact directly by integrin-mediated interactions with vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 or intercellular adhesion molecule 2 (ICAM-1 and ICAM-2, respectively) without the need for a specialized capture phase. Furthermore, VCAM-1 itself can directly capture flowing lymphocytes by supporting rolling adhesion, particularly under low-shear stress (Berlin, Berg et al. 1993; Lalor, Shields et al. 2002). Similarly, higher expression of these adhesion molecules have been found in the liver with cHCV infections (Kaplanski, Maisonobe et al. 2005). It is possible that molecules other than selectins or VCAM-1 act as tethering receptors in the liver. One contender for this role is vascular adhesion protein 1 (VAP-1), a homodimeric transmembrane protein that has been shown to support lymphocyte adhesion to high endothelial vessels in lymph nodes (Salmi, Adams et al. 1998; Salminen, Smith et al. 1998; Kurkijarvi, Yegutkin et al. 2000). Furthermore, MAdCAM-1 is also present on hepatic endothelium and support lymphocyte adhesion (Grant, Lalor et al. 2001).

*Triggering and arrest of lymphocytes by chemokines:*

Chemokines need to be constitutively expressed during immune surveillance in the normal liver. Some of the chemokines like CCL2, CCL3, CCL4, CCL5 and CXCL8, CXCL9, CXCL10, CXCL11, CXCL12 have been detected in the non-inflamed liver and studies suggest that lymphocytes infiltrating non-diseased liver express similar chemokine receptors to those entering the inflamed liver, indicating that recruitment under physiological conditions is likely to be equivalent to a state of mild or controlled inflammation (Shields, Morland et al. 1999; Zeremski, Petrovic et al. 2007).

CC chemokines like CCL2, CCL3, CCL4, CCL5, CCL8, CCL19, CCL21 and their receptors CCR1, CCR2, CCR5 and CCR7 are expressed in the normal liver and elevated in the cHCV infection (Yoong, Afford et al. 1999; Grant, Goddard et al. 2002; Charo and Ransohoff 2006; Zeremski, Petrovic et al. 2007). The different patterns of chemokine expression within portal tracts and the liver parenchyma provide further evidence for specific pathways of lymphocyte recruitment to the liver (Adams, Hubscher et al. 1996; Hubscher 1998; Salmi, Adams et al. 1998). Portal inflammation is a frequent finding in many inflammatory liver diseases and the portal tract is the main site at which lymphocytes are found in the normal liver (Adams, Hubscher et al. 1996; Hubscher 1998; Salmi, Adams et al. 1998). CCL2, CCL8/CCR2 which are considered as monocyte chemotactic chemokines and are highly expressed in the portal

tract in HCV inflamed liver (Marra, DeFranco et al. 1998; Charo and Ransohoff 2006). Moreover, CCL2 has a link to the degree of liver fibrosis during chronic HCV liver diseases via hepatic stellate cells which are key players in liver fibrosis (Marra, Romanelli et al. 1999; Hellier, Frodsham et al. 2003). CC chemokines like CCL3 and CCL4 are expressed predominantly by vascular endothelium within portal tracts in both normal and inflamed liver (Zeremski, Petrovic et al. 2007). In HCV infected livers, inflammation and fibrosis are mainly located in the portal areas (Shields, Morland et al. 1999). Inflammation at this anatomic site may be attributed in part to the upregulation of the chemokines CCL3, CCL4 and CCL5 in the portal tracts and to the recruitment of CCR5 positive cells to these areas (Shields, Morland et al. 1999; Boisvert, Kunkel et al. 2003). HCV induce CCL5 expression by some cancer cell lines and CD8<sup>+</sup> T cells (Soo, Garzino-Demo et al. 2002; Nattermann, Nischalke et al. 2004). It has been demonstrated that hepatic expression of CCL21 promotes the development of portal associated lymphoid tissue in the chronic liver diseases (Grant, Goddard et al. 2002). Moreover, CCL21 was shown to be upregulated in lymphoid like structures formed in the inflamed liver of HCV patients and in the portal tracts of these livers. It was further suggested that CCL21 modulates CCR7 positive lymphocyte recruitment and fibrosis in chronic HCV infection (Bonacchi, Petrai et al. 2003; Heydtmann, Hardie et al. 2006). Taken together it is suggested that CC chemokine generally may responsible for the recruitment of lymphocytes in the portal tract and might be associated with fibrosis and contribute to establishment of lymphoid-like organs within the liver.

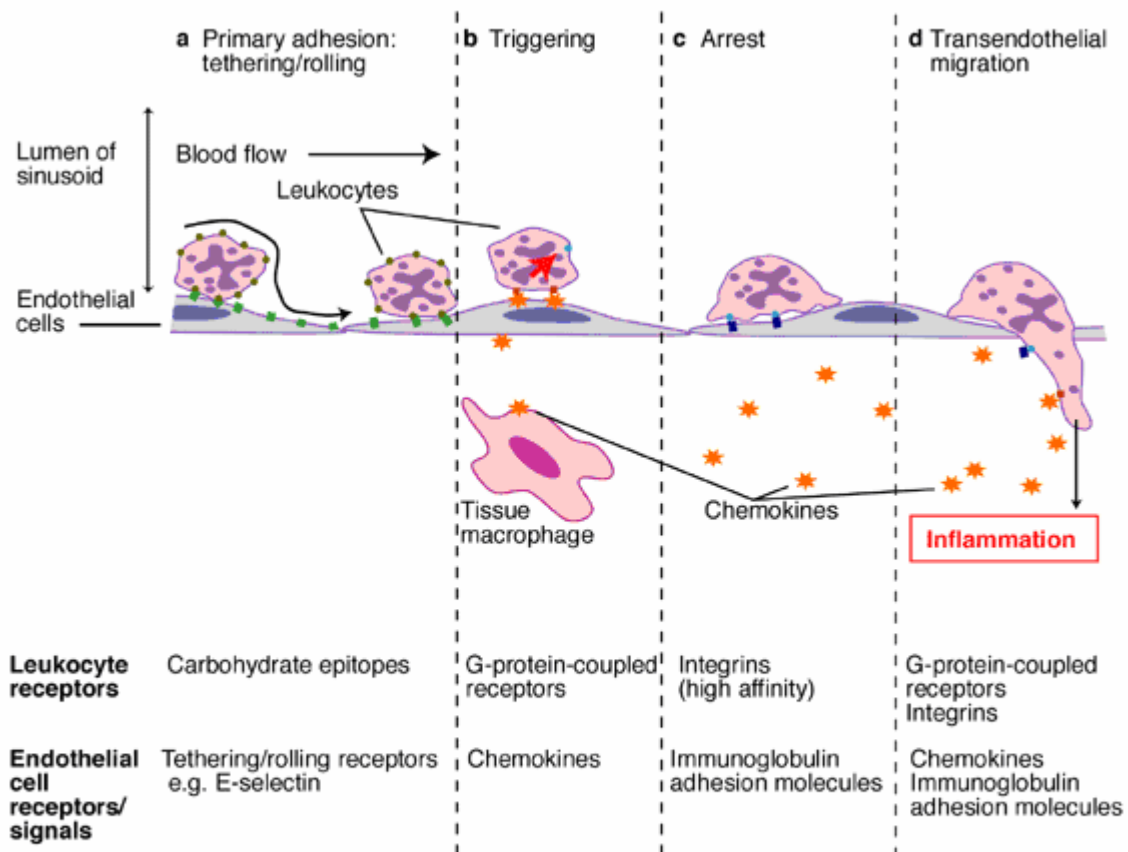
CXC chemokines like CXCL6, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL16 and their receptors CXCR3 CXCR4 and CXCR6 have been reported to express in the normal and cHCV infected liver (Wald, Weiss et al. 2007; Zeremski, Petrovic et al. 2007). The CXC chemokines CXCL9 (MIG), CXCL11 (ITAC) and CXCL10 (IP-10) are preferentially expressed by sinusoidal endothelium (Shields, Morland et al. 1999). Although CXCL9 can be detected in the normal liver but its expression is increased greatly in inflammation. Furthermore, both CXCL10 and CXCL11 are detected on sinusoidal endothelium in the inflamed liver. CXCL10 secretion by sinusoidal endothelial cells is stimulated by IFN- $\gamma$  and TNF- $\alpha$  *in vitro* (Narumi, Tominaga et al. 1997; Shields, Morland et al. 1999; Narumi, Yoneyama et al. 2000; Tamaru, Nishioji et al. 2000; Helbig, Ruzskiewicz et al. 2004), indicating that such CXC chemokines might play a specific role in the recruitment of T cells to the hepatic parenchyma via the sinusoids. The chemokine receptor CXCR3 (receptor for CXCL9, CXCL10, and CXCL11) is highly expressed on activated effector T cells, and is

closely linked to Th1-type immune responses (Qin, Rottman et al. 1998). Liver-infiltrating T cells express much higher levels of CXCR3 and CCR5 than circulating T cells (Coulin, Power et al. 1997; Shields, Morland et al. 1999). Thus, CXCR3 is involved in the recruitment of activated lymphocytes to the inflamed liver. Nevertheless, additional chemokine receptors such as CCR5 and CXCR6, which are also expressed on activated T cells, may also contribute to their recruitment/retention in the inflamed liver (Boisvert, Kunkel et al. 2003; Heydtmann, Lalor et al. 2005). A recent study indicated that intrahepatic CD161<sup>+</sup>CD8<sup>+</sup> HCV-specific T cells produce proinflammatory cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) but contain scanty amounts of cytolytic molecules (granzyme B and perforin) and proliferate poorly *in vitro*. Interestingly, the expression of CD161 on CD8<sup>+</sup> T cells was tightly linked to that of CXCR6 a chemokine receptor with a major role in liver homing (Northfield, Kasprovicz et al. 2008). CXCR3<sup>+</sup>, Th1-type effector cells were shown to produce multiple pro-inflammatory cytokines, chemokines and cytotoxic factors such as IFN- $\gamma$ , TNF- $\alpha$ , CCL5 and perforin (Prezzi, Casciaro et al. 2001; Leroy, Vigan et al. 2003). It is therefore suggested that, in addition to their antiviral potential, CXCR3<sup>+</sup> effector cells may also promote liver inflammation and damage during chronic HCV infection. Interestingly, the intra-hepatic levels of CXCL11 and CXCL10 were reported to correlate with HCV disease severity (Apolinario, Majano et al. 2002; Harvey, Post et al. 2003; Helbig, Ruszkiewicz et al. 2004). In humans, CXCL12 is mainly expressed by stromal cells in the bone marrow, in B cells zones of lymphoid follicles, in the spleen, by epithelial cells in the lung, and by bile duct epithelial cells in the liver (Coulomb-L'Hermin, Amara et al. 1999; Gonzalo, Lloyd et al. 2000). Moreover, CXCL12 was upregulated in the endothelium of neo-blood vessels formed in active inflammatory foci and along the fibrotic septi. Complementing these findings, over 50% of liver infiltrating T cells, NK T and NK cells expressed CXCR4. Moreover, increased expression of CXCR4 has been detected at active inflammatory foci resembling lymphoid structures formed in the cirrhotic livers (Wald, Pappo et al. 2004). These observations support a role of CXCL12 in recruitment of immune cells to the chronically inflamed liver and suggest that CXCL12 may be involved in the formation of lymphoid-like organs in the cirrhotic liver and might also involve in fibrosis. It has been demonstrated that CXCL16 is expressed by bile ducts epithelial cells as well as by hepatocytes and in the portal tracts of the HCV inflamed liver, while liver infiltrating CD4, and CD8 T cells and NK cell express CXCR6 (Boisvert, Kunkel et al. 2003; Wang, Holmes et al. 2004; Heydtmann, Lalor et al. 2005), suggesting that this receptor may promote the recruitment of Th1 type cells. Moreover, mRNA levels of the chemokines CXCL6 and CXCL8 and of the membrane bound chemokine CX3CL1, were also found to be

elevated. Interestingly, increased expression of CXCR4 mRNA, the receptor for the homeostatic chemokine CXCL12, was also observed (Asselah, Bieche et al. 2005; Bieche, Asselah et al. 2005). These studies indicate that chemokines and chemokine receptors are key players in HCV-associated liver inflammation. Moreover, mRNA levels of the chemokines CXCL6 and CXCL8 were also found to be elevated in the liver of HCV infected patients (Asselah, Bieche et al. 2005; Bieche, Asselah et al. 2005). In addition, these chemokines and their receptors are involved in extracellular matrix production, in extracellular matrix remodeling, and in cell junction contributing in liver fibrosis in these patients (Liang, Zhong et al. 2007). One recent study shows that the fractalkine (CX3CL1) receptor CX3CR1 is a susceptible gene for hepatic fibrosis in HCV infection (Wasmuth, Zaldivar et al. 2008). In summary, CXC and CX3C chemokines are involved in the liver inflammation and fibrosis.

#### *Transendothelial migration:*

Once chemokine signals have been received by adherent lymphocytes newly expressed or activated integrin molecules can bind to their Ig counter-receptors on the endothelium for firm adhesion. Immobilized CCL21, CCL25, CCL28, CXCL12, and CXCR3 convert rolling adhesion to static arrest on MAdCAM-1 and VCAM-1 by activating lymphocyte integrins (Wong, Johnston et al. 1997; Yoong, McNab et al. 1998; Curbishley, Eksteen et al. 2005; Miles, Liaskou et al. 2008). Hepatic sinusoidal endothelial cells form a discontinuous barrier lacking tightly regulated junctions. Lymphocyte transendothelial migration is mediated by CD31 in many tissues. Liver expresses much lower levels of CD31 than other vascular endothelium, suggesting a minimal roll of CD31 in leukocyte transmigration in the liver (Muller 1995; Berman, Xie et al. 1996). CCL21 and CXCL12 trigger a motile phenotype characterized by lamellipodia and uropod formation. Thus VCAM-1, MAdCAM-1 and CXCR3 operate independently to support lymphocyte adhesion from flow, and chemokines may act in concert with one chemokine (like CCL25, CCL28) triggering integrin-mediated arrest and a second chemokine (CCL21 and CXCL12) promoting motility and transendothelial migration (Miles, Liaskou et al. 2008). Moreover, CXCR3 not only promotes the adhesion of effector T cells to endothelium from flow but also drove transendothelial migration in the liver (Curbishley, Eksteen et al. 2005). CXCL12 (SDF) might also have a role in maintaining lymphocytes within portal tracts (Buckley, Amft et al. 2000; Goddard, Williams et al. 2001; Yoneyama, Matsuno et al. 2001).



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**Fig. 11. Adhesion of leukocytes to endothelial cells under conditions of blood flow.** (a) Primary adhesion. Free-flowing leukocytes in the circulation are captured by tethering or rolling receptors (such as E-selectin; shown here in green) expressed on endothelial cells. (b) Triggering. Once captured, chemokine signals (orange) localized to the luminal side of the endothelial surface are detected by specific G-protein-coupled receptors (red) expressed on the leukocyte. (c) Arrest. This results in a conformational change in leukocyte integrin molecules (light blue), which are converted to a high-affinity state, permitting firm adhesion to immunoglobulin adhesion molecules (dark blue) expressed by endothelial cells. (d) Transendothelial migration. Chemokine recognition also results in cytoskeletal reorganization within the adherent leukocyte, which facilitates migration across the endothelial monolayers and into tissue. The molecules that mediate migration have not been precisely defined but are likely to be members of the integrin and immunoglobulin families. Once within the tissue, the leukocyte follows a chemotactic gradient of chemokine signals towards the site of inflammation (Lalor and Adams 2002).

### Chemokines and Hepatocellular carcinoma.

For lymphocytes to mount an immune response against a solid tumor, they must first be able to enter in the tumor tissue and then recognize and respond to the tumor antigens. HCC contains two distinct types of endothelium; sinusoidal-like endothelial channels that run between the tumor cells and vascular endothelium on tumor vessels. Both of these endothelia stained strongly for ICAM-1 and VAP-1 which support the adhesion of tumor-infiltrating

lymphocytes to tumor endothelium in human hepatocellular carcinoma (Volpes, van den Oord et al. 1992; Steinhoff, Behrend et al. 1993; Adams, Hubscher et al. 1996; McNab, Reeves et al. 1996; Yoong, McNab et al. 1998). TIL binding to VAP-1 in HCC is also L-selectin independent because very little L-selectin is expressed by TIL (Salmi and Jalkanen 1996; Salmi, Tohka et al. 1997).

*Chemokines and HCC progression:*

HCC cells, as well as other tumors produce chemokines, which, in many cases play an important role in tumor progression. The chemokine CXCL8, a powerful chemoattractant for neutrophils with angiogenesis action, is significantly correlated to the tumor size and stage suggests that CXCL8 may be involved in the progression of HCC. Furthermore, CXCL8 may be a useful biological marker of tumor invasiveness and an independent prognostic factor for patients with HCC (Akiba, Yano et al. 2001; Shin, Choi et al. 2002; Ren, Poon et al. 2003).

The chemokine receptor CXCR4 and its ligand CXCL12 have been recognized as potent targets in the cancer treatment. CXCR4/CXCL12 axis was found to play a critical role in the progression and development of various tumors including HCC (Muller, Homey et al. 2001), prostate (Staller, Sulitkova et al. 2003; Darash-Yahana, Pikarsky et al. 2004; Schimanski, Bahre et al. 2006) However, HCC tumor cells *in vivo* do not express CXCL12 (Shibuta, Begum et al. 1997; Mitra, Shibuta et al. 1999; Mitra, De et al. 2001; Shibuta, Mori et al. 2002), but is detected in sinusoidal endothelial cells in HCC tissue, and their expressions are significantly higher than in non-HCC tissues confirming its role in HCC progression through neoangiogenesis (Li, Gomez et al. 2007).

The chemokine CCL20 attracts immature dendritic cells and lymphocytes and is thought to play a role in the pathogenesis of inflammation and carcinogenesis. CCR6 is the receptor for CCL20. During chronic HCV infection, CCL20 is expressed at sites of liver necrosis while lymphocytes infiltrating these livers were shown to express moderate levels of CCR6, possibly indicating that CCL20/CCR6 pathway is also involved in augmenting liver inflammation (Shimizu, Murata et al. 2001; Boisvert, Kunkel et al. 2003). Furthermore, low CCL20 levels are also expressed by HCC and CCR6<sup>+</sup> immune cells were shown to accumulate in HCC (Liu, Poon et al. 2004; Liu, Poon et al. 2005). Nevertheless, in HCC patients, significant overexpression of CCL20 in the tumor surrounding tissues and marked



overexpression of the corresponding receptor CCR6 in the tumor-invading front was found. Moreover, CCL20 expression correlated with tumor grade, thus suggesting that CCL20/CCR6 axis is involved in hepatocarcinogenesis (Yamauchi, Akbar et al. 2002; Yamauchi, Akbar et al. 2003; Rubie, Frick et al. 2006).

NK cells as well as memory and activated T cells express high levels of the chemokine receptor CXCR3 (Wald, Pappo et al. 2004). Interestingly, significant higher concentration of CXCL10 was found in HCC tumors than non-involved adjacent liver tissues and its high levels in the blood and tumors caused lymphocyte dysfunction and may therefore impair the immune defense mechanisms against the tumor (Liu, Poon et al. 2005). Likewise, expansion of tumor-derived lymphocytes in recombinant IL-2 increased expression of CXCR3. The corresponding chemokines were detected on vascular endothelium (CXCL9, CXCL8, CCL3, CCL4) and sinusoidal endothelium (CXCL9, CCL3, CCL4) in hepatocellular carcinoma. The relevant chemokine ligands, particularly CXCL9 and CXCL10, are expressed in hepatocellular carcinoma (HCC) and play a role in the recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> cells in these tumors (Taub, Longo et al. 1996; Yoong, Afford et al. 1999; Hirano, Iwashita et al. 2007). In contrast, CD4<sup>+</sup> and CD8<sup>+</sup> cells from HCC patients show reduce expression of CXCR3 and exhibit diminished chemotactic responses towards CXCR10 *in vitro* as compared to T cells derived from healthy controls (Liu, Poon et al. 2005). It has also been shown that CCR6 and CXCR3 were expressed on both NKT and NK cells in TIL and NIL (Kim, Johnston et al. 2002; Johnston, Kim et al. 2003; Maghazachi 2003) suggesting that expression of these chemokine receptors may mediate the preferential localization of NKT and NK in the liver to assist host defense against pathogens and tumors. High expression of CCR5 could only be detected on NK and NKT from the non-tumor liver. This might indicate that CCR5 is not the key for recruitment of NK and NKT cells into the tumor. On the other hand, Vgamma9Vdelta2 T lymphocytes are reported to participate in the anti-tumor immune surveillance in human (Sicard, Al Saati et al. 2001). They expressed NKG2D and CD161 and the proinflammatory CXCR3 and CCR5 chemokine receptors (Bouet-Toussaint, Cabillic et al. 2008), indicating the therapeutic potential of these cells in HCC.

CCL21 (SLC) induced a significant delay of tumor progression and increased infiltration of DCs and activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells into the tumor site in murine hepatocellular carcinoma model. In addition, SLC treatment was also found to reduce tumor growth in nude mice, most likely due to inhibition of neoangiogenesis (Liang, Zhong et al. 2007). In addition,

lymphotactin (XCL) gene-modified dendritoma induces T-cell proliferation and strong CTL reaction against allogenic HCC cells (Sheng and Zhang 2007).

*Chemokines and HCC metastasis and recurrence:*

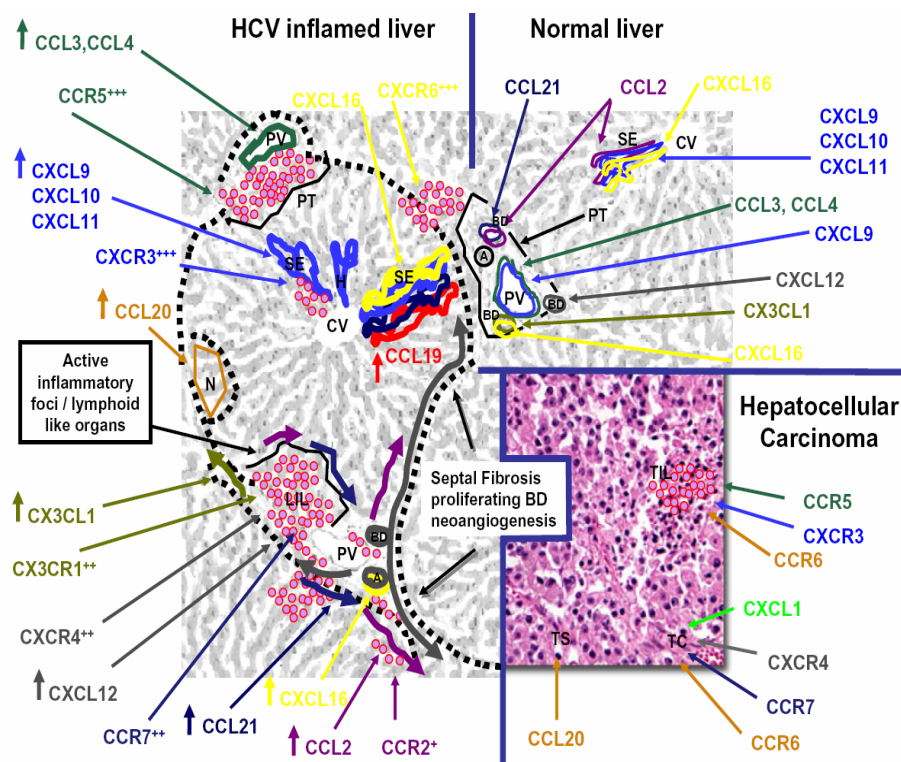
Chemokines also play an important role in tumor metastasis and recurrence. It has been demonstrated that when CCR6 high HepG2 cells were stimulated with MIP-3 $\alpha$  (CCL20), they migrated in a dose-dependent manner, and formation of pseudopodia was observed. These phenomena were not observed in CCR6 low cells. In addition, the incidence of metastasis was higher in the high CCR6 expression HCC group while the disease-free survival was significantly poorer in the high CCR6 expression group, showing critical roll of CCR6 in metastasis and survival in HCC patients (Uchida, Iwashita et al. 2006). CXCR3 expression was indicated in the HCC cell lines with metastatic potential but not in HCC cell lines without metastatic potential. Its expression is significantly correlated to the invasiveness of the tumors and survival rates of HCC patients (Shi, Guo et al. 2006). CCR1 has an important role in the recruitment of leukocytes to the site of inflammation. However, it has also been indicated that CCR1 has an important role in HCC invasion (Wu, Fan et al. 2007).

In contrast, the expression levels of chemokine receptors CCR10, CXCR4 and CXCR6 on different HCC cell lines decrease gradually as the metastatic potential of these cell lines increase, suggesting some sort of anti-metastatic roll of these chemokine receptors (Xue, Chen et al. 2007). Both fractalkine (CX3CL1) and CX3CR1 were up-regulated during chronic injury, in areas of portal and lobular inflammation (Efsen, Grappone et al. 2002). Tumors with high expression of both fractalkine and CX3CR1 had significantly fewer intra- and extrahepatic recurrences. Patients with tumors that expressed both had a significantly better prognosis in terms of disease-free and overall survival, suggesting that the fractalkine-CX3CR1 system plays a pivotal role in the prognosis of patients with HCC (Matsubara, Ono et al. 2007).

**Chemokines and cytokines.**

Type-I interferons as well as TNF- $\alpha$  and IL-1 $\beta$  have all been reported to upregulate either directly or indirectly the expression of the chemokines CCL3, CCL4 and CCL5, these chemokines being potent ligands of the chemokine receptors CCR5 and CCR1 (Kakumu, Okumura et al. 1997; Zhang, Guo et al. 2003). Similarly, Type II interferons are recognized as

the most potent inducers of the chemokines CXCL9 and CXCL10, which bind to the chemokine receptor, CXCR3 (Farber 1997). Moreover, IL-4 was found to enhance the cell-surface expression of CXCR4 on resting T-cells, whereas the receptor is down-regulated after T-cell stimulation by CD28 or CD3 and CD2 (Jourdan, Abbal et al. 1998). IL-2 and IL-15 induce the expression of CCR1 on activated T cells (Loetscher, Seitz et al. 1996; Perera, Goldman et al. 1999), whereas IL-10 selectively up-regulated the expression of CCR1 in human monocytes by prolonging mRNA half-life (Sozzani, Ghezzi et al. 1998). CCR6 mRNA has been shown to be up-regulated by treatment with IL-2 (Baba, Imai et al. 1997; Greaves, Wang et al. 1997). However, another study contradict this finding (Liao, Rabin et al. 1999) and the effect of IL-2 on CCR6 expression remains uncertain. The expression of CCR2 mRNA in monocytes has been found to be decreased by both IFN- $\gamma$  and lipopolysaccharide (Sica, Sacconi et al. 1997).



**Fig. 12. Chemokine and chemokine receptor expression patterns in the normal and HCV-inflamed liver and in hepatocellular carcinoma.** A scheme was designed to illustrate the anatomic location of chemokines and chemokine receptors expressed in the normal and HCV-inflamed liver and in hepatocellular carcinoma. The presented data is based on literature review of immunohistochemical analysis of chemokine expression patterns in the liver and on fluorescent immunostaining followed by flow cytometry analysis of liver-derived lymphocytes. Chemokine/chemokine-receptor pairs are marked with the same color. A, artery; BD, bile duct epithelial cells; CV, central vein; H, hepatocytes; LIL, liver infiltrating lymphocytes; N, necrotic area; PT, portal tracts; PV, portal vein; SE, sinusoidal endothelium; TC, tumor cells; TIL, tumor infiltrating lymphocytes; TS, tumor surrounding tissue; +, indicates that up to 30% of LILs expressed the receptor; ++, indicates 30–60% of LILs expressed the receptor; +++, indicates more than 60% of LILs expressed the receptor (Wald, Weiss et al. 2007).

**MATERIALS  
AND  
METHODS**

## Materials and Methods

### Patients.

Patients with histologically proven HCC who had undergone curative hepatectomy and patients with histologically proven persistent HCV infection with cirrhosis (Cirrhosis C) in the University Hospital, Grenoble, between 1999 and 2008 were included in this study. Among HCC cases, half went with HCV (HCC-HCV) and half with alcohol (HCC-OH). All HCC-HCV and Cirrhosis C patients were anti-HCV antibody positive by two different third generation ELISA tests and had detectable serum HCV-RNA by PCR (mono-infected HCV group). Exclusion criteria included co-infection with human immunodeficiency virus, hepatitis B virus or hepatitis delta virus, other causes of liver disease, alcohol consumption higher than 30 g/day, inflammatory syndrome, previous antiviral treatment and previous liver transplantation. All HCC-OH patients were anti-HCV or anti-HBV antibody negative. Patients with cirrhosis C underwent needle liver biopsy as a part of their diagnostic evaluation. The main characteristics of all the patients are shown in demographic tables in the results. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by our institutional review board. Patients were enrolled after taking their written informed consent.

### Immunohistochemical Staining.

#### *1. Immunohistochemical staining of paraffin-embedded tissues:*

Immunohistochemical study of the markers CD3, CD4, CD8, CD20, FoxP3 and Ki67 (Table 4) was performed on paraffin-embedded, formalin-fixed liver tissues and processed on an automated immunostainer (Nexes, Ventana, Tucson, AZ, USA), except for CD4 (processed manually). Briefly, consecutive sections of liver tissue blocks were cut at 4 $\mu$ m, deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was done by heating slides in adequate buffer using water bath or microwave oven. Non specific protein binding were blocked by 30 minutes incubation in phosphate-buffered saline (PBS), pH 7.4, with 2% milk. Endogenous peroxidase activity was blocked by 1% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 5 min. These treatments were alternated with rinses with PBS (pH 7.4). Slides were then processed on the Nexes immunostainer, starting with an automatic incubation with the primary antibodies followed by an immunodetection with DAB (diaminobenzidine) I-view kit, according to the manufacturer's recommendations (Ventana, Tucson, AZ, USA). For CD4

immunostaining, after incubation with primary antibody, slides were washed with PBS (pH 8.6), sur-fixed five minutes with 0.005% glutaraldehyd in PBS pH 8.6, washed once more in PBS pH 8.6 and then incubated with secondary antibody by using an Envision system (labelled polymer HorseRadish Peroxidase anti-mouse antibody, ref K4000, Dako, Glostrup, Denmark). This was followed by a rinse in PBS (pH 8.6), a second sur-fixation in glutaraldehyd, another rinse in PBS (pH 8.6), Diaminobenzidine chromogen staining and counterstaining with haematoxylin.

2. *Immunohistochemical staining of frozen tissue paraffin-embedded tissues:*

CD123, CD56, TCRgd (Table 4) was performed on 10 µm thick frozen tissue sections, and processed manually. Frozen sections stored at – 70°C were defrosted for 30 minutes at room temperature, fixed in a first bath of cold pure acetone (10 minutes at – 20°C) then in a second bath of acetone with 1% H<sub>2</sub>O<sub>2</sub> (5 minutes at 4°C). They were then briefly rinsed in pure acetone, dried and washed in PBS 7.4. Non specific protein binding were blocked by 30 minutes incubation in phosphate-buffered saline (PBS) pH 7.4 with 2% milk. Further steps (incubation with primary and secondary antibodies, amplification and revelation) were similar to those described for manually CD4 immunodetection. As a negative control, non immunized mouse or rabbit immunoglobulin was substituted to the primary antibody.

3. *Immunopositive cells counting:*

Immunopositive cells were counted in 40 randomly selected fields per slide on a computer screen with the help of automatic software LUCIA (Laboratory Universal Computer Image Analysis, version 5.0, Laboratory Imaging, Prague, Czech) at high magnification (X400). All positive cells with above mentioned antibodies were independently counted by two observers with 95% concordance. Cells in the tumoral and cirrhotic areas were counted separately, as well as their localization in the fibrous tissue (portal tracts and fibrous septa) and the parenchymatous nodules and their number was expressed per field at magnification X400.

Table 4. **Characteristics of antibodies used in immunohistochemical staining.**

Antigen	Pre-treatment for antigen retrieval	Dilution	Clone	Source
<i>CD3</i>	Citrate buffer, pH 6, Water bath 100°C, 1h	1:100	polyclonal	Dako, Glostrup, Denmark
<i>CD4</i>	Tris-citrate buffer, pH 9, Microwave oven 3x5 min	1:75	4B12	Novocastra, Newcastle, UK
<i>CD8</i>	Citrate buffer, pH 6, Water bath 100°C, 1h	1:100	C8/144B	Dako, Glostrup, Denmark
<i>CD20</i>	Citrate buffer, pH 6, Water bath 100°C, 1h	01:50	L26	Dako, Glostrup, Denmark
<i>FoxP3</i>	Tris-citrate buffer with 0.02% saponin, pH 9, Microwave oven 15 min	01:20	mAbcam 22510	Abcam, Cambridge, UK
<i>Ki67</i>	Tris-citrate buffer, pH 9.15 Water bath, 1h	01:50	Ki S5	Dako, Glostrup, Denmark
<i>CD123</i>	Non	1:300	6H6	Biolegend, San Diego, CA
<i>CD56</i>	Non	1:200	1B6	Novocastra, Fremont, CA
<i>TCR<math>\gamma\delta</math></i>	Non	1:200	B1	BD Pharmigen, Oxon, UK

#### **Immunohistochemical Staining with anti-ING2 (inhibitor of growth) antibody.**

ING2 is a member of tumor suppressor gene. We also performed immunohistochemical staining with anti-ING2 (provided and manufactured by Mr. Rémy PEDEUX, IAB, INSERM-UJF U823, Grenoble) antibody. Briefly, 4  $\mu\text{m}$  thick tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. Heat induced antigen retrieval was performed in Tris-citrate buffer with 0.02% saponin for 15 minutes in microwave. Non specific protein binding were blocked by 30 minutes incubation in phosphate-buffered saline (PBS), pH 7.4, with 2% milk. Endogenous peroxidase activity was blocked by 1% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) for 5 min. These treatments were alternated with rinses with PBS (pH 7.4). Slides were then processed, starting with incubation with the primary antibodies followed by an immunodetection with DAB (diaminobenzidine) I-view kit, according to the manufacturer's recommendations (Ventana, Tucson, AZ, USA). After incubation with primary antibody, slides were washed with PBS (pH 8.6), sur-fixed five minutes with 0.005% glutaraldehyd in PBS pH 8.6, washed once more in PBS pH 8.6 and then incubated with secondary antibody by using an Envision system (labelled polymer HorseRadish Peroxidase anti-mouse antibody, ref K4000, Dako, Glostrup, Denmark). This was followed by a rinse in PBS (pH 8.6), a second sur-fixation in glutaraldehyd, another rinse in PBS (pH 8.6), Diaminobenzidine chromogen staining and counterstaining with haematoxylin.

## **Real-Time Quantitative RT-PCR analysis.**

### *1. Extraction of total RNA:*

Total RNA was extracted from liver specimen (approximately 5-10mg) by using the homogenization of tissues by the MagnaLyser System (Roche Diagnostics, Meylan, France) in the presence of silica balls. After centrifugation, 600  $\mu$ l of 70% ethanol was added in the aliquot. The mixture was filtered on a silica column and was digested by DNaseI in order to eliminate the residual DNA for 15 min at room temperature. Then, the precipitation of the RNA was performed in the presence of ethanol and was followed by two washings. The total RNA was obtained in 50  $\mu$ l sterile H<sub>2</sub>O and preserved at -80°C.

### *2. Preparation of cDNA:*

Single-stranded cDNA was prepared with 1 $\mu$ g of total RNA by using the SuperScript II Reverse transcriptase (Gibco BRL, Life Technologies) in a final volume of 20  $\mu$ l. The samples of cDNA thus obtained were stored at -20°C.

### *3. RT-PCR analysis:*

Primer pairs and probes for quantification of CD4, CD8 $\alpha$ , CD8 $\beta$ , IL-6, IL-10, IFN- $\gamma$ , TGF- $\beta$ , perforine and RANTES genes were performed by using ProbeFinder Software for Human (version 2.35) (Roche Diagnostics, Meylan, France) and were synthesized by Genset (Paris, France). The sequence and main characteristics of each primer and probe were summarized Table 5. The quantification of the transcripts from the liver samples was performed by using the LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche Diagnostics, Meylan, France). Target cDNA sequences were amplified with the LightCycler<sup>®</sup> 480 Probes Master and were detected with UPL (Universal ProbeLibrary) Probes. Briefly, each PCR reaction mixture contained: 1X of LightCycler<sup>®</sup> 480 Probes Master (Roche Diagnostics, Meylan, France), 0.5  $\mu$ M of each primer, 0.1 $\mu$ M of appropriate probe and 2.5 $\mu$ L of cDNA preparation, in a final volume of 10 $\mu$ L. After an initial denaturation at 95°C for 10 min, PCR reactions were cycled 35-40 times as follows; 10 s at 95°C, annealing step 15 s at 60°C (for all primers and probes) and 10 s at 72°C. Fluorescence was measured once at each 60°C step and monitored continuously during the melting process. Data were analyzed with the LightCycler<sup>®</sup> 480 Software. The detection format was set to “Mono Color Hydrolysis Probe” and the Second Derivative Maximum Method was used for absolute quantification. The housekeeping gene used for normalization of the values was the Hypoxanthine Phosphoribosyl Transferase (HPRT). The



results were expressed as the log of the number of amplicons ( $2^n - 2n$ ,  $n$  = number of cycles) of the targeted transcripts.

**Table 5. Primers and Universal probe Library (UPL) Sequences used in the LightCycler® 480 Real-Time Quantitative PCR System.**

Gene		Primer Sequences	Product Length	UPL sequences
CD8 $\alpha$	F	cgccctgtgatatcatctgg	108 nt	ttctctcg
	R	caaacacgtcttcggttcc		
CD8 $\beta$	F	caggccagagaccagaa	84 nt	gcctgctg
	R	gaaaccagcagaaccaggac		
FoxP3	F	ctacgccacgctc atccgctgg	260 nt	gctccaga
	R	cccagcagggttccaacccta		
IFN- $\gamma$	F	ggcatttgaagaattggaaag	112 nt	cagagcca
	R	ttggatgctctgtctatctt		
IL-6	F	cacattcctggctgctgga	88 nt	ctcctctg
	R	cagcttcacgtctcttga		
IL-10	F	tgggggagaacctgaagac	62 nt	ggctgagg
	R	acagggaagaaatc gatgaca		
TGF- $\beta$ 3	F	aagaagcgggctttggac	60 nt	ttcctgac
	R	cacacagcagttc tctcca		
Perforine	F	cttggctcactgcaac ctc	105 nt	ttctctcg
	R	aaaattagctggcatgggtg		
RANTES	F	cctcattgctactgcccctct	63 nt	ctgcctcc
	R	ggtgtggtgtccgaggaata		
HPRT	F	gaccagtcacacagggacat	95 nt	tgggtggag
	R	gtgtcaattatatcttcacaatcaag		

F = Forward primer R = Reverse primer

### Peripheral blood mononuclear cells (PBMCs) isolation.

The peripheral blood was collected in the presence of heparin. The Ficoll-Hypaque method was used to insulate the mononuclear cells from the blood. According to this procedure, PBMCs are isolated by density centrifugation over Ficoll gradients. While the polynuclear cells and the red blood corpuscles are pellet down. The mononuclear cells are collected form a ring with the interface between aqueous-medium and Ficoll.

### Isolation of intrahepatic and intratumoral lymphocytes.

The intrahepatic and intratumoral lymphocytes were isolated from the pieces of tumor and liver adjacent to the tumor taken after resection of the tumor from the department of cellular pathology CHU Grenoble. These pieces were thoroughly washed by three times with RPMI 1640 mixed with Gentamycine. Then, they were crushed into very small pieces with sterile scalpel in a complete medium (10ml). 10% collagenase (Roche Diagnostics, Mannheim, Germany) was added in the medium followed by 100 $\mu$ l DNase to avoid the cell clusters and

placed the mixture in the incubator at 37°C for 30 minutes. The suspension was filtered with 100µm filter in 50ml falcon tube then 10%BSA (bovine serum albumen) was added. Cells were pelleted down and then washed by two times in 10ml of complete medium with 10%BSA. The cells were then counted by Coulter counter (97R&D007). Washed cells were then frozen in 50% BSA in RPMI 1640 and 20% DMSO (Dimethyl sulfoxide) with 1:1 ratio. Finally, the isolated cells were transferred in liquid nitrogen for further use.

### **Immunophenotyping by flow cytometry.**

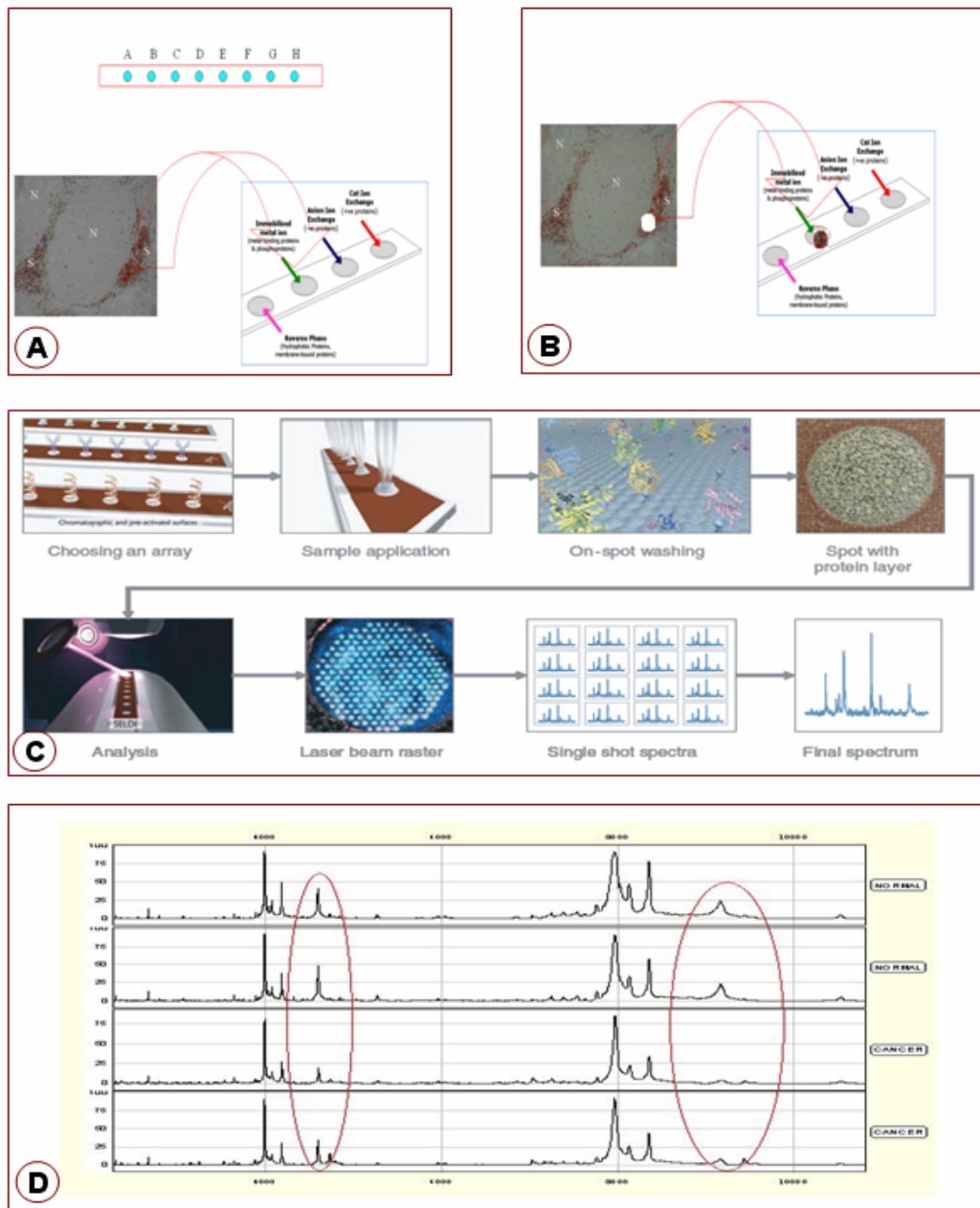
For immunophenotyping, different antibodies used and protocols are described in the articles presented in the part of results. In brief, the cells were incubated with a mixture of surface antibodies with different fluorochromes at 4°C for 30 minutes in the darkness. This was followed by two washings with PBS (phosphate buffer saline) containing 3% of fetal bovine serum before flow cytometric analysis. For indirect immunophenotyping, the cells were initially incubated with a non-conjugated primary antibody and then after washing, incubated with F(ab')<sub>2</sub> goat anti-Immunoglobulin of mouse conjugated with FITC (Fluorescein isothiocyanate). For intracellular markings, the cells were initially marked with the surface antibodies and then permeabilized (Cytotfix/Cytoperm buffer, BD), washed (Perm/Wash buffer) and labelled for the intracellular molecule of interest for 15 min at 4°C then washed twice.

The multi-parametric analyses were carried out by using cytometer allowing the analyses of four colors (FACSCalibur, Becton Dickinson, Mountain View, CA: FL-1: FITC; FL-2: EP + IP; FL-3: PE/Cy5 + IP; FL-4: APC) with the help of CellQuest software (Becton Dickinson). The dead cells were excluded by electronic selection in the presence of propidium iodide (PI) or according to the parameters of size and granularity (FSC and SC). The necessary compensations for the different fluorochromes were made with the cells marked separately for each fluorochrome. According to the conditions, 20,000 to 200,000 events were acquired.

### **SELDI-TOF MS for HCC liver samples.**

To identify the most discriminating tissue protein profiles between HCC-HCV and HCC-OH patients, we used SELDI-TOF MS (Surface-Enhanced Laser Desorption/ionization Time-Of-Flight Mass Spectrometry). The procedure is shown in Fig. 12. In brief, three types ProteinChip Array were used; Hydrophobic, Cationic and Anionic. Tissues were cut at 10µm of thickness by microtome then about 2mm<sup>2</sup> of selected tissue was placed on specific spots of each chip. Two spots were selected for each type of tissue. Then the chips left for drying for

one hour at room temperature. The chips then washed after fixing on stand by using 200 $\mu$ l of each solution [i.e. For Anionic Chip (Q10); 10ml deionizer water + 1ml Tris pH 8 (100mM), For Hydrophobic Chip (H50); 10% Acetonitril + 0.1 Trifluoroacetic acid (TFA), For Cationic Chip (CM10); Sod. Acetate, pH4, 100mM]. The wells were then covered with plastic sheet and the plate was agitated on agitator for 25 minutes. Then, the solution was thrown and the plat was dried. The samples were washed two times more with same procedure. Then separate the stand and left the plat for drying. 0.8 $\mu$ l of EAM (Energy Absorbing Matrix provided by the manufacturer) right on the spot (on chip) and leave for drying for 2 minutes. The plat then analyzed with the CIPHERGEN Protein-Chip Reader (model PBS II). The mass spectra of proteins were generated by using an average of 195 laser shots at a laser intensity of 225 to 250 arbitrary units. For data acquisition of low-molecular weight proteins, the detection size range was between 3 and 30 kd. Detector sensitivity was set at 10 and laser intensity was set at 225. For the high-molecular weight proteins, the detection size range was between 30 and 150 kd. The detector sensitivity was set at 10 and the laser intensity was set at 250. The mass-to-charge ratio ( $m/z$ ) of each of the proteins captured on the array surface was determined according to externally calibrated standards (CIPHERGEN Biosystems).



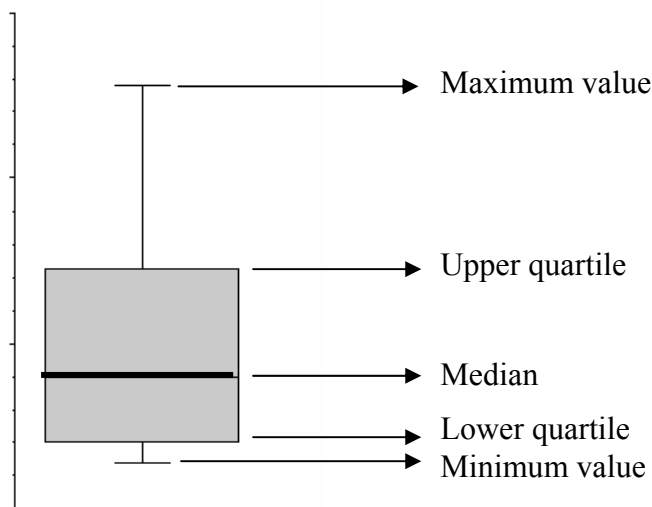
**Figure 13.** The experimental steps for SELDI-TOF MS.

**A & B.** After choosing an array from a selection of chromatographic and preactivated ProteinChip Arrays, samples are applied and incubated on the spots. **C.** On-spot washing ensures efficient sample cleanup, and the spot surfaces allow the formation of a homogenous layer of cocrystallized proteins and matrix compounds. In the ProteinChip Reader, a laser beam is directed on the spot causing desorption and ionization of the proteins. A defined laser beam raster is used to selectively cover the entire spot surface and allows repeated reading of a single spot without using the same positions twice. **D.** Multiple spectra from a statistically meaningful area are then averaged in a final spectrum in which the mass-to-charge ratios of the ionized proteins are given and a good correlation between signal intensities and analyzed concentration is achieved for the different peptides and proteins in the sample (Vorderwulbecke 2005).

## Statistical methods.

The non-parametric Mann-Whitney U-test was used to analyze the comparisons between the different groups of patients and control, while the non-parametric Wilcoxon test was used to analyze the comparisons between the two different parameters in the same individuals. The non-parametric tests are suitable when the size of the analyzed samples is small, when the size of the two populations to be analyzed is different or when the nature of the distribution is not known. Statistical analysis was performed by using the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). To evaluate correlation between the two variables, the 2-tailed Spearman's correlation coefficient was used. P values less than 0.05 were regarded as statistically significant.

The types of graphs used were "box-plot" which illustrates the distribution of the values obtained. The box-plot gives five principal information i.e. median value, maximum value, minimum value, upper quartile (where 25% of values are located) and lower quartile (where 75% of values are located).



# RESULTS

## **Article 1**

### **Clinicopathological study of the Intra-hepatic lymphocytes populations during HCV related Hepatocellular carcinoma**

Muhammad Ramzan, Nathalie Sturm, Marie-Ange Thélu, Xavier Camous, Tania Dufeu-Duchesne, Christiane Guillermet, Alice Cheveau, Marie-Noëlle Hilleret, Jean-Pierre Zarski, Evelyne Jouvin-Marche, Patrice N Marche & Vincent Leroy

Submitted article

**Article 1**  
**Clinicopathological study of the Intra-hepatic lymphocytes populations during HCV related Hepatocellular carcinoma**

Chronic HCV infection is always accompanied by the immune cells infiltration in the liver. These infiltrated immune cells are characterized by T cells, B cells, NK cells, NKT cells,  $\gamma\delta$ T cells and dendritic cells along with macrophages and polynuclear cells. It has been observed that when chronic HCV leads to HCC, the number of all the infiltrated immune cells tend to increase in the liver. Moreover, this cellular infiltrate is also present at varying degree inside the tumors.

Much has been studied regarding the relative density and functionality of cytotoxic T lymphocytes and helper T lymphocytes in the chronic HCV infections and also in HCC up to certain extent. We also have some reports on NK and NKT cells in these diseases. Specially, the knowledge regarding most of the liver and tumor infiltrate in HCC is rare and fragmentary. In this article, we studied almost all the important phenotypes of the immune cells and their precise histological location in the liver and tumor. The relative comparison of these infiltrated cells in the non-involved liver of HCV related HCC (viral HCC) with non-involved liver of alcohol related HCC (non-viral HCC) and in the liver with chronic HCV have also been studied. This comparison gives us changes in the relative densities of different infiltrated cells in the course of disease progression i.e. from chronic HCV to HCC and also the impact of presence or absence of HCV. Moreover, it provides a picture of relative densities of tumor infiltrating cells in the two different tumors i.e. HCV related and alcohol related HCC. This article also shows some important correlations between the different immune cells populations inside the tumor and in the non-involved liver outside the tumor in the same group and between the groups. In addition, the relationships among the tumor aggressivity, tumor recurrence and tumor infiltrating cells have also been studied.

Cytokines and chemokines are very important in the microenvironments of the tumors and in the tissues near to tumors. This article also represents a brief picture of some important pro-inflammatory and anti-inflammatory cytokines and chemokines in the tumor and in the non-involved liver in different groups of patients. Moreover, their correlations with some important clinical parameters provide some useful information. In brief, this article illustrates a detail picture of relative densities of different infiltrating cells population, cytokines and chemokines in the tumor microenvironments.



Tittle

## **Clinicopathological study of the Intra-hepatic lymphocytes populations during HCV related Hepatocellular carcinoma**

**Muhammad Ramzan, Nathalie Sturm, Marie-Ange Thélu, Xavier Camous, Tania Dufeu-Duchesne, Christiane Guillermet, Alice Cheveau, Marie-Noëlle Hilleret, Jean-Pierre Zarski, Evelyne Jouvin-Marche, Patrice N Marche & Vincent Leroy**

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## **Abstract.**

We compared immunological and clinicopathological features in tumoral and cirrhotic areas of 12 HCV related hepatocellular carcinomas (HCC-HCV), 12 alcohol related HCC (HCC-OH) and 23 HCV related cirrhosis (Cirrhosis C). Liver infiltrating cells ( $CD3^+$ ,  $CD4^+$ ,  $CD8^+$ ,  $CD20^+$ ,  $CD56^+$ ,  $TCR\gamma\delta^+$ ,  $FoxP3^+$  and  $CD123^+$ ) were significantly higher ( $p<0.05$ ) in the cirrhotic areas as compared to the tumoral areas in both cancerous groups. The tumoral expressional levels of  $CD8\beta$  in HCC-HCV group and  $CD8\alpha$  and  $IFN-\gamma$  in HCC-OH group were significantly high ( $p<0.05$ ) as compared to the cirrhotic areas. Comparisons between cancerous cirrhotics showed that  $CD3^+$ ,  $CD4^+$ ,  $CD8^+$ ,  $CD20^+$ ,  $FoxP3^+$ ,  $CD123^+$  and almost  $TCR\gamma\delta^+$  cells ( $p=0.089$ ) and  $CD3/CD20$ ,  $FoxP3/CD4$  cell ratio were significantly higher ( $p<0.05$ ) in cirrhosis HCC-HCV than cirrhosis HCC-OH. Expression levels of  $IFN-\gamma$ ,  $CD8\alpha$  and perforin were significantly lower ( $p<0.05$ ) in cirrhosis HCC-HCV than cirrhosis HCC-OH. In addition,  $CD8^+$  cells were correlated with  $FoxP3^+$  cells in the parenchymatous nodules in cirrhosis HCC-HCV, while the expression levels of  $CD8\alpha$  with  $IFN-\gamma$  and perforin in cirrhosis HCC-OH. Results concerning cirrhosis HCC-HCV versus Cirrhosis C showed that  $CD3^+$ ,  $CD4^+$ ,  $CD20^+$  and  $CD8^+$  cells were significantly higher ( $p<0.05$ ) in cirrhosis HCC-HCV than Cirrhosis C, but  $CD56^+$  cells ( $p=0.067$ ) and  $CD3/CD20$  cell ratio were significantly lower ( $p<0.05$ ) in cirrhosis HCC-HCV.  $TGF-\beta$  and  $IFN-\gamma$  were correlated in Cirrhosis C. No difference was observed inside the tumours of both HCC groups, at cellular and mRNA expression levels, except  $CD8\alpha$  which was significantly lower ( $p<0.05$ ) in HCC-HCV group. Taking 24 HCC together, intra-tumoral  $FoxP3^+$  cells correlated with  $CD4^+$  and  $CD8^+$  cells and tumoral expression levels of  $FoxP3$  correlated with  $CD8\alpha$ ,  $CD8\beta$ ,  $IFN-\gamma$ , while they inversely correlated with tumoral expression levels of perforin. Positive correlations of  $CD4^+$  cells with  $CD8^+$  cells,  $CD56^+$  cells with  $TCR\gamma\delta^+$  cells, perforin with  $IFN-\gamma$  and RANTES have also been observed. It is important to note that  $CD3^+$  cells were almost always correlated with  $CD20^+$

cells in all areas in all groups. Concerning with relationships between immunity and tumour aggressivity, we observed that ALT, AFP and Metavir-activity were correlated with CD3<sup>+</sup>, CD4<sup>+</sup> and TCRγδ<sup>+</sup> cells in HCC-HCV group. Tumour size was correlated with age in both cancerous groups and with FoxP3 expression in HCC-HCV group. Tumour necrosis was significantly higher (p<0.05) in HCC-OH than HCC-HCV group and was inversely correlated with age and the expression levels of RANTES in both groups. Ki67 labelling index was correlated with ALT, AFP, PT and viremia, but, inversely correlated with tumoral perforin. FoxP3<sup>+</sup> cells in both types of tumours were negatively correlated with bilirubin. Tumour recurrence was significantly higher (p<0.05) in HCC-HCV than HCC-OH group. In recurrent patient, CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup> and FoxP3<sup>+</sup> cells were significantly higher (p<0.05) in the cirrhotic areas, while CD123<sup>+</sup> cells and FoxP3<sup>+</sup>/CD4<sup>+</sup> cell ratio were significantly higher (p<0.05) in the tumoral areas. Overall, high number of infiltrating immune cells outside and inside of the tumours seems to be less active and associated with tumour recurrence and poor prognosis. These results might be contributed towards the development of an effective immunotherapy to prolong survival and reduce recurrence after surgery in HCC patients.

## **Introduction:**

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with increasing incidence and low therapeutic options [1-4]. Most HCC occur in chronic hepatitis and in cirrhotic livers with a marked increase in liver-infiltrating lymphocytes (LILs) and tumour-infiltrating lymphocytes (TILs) [5, 6]. Several studies have shown that TILs seem to be related with good post-operative survival rates in different cancers including HCC [7-11], but, others have different conclusions [12, 13]. Liver inflammatory infiltrate includes mainly T-lymphocytes (CD3<sup>+</sup>), i.e. helper (CD4<sup>+</sup>) and suppressor (CD8<sup>+</sup>) T lymphocytes, regulatory T cells, immature dendritic cells (DCs), macrophages, few natural killer (NK) cells, B lymphocytes, plasma cells and neutrophils [7, 10, 11, 14-20]. Although, cases of spontaneous regression have been reported [21] but in general, a continued tumour growth is observed despite the infiltration of TILs including tumour-specific T cells within and surrounding the tumours. This suggests a failure of the immune control, which might be due to the partial antigen masking, failure of antigen processing, inadequate costimulation, and/or direct suppression of the effector [20, 22-24].

Cytokines and chemokines are critical to the functioning of both innate and adaptive immune responses and determine the balance between T<sub>H</sub>1 and T<sub>H</sub>2 lymphocytes. A strong pro-inflammatory immune response, associated with T<sub>H</sub>1 lymphocytes, has been observed in those patients who clear acute HCV infection, whereas, a blunted pro-inflammatory immune response and a more pronounced anti-inflammatory immune response, associated with T<sub>H</sub>2 lymphocytes, has been described in patients with chronic HCV infection [25]. A bias towards a T<sub>H</sub>2 immune response has been noted in chronic HCV infections and HCC which is characterised by the low productions of IFN- $\gamma$  and IL-2 and high productions of IL-4, IL-10 and TGF- $\beta$  [20, 25-28]. Furthermore, high levels of certain chemokines [MIP3- $\alpha$  (CCL-20), IP-10 (CXCL-10), Mig (CXCL-9), RANTES (CCL-5)] and their receptors [CXCR-3, CCR-5]

have also been reported in the serum and liver of the patients with chronic HCV infections and HCC [29-32]. These features facilitate the recruitment of the immune cells such as T and B lymphocytes, NK cells and dendritic cells at the site of infection. Overall, the data of LILs and TILs in HCC patients is rare and scattered. In the present study, we analysed phenotypic and functional markers of LILs and TILs along with clinical and histological characteristics of HCV related HCC patients, in comparison with alcoholic related HCC patients and HCV related cirrhotic patients without cancer.

### **Patients and Methods:**

#### ***Patients.***

Twenty four patients with histologically proven HCC who had undergone curative hepatectomy and twenty three with histologically proven persistent HCV infection with cirrhosis (Cirrhosis C) in the University Hospital, Grenoble, between 1999 and 2007 were included in this study. Among 24 HCC cases, 12 went with HCV (HCC-HCV) and 12 with alcohol (HCC-OH). All HCC-HCV and Cirrhosis C patients were anti-HCV antibody positive by two different third generation ELISA tests and had detectable serum HCV-RNA by PCR (mono-infected HCV group). Exclusion criteria included co-infection with human immunodeficiency virus, hepatitis B virus or hepatitis delta virus, other causes of liver disease, alcohol consumption higher than 30 g/day, inflammatory syndrome, previous antiviral treatment and previous liver transplantation. All HCC-OH patients were anti-HCV or anti-HBV antibody negative. Patients with cirrhosis C underwent needle liver biopsy as a part of their diagnostic evaluation. The main characteristics of all the patients are shown in Table 1. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by our institutional review board. Patients were enrolled after giving their written informed consent.

### *Immunohistochemical Staining:*

Immunohistochemical study of the markers CD3, CD4, CD8, CD20, FoxP3 and Ki67 (**Table 3**) was performed on paraffin-embedded, formalin-fixed liver tissues and processed on an automated immunostainer (Nexes, Ventana, Tucson, AZ, USA), except for CD4 (processed manually). Briefly, consecutive sections of liver tissue blocks were cut at 4µm, deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was done by heating slides in adequate buffer using water bath or microwave oven. Non specific protein binding were blocked by 30 minutes incubation in phosphate-buffered saline (PBS), pH 7.4, with 2% milk. Endogenous peroxidase activity was blocked by 1% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 5 min. These treatments were alternated with rinses with PBS (pH 7.4). Slides were then processed on the Nexes immunostainer, starting with an automatic incubation with the primary antibodies followed by an immunodetection with DAB (diaminobenzidine) I-view kit, according to the manufacturer's recommendations (Ventana, Tucson, AZ, USA). For CD4 immunostaining, after incubation with primary antibody, slides were washed with PBS (pH 8.6), sur-fixed five minutes with 0.005% glutaraldehyd in PBS pH 8.6, washed once more in PBS pH 8.6 and then incubated with secondary antibody by using an Envision system (labelled polymer HorseRadish Peroxidase anti-mouse antibody, ref K4000, Dako, Glostrup, Denmark). This was followed by a rinse in PBS (pH 8.6), a second sur-fixation in glutaraldehyd, another rinse in PBS (pH 8.6), Diaminobenzidine chromogen staining and counterstaining with haematoxylin.

CD123, CD56, TCRgd (**Table 3**) was performed on 10 µm thick frozen tissue sections, and processed manually. Frozen sections stored at – 70°C were defrosted for 30 minutes at room temperature, fixed in a first bath of cold pure acetone (10 minutes at – 20°C) then in a second bath of acetone with 1% H<sub>2</sub>O<sub>2</sub> (5 minutes at 4°C). They were then briefly rinsed in pure acetone, dried and washed in PBS 7.4. Non specific protein binding were blocked by 30

minutes incubation in phosphate-buffered saline (PBS) pH 7.4 with 2% milk. Further steps (incubation with primary and secondary antibodies, amplification and revelation) were similar to those described for manually CD4 immunodetection. As a negative control, non immunized mouse or rabbit immunoglobulin was substituted to the primary antibody.

Immunopositive cells were counted in 40 randomly selected fields per slide on a computer screen with the help of automatic software LUCIA (Laboratory Universal Computer Image Analysis, version 5.0, Laboratory Imaging, Prague, Czech) at high magnification (X400). All positive cells with above mentioned antibodies were independently counted by two observers with 95% concordance. Cells in the tumoral and cirrhotic areas were counted separately, as well as their localization in the fibrous tissue (portal tracts and fibrous septa) and the parenchymatous nodules and their number was expressed per field at magnification X400.

#### ***Real-Time Quantitative RT-PCR analysis.***

Total RNA was extracted from frozen liver tissues (5-10mg) by the homogenization of the tissues in silica balls using MagnaLyser System (Roche Diagnostics, Meylan, France). After centrifugation, 600  $\mu$ l of 70% ethanol was added in the aliquot. The mixture was filtered on a silica column and residual DNA was digested by DNase I for 15 min at room temperature. Then, the precipitation of the total RNA was performed in the presence of ethanol followed by two washings. Single-stranded cDNA was prepared with 1 $\mu$ g of total RNA by using the Superscript II Reverse transcriptase (Gibco BRL, Life Technologies) in a final volume of 20  $\mu$ l. Primer pairs and probes for quantification of CD4, CD8 $\alpha$ , CD8 $\beta$ , IL-6, IL-10, IFN- $\gamma$ , TGF- $\beta$ , perforine and RANTES genes were designed by using ProbeFinder Software for Human (version 2.35) (Roche Diagnostics, Meylan, France) and were synthesized by Genset (Paris, France). The sequence and main characteristics of each primer and probe were summarized **Table 2**. The quantification of the transcripts from the liver samples was performed by using the LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche Diagnostics, Meylan, France). Target

cDNA sequences were amplified with the LightCycler<sup>®</sup> 480 Probes Master and were detected with UPL (Universal ProbeLibrary, City, Country) Probes. Briefly, each PCR reaction mixture contained: 1X of LightCycler<sup>®</sup> 480 Probes Master (Roche Diagnostics, Meylan, France), 0.5 μM of each primer, 0.1μM of appropriate probe and 2.5μL of cDNA preparation, in a final volume of 10μL. After an initial denaturation at 95°C for 10 min, the PCR reactions were cycled 35-40 times as follows; 10 s at 95°C, annealing step 15 s at 60°C (for all primers and probes) and 10 s at 72°C. Fluorescence was measured once at each 60°C step and monitored continuously during the melting process. Data were analyzed with the LightCycler<sup>®</sup> 480 Software. The detection format was set to “Mono Colour Hydrolysis Probe” and the Second Derivative Maximum Method was used for absolute quantification. The housekeeping gene used for normalisation of the values was the Hypoxanthine Phosphoribosyl Transferase gene (HPRT). The results were expressed as the log of the number of amplicons ( $2^n - 2n$ , n = number of cycles) of the targeted transcripts.

### ***Statistical methods.***

The non-parametric Wilcoxon and Mann-Whitney U-test were performed to compare quantitative variables. Within group, comparisons were performed by using the 2-tailed Spearman's correlation coefficient. Statistical analysis was performed by Principal Component Analysis (PCA) and linear univariate and multivariate regression analysis. P values less than 0.05 were considered significant. Statistical analysis was performed by using the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).



## **Results.**

### ***Clinical Parameters.***

The main characteristics of the patients are shown in **table 1**. The mean age of Cirrhosis C group was significantly lower than both HCC-HCV ( $p<0.05$ ) and HCC-OH ( $p<0.05$ ) groups but did not differ between HCC-HCV and HCC-OH groups. The mean serum AFP level was significantly higher in HCC-HCV group as compared to HCC-OH group ( $p<0.05$ ). Likewise, the mean serum ALT level was significantly higher in HCC-HCV and Cirrhosis C than HCC-OH group ( $p<0.005$ ) but did not differ significantly between HCC-HCV and Cirrhosis C groups. Similarly, prothrombin time was higher ( $p=0.060$ ) in HCC-HCV than HCC-OH group while the serum bilirubin level did not change among all the three groups. Among HCV infected patients the serum viral load in HCC-HCV and Cirrhosis C groups did not vary significantly but it was correlated with the high levels of ALT in both HCC-HCV ( $r=0.460$ ,  $p=0.031$ ) and Cirrhosis C (&  $r=0.845$ ,  $p=0.017$ ) groups and to lesser extent with the serum levels of AFP in cirrhosis C group ( $r=0.607$ ,  $p=0.063$ ).

### ***Histological Parameters.***

All 24 HCC developed in cirrhotic (Metavir F4 stage) liver. Most of HCV infected patients in both Cirrhosis C and HCC-HCV group had moderate or high necro-inflammatory activity (Metavir A2 and A3) (**Table 1**). It is noteworthy that Metavir-activity (Metavir A2) score was correlated positively with higher ALT levels in HCC-HCV group ( $r=0.751$ ,  $p=0.008$ ) and up to some extent in Cirrhosis C group ( $r=0.374$ ,  $p=0.086$ ). Moreover, a strong correlation has also been observed between Metavir-A score and AFP levels ( $r=0.796$ ,  $p=0.003$ ) in HCC-HCV group. Most tumours were well or moderately differentiated and unifocal in both HCC groups. There was no difference in the tumour size, capsule formation and proliferative index (Ki67) in both HCC groups. We observed that tumour necrosis was significantly lower in HCC-HCV than HCC-OH group ( $p<0.05$ ). Tumour recurrence, diagnosed on tumour

regeneration and clearly identified on images and elevated tumour marker levels, was significantly higher in HCC-HCV than HCC-OH (50% vs 8.3%,  $p<0.05$ ) group and was observed within two years after surgery.

#### ***Intrahepatic and intratumoral immune cells.***

All the cellular immunomarkers including in our study i.e. CD3, CD4, CD8, CD20, CD56, FoxP3, TCR $\gamma\delta$ , and CD123 were higher ( $p<0.05$ ) outside the tumour than inside in both HCC-HCV and HCC-OH groups (**Fig.1**). Particularly, we noted that all the immune cells were high in number in the fibrous areas including lymphoid aggregates, portal tracts and the fibrous septa (**Fig. 2**). So, when we compared the relative density of the immunopositive cells inside the tumour and in the parenchymatous nodules, no any significant difference was noticed except for FoxP3<sup>+</sup> cells which were significantly higher inside the tumours in both HCC-HCV and HCC-OH groups ( $3.04\pm 1.3$  vs  $0.9\pm 0.3$  &  $3.3\pm 2.4$  vs  $0.6\pm 0.5$ ,  $p < 0.05$ ). Moreover, we did not observe any significant difference in the expression levels of all the gene markers (CD8 $\alpha$ , CD8 $\beta$ , IL-6, IL-10, IFN- $\gamma$ , TGF- $\beta$ , perforine and RANTES) except CD8 $\beta$  which were significantly higher ( $p<0.05$ ) outside the tumour than inside in HCC-HCV group and CD8 $\alpha$ , IFN- $\gamma$  and perforine were significantly higher ( $p<0.05$ ) outside the tumour than inside in HCC-OH group. The only two correlations we noted between inside and outside of the tumour were the number of CD56<sup>+</sup> cells in HCC-OH group ( $r=0.731$ ,  $p=0.007$ ) and the expression levels of IFN- $\gamma$  in HCC-HCV group ( $r=0.832$ ,  $p=0.001$ ). Generally, the immunopositive cells are higher inside the cirrhotic tissue than inside the tumour, but, with few exceptions, the gene expression levels of functional immunomarkers do not change a lot.

#### ***Comparison between the cirrhotic areas of HCC-HCV and HCC-OH groups.***

Most of the immune cells including CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup>, FoxP3<sup>+</sup> and CD123<sup>+</sup> cells were significantly higher (1.4 to 2.8 folds  $-p<0.05$ ) in cirrhosis HCC-HCV than cirrhosis HCC-OH (**Fig. 3**). TCR $\gamma\delta$ <sup>+</sup> cells were also nearly significantly higher ( $p=0.089$ ) in cirrhosis HCC-

HCV but no difference was observed for CD56<sup>+</sup> cells. It is noteworthy that this difference was observed in the portal tracts and fibrous septa for all the above mentioned immunomarkers except CD4 and FoxP3 which also differed significantly ( $p<0.05$ ) in the parenchymatous nodules. Moreover, CD3/CD20 (median values 3.9 vs 2.1 -  $p<0.005$ ) and FoxP3/CD4 (median values 0.17 vs 0.08 -  $p<0.005$ ) ratios were significantly higher ( $p<0.05$ ) in cirrhosis HCC-HCV, indicating that high proportion of T cells and FoxP3<sup>+</sup> regulatory T cells were more evident in the cirrhotic areas of HCC-HCV group than HCC-OH group. A positive correlation between CD3<sup>+</sup> and CD20<sup>+</sup> cells have been found in both groups but it was restricted to the parenchymatous nodules ( $r=0.776$ ,  $p=0.003$ ) in cirrhosis HCC-HCV and fibrous septa ( $r=0.678$ ,  $p=0.015$ ) in cirrhosis HCC-OH. It is an establish fact that CD8<sup>+</sup> cells are important in the viral and tumour control and FoxP3<sup>+</sup> cells reduce their effector function. We also observed a positive correlation between of CD8<sup>+</sup> and FoxP3<sup>+</sup> cells ( $r=0.602$ ,  $p=0.038$ ) in the parenchymatous nodules in cirrhosis HCC-HCV.

Concerning with the gene expression levels, we noticed that IFN- $\gamma$ , CD8 $\alpha$  and perforine expression levels were significantly lower ( $p<0.05$ ) in cirrhosis HCC-HCV while the expression levels of the others did not differ. CD8/perforine ratio (0.6 vs 17.3 -  $p<0.001$ ) was also significantly lower in cirrhosis HCC-HCV than HCC-OH. Moreover, we found that the expression level of CD8 $\alpha$  was correlated with IL-6 ( $r=0.703$ ,  $p=0.035$ ) in cirrhosis HCC-HCV, while it was correlated with the expression levels of IFN- $\gamma$  ( $r=0.776$ ,  $p=0.003$ ) and perforine ( $r=0.552$ ,  $p=0.063$ ) in cirrhosis HCC-OH. Altogether, these results show that a large numbers of immune cells are present outside of the tumour in the cirrhotic areas of HCC-HCV groups but they are less active as compared to HCC-OH group.

#### ***Comparison between tumoral areas of HCC-HCV and HCC-OH groups.***

Interestingly, immunohistochemical study did not show any significant difference inside the tumoral areas in both HCC-HCV and HCC-OH groups (**Fig.4**). In addition, the expression

level of all the gene markers did not differ more except CD8 $\alpha$  which was significantly lower ( $p<0.05$ ) in the tumour of HCC-HCV group. So, we pooled 24 HCC and investigated certain interesting correlations among the immunological markers at cellular and mRNA expression levels. FoxP3<sup>+</sup> cells are very important in the microenvironments of the tumours. In order to study the role of these cells, we observed that FoxP3<sup>+</sup> cells were correlated with CD4<sup>+</sup> and CD8<sup>+</sup> cells (**Fig.5a,b**). At transcriptional level, we also observed that FoxP3 was correlated with CD8 $\alpha$ , CD8 $\beta$  and IFN- $\gamma$  (**Fig.5c,d,e**), while they were inversely correlated with perforine ( $r= -0.452, p=0.045$ ) in the tumoral areas. We also found that CD20<sup>+</sup> cells were correlated with CD3<sup>+</sup> ( $r=0.699, p=0.002$ ), CD4<sup>+</sup> ( $r=0.699, p=0.000$ ) and CD8<sup>+</sup> cells ( $r=0.543, p=0.006$ ) and CD4<sup>+</sup> cells with CD8<sup>+</sup> cells ( $r=0.574, p=0.003$ ). In addition, CD56<sup>+</sup> cells were also correlated ( $r=0.623, p=0.001$ ) with TCR $\gamma\delta$ <sup>+</sup> cells and the expression levels of perforine were correlated with those of both IFN- $\gamma$  ( $r=0.473, p=0.035$ ) and RANTES ( $r=0.790, p=0.000$ ).

In summary, despite a great variation in infiltrating immune cells outside the tumour in both HCC groups, they did not vary inside, indicating that transformed hepatocytes might behave similarly towards the immune response, regardless of presence or absence of the virus.

#### ***Comparison between cirrhotic areas of HCC-HCV and Cirrhosis C group.***

In order to identify the immunomarkers associated with carcinogenesis, we compared HCV-related cirrhosis which developed cancer (cirrhosis HCC-HCV) and HCV-related cirrhosis without cancer (Cirrhosis C). We found that CD3<sup>+</sup> ( $p<0.05$ ), CD4<sup>+</sup> ( $p<0.05$ ), and CD20<sup>+</sup> cells ( $p<0.05$ ) were significantly higher in the cirrhosis HCC-HCV group (**Fig.6**). For CD8<sup>+</sup> cells, this difference was limited to the parenchymatous nodules ( $p<0.05$ ). In contrast, CD56<sup>+</sup> cells were nearly significantly lower ( $p=0.067$ ) in cirrhosis HCC-HCV than Cirrhosis C. In Cirrhosis C group, positive correlations have been observed between CD3<sup>+</sup> and CD20<sup>+</sup> cells ( $r=0.746, p=0.001$ ) and between CD4<sup>+</sup> and CD8<sup>+</sup> cells ( $r=0.539, p=0.038$ ). At the expressional levels,

we observed that IL-6 ( $0.9 \pm 2.0$  vs  $1.2 \pm 0.8$ ,  $p < 0.05$ ) and TGF- $\beta$  ( $0.001 \pm 0.0$  vs  $0.2 \pm 0.2$ ,  $p < 0.001$ ) were significantly lower in cirrhosis HCC-HCV than Cirrhosis C group. A positive correlation was also observed between TGF- $\beta$  and IFN- $\gamma$  ( $r = 0.900$ ,  $p = 0.037$ ) in Cirrhosis C group. Generally, more liver infiltrating cells have been observed in HCV-related cirrhosis which develop cancer than in HCV-related cirrhosis without cancer but some types of immune cells and cytokines (CD56<sup>+</sup> cells, IL-6, TGF- $\beta$ ) were higher in Cirrhosis C group.

***Relationship between tumoral immunity and tumoral aggressivity.***

To investigate the immune response with respect to the degree of tumour aggressivity, some interesting correlations have been found outside and inside of the tumours in both groups i.e. HCC-HCV and HCC-OH. Outside the tumours, serum ALT levels in both types of HCC were correlated positively with CD3<sup>+</sup> cells in parenchymatous nodules ( $r = 0.692$ ,  $p = 0.018$  &  $r = 0.665$ ,  $p = 0.025$ ), while, they also correlated positively with CD3<sup>+</sup> ( $r = 0.692$ ,  $p = 0.018$ ), CD4<sup>+</sup> ( $r = 0.642$ ,  $p = 0.033$ ) and TCR $\gamma\delta$ <sup>+</sup> cells ( $r = 0.692$ ,  $p = 0.018$ ) in the parenchymatous nodules of HCC-HCV group and with CD4<sup>+</sup> cells ( $r = 0.661$ ,  $p = 0.027$ ) present in the exterior of the tumour in HCC-OH group. Serum levels of AFP were correlated with CD3<sup>+</sup> ( $r = 0.670$ ,  $p = 0.017$ ), CD4<sup>+</sup> ( $r = 0.646$ ,  $p = 0.023$ ) and TCR $\gamma\delta$ <sup>+</sup> cells ( $r = 0.657$ ,  $p = 0.020$ ) in the parenchymatous nodules in HCC-HCV group. Moreover, Metavir activity score was also correlated with CD3<sup>+</sup>, CD4<sup>+</sup>, CD20<sup>+</sup>, and TCR $\gamma\delta$ <sup>+</sup> cells present only in the parenchymatous nodules in HCC-HCV group (**Fig.7a,b,c,d**).

Ki-67 labelling index is another factor which determines the tumour aggressivity and we found that it was correlated with ALT ( $r = 0.463$ ,  $p = 0.030$ ), AFP ( $r = 0.566$ ,  $p = 0.004$ ) and PT ( $r = 0.449$ ,  $p = 0.032$ ). Interestingly, in HCC-HCV group, it was negatively correlated with tumoral expression levels of perforine ( $r = -0.729$ ,  $p = 0.017$ ) and positively correlated with viremia ( $r = 0.809$ ,  $p = 0.005$ ) but no such correlations have been found in HCC-OH group. Tumour size was positively correlated with age ( $r = 0.417$ ,  $p = 0.042$ ) in HCC patients,

while, in HCC-HCV group, it was also correlated positively with the tumoral expression levels of FoxP3 ( $r=0.612$ ,  $p=0.045$ ). Moreover, we observed that FoxP3<sup>+</sup> cells in both types of the tumours were positively correlated with PT ( $r=0.433$ ,  $p=0.039$ ), while they were inversely correlated with serum bilirubin ( $r= -0.633$ ,  $p=0.001$ ). Necrosis was significantly higher ( $p<0.05$ ) in HCC-OH than HCC-HCV group. Moreover, we found that the percentage of tumour necrosis was inversely correlated with age ( $r = -0.417$ ,  $p = 0.042$ ), CD4<sup>+</sup> cells ( $r = -0.447$ ,  $p = 0.028$ ) and RANTES expression level ( $r = -0.469$ ,  $p = 0.024$ ) in the tumour of HCC patients. Vascular invasion was also positively correlated with the expression level of CD8 $\alpha$  ( $r = 0.618$ ,  $p = 0.006$ ) in the tumour of both HCC patients. Taking altogether, the factors which increase tumour aggressivity (e.g. tumour size, Ki-67 labelling index, AFP) are positively correlated with age, viremia, different types of lymphocytes outside the tumours and the expression level of FoxP3 inside the tumour, while, the factor which decrease tumour aggressivity (e.g. tumour necrosis) is negatively correlated with the expression levels of perforine in the tumour.

#### ***Tumour recurrence in HCC.***

Regarding tumour recurrence in 24 HCC patients, we observed recurrence in 6 HCC-HCV and only one in HCC-OH group. CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup> and FoxP3<sup>+</sup> cells were significantly higher outside the tumour in 7 recurrent patients as compared to the 17 non-recurrent patients (**Fig.8**). Particularly, FoxP3<sup>+</sup> cells were significantly higher in the parenchymatous nodules in the recurrent patients ( $53.5\pm 24.6$  vs  $23.9\pm 19.7$  - $p<0.05$ ). In addition, FoxP3<sup>+</sup>/CD4<sup>+</sup> cell ratio was significantly higher inside the tumour in recurrent patients ( $0.2\pm 0.1$  vs  $0.1\pm 0.1$  - $p<0.05$ ). Specially, in HCC-HCV group, we also found a significant higher CD8<sup>+</sup> cells outside the tumour ( $136.3\pm 26.7$  - $p<0.05$ ), and a FoxP3<sup>+</sup>/CD4<sup>+</sup> cell ratio inside the tumour in recurrent patients ( $0.3\pm 0.1$  vs  $0.2\pm 0.2$  - $p=0.034$ ). Tumoral and peri-tumoral macrophages have been thought to involve in the tumour progression, metastasis and recurrence in many type of

cancer including HCC [33, 34]. Here, we also found that CD123<sup>+</sup> cells were higher in recurrent patients than non-recurrent patients ( $p=0.089$ ). Altogether, we reported here that high numbers of different infiltrating cells specially CD8<sup>+</sup> and Foxp3<sup>+</sup> cells outside the tumour and high numbers of CD123<sup>+</sup> and FoxP3<sup>+</sup> cell inside the tumour might be the risk factors for tumour recurrence in HCC patients.

## **Discussion.**

Our results showed that when hepatocellular carcinoma developed in chronic inflammatory liver disease, a large number of immune cells have been infiltrated in the tumoral as well as peri-tumoral cirrhotic liver. However, as partially reported by others [35], both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, NK and NKT cells,  $\gamma\delta$  T cells, FoxP3<sup>+</sup> regulatory T and dendritic cells and/or macrophages (CD123<sup>+</sup> cells) are many folds higher in the non tumoral areas than tumoral areas, suggesting that along with other factors previously proposed [20, 22, 23], most of the immune cells are fail to enter into the tumours.

Liver cirrhosis is a major clinical risk factor for HCC development and 70–90% of HCC developed in cirrhotic patients [36, 37]. Our study focus on cancer developed in HCV or alcohol related cirrhotic liver. Our data demonstrated that most of the infiltrating immune cells (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup>, FoxP3<sup>+</sup>, TCR $\gamma\delta$ <sup>+</sup>, and CD123<sup>+</sup> cells) are significantly higher in HCV related cirrhosis with cancer than alcohol related one. It is noteworthy that this difference was evident only in the portal tracts, lymphoid aggregates and fibrous septa, but did not observe in the parenchymatous nodules. Not surprisingly, we observed more lymphoid aggregates in HCV related cirrhosis than alcoholic cirrhosis, a feature already reported as a character of HCV infection [38-40]. Concerning HCV related cirrroses, we noted that CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells are significantly higher in those which develop cancer. Overall, our results showed that immune cells are lowest in cirrhosis HCC-OH. It seems that the higher numbers of infiltrating lymphocytes in Cirrhosis C may be due to presence of HCV and their highest numbers in cirrhosis HCC-HCV might be due to the presence of both HCV and HCC. NK and NKT cells (CD56<sup>+</sup> cells) are the main player against anti-tumoral immunity along with cytotoxic T lymphocytes (CD8<sup>+</sup> cells) [41]. A low frequency of CD56<sup>+</sup> cells has been reported in the liver of chronic HCV infection [42, 43]. We reported a significant higher numbers of CD56<sup>+</sup> cells in Cirrhosis C than in both cancerous cirrhosis groups but CD56<sup>+</sup>



cells are the only immune cells which do not differ significantly between the two etiologies. These results suggest that HCC lowers the frequency of CD56<sup>+</sup> liver infiltrating lymphocytes independent of the presence or absence of the virus. Moreover, we also observed that CD3<sup>+</sup> cells are always correlated with CD20<sup>+</sup> cells in the three types of cirrhosis we studied indicating that whenever and wherever T cells present that always correspond with B cells.

Increased Foxp3<sup>+</sup> T-lymphocytes in different cancer including HCC and in chronic hepatitis C have been demonstrated and their presence is a predictor of reduced survival [19, 26, 44-46]. Our data demonstrated that FoxP3<sup>+</sup>/CD4<sup>+</sup> cells ratio is significantly higher in cirrhosis HCC-HCV as compared to the cirrhosis HCC-OH. Increased regulatory T cells correlate with CD8<sup>+</sup> T-cell impairment and poor survival in hepatocellular carcinoma patients [47-49]. Moreover, the site of action of CD8<sup>+</sup> cytotoxic T cells is also nodular parenchyma where they kill virus infected or transformed hepatocytes. Interestingly, we observed that Foxp3<sup>+</sup> cells are significantly higher in the nodular parenchyma of cirrhosis HCC-HCV where they have a positive correlation with CD8<sup>+</sup> cells, suggesting that FoxP3<sup>+</sup> regulatory T cells might inhibit CD8<sup>+</sup> cytotoxic T cells at the site of action perhaps in the necrotic lesions.

As for as the functional properties of these infiltrating lymphocytes are concern, we observed a significant low expression levels of IFN- $\gamma$ , perforine and CD8 $\alpha$  in the cirrhosis HCC-HCV than in cirrhosis HCC-OH, and CD8 $\alpha$  positively correlated with both IFN- $\gamma$  and perforine in cirrhosis HCC-OH only. This interesting picture demonstrates that a high numbers of immune cells present in cirrhosis HCC-HCV are less active than a low numbers present in cirrhosis HCC-OH and this attenuation might be at the level of CD8 $\alpha$  expressing cell population. We also found low expression levels of IL-6 and TGF- $\beta$  in cirrhosis HCC-HCV as compared to Cirrhosis C, indicating a counterbalance in between pro-inflammatory and anti-inflammatory cytokines in HCV related cirrhosis.

Our study also provides a new data on the comparative profile of the tumour infiltrating lymphocytes (TILs). There is no difference in between their number and expression level in both HCC-HCV and HCC-OH groups, except the expression level of CD8 $\alpha$  which is significantly higher in HCC-OH tumour. This could indicate that the tumour microenvironments might behave similarly towards the immune response regardless of presence or absence of the virus. One crucial role of T-regulatory cells in anti-tumour immunity is the inhibition of the cytotoxic functions of CD8 $^+$  lymphocytes [18, 47-49]. Here, we found a positive correlation between intra-tumoral FoxP3 $^+$  and CD8 $^+$  cells and also between the intra-tumoral expression levels of FoxP3 and CD8 $\alpha$  and CD8 $\beta$ . It is noteworthy that this correlation is stronger with CD8 $\alpha$  than CD8 $\beta$ , indicating that the regulatory functions of FoxP3 $^+$  T cells are more strong towards CD8 $\alpha$  expressing cytotoxic cells. In addition, the expression levels of FoxP3 also correlate with those of IFN- $\gamma$  in the tumours, suggesting another counterbalance of pro-inflammatory immune response. Still another, negative correlation have been found in between the expression levels of FoxP3 and perforine, confirming the anti-effector role of FoxP3 cells in the tumour microenvironments. Our data also represents some interesting positive correlation in HCC tumours, such as CD3 $^+$  with CD20 $^+$  cells, CD4 $^+$  with CD8 $^+$  cells and CD56 $^+$  with  $\gamma\delta$ T cells, showing certain synergy of these immune cells in the tumour microenvironments. The expression levels of CD8 $\alpha$  are high in HCC-OH tumour. To further investigate their role in these tumours, we found that their expression levels are correlated with the expression levels of RANTES as well as with perforine and FoxP3. In summary, it seems that RANTES may attract preferentially CD8 $\alpha$  cells which might be more cytotoxic but counterbalance by FoxP3 $^+$  cells in the microenvironments of HCC.

Serum levels of ALT, AFP and bilirubin are the indicators of liver injury and regeneration and were reported high during HCC [50-52]. In our study, we found a positive correlation

between ALT and CD3<sup>+</sup> cells in both groups, while, it correlates with CD4<sup>+</sup> cells in HCC-OH group and TCR $\gamma\delta$ <sup>+</sup> cells in HCC-HCV group. Notably all these correlations were restricted to the parenchymatous nodules and no correlation have been found in the fibrous areas. It has been reported that cancer specific CD4<sup>+</sup> cytotoxic T lymphocytes exist in both human and murine HCC [53, 54] and these cells might exist outside of the tumour which make an increase in ALT levels by killing occasional transformed cells outside the tumours. Moreover,  $\gamma\delta$ T cells seem to be very important in HCV related HCC immunosurveillance. Metavir activity score represents the disease gravity and the activeness of the immunity. Our results showed a regular increase in CD3<sup>+</sup>, CD4<sup>+</sup>, CD20<sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> cells in the parenchymatous nodules with an increase in Metavir activity score in HCC-HCV group, indicating again the cytotoxic role of CD4<sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> cells along with CD20<sup>+</sup> cells. Here, we also found that FoxP3<sup>+</sup> cells in the tumours of both HCC groups were inversely correlated with the serum bilirubin, indicating the regulatory T cells lessen the liver damage up to certain extents.

Among the histological parameters, tumour size is positively correlated with age and the expression levels of FoxP3 in the HCV related tumours, suggesting that these parameters are negative regulators of the tumour immunity. Interestingly, our data shows inverse correlations among percentage of necrosis and age, CD4<sup>+</sup>, CD20<sup>+</sup> cells and with the expression levels of RANTES in HCC tumours, confirming that the reduction of intratumoral immunity is evident with the passage of age. In addition, RANTES may attract immune cells such as CD4<sup>+</sup> and CD20<sup>+</sup> cells but somehow they might be more regulatory in nature than cytotoxic in the tumour microenvironments. This hypothesis is supported by the notion that a large numbers of CD4<sup>+</sup> cells are regulatory T cells which can directly suppress B cell immunoglobulin response [55, 56]. Ki67 labelling index is another parameter which represents tumour progression [57]. It correlates positively with the serum levels of AFP, ALT and PT in both groups indicating that high serum levels of certain clinical parameters are the indicators of

tumour progression. We also demonstrate a strong positive correlation between Ki67 and viremia, suggesting that HCV and its certain protein might involve in HCC growth, as already reported [37]. Interestingly, we found an inverse correlation between Ki67 and the expression levels of intra-tumoral perforine, indicating that attenuated immune response may favour tumour growth.

Tumour recurrence following liver resection for HCC is common and a major cause of death [58]. Multiple risk factors for tumour recurrence e.g. AFP, AST, tumour size, multifocality, portal invasion, HCV, steatosis, cirrhosis and histological activity have been reported in HCC patients [59-63]. We have also found a significantly higher recurrence in HCC-HCV group as compared to HCC-OH group. Interestingly, we found that CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup>, FoxP3<sup>+</sup> cells in HCC, and CD8<sup>+</sup> cells particularly in HCC-HCV group, are significantly higher outside the tumour in recurrent patients than non-recurrent patients, suggesting that the high number of liver infiltrating cells outside the tumour are not beneficial and could be related to recurrence. In contrast, high numbers of tumour infiltrating cells have been reported to link with better prognosis [11]. Recently, it has been reported that high number of tumour infiltrating regulatory T cells is an independent predictive factor of tumour recurrence after hepatic resection for HCC [19]. We confirmed here that a significant higher FoxP3/CD4 cell ratio is present in the tumours of recurrent patients than non-recurrent patients. Tumoral and peri-tumoral macrophages have been thought to involve in the tumour progression, metastasis and recurrence in many type of cancer including HCC [33, 34, 64]. Moreover, plasmacytoid dendritic cells (pDCs) were also thought to correlate with adverse outcome in breast cancer [65]. For the first time, we report higher number of CD123<sup>+</sup> cells in the tumours of recurrent patients than non-recurrent patients, indicating that tumoral pDCs/macrophages are also involved in tumour recurrence in HCC. Taking together, these results show that greater is the infiltrating cells outside HCC, greater might be the recurrence, while, high numbers of

regulatory T cells and pDCs/macrophages cells inside the tumour also seem to associated with tumour recurrence.

Overall, very high numbers of infiltrating lymphocytes outside the tumours seems to be attenuated in their antitumoral function and are more regulatory, particularly in the presence of chronic HCV infection. Moreover, they are associated with tumour recurrence and poor prognosis. The tumour infiltrating lymphocytes are low in numbers and also looking dysfunctional. A concomitant stimulation of these lymphocytes along with functional depletion of regulatory T cells might be an effective immunotherapy to prolong survival and reduce recurrence after surgery.

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## Figure legends

**Fig. 1.** Quantifications of immunopositive cells in the non-tumoral cirrhotic tissues verses tumoral tissues in 24 HCC patients. All the above mentioned markers are many folds high in the non-tumoral cirrhotic tissues (outside the tumour) as compared to tumoral tissues in all HCC patients.

**Fig. 2. a.** CD3<sup>+</sup> cells in fibrous septa (S) (including lymphoid aggregates and portal tract) and in parenchymatous nodules (N) outside the tumour. **b.** CD3<sup>+</sup> cells in the tumoral area. **c.** FoxP3<sup>+</sup> cells in fibrous septa (S) and in the tumoral areas (T). **d.** CD56<sup>+</sup> cells in fibrous septa (S) and in the tumoral areas (T). **e.** TCR $\gamma\delta$ <sup>+</sup> cells in fibrous septa (S) and in the tumoral areas (T). **f.** CD123<sup>+</sup> cells in fibrous septa (S) and in tumoral areas (T). In general, all the markers studied are many folds high outside the tumours than inside.

**Fig. 3.** Comparison between the cirrhotic areas outside the tumour in HCC-HCV and HCC-OH groups showing that most of the liver infiltrating cells are high in density in cirrhosis HCC-HCV than cirrhosis HCC-OH. (Vertical axes represent the numbers of immunopositive cells/field (400X).

**Fig. 4.** Comparison between the tumoral areas of HCC-HCV and HCC-OH groups showing that the tumour infiltrating cells do not vary a lot. (Vertical axes represent the numbers of immunopositive cells/field (400X).

**Fig. 5 a,b.** Numbers of FoxP3<sup>+</sup> cells are correlated with CD4<sup>+</sup> and CD8<sup>+</sup> cells in the tumours of both HCC groups. **c, d, e.** The expression levels of FoxP3 are correlated with the expression levels of CD8 $\alpha$ , CD8 $\beta$  and IFN- $\gamma$  in the tumours of both HCC groups.

**Fig. 6.** Comparison between the cirrhotic areas outside the tumour in HCC-HCV and cirrhotic areas of Cirrhosis C group showing that CD3<sup>+</sup>, CD4<sup>+</sup> and CD20<sup>+</sup> cells are high in cirrhosis HCC-HCV while CD56<sup>+</sup> cells are high in Cirrhosis C group. (Vertical axes represent the numbers of immunopositive cells/field (400X).

**Fig. 7. a,b,c,d.** Metavir-activity score is correlated with the number of CD3<sup>+</sup>, CD4<sup>+</sup>, CD20<sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> cells in the parenchymatous nodules in HCC-HCV group.

**Fig. 8.** Many types of liver infiltrating cells such as CD3, CD4, CD8, CD20 and FoxP3 positive cells are significantly high in the cirrhotic tissues outside the tumours in tumour recurrent patients than tumour non-recurrent patients

Table 1. Demographic Data of Patients

	HCC-OH (N=12)	HCC-HCV (N=12)	Cirrhosis C (N=23)
<i>Gender (male)</i>	11	8	16
<i>Age (y)</i>	60.4±6.9*	64.1±11.9*	53.9±7.9
<i>Child-Pugh score</i>	A 10	11	22
<i>Alanine aminotransferase (ALT) (UI/ml)</i>	40.2±21.4	93.4±54.6	196.7±190.8
<i>Viral load (log-UI/ml)</i>		5.1±1.5	5.8±1.0
<i>Prothrombin Time (%)</i>	68.8±16.9	82.9±12.7	83.0±15.9
<i>Bilirubin (µm/l)</i>	54.4±69.7	16.9±7.7	17.4±9.9
<i>α-Fetoprotein (AFP)(µg/l)</i>	104.9±322.7	349.1±1094.9*	24.9±46.9
<i>Metavir-Activity score</i>	1 2 3	3 4 5	0 10 12
<i>Necrosis (%)</i>	26.25±27.2*	15.4±31.2	
<i>Tumour Capsule (present)</i>	7	7	
<i>Vascular invasion (present)</i>	6	3	
<i>Tumour count/patient</i>			
	Unifocal	9	11
	Multifocal	3	1
<i>T Size (mm)</i>	36.2±14.7	38.3±16.4	
<i>T Differentiation (WHO classification)</i>			
	Well	6	3
	Moderate	5	8
	Poor	1	1
<i>Ki67 labelling index (%)</i>	16.0±17.9	23.3±14.9	
<i>Recurrence (present)</i>	1	6*	

\* p &lt; 0.05

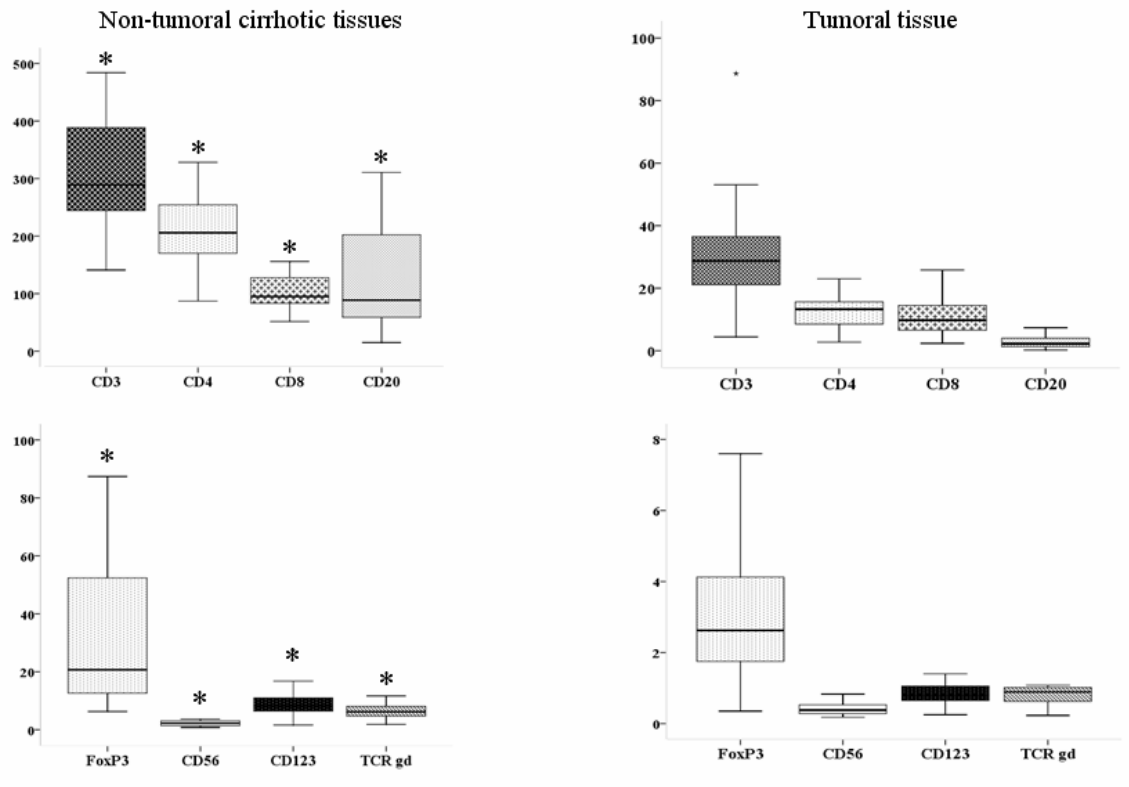
**Table 2. Primers and Universal probe Library (UPL) Sequences used in the LightCycler® 480 Real-Time Quantitative PCR System**

Gene		Primer Sequences	Product Length	UPL sequences
CD8 $\alpha$	F	<u>egcctgtgatatctacatctgg</u>	108 nt	<u>ttctcctg</u>
	R	<u>caaacacgtcttcggttc</u>		
CD8 $\beta$	F	<u>cagccagagaccagaa</u>	84 nt	<u>gcctcctg</u>
	R	<u>gaaaccagcagaaccaggac</u>		
FoxP3	F	<u>ctagccacgctcatccgctgg</u>	260 nt	<u>gctccaga</u>
	R	<u>cccagcaggtttccaacccta</u>		
IFN- $\gamma$	F	<u>ggcatttgaagaatggaaag</u>	112 nt	<u>cagagcca</u>
	R	<u>tttgatgctctggtcatctt</u>		
IL-6	F	<u>cacattcctggttctctga</u>	88 nt	<u>ctctcctg</u>
	R	<u>cagcttcacgctctcttga</u>		
IL-10	F	<u>tgggggagaacctgaagac</u>	62 nt	<u>ggctgagg</u>
	R	<u>acaaggaaataatgatgaca</u>		
TGF- $\beta$ 3	F	<u>aagaagcggccttggac</u>	60 nt	<u>ttctcctg</u>
	R	<u>cacacagcagttctcctcca</u>		
Perforine	F	<u>cttgctcactgcaacctc</u>	105 nt	<u>ttctcctg</u>
	R	<u>aaaattagctggcatgctg</u>		
RANTES	F	<u>ectcattgctactgcctct</u>	63 nt	<u>ctgcctcc</u>
	R	<u>ggtgtggtctccagggaata</u>		
HPRT	F	<u>gaccagtcacaagggacat</u>	95 nt	<u>tgctggag</u>
	R	<u>gtgtcaattatcttcacacaatcaag</u>		

F=Forward primer R=Reverse primer

**Table 3: Characteristics of antibodies used**

Antigen	Pre-treatment for antigen retrieval	Dilution	Clone	Source
<i>CD3</i>	Citrate buffer, pH 6, Water bath 100°C, 1h	1:100	polyclonal	<u>Dako, Glostrup, Denmark</u>
<i>CD4</i>	<u>Tris-citrate buffer, pH 9, Microwave oven 3x5 min</u>	1:75	4B12	<u>Novocastra, Newcastle, UK</u>
<i>CD8</i>	Citrate buffer, pH 6, Water bath 100°C, 1h	1:100	C8/144B	<u>Dako, Glostrup, Denmark</u>
<i>CD20</i>	Citrate buffer, pH 6, Water bath 100°C, 1h	01:50	L26	<u>Dako, Glostrup, Denmark</u>
<i>FoxP3</i>	<u>Tris-citrate buffer with 0.02% saponin, pH 9, Microwave oven 15 min</u>	01:20	<u>mAbcam 22510</u>	<u>Abcam, Cambridge, UK</u>
<i>Ki67</i>	<u>Tris-citrate buffer, pH 9.5 Water bath, 1h</u>	01:50	Ki S5	<u>Dako, Glostrup, Denmark</u>
<i>CD123</i>	Non	1:300	6H6	<u>Biolegend, San Diego, CA</u>
<i>CD56</i>	Non	1:200	1B6	<u>Novocastra, Fremont, CA</u>
<i>TCR<math>\gamma\delta</math></i>	Non	1:200	B1	<u>BD Pharmigen, Oxon, UK</u>



**Fig. 1.**

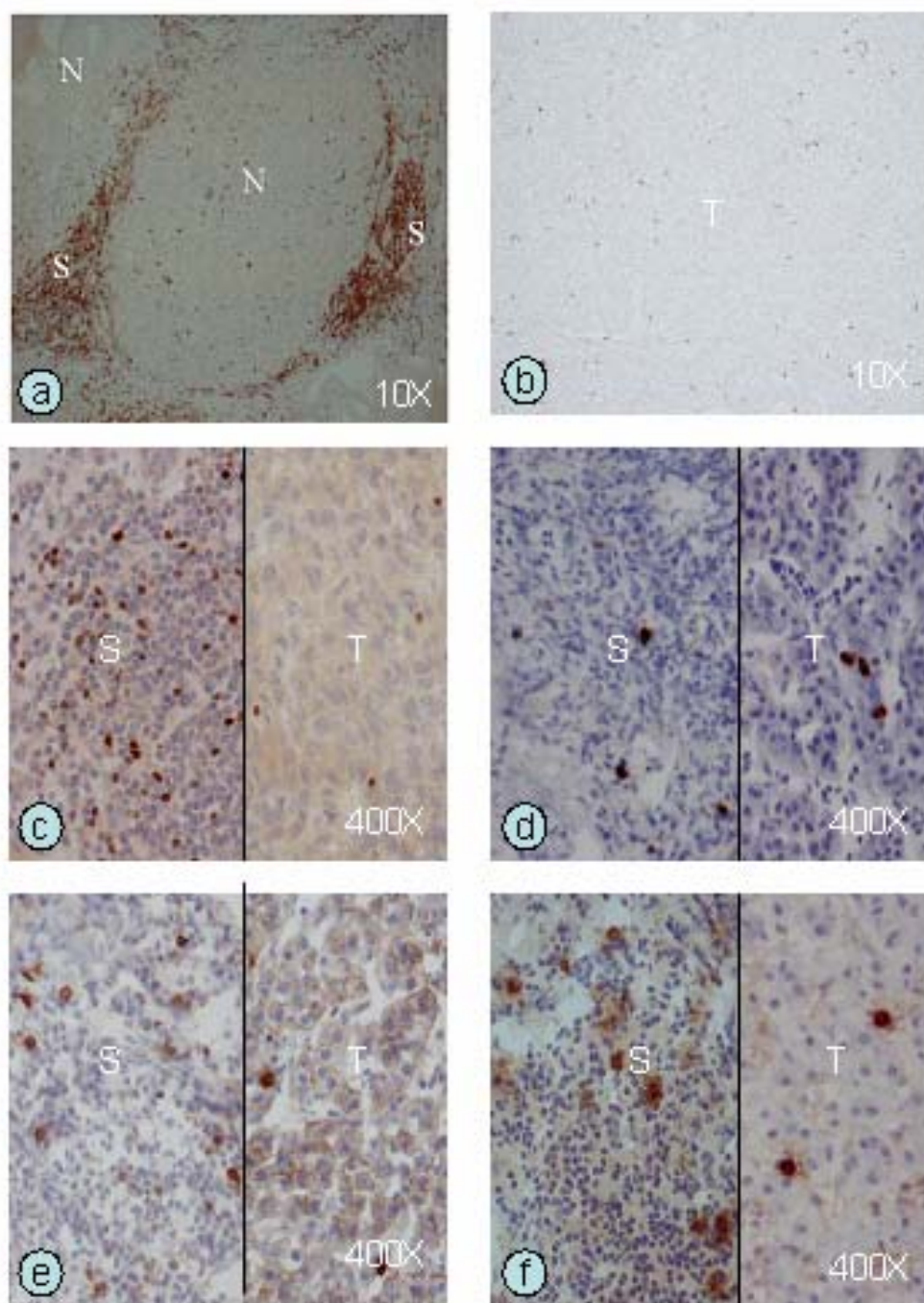


Fig. 2

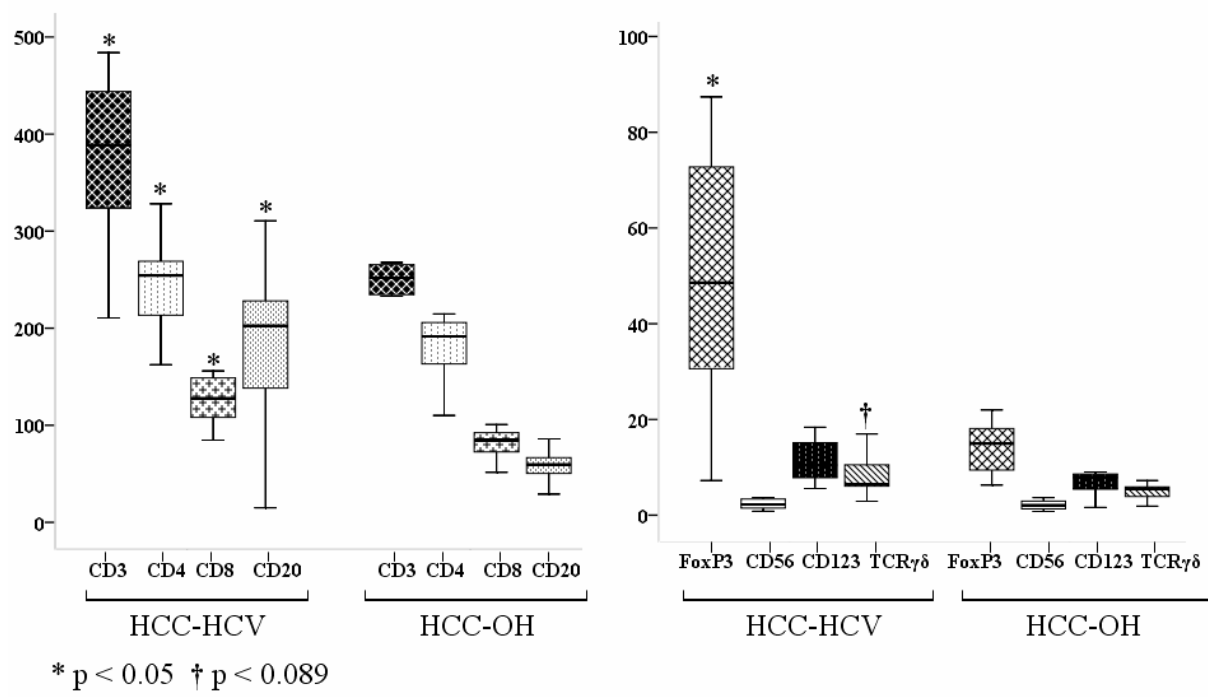


Fig. 3.

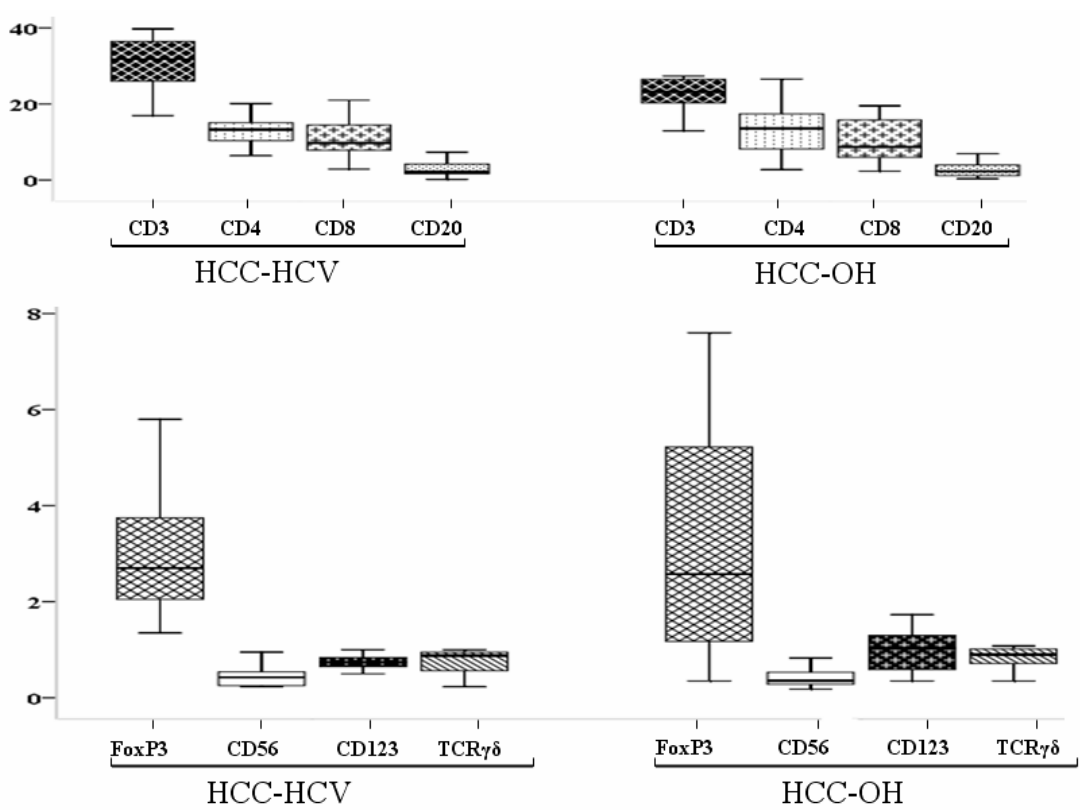
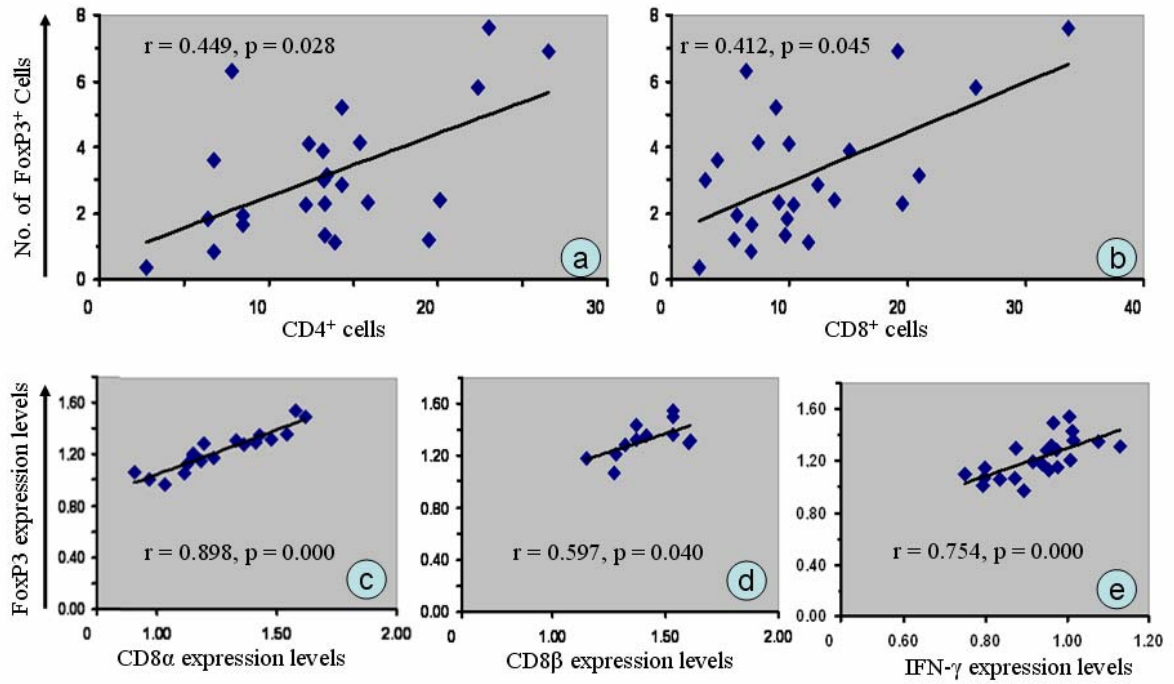
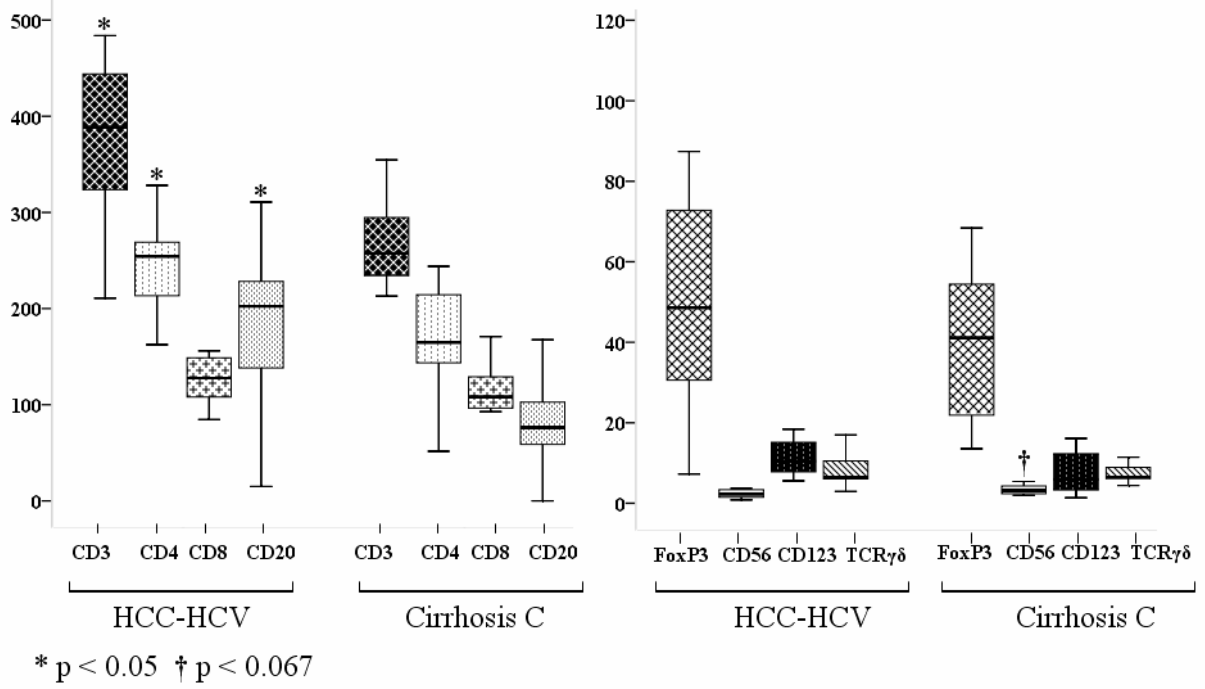


Fig. 4.



**Fig. 5 a.b.**



**Fig. 6.**



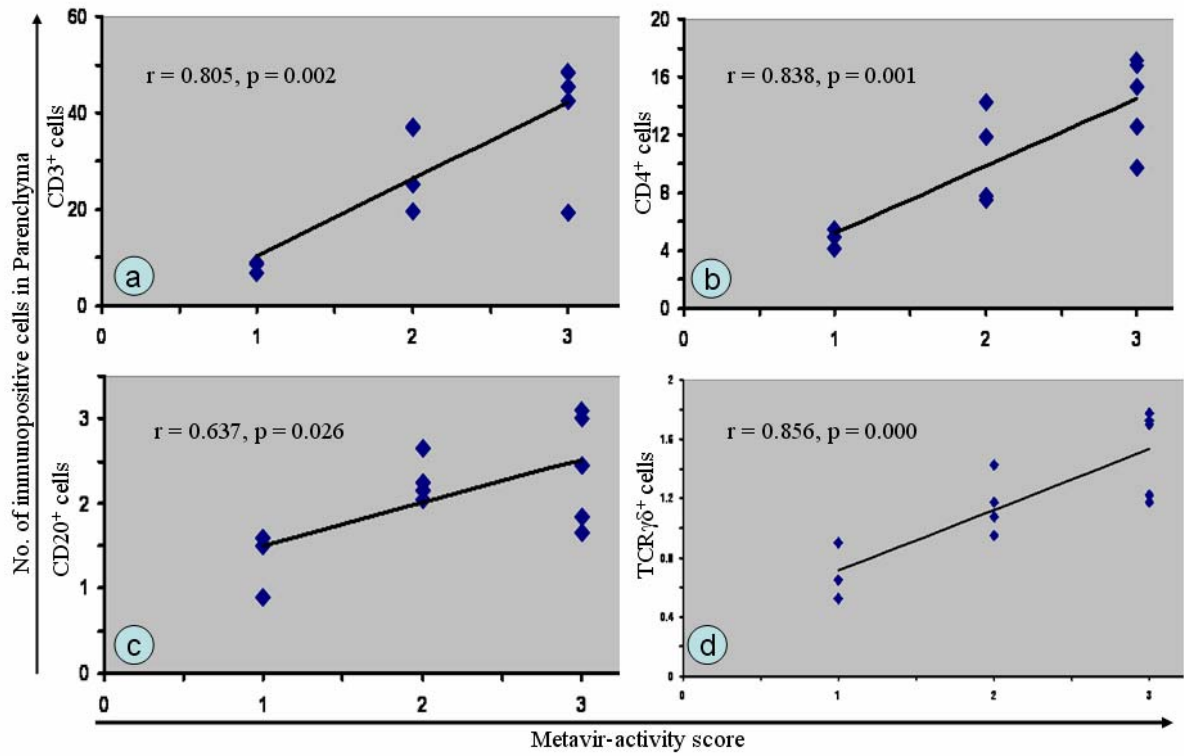


Fig. 7. a,b,c,d.

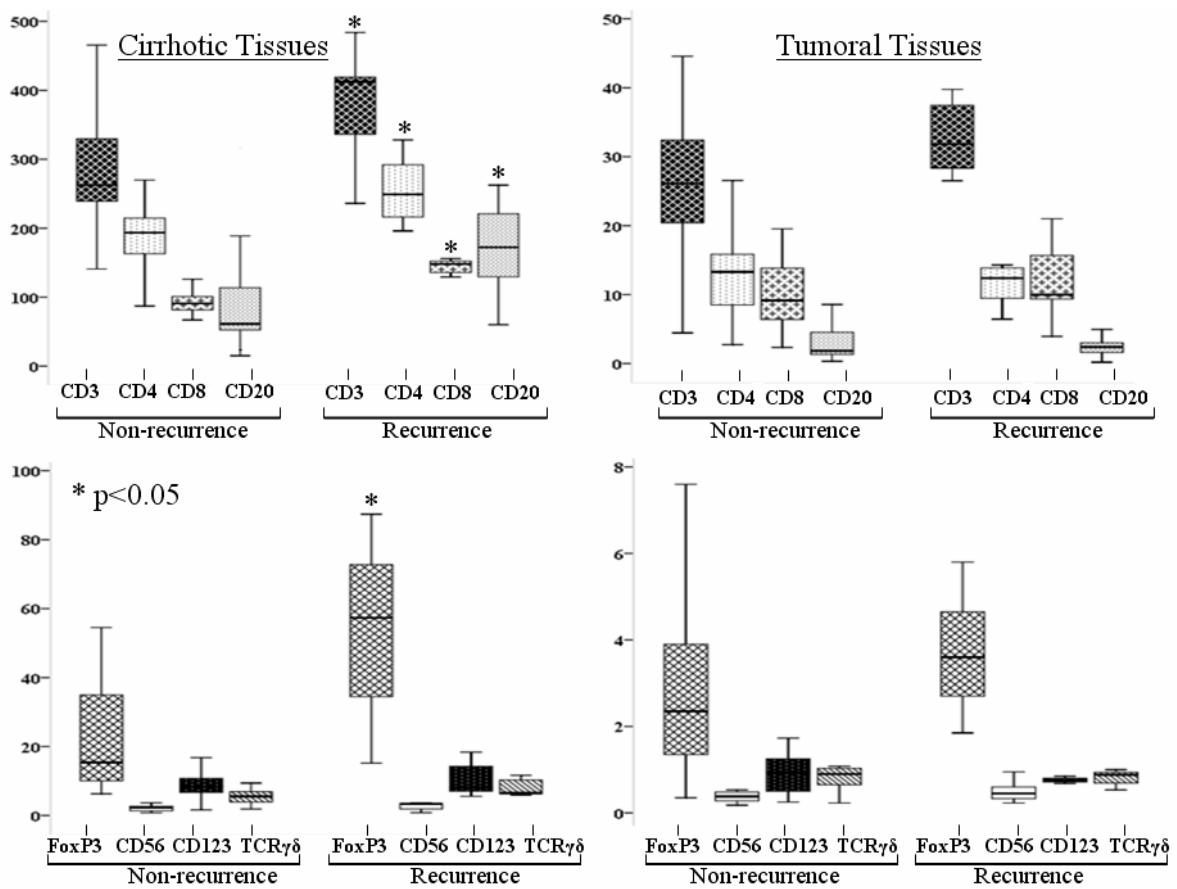


Fig. 8

## Article 2

### **Fine Characterization of Intrahepatic NK cells Expressing Natural Killer Receptors in Chronic HCV and HBV Infections**

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Submitted article

## **Article 2**

### **Fine Characterisation of Intrahepatic NK cells Expressing Natural Killer Receptors in Chronic HCV and HBV Infections**

Natural Killer cells (NK cells) are a type of cytolytic lymphocyte that constitutes a major component of the innate immune system. These cells play a major role in the rejection of tumors and cells infected by viruses. NK cells hold the potential to play a vital role in controlling HCV replication in hepatic cells (Li, Zhang et al. 2004). It is known that, both HCV and HBV impair human and murine NK cell activity. HCV envelope protein E2 can bind to molecules CD81 present on NK cells and can strongly inhibit the activation, proliferation, cytokines secretion and lytic capacities of these cells. Moreover, NS3 and NS4A also inhibits NK cells activation (Taylor, Shi et al. 1999). During chronic HCV infection, the inflamed liver microenvironment is characterized by an enhanced expression level of HLA-E on multiple cell types which is stabilized by its binding with HCV core peptide (Nattermann, Nischalke et al. 2005) while, NKG2A/CD94 inhibitory heterodimer recognizes non classical HLA-E molecules. Although the numbers of NK cells in chronic HCV infection are reduced significantly as compared to healthy individuals, several studies also suggest the reduction of their cytolytic capacity. Thus, along with reduction of NK cells density most of the factors also favor NK cells inhibition in the liver of chronic HCV infected patients.

For our part, we wished to examine the distribution of NK cells population as well as possible associations between the distribution of these cells and the clinical parameters among the chronic HCV and HBV patients. We observed a significant reduction in NK cell frequency and a quantitative imbalance of CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets within the total NK cells population in the liver and blood of chronic HCV patients. Moreover, NK cells expressing CD158a,h and CD158b,j cell markers were significantly lower among intrahepatic lymphocytes than peripheral blood cells in chronic HCV & HBV patients. In chronic HCV patients, intrahepatic CD56<sup>+</sup> cells tended to localize preferentially outside of the necrosis as the necroinflammatory activity increase and the circulating CD3<sup>-</sup>CD56<sup>bright</sup> NKG2A<sup>+</sup> cells correlated positively with the extend of necro-inflammation and fibrosis. Moreover, the frequency of circulating CD3<sup>-</sup>CD56<sup>dim</sup>NKG2A<sup>+</sup> cells correlated negatively with the viral load in chronic HCV and HBV patients. As a whole, this article provides the information regarding numbers of NK cells, their distribution and subsets and their correlation with viral loads in the patients with chronic viral hepatitis.

# **Fine Characterisation of Intrahepatic NK cells Expressing Natural Killer Receptors in Chronic HCV and HBV Infections**

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## Summary

The role of cells mediating the innate immune response in the liver of patients chronically infected by either HCV or HBV remains to be fully documented. Here, we analysed in chronically infected HCV or HBV patients the intrahepatic NK cells and their subsets expressing either NKG2A or CD158a,h<sup>+</sup> and CD158b,j<sup>+</sup> by flow cytometry and immunohistochemistry. A significant reduction in NK cells frequency and a quantitative imbalance between the CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets were observed in HCV<sup>+</sup> patients as compared to HBV<sup>+</sup> patients, underlining that the inflammatory environment is not the only cause of these phenomena. The proportions of NK cells expressing CD158a,h<sup>+</sup> and CD158b,j<sup>+</sup> were significantly lower among intrahepatic lymphocytes than to the peripheral blood cells in HCV<sup>+</sup> patients and to the liver of HBV<sup>+</sup> patients. The frequencies of NKG2A expressing NK cells were significantly low in the blood and liver of HBV<sup>+</sup> patients as compared to HCV<sup>+</sup> patients. In HCV<sup>+</sup> patients, i) intrahepatic CD56<sup>+</sup> cells tended to localize preferentially outside of the necrosis as the necroinflammatory activity increase; ii) the circulating CD3<sup>-</sup>CD56<sup>bright</sup> NKG2A<sup>+</sup> cells correlated positively with the extend of necro-inflammation and fibrosis, and the frequency of circulating CD3<sup>-</sup>CD56<sup>dim</sup>NKG2A<sup>+</sup> cells correlated negatively with the viral load. In HBV<sup>+</sup> patients, the intrahepatic CD3<sup>-</sup>CD56<sup>dim</sup> NKG2A<sup>+</sup> cells correlated negatively with the viral load. In conclusion, the host innate defence is critical in both infections, especially CD3<sup>-</sup>CD56<sup>dim</sup>NKG2A<sup>+</sup> and CD3<sup>-</sup>CD56<sup>bright</sup> NKG2A<sup>+</sup> cells which may be of great interest for disease monitoring.

## 1. Introduction:

Hepatitis C virus (HCV) and hepatitis B virus (HBV) infections are the most common causes of liver disease worldwide. They have distinct replication mechanisms and kinetics (10).

However, they share several aspects of pathogenesis, natural history, and adaptive immune responses. Both viruses are preferentially hepatotropic, not directly cytopathic, and capable of provoking acute and chronic necro-inflammatory liver injury (31).

The various strategies that many viruses have evolved to evade natural killer (NK) cell effector functions illustrate the central role for these cells in early host defence against viral infections (19, 27). It is known that, both HCV and HBV, impair human and murine NK cell activity (5, 34). HCV may inhibit the activation of Interferon Regulatory Factor (IRF) (8) and also interferes with the function of PKR kinase (33) which can activate NK cells and may play a role in the efficient production of type 1 Interferons (IFNs). In the case of HBV, both IRF and PKR individually appear to mediate signals that modulate viral replication in IRF<sup>(-/-)</sup> or PKR<sup>(-/-)</sup> transgenic mice (11). In contrast to other viruses, HCV and HBV induce a sustained HLA class I expression on infected cells (13, 37). The inhibitory effect of NK cells on HCV replication in cultured human hepatocytes (17) suggests that NK cells, in particular, might be able to contribute to the control of these infections. On the other hand, in the early phase of HBV infection, it has been demonstrated that the host's response to HBV mediates the activation of NK cells which produce IFN- $\gamma$  and are able to lyse infected cells by TRAIL-mediated death of hepatocytes (7).

Multiple studies were conducted regarding the functional potential of NK cells in the persistent HCV and HBV infections (2, 6, 18, 21, 25) but the evidence of an NK cell defect and their role in these chronic infections remains to debate. It is established that NK cell

frequencies in HCV infected patients are lower than that observed in the uninfected population (9, 21, 25). According to the surface expression level of CD56, NK cells can be divided into two subsets with important functional distinctions. The vast majority of NK cells displays a moderate level of CD56 (CD56<sup>dim</sup>) with a marked cytotoxicity potential, while, a small proportion harbors a high CD56 expression level (CD56<sup>bright</sup>) having a greater capacity to secrete cytokines (4, 14). Recently, it was reported that the frequency of peripheral blood cytolytic CD56<sup>dim</sup> NK cells is decreased in HCV infected patients (25). In our knowledge, less is known about examination of their population complexity, in particular, in term of subsets defined by HLA class I specific NK receptors (NKR) expression. It is noteworthy that NKRs are subdivided into two major families, a first one is composed of killer immunoglobulin-like receptors (KIR), such as CD158 molecules, LIR1/ILT2 (leukocyte Ig-like receptor 1/Ig-like transcript 2) which recognize classical HLA-A, HLA-B and HLA-C class I molecules and a second one includes C type lectin like molecules, such as, NKG2A that associates with CD94 to form inhibitory heterodimer recognizing non classical HLA-E molecules.

In the present study, we examined the expression of HLA class I reactive NKRs: the immunoglobulin-like KIRs and the C-type lectin NKG2A, in the blood and liver-infiltrating NK cells from patients with persistent HCV and HBV infection. We then searched for a possible relationship between CD56<sup>dim</sup>/CD56<sup>bright</sup> subset distribution and the clinical parameters in these infectious diseases. In addition, we performed detailed histological studies on liver biopsies from chronic HCV and HBV patients to evaluate quantitatively precise location of CD56<sup>+</sup> cells in the different histological areas.

## **2. Patients and Methods**

### ***2.1 Patients.***

Twenty eight patients (n=28) with chronic hepatitis C and nineteen (n=19) with chronic hepatitis B seen in the University Hospital, Grenoble, between 2005 and 2007 were included

in this study. Their main characteristics are shown in Table 1. Their biological and physioclinical parameters were analysed as previously described (1). For Immunohistochemical studies, HCV<sup>+</sup> patients (n=6) and HBV<sup>+</sup> patients (n=6) patients were selected for low necro-inflammatory grading (Metavir-A1, 3 patients) and high necro-inflammatory grading (Metavir-A3, 3 patients) in the French Metavir Scoring System. Blood samples from healthy volunteers (n=18) were obtained from the regional Etablissement Français du Sang (La Tronche, France).

### *2.2 Isolation of intra-hepatic cells.*

Liver biopsies obtained by using 1.5 mm diameter disposable biopsy needle were divided for histopathological examination (Metavir scoring system) and immunological analysis. Cells were experimented as described previously (16, 35).

### *2.3 Monoclonal antibodies, Immunostaining and Flow Cytometry.*

The monoclonal antibodies used for immunophenotyping were anti-CD3-R-Phycoerythrin (PE) Cy5 (UCHT1), and anti-CD56-PE (NCAM16.2), from Becton-Dickinson Biosciences (Le Pont de Claix, France). Anti-CD158a,h+-PE (EB6), anti-CD158b1,b2,j-PE (GL183), anti-NKG2A(CD159A)-PE (Z199) were obtained from Beckman Coulter France (Villepinte, France). Immunostaining and flow cytometric analysis were done as described previously (1, 28).

### *2.4 Immunohistochemical Staining.*

Immunohistochemical staining was performed with a mouse monoclonal anti-CD56/NCAM (Clone 1B6 Novacastra, Newcastle, United Kingdom) antibody. In brief, consecutive sections from formalin-fixed, paraffin embedded liver tissue blocks were cut at 4µm, deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was done by heating slides for one hour in Tris-citrate buffer (pH 9.15) at 98°C in water bath.



Endogenous peroxidase activity was blocked by 1% hydrogen peroxide for 5 min. Slides were then processed on an automated immunostainer (Nexes, Ventana, Tucson, AZ, USA), starting with an incubation with a primary anti-CD56 antibody (1:40) for 32 minutes, followed by an immunodetection with DAB (diaminobenzidine) I-view Kit (Ventana Medical Systems, Tucson, Arizona, USA), according to the manufacturer's recommendations. Then slides were counterstained with haematoxylin. CD56<sup>+</sup> cells were counted in 40 randomly selected fields per slide on a computer screen with the help of automatic software LUCIA (Laboratory Universal Computer Image Analysis, version 5.0, Laboratory Imaging, Prague, Czech) at high magnification (X400) (16). Several parameters were scored separately including total lymphocytes, CD56 positive cells, association of CD56<sup>+</sup> cells with piecemeal necrosis or lobular necrosis as well as their localization in the fibrotic tissues and in the nodular parenchyma. The numbers of cells were expressed as Unit defined as 40 fields (400X) equivalent of an area of 2.74 mm<sup>2</sup>.

### *2.5 Statistical methods.*

The non-parametric Wilcoxon and Mann-Whitney U-tests were performed for comparisons between the groups of patients and controls. Within group, comparisons were performed by using the 2-tailed Spearman's correlation coefficient. When multiple comparisons were done, the p value was corrected by multiplying it by the number of comparisons. *P* values less than 0.05 were considered significant. Statistical analysis was performed by using the SPSS 15.0 software (SPSS Inc., Chicago, IL).

## **3. Results**

### *3.1 Decreased frequency of blood and intra-hepatic NK cells in HCV infected patients.*

The flow cytometric gating strategy used to identify the different subsets of NK cells is depicted in **Fig1**. Phenotypic analysis of freshly isolated PBMCs from HCV-infected

patients demonstrated that the proportion of blood CD3<sup>-</sup>CD56<sup>+</sup> NK cells relative to whole lymphocyte populations was significantly decreased as compared to the blood samples from healthy donors (median value: 8.6 % vs 13.3 %,  $p < 0.05$ ) (**Fig.2A**). Furthermore, examination of the CD3<sup>-</sup>CD56<sup>+dim</sup> and CD3<sup>-</sup>CD56<sup>bright</sup> cells distribution among blood NK cells revealed that the frequency of CD3<sup>-</sup>CD56<sup>bright</sup> cells was significantly increased (median value: 10.0 % vs 6.0%,  $p < 0.05$ ) (**Fig.2B**) while the frequency of CD3<sup>-</sup>CD56<sup>+dim</sup> cells reduced significantly (median value: 90.0 % vs 94.0 %,  $p < 0.05$ )(**Fig.2C**) in HCV-infected patients as compared to controls. Our above mentioned results are similar to the findings described previously by other investigators (9, 21, 25). It is worth noting that no such difference between HBV infected patients and controls was found in the frequencies of CD3<sup>-</sup>CD56<sup>+</sup> NK cells (median value: 12.3% vs 13.3%), CD3<sup>-</sup>CD56<sup>bright</sup> cells (median value: 6.0% vs 6.0%), and CD3<sup>-</sup>CD56<sup>dim</sup> cells (median value: 94.0% vs 94.0%).

Similar analysis conducted on liver infiltrating lymphocytes recovered from the biopsies of HCV<sup>+</sup> patients revealed that the proportion of total CD3<sup>-</sup>CD56<sup>+</sup> NK cells appeared dramatically lower than that of their blood counterparts (median value: 3.0 % vs 8.6%,  $p < 0.005$ ) as shown in **Fig.2A**. Again, this feature was not found in HBV<sup>+</sup> patients where the proportion of intra-hepatic CD3<sup>-</sup>CD56<sup>+</sup> NK cells was similar to that of their blood counterparts (15.2% vs 12.3%, *NS*) and thus significantly higher than that observed in the liver from HCV<sup>+</sup> patients (median value: 15.2% vs 3.0%,  $p < 0.005$ ). A low number of intrahepatic CD3<sup>-</sup>CD56<sup>+</sup> cells excluded the possibility to analyze with confidence the proportion of CD3<sup>-</sup>CD56<sup>bright</sup> vs CD3<sup>-</sup>CD56<sup>dim</sup> cells in HCV<sup>+</sup> patients. Nevertheless, this analysis was conducted in the liver of HBV<sup>+</sup> patients and revealed that the proportion of CD3<sup>-</sup>CD56<sup>bright</sup> cells was significantly higher (median value: 6.0% vs 19.5%,  $p < 0.005$ ) than those observed in the periphery while the proportion of CD3<sup>-</sup>CD56<sup>dim</sup> cells was significantly lower

in the liver (median value: 94.0% vs 80.5%,  $p < 0.005$ ) as compared to the periphery (**Fig 2B,C**).

### *3.2 Intra-hepatic localization of CD56<sup>+</sup> cells in HCV and HBV infected patients.*

In order to further characterize the CD56<sup>+</sup> cells in the liver, immunohistochemistry was used to precisely quantify and localize them in different intra-hepatic areas, namely, nodular parenchymatous areas and necro-inflammatory lesions. For this analysis, we selected 6 chronic HCV<sup>+</sup> patients with A1 (n=3) and A3 (n=3) Metavir scores and 6 chronic HBV<sup>+</sup> patients strictly matched with the histological characteristics.

Histological slices were stained with an anti-CD56 antibody, revealing the presence of CD56<sup>+</sup> cells essentially in piecemeal and lobular necrosis as shown in **Fig 3 A, B, C, D** which depicts a representative example. The summary of the CD56<sup>+</sup> cell quantifications among all the 12 cases is presented in **Fig 3** where the cell density was evaluated by number of cells in a given unit of surface (equivalent of 2.74mm<sup>2</sup>) at high magnification (X400). The values of CD56<sup>+</sup> cells density were significantly lower in the liver of HCV<sup>+</sup> patients than in HBV<sup>+</sup> patients (69 vs 134 -  $p < 0.05$ ) (**Fig 3E**). Strikingly the density of CD56<sup>+</sup> cells was significantly higher in HCV<sup>+</sup> patients than in HBV<sup>+</sup> patients (on average 2122 vs 583 cells/U,  $p < 0.005$ ). So, the percentage of CD56<sup>+</sup> cells among the total infiltrating immune cells was significantly lower in HCV<sup>+</sup> patients than in HBV<sup>+</sup> patients (3.6% vs 18.4%,  $p < 0.005$ ).

Considering the localization of CD56<sup>+</sup> cells in the different histological areas, we observed that i) CD56<sup>+</sup> cells density was significantly lower in parenchymatous areas in HCV<sup>+</sup> patients than HBV<sup>+</sup> patients (28 vs 114,  $p < 0.005$ ) (**Fig 3F**); and ii) CD56<sup>+</sup> cells density was rare inside the necroinflammatory lesions (piecemeal necrosis and lobular necrosis) in both HCV<sup>+</sup> and HBV<sup>+</sup> patients. However, they were significantly higher in HCV<sup>+</sup> patients as compared to HBV<sup>+</sup> patients (9 vs 2,  $p < 0.005$ ) (**Fig 3G**).

### *3.3 Relationship between blood and intra-hepatic CD3 CD56<sup>+</sup> NK cells in necroinflammatory lesions*

In HCV<sup>+</sup> patients, we tried to search for a possible association among CD3<sup>+</sup>CD56<sup>+</sup> NK cells and biochemical parameters including necroinflammatory grade, ALT serum levels and HCV viral load, but no significant correlation was found considering blood NK cells. Moreover, the very low number of CD56<sup>+</sup> cells found in HCV<sup>+</sup> liver biopsies excluded the possibility to analyse these cells by flow cytometry. However, we took advantage of the enumeration of the CD56<sup>+</sup> cells in the different areas of the liver by immunohistochemistry to search for possible association between CD56<sup>+</sup> cells and progression of the disease (**Fig.3**). We observed, in both pathologies, that CD56<sup>+</sup> cells density remained constant whatever was the necro-inflammatory grading. In contrast, the CD56<sup>-</sup> cells density increased from A1 to A3 Metavir score, in both HCV<sup>+</sup> and HBV<sup>+</sup> patients (**Table 2**). Thus, the proportion of CD56<sup>+</sup> cells among total lymphocytes decreased from A1 to A3 Metavir in both HCV<sup>+</sup> and HBV<sup>+</sup> groups. The CD56<sup>+</sup> cells density inside the necro-inflammatory lesions was higher in Metavir-A1 than in Metavir-A3 patients in HCV<sup>+</sup> groups but such difference was not found in HBV<sup>+</sup> groups. The vast majority of CD56<sup>+</sup> cells were located outside the necro-inflammatory lesions in both in HCV<sup>+</sup> and in HBV<sup>+</sup> groups. Taken together, in the liver of HCV<sup>+</sup> patients, as the necro-inflammatory activity increased, CD56<sup>+</sup> cells were tended to localize preferentially outside of the necroinflammatory lesions.

#### *3.4 Analysis of HLA class I specific receptors expression by NK cells.*

As HLA class I specific inhibitory NK cell receptors can greatly modulate NK cell activity (15, 23, 24), we analyzed the surface expression of members of KIRs family of NKRs (CD158a/h and CD158b/j KIRs) and of NKG2A by NK cells from PBMCs and liver of HCV<sup>+</sup> and HBV<sup>+</sup> patients. The overall examination of blood lymphocytes for the above said markers did not reveal any distinction between HCV<sup>+</sup> patients and controls. However, we detected a significant decrease of blood NKG2A<sup>+</sup> NK cells in HBV<sup>+</sup> patients as compare to HCV<sup>+</sup>

patients (median value 54.5% vs 24.7%,  $p < 0.001$ ) and control (median value 55.4% vs 24.7%,  $p < 0.001$ ) as shown in **Fig 4A**.

As far as HLA class I specific NKRs expression by intra-hepatic NK cells is concerned, we noticed that the frequencies of both CD158a,h<sup>+</sup> and CD158b,j<sup>+</sup> NK cells were significantly higher among blood lymphocytes relative to the liver infiltrating lymphocytes in HCV<sup>+</sup> patients (mean values: 19.2% vs 7.6% -  $p < 0.001$ , 30.0% vs 15.0%  $p < 0.001$ , respectively) (**Fig 4B,C**), whereas NKG2A was expressed at a comparable frequency by liver and blood NK cells (58.0% vs 54.5%, *NS*) (**Fig 4A**). In contrast, in the liver of HBV<sup>+</sup> patients, the frequency of both CD158a,h<sup>+</sup> and CD158b,j<sup>+</sup> NK cells appeared similar to those observed in the blood (mean values: 26.3% vs 27.5% - *NS*, 30.9% vs 28.6% - *NS*, respectively) (**Fig 4B,C**).

In HCV<sup>+</sup> patients, the frequency of intrahepatic CD158a,h<sup>+</sup> and CD158b,j<sup>+</sup> NK cells appeared significantly lower than those observed in HBV<sup>+</sup> patients (7.6% vs 27.5%,  $p < 0.001$  and 15.0% vs 28.6% -  $p < 0.005$  respectively). Inversely, the frequency of intrahepatic NKG2A<sup>+</sup> NK cells was significantly higher in HCV<sup>+</sup> than in HBV<sup>+</sup> patients (58% vs 26.1% -  $p < 0.005$ ). Taken together, our findings revealed striking modifications in the tissue distribution of these NK cell subsets in the patients with persistent HCV or HBV infection. In particular, our data pointed out a reduction of the proportion of CD158a,h<sup>+</sup> and CD158b,j<sup>+</sup> liver infiltrating NK lymphocytes among HCV<sup>+</sup> patients at different stages of the viral infections even in the patients with low Metavir Activity score (data not shown) and an alteration of intra-hepatic NKG2A<sup>+</sup> NK cells in HBV<sup>+</sup> patients.

### *3.5 Analysis of the frequencies of CD3<sup>+</sup>CD56<sup>dim</sup> NK cells subsets with clinical parameters in HCV<sup>+</sup> and HBV<sup>+</sup> patients.*

Looking at CD3<sup>+</sup>CD56<sup>dim</sup> and CD3<sup>+</sup>CD56<sup>bright</sup> populations of blood NK cells according to HLA class I specific NK cell receptor expression, we found that the percentage of CD3<sup>+</sup>

CD56<sup>bright</sup> NK cells with NKG2A expression was positively associated with the severity of both necroinflammation ( $r = 0.545$ ,  $p < 0.01$ ) and fibrosis ( $r = 0.454$ ,  $p < 0.05$ ) in HCV<sup>+</sup> patients. Moreover, as shown in **Fig 5**, the number of blood CD3<sup>-</sup>CD56<sup>bright</sup> NKG2A<sup>+</sup> cells increased with the severity of the HCV disease. It is important to note that the correlations mentioned above between the subsets of NK cells and necroinflammation were not found in the case of HBV<sup>+</sup> patients.

Concerning HCV<sup>+</sup> patients, we observed a negative association ( $r = -0.540$ ,  $p < 0.01$ ) between the frequency of CD3<sup>-</sup>CD56<sup>+</sup> NKG2A<sup>+</sup> cells and the serum viral load (**Fig 6A**). This comparable negative correlation was also detectable in these patients when the analysis was restricted to the blood CD3<sup>-</sup>CD56<sup>dim</sup> subset ( $r = -0.441$ ,  $p < 0.05$  (**Fig 6B**)). In HBV<sup>+</sup> infected patients, we did not observe correlation between viral load and peripheral NK cells subsets. However, in the six HBV infected patients in whom viral load and intrahepatic flow cytometric data were available, a negative correlation between intra-hepatic CD3<sup>-</sup>CD56<sup>+</sup> or CD3<sup>-</sup>CD56<sup>dim</sup>NKG2A<sup>+</sup> and viral load ( $r = -0.900$ ,  $p < 0.05$ ) was found.

#### **4. Discussion**

In our study, we found that the pool of NK cells in the liver from HCV<sup>+</sup> patients was low at any stage of the disease as compared to HBV<sup>+</sup> patients. We observed differential localization of intra hepatic CD56<sup>+</sup> cells in the different histological areas in both viral infections. We demonstrated clearly that the liver infiltrating lymphocytes (CD56<sup>-</sup> cells) tend to increase from low necroinflammatory activity to high necroinflammatory activity but strikingly, we did not detect any increase in CD56<sup>+</sup> cells in both groups as the disease progress. Generally, we observed an increasing trend of CD56<sup>+</sup> cells with the progression of disease towards outside of the necroinflammatory lesions in HCV infection, whereas, they remain constant in HBV infection.

Ours data confirm a significant reduction in NK cell frequency and a quantitative imbalance of the CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets within the total NK cells population as reported by others (9, 21). It has also been reported in HCV infections and under proinflammatory conditions that CD3<sup>-</sup>CD56<sup>dim</sup> NK cells are more susceptible to apoptosis than CD3<sup>-</sup>CD56<sup>bright</sup> NK cells and thus contributed to an altered maintenance of CD3<sup>-</sup>CD56<sup>dim</sup> NK cells in the infectious diseases (32). Moreover, even in the healthy individuals, there are clearly more apoptotic activated CD56<sup>dim</sup> cells among the blood NK cells than their CD3<sup>-</sup>CD56<sup>bright</sup> counterparts (18) suggesting that a differential susceptibility to cell death is a natural feature of these two subsets. Taking into account, we did not observe either decrease of CD56<sup>+</sup> cells nor difference in CD3<sup>-</sup>CD56<sup>bright</sup>/CD3<sup>-</sup>CD56<sup>dim</sup> ratio in HBV<sup>+</sup> patients, this suggests that the proinflammatory environment is not sufficient to explain that altered CD3<sup>-</sup>CD56<sup>dim</sup>/CD3<sup>-</sup>CD56<sup>bright</sup> ratio in HCV<sup>+</sup> group. The clear difference in NK cell frequencies and the expression of differential CD3<sup>-</sup>CD56<sup>dim</sup>/CD3<sup>-</sup>CD56<sup>bright</sup> ratio in the two viral infections, strongly supports that HCV itself may be able to alter significantly the activation, proliferation and cytotoxicity of NK cells (5, 29, 34). Anti-fibrotic activity of NK cells is well established in mice (22, 30) and NK cell activity is negatively correlated with liver fibrosis in the patients with chronic hepatitis C infection (25). Therefore, the low number of CD56<sup>+</sup> cells and/or alteration in the ratio of CD3<sup>-</sup>CD56<sup>dim</sup>/CD3<sup>-</sup>CD56<sup>bright</sup> subsets expressing NKG2A in the liver of HCV<sup>+</sup> patients may favour the fibrosis.

NK cell activation may be impaired and/or prevented by cell surface inhibitory receptors expressed by NK cells (20). We did not observe any significant change in the frequencies of both CD158a,<sup>h</sup><sup>+</sup> and CD158b,<sup>j</sup><sup>+</sup> cells in the blood of HCV<sup>+</sup> and HBV<sup>+</sup> patients as depicted by others in the patients of chronic HCV and hepatocellular carcinoma (9, 36). Here we report a strong alteration in the frequencies of CD3<sup>-</sup>CD56<sup>+</sup>NK cells expressing CD158a,<sup>h</sup><sup>+</sup> or CD158b,<sup>j</sup><sup>+</sup> in the liver of HCV<sup>+</sup> patients but not in HBV<sup>+</sup> patients. To date, the

role of these subsets of NK cells in the liver has not been clearly determined, even in some cases where they represent a significant proportion of intrahepatic NK cells in the healthy donors (36). As the  $CD3^+CD56^+CD158a,h^+$  and  $CD158b,j^+$  are more expressed in  $CD3^+CD56^{dim}$  than in  $CD3^+CD56^{bright}$  (3, 9), the decrease of these subsets in the liver of HCV<sup>+</sup> patients, again underlines that HCV may interfere with the activation or differentiation of  $CD3^+CD56^{dim}$  cells subset in the liver which is generally more cytotoxic. In type 2 transmembrane proteins, no significant change in the frequencies of blood and liver infiltrating NKG2A<sup>+</sup> cells were detected in HCV<sup>+</sup> patients, while, they are significantly reduced in blood and liver compartments of HBV<sup>+</sup> patients. Taken together, our results show that both viruses interfere differentially on the NK cells pool: HCV targets type 1 transmembrane proteins whereas HBV targets type 2 transmembrane proteins. However, during persistent HCV infection, the inflamed liver microenvironment is characterized by an enhanced expression level of HLA-E on multiple cell types which is stabilized by its binding with HCV core<sub>35-44</sub> peptide (26), while, NKG2A/CD94 inhibitory heterodimer recognizes non classical HLA-E molecules. HCV E2 also has a capacity to negatively regulate the activation and cytotoxicity of NK cells (5, 29, 34), suggesting that HCV may also target indirectly type 2 transmembrane proteins.

Statistical analysis point out that only the blood  $CD3^+CD56^{bright}NKG2A^+$  cells are positively correlated with necroinflammatory activity score in HCV<sup>+</sup> patients, but such correlation is not found in HBV<sup>+</sup> patients. In spite of a marked decrease in the blood  $CD3^+CD56^{dim}$  subset in HCV<sup>+</sup> patients, the cytolytic killing of the  $CD3^+CD56^+$  cells are maintained in the peripheral compartment (18). Possibly, the positive association we found between the percentage of NKG2A-expressing  $CD3^+CD56^{bright}$  cells and the severity of necroinflammation Metavir scores could suggest that the proinflammatory context associated with enhanced fibrosis favours the preferential survival of NKG2A<sup>+</sup> cells among  $CD3^+CD56^{bright}$  NK cells in



HCV<sup>+</sup> patients. This is consistent with the fact that NKG2A expression correlates with CD94 and that high level of CD94 expression (a well established feature of CD3<sup>-</sup>CD56<sup>bright</sup> NK cells) is associated with an enhanced resistance of NK lymphocytes to cell death (12). One can argue that under the constant inhibition exerted by HCV and some cell-cell interaction (s) and/or local cytokine productions in the inflamed liver, a certain level of selective persistence/retention of particular intrahepatic NK cells subgroup such as CD3<sup>-</sup>CD56<sup>bright</sup> which could not differentiate beyond the CD3<sup>-</sup>CD56<sup>bright</sup> stage but might acquire a higher cytotoxic activity. In summary, as our analysis point out that high score of necroinflammatory activity is associated with high frequency of CD3<sup>-</sup>CD56<sup>bright</sup>NKG2A<sup>+</sup> cells in the peripheral blood of HCV<sup>+</sup> patients, we propose that this CD3<sup>-</sup>CD56<sup>bright</sup>NKG2A<sup>+</sup> cells subpopulation has a great interest in the histological evaluation in the persistent HCV infection.

Here we found a negative correlation between CD3<sup>-</sup>CD56<sup>+</sup>NKG2A<sup>+</sup> cells and viral load in HCV<sup>+</sup> and HBV<sup>+</sup> patients. To pin point this correlation, we observed that the viral load was correlated negatively with CD3<sup>-</sup>CD56<sup>dim</sup>NKG2A<sup>+</sup> cells subset in both infections, suggesting that antiviral property of NK cells might be restricted to NKG2A expressing CD3<sup>-</sup>CD56<sup>dim</sup> cells subset

In summary, our data pointed out a consistent low frequency of the liver CD56<sup>+</sup> cells, predominantly localised outside of necroinflammatory lesions in HCV infection, suggesting that the limited pool of intrahepatic CD56<sup>+</sup> cells is inefficient to clear the virus. Since it was well established that NK cells have an anti fibrotic activity, the reduced number of NK cells might favour fibrogenesis in HCV<sup>+</sup> patients. Unexpectedly, as high score of necro-inflammatory activity correlated with high frequency of CD3<sup>-</sup>CD56<sup>bright</sup>NKG2A<sup>+</sup> cells, this particular population might be a new marker for grading of the disease in HCV infection. Interestingly, CD3<sup>-</sup>CD56<sup>dim</sup>NKG2A<sup>+</sup> subset seems to be very important for virus control in both diseases and might be used as therapeutic target to control liver cirrhosis and

hepatocellular carcinoma in the future. In HBV infections, a differential CD3<sup>-</sup>CD56<sup>bright</sup>/CD3<sup>-</sup>CD56<sup>dim</sup> ratio and low frequency of CD3<sup>-</sup>CD56<sup>+</sup>NKG2A<sup>+</sup> cells in the liver have been observed which highlighted that both viruses may use some different strategy to target immune system.

## Figure and Table Legends:

**Table 1.** Demographic, biochemical and clinical characteristics of chronic HCV and HBV infected patients studied by flow cytometry

**For supplement Data: Table 1.** Demographic, biochemical and clinical characteristics of chronic HCV and HBV infected patients studied by Immunohistochemical staining.

**Table 2.** Expression of CD56<sup>+</sup> cells by immunohistochemistry among chronic HCV patients (n=6) and chronic HBV patients (n=6) having low necroinflammatory grading (Metavir-A1, 3 patients) and high necroinflammatory grading (Metavir-A3, 3 patients) in the French Metavir Scoring System.

### Figure 1. Expression of Natural Killer Receptors (NKR) by NK cells

A representative four-color flow cytometry analysis for peripheral blood lymphocytes is shown for a patient chronically infected by the Hepatitis C virus.

**A)** Dot plot representation of CD3 and CD56 double staining used to estimate the frequency of NK cells (boxed CD3-CD56<sup>+</sup>); **B)** Histogram of the CD56<sup>+</sup> cells defining the CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells (CD3-CD56<sup>+</sup>); **C)** CD158a,h; **D)** CD158b,j and **E)** NKG2A expressions respectively.

### Figure 2. Frequency of blood and liver NK cells in HCV<sup>+</sup> and HBV<sup>+</sup> patients

The frequency of **(A)** NK cells (CD3-CD56<sup>+</sup>) **(B)** CD56<sup>dim</sup> within CD3-CD56<sup>+</sup> cells **(C)** CD56<sup>bright</sup> within CD3-CD56<sup>+</sup> cells was evaluated by immunostaining and flow cytometry in the blood of healthy control (n=18) and patients with chronic Hepatitis C (HCV<sup>+</sup>)(n=28) and B (HBV<sup>+</sup>)(n=19); and in intrahepatic lymphocytes for HCV<sup>+</sup> (n=14) and HBV<sup>+</sup> (n=6) patients. Data is represented as boxplots showing medians (horizontal lines), upper and lower quartiles (boxes) and extreme values (whiskers).

### Figure 3. Immunohistochemical detection and quantification of CD56<sup>+</sup> cells in the chronic HCV and HBV patients.

**(A)** CD56<sup>+</sup> cells are present in the Fibrotic area (indicated by F and red arrows) without localization in the Piecemeal Necrosis (PN) (Original magnification X100). **(B)** CD56<sup>+</sup> cells are present in the Fibrotic area and Parenchyma (indicated by P and red arrows) with the localization in Piecemeal Necrosis (Original magnification X100). **(C)** Localization of CD56<sup>+</sup> cells in the Piecemeal Necrosis (Original magnification X400). **(D)** Localization of CD56<sup>+</sup> cells in the Lobular Necrosis (LN) (Original magnification X400). **(E)** Total CD56<sup>+</sup> cells in both groups of patients. **(F)** CD56<sup>+</sup> cells in Parenchyma in both groups of patients. **(G)** CD56<sup>+</sup> cells in necrosis (Piecemeal and Lobular necrosis) in both groups of patients. In (E), (F) and (G), all the values represent a density of CD56<sup>+</sup> cells/unit area = 2.74mm<sup>2</sup> and the horizontal bar represents median values.

### Figure 4. Frequency of NKR<sup>+</sup> cells within the total NK population (CD3 CD56<sup>+</sup>)

The frequency of **(A)** NKG2A<sup>+</sup>, **(B)** CD158a,h<sup>+</sup> and **(C)** CD158b,j<sup>+</sup> cells within the total NK cells population (CD3-CD56<sup>+</sup>) was analysed in blood lymphocytes for healthy control, HCV<sup>+</sup> and HBV<sup>+</sup> patients and in intrahepatic lymphocytes for HCV<sup>+</sup> and HBV<sup>+</sup> patients by the

immunostaining and flow cytometry.

**Figure 5. Correlation between CD3-CD56<sup>bright</sup>NKG2A<sup>+</sup> cells and Metavir Activity Score from PBMCs of HCV<sup>+</sup> Patients**

Wilcoxon statistical tests were performed between Metavir Activity Scores from A1(n=12) /A2(n=9) –  $z=-1.388$ - $p=0.169$  and A1(n=12) /A3(n=4) –  $z=-2.425$ - $p=0.013$  and A2(n=9) 26/A3(n=4) –  $z=-2.472$   $p=0.011$ ]. \* For number of patients displaying enough labelled cells to be included in the analysis.

**Fig 6A. Correlation between CD3-CD56<sup>+</sup>NKG2A<sup>+</sup> cells and viral load from PBMCs of HCV<sup>+</sup> Patients**

(Spearman statistical tests were performed between viral load from 26 patients - Rho de Spearman: - 0.540- $p=0.008$ ).

**Fig 6B. Correlation between CD3-CD56<sup>dim</sup>NKG2A<sup>+</sup> cells and Viral Load from PBMCs of HCV<sup>+</sup> Patients**

(Spearman statistical tests were performed between viral loads from 26 patients - Rho de Spearman: - 0.441- $p=0.035$ ).

**Additional Table 1. Demographic, biochemical and clinical characteristics of chronic HCV and HBV infected patients studied by Immunochemical staining**

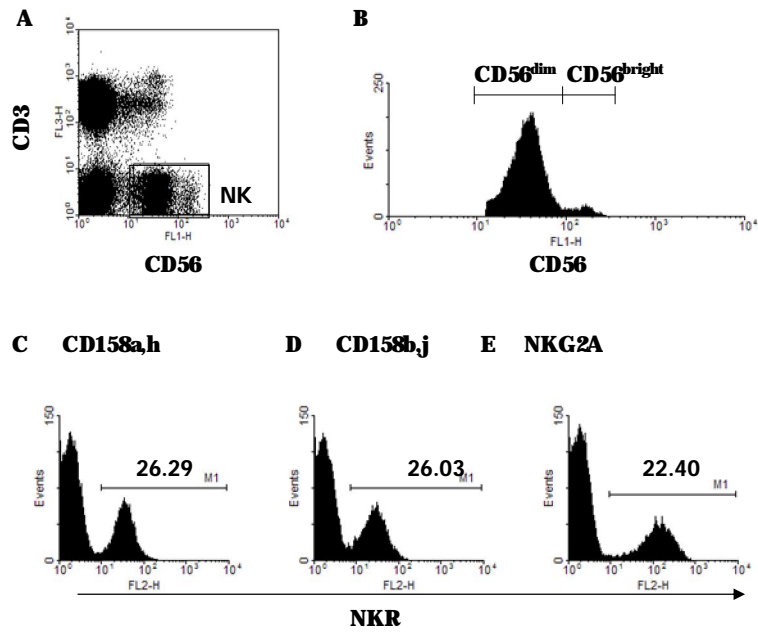
	HCV+ patients (n=6)	HBV+ patients (n=6)
<b>Demography</b>		
Male, n(%)	5 (83%)	3 (50%)
Age (y) *	56 (43-73)	41 (20-64)
Plasma Viral Load (logIU/mL)	4.2±1.7	5.3± 2.4
Serum ALT (U/L)	76.0± 47.4	63.7± 58.9
<b>Liver Histology**</b>		
Metavir Activity Score, n(%)	n=6	n=6
0	0	0
1	3 (50%)	3 (50%)
2	0	0
3	3 (50%)	3 (50%)
unknown	-	-
Metavir Fibrosis Score, n(%)	n=6	n=6
0	0	1 (17%)
1	0	3 (50%)
2	0	1 (17%)
3	1 (17%)	1 (17%)
4	5 (83%)	0
unknown	-	-

\* Median (Range)

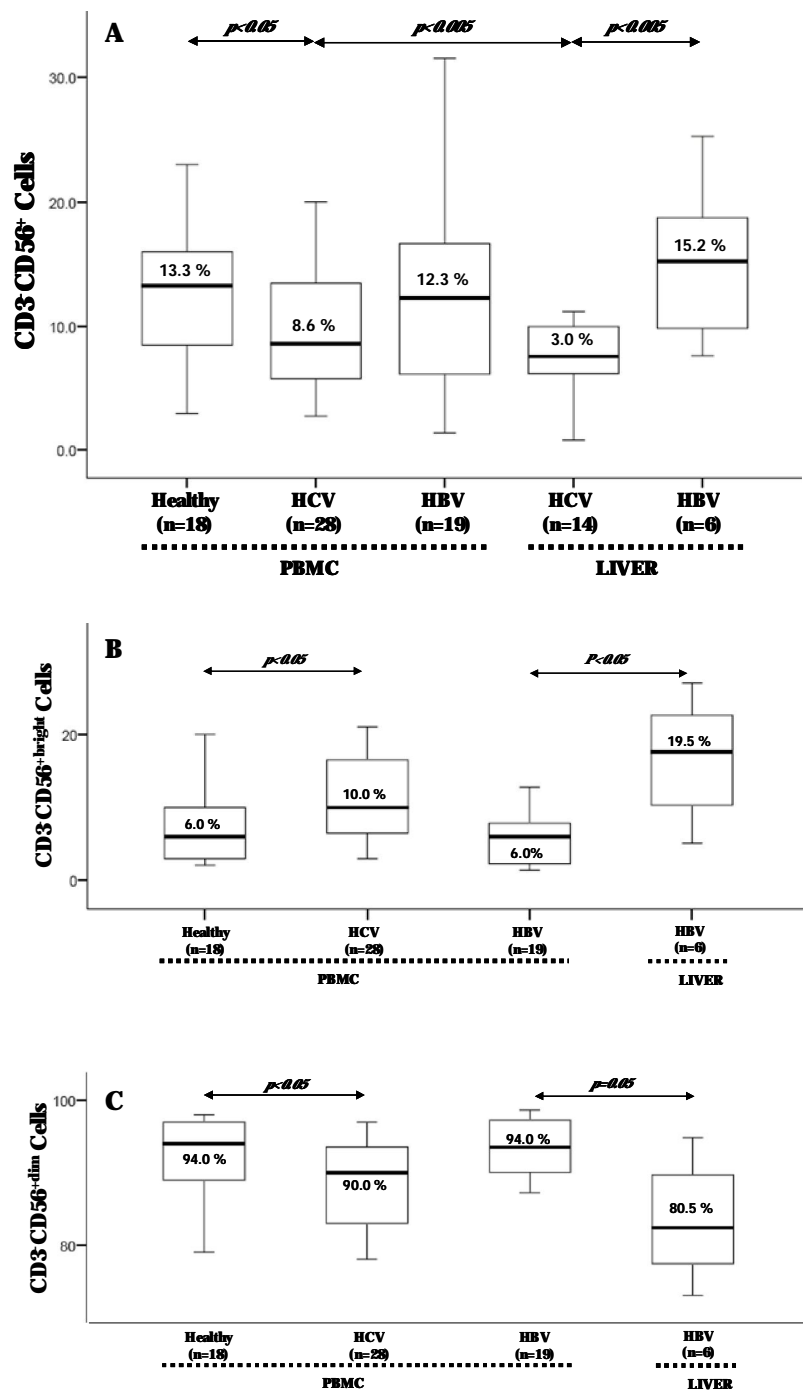
\*\* Number of cases / total (%)

\*\*\* Note that for technical reasons (N° of cells available), the entire set of experiments described could not be performed for every single patient

ALT : Alanine AminoTransferase

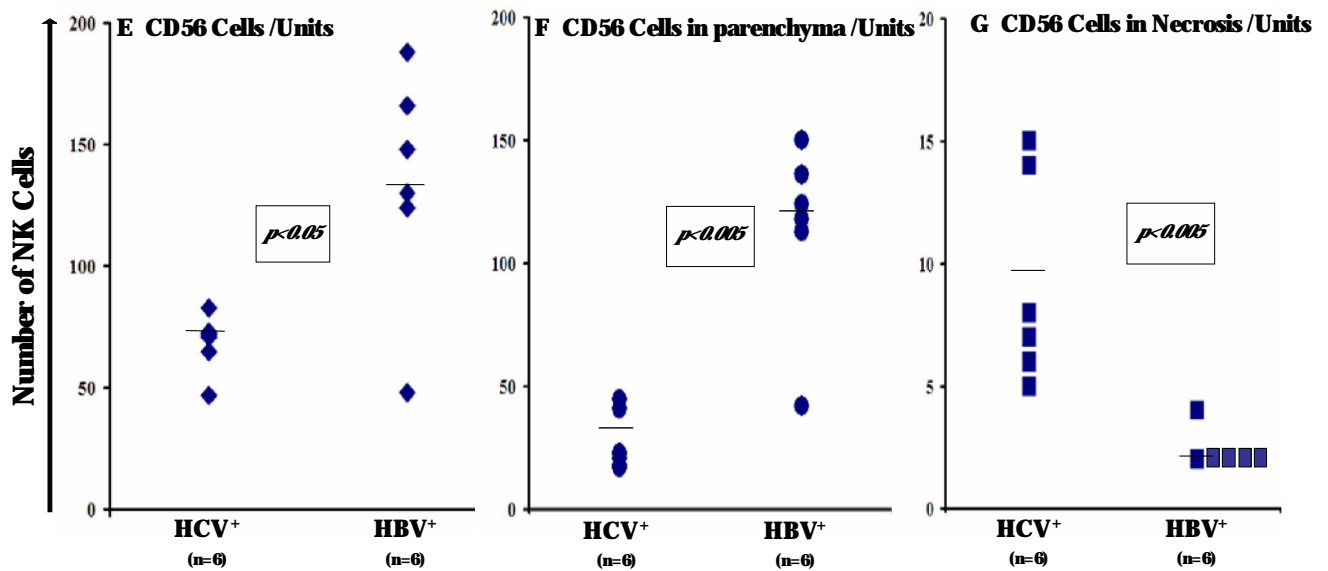
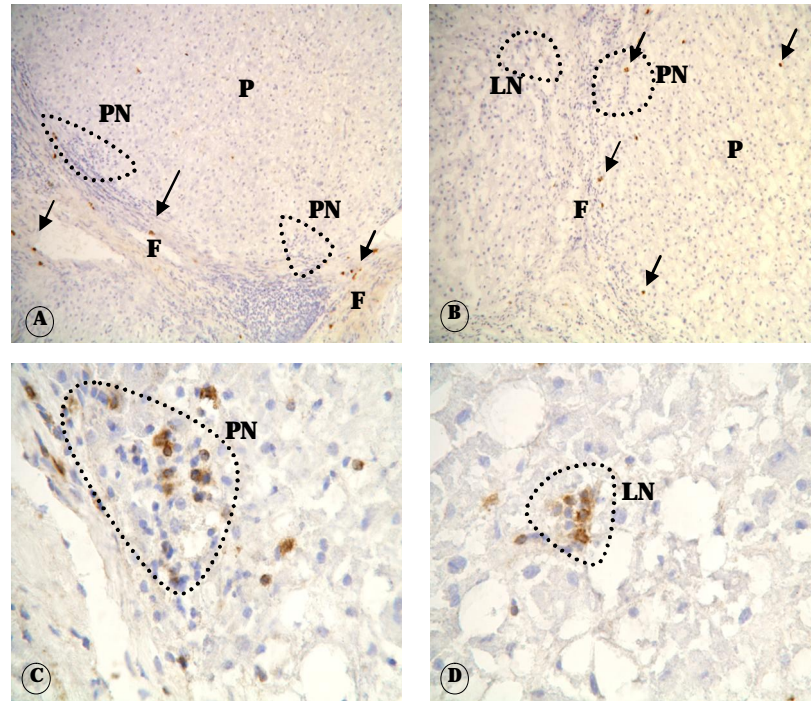


**Fig. 1.**



**Fig 2**

**Fig.3**

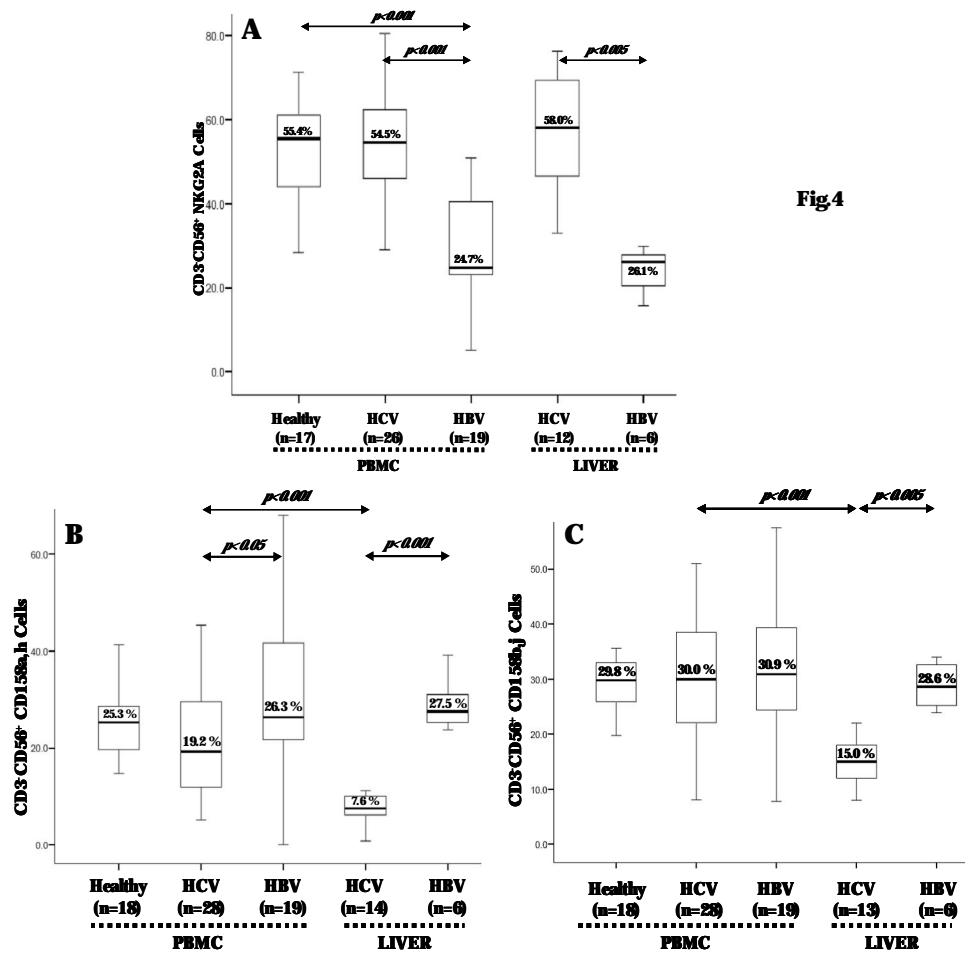


**Table 2 Expression of CD56+ cells by immunohistochemistry among cirrhosis C patients and cirrhosis B patients having low necro-inflammatory grading (Metavir-A1, 3 patients) and high necro-inflammatory grading (Metavir-A3, 3 patients) in the French Metavir Scoring System.**

	Cirrhosis C		Cirrhosis B	
	Metavir A1 n=3	Metavir A3 n=3	Metavir A1 n=3	Metavir A3 n=3
<b>CD56<sup>+</sup> Cells</b>	<b>68</b>	<b>69</b>	<b>134</b>	<b>134</b>
<b>CD56<sup>+</sup> Cells Lymphocytes</b>	<b>1501</b>	<b>2744</b>	<b>531</b>	<b>635</b>
<b>CD56<sup>+</sup> Cells/Lymphocytes (%)</b>	<b>4.6</b>	<b>2.6</b>	<b>20.3</b>	<b>16.4</b>
<b>CD56<sup>+</sup> Cells in Parenchyma</b>	<b>36</b>	<b>19</b>	<b>129</b>	<b>99</b>
<b>CD56<sup>+</sup> Cells outside Necrosis</b>	<b>55</b>	<b>63</b>	<b>132</b>	<b>131</b>
<b>CD56<sup>+</sup> Cells inside Necrosis</b>	<b>12</b>	<b>6</b>	<b>2</b>	<b>3</b>

All the parameters were expressed as / Unit - 1Unit = 40 fields (400X) = 2.74mm<sup>2</sup>





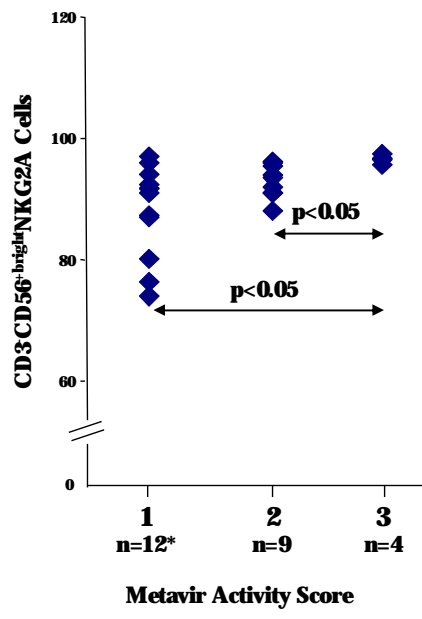
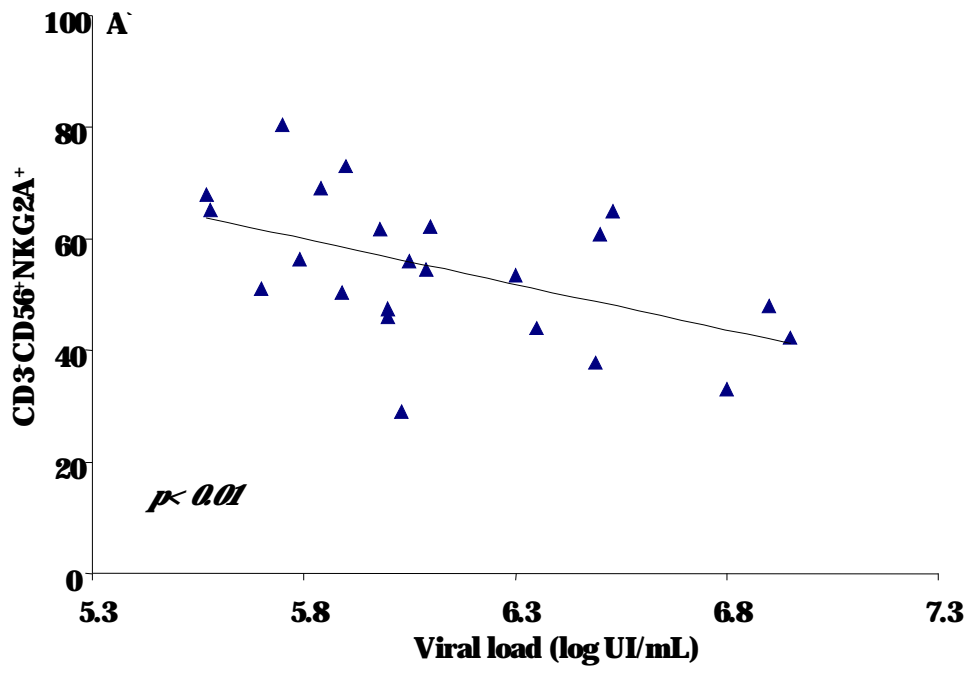
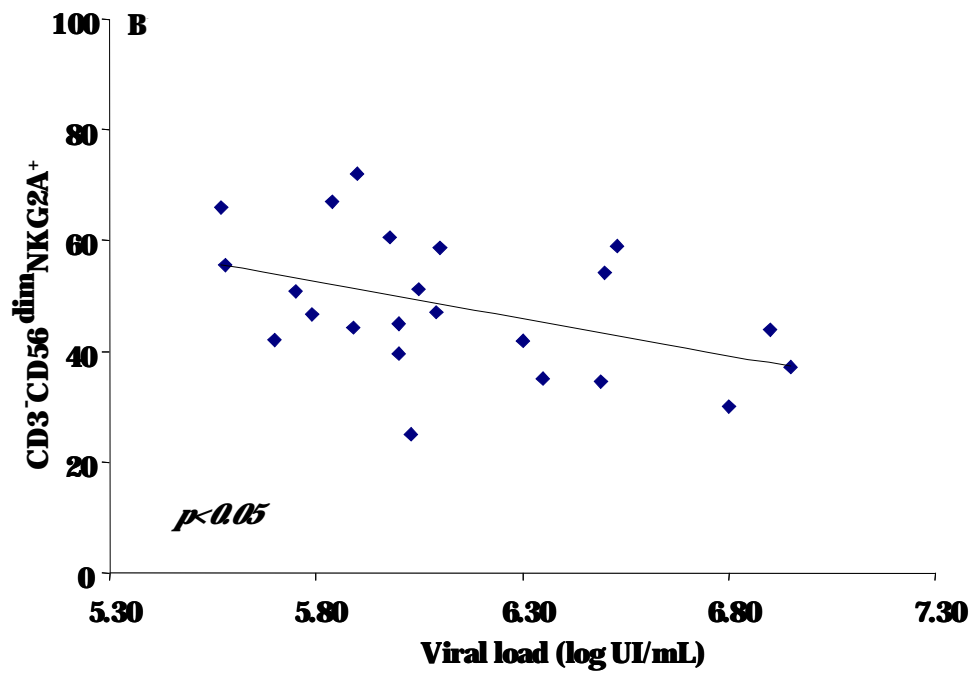


Fig 5



**Fig 6A**



**Fig 6B**

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### **Article 3**

#### **Critical Role of Intra-hepatic CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in HCV infected patients**

Nathalie Sturm, Marie-Ange Thélu, Xavier Camous, Guéorgui Dimitrov, Tania Dufeu-Duchesne, Muhammad Ramzan, Christiane Guillermet, Philippe Arvers, Paula Bonorino, Vincent Leroy, Patrice N. Marche and Evelyne Jouvin-Marche<sup>1,2</sup>.

Ready for submission

### Article 3

#### Critical Role of Intra-hepatic CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in HCV infected patients

Regulatory T cells (T<sub>reg</sub>) seem to be very important in the disease caused by hepatitis C virus (HCV) including HCC. In chronic HCV infection, more circulating T<sub>reg</sub> have been observed than uninfected people. Liver biopsies obtained from the liver of HCV infected patients show an inverse correlation between the number of T<sub>reg</sub> cells in the periphery and the histological inflammatory score (Cabrera, Tu et al. 2004). Depletion of T<sub>reg</sub> enhances antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells proliferation and response. Moreover, when the natural T<sub>reg</sub> cell 'pressure' is removed, the effector function of CD8<sup>+</sup> T cells is restored and persistent viral loads are reduced considerably (Dittmer, He et al. 2004), suggesting that T<sub>reg</sub> are likely to play a critical role in peripheral and intrahepatic immune regulation in chronic HCV disease. Peptide specific proliferation as well as INF- $\gamma$  production of HCV-specific CD8<sup>+</sup> T cells was inhibited by CD4<sup>+</sup>CD25<sup>+</sup> T cells required direct cell-cell contact for their suppressor functions, and was independent of IL-10 and TGF- $\beta$  (Cabrera, Tu et al. 2004; Boettler, Spangenberg et al. 2005), indicating that T<sub>reg</sub> may play a significant role in the dysfunction of HCV-specific T cell response and require cell-cell contact for their suppressor functioning. T<sub>reg</sub> activation is demonstrated by enhanced IL-10 or TGF- $\beta$  production which may play an important role in the suppressive function of T<sub>reg</sub>. In conclusion, T<sub>reg</sub> are the important immune cells population which inhibit the cytolytic functions of effector cells.

In this article we studied CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory cells in the liver and blood of chronically HCV infected patients. FoxP3<sup>+</sup> cells in the necroinflammatory lesions were always predominantly associated with CD8<sup>+</sup> T cells. We also found that FoxP3 expression and FoxP3<sup>+</sup> cell count both were correlated positively with CD8<sup>+</sup> cell count and CD8 expression in the livers of these patients, indicating the role of T<sub>reg</sub> in the suppression of CD8<sup>+</sup> cell in the necroinflammatory lesions. In addition, CD8<sup>+</sup> cell count was correlated positively with the Metavir-fibrosis and Metavir-activity score, while FoxP3<sup>+</sup> cell count in the necroinflammatory lesions was also correlated positively with Metavir-fibrosis score. Globally, this article provides some important insights regarding interactions between T<sub>reg</sub> and CD8<sup>+</sup> cells and their interaction with the pro and anti-inflammatory cytokines in the liver of chronic HCV infected patients.



## **Critical Role of liver CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in Chronic Hepatitis C.**

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**Running Title:** Liver Treg in chronic Hepatitis C

**Key words :** FoxP3, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>, regulatory T cell, HCV, HBV, Liver

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**Abbreviations:** Hepatitis C virus (HCV), regulatory T cells (T<sub>regs</sub>), Principal Component Analysis (PCA)

*Regulatory T cells in chronic Hepatitis C*

**ABSTRACT**

In chronic hepatitis C, specific T-cell responses are often dysfunctionnal. *In vitro* data point out that regulatory T cells ( $T_{\text{regs}}$ ) are able to suppress HCV-specific lymphocytes proliferation and cytokines secretion but their implication in this pathology is still debated. In order to document this point, we studied  $\text{FoxP3}^+$   $T_{\text{regs}}$  in the liver of patients with chronic hepatitis C. Flow cytometry data showed the presence of  $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$  T cells in liver compartment while intra-hepatic  $\text{CD8}^+\text{CD25}^+\text{FoxP3}^+$  T cells were scarce. Precise immunohistochemical localization showed that these cells are present in necro-inflammatory areas in close contact with  $\text{CD8}^+$  lymphocytes, suggesting that  $T_{\text{regs}}$  mediated inhibition of  $\text{CD8}^+$  T cell proliferation required direct cell-cell contact at the inflammatory site. The analysis of cytokines revealed a strong correlation between CD8, FoxP3 and IL-10, defining four distinct gene clusters: CD8/FoxP3, CD8/IL-10, TGF- $\beta$ /IL-10 and TNF- $\alpha$ /TGF- $\beta$ . No correlation was found between serum viral load and any immune markers. Interestingly, the ratio of  $\text{CD8}^+/\text{FoxP3}^+$  cells remained similar in both moderate and mild fibrosis (Metavir  $F \leq 2$ ) but differed significantly in severe fibrosis ( $F \geq 3$ ). In conclusion, our results support that a balance between  $\text{FoxP3}^+$ ,  $\text{CD8}^+$  cells and IL-10, establishing an equilibrium between the host and virus, which contributes to the viral persistence and to the prevention from immunopathological liver tissue damages.

## INTRODUCTION

In chronic Hepatitis C virus (HCV) infection, blood CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses have limited impact on disease course (1-3) but intra-hepatic T cells lymphocytes seem to control disease pathogenesis (2, 4-6). Therefore, regulation of intra-hepatic T cell response in HCV patients may have important implication not only in determining spontaneous clearance but also in disease progression during persistence infection (7).

It is well known that CD4<sup>+</sup>CD25<sup>+</sup> T cells are activated by microbial infections and this leads to an overall increase of anti-inflammatory cytokines. Generally, the increase in these cytokines induce the suppressive functions of CD4<sup>+</sup>CD25<sup>+</sup> natural regulatory T cells (T<sub>regs</sub>) (8-12) and could also promote the local survival or retention of natural T<sub>regs</sub> cells (13). Taken together, infections disturb balance between natural T<sub>regs</sub> cells and effector lymphocytes, thereby alter immune response and contribute to the progression of disease.

Several studies demonstrated that regulatory T cells defined as CD4<sup>+</sup>CD25<sup>+</sup> played a key role in the disease caused by hepatitis C virus by limiting the magnitude of effector responses (9, 14). It was shown that HCV patients had a higher number of T<sub>regs</sub> cells in peripheral blood than healthy people (15, 16). When the natural T<sub>regs</sub> cells pressure was removed, the effector function of CD8<sup>+</sup> T cells was restored and viral load was reduced considerably (17). Depletion of CD4<sup>+</sup>CD25<sup>+</sup> cells enhanced antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T proliferation and response (9, 16, 18).

In chronic hepatitis C, most of the available studies were focused on peripheral natural T<sub>regs</sub> that probably did not reflect the precise composition and functions of these cells in the liver. Furthermore, the intrahepatic T<sub>regs</sub> cells in HCV patients were identified by the expression of CD4 and CD25. Nowadays, T<sub>regs</sub> cells are further characterized by the expression of the transcriptional factor Forkhead box Protein 3 (FoxP3), allowing a better discrimination among the various lymphocyte populations (19, 20). Recently, much higher

percentages of CD4<sup>+</sup>CD25<sup>+</sup> and FoxP3<sup>+</sup> cells were observed in liver than in the blood of HCV-infected patients (14, 21, 22).

Studies in humans and mice pointed out that the different subsets of CD8<sup>+</sup> T cells possessed regulatory ability (23-25). During *in vitro* stimulation, MHC class-I restricted FoxP3<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup> T cells were identified both with non-specific antigen and with virus-specific peptides (6, 26, 27). Nevertheless, the frequency and biological roles of CD8<sup>+</sup>FoxP3<sup>+</sup>T cells in humans remain less understood than their counterpart CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>T cells. Concerning HCV patients, *in vitro* stimulation of human PBMC with HCV peptides revealed the presence of both virus-specific CD8<sup>+</sup>FoxP3<sup>-</sup> and CD8<sup>+</sup>FoxP3<sup>+</sup> T cells. CD8<sup>+</sup>FoxP3<sup>+</sup>T cells, expressing phenotypical markers of T<sub>regs</sub>, displayed a proliferation capacity and a cell-cell contact dependent suppressive activity (23).

Comparison of CD4<sup>+</sup>CD25<sup>+</sup> FoxP3<sup>+</sup> T<sub>regs</sub> between infected and uninfected HCV patients showed that they were phenotypically, functionally and genetically indistinguishable (14, 21). Among HCV infected patients, HCV-specific FoxP3<sup>+</sup> T<sub>regs</sub> were detected in the blood and had the capacity to suppress HCV-specific T cells (21).

In order to examine the implication of intrahepatic T<sub>regs</sub> during different grades/stages of chronic hepatitis C disease, we studied first, the frequency of these cells by flow cytometry, second, the precise histological localization of these cells by immunohistochemistry on liver tissue sections and third, the expression levels of cytokines in liver biopsies by RT-PCR, and compared clinical, biochemical and histological parameters. This allows defining precise phenotype of T<sub>regs</sub> cells and addressing their role, specifically in regard to CD8<sup>+</sup> T cell functions in chronic hepatitis C.

## RESULTS

### Detection and quantification of intrahepatic CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells

The presence and the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T lymphocytes, especially CD8<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>T lymphocytes, in the human liver with infectious diseases are not fully documented until now. In order to document this point, flow cytometric analysis was carried out from fresh liver biopsies and blood samples of HCV infected patients. The flow cytometry gating strategy used to characterize the different subsets of CD4<sup>+</sup> and CD8<sup>+</sup> cells are depicted in **Fig1A**. This permits to determine the frequencies of cells expressing CD4, FoxP3 or CD25 and CD8, FoxP3 or CD25 and the frequencies of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells or CD8<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells. This was achieved on 6 liver biopsies with a significant number of lymphocytes to conduct this analysis.

As expected (5), in the liver of the 6 HCV infected patients, the frequency of the CD8<sup>+</sup> T cells (32.5% ± 5.5%) was higher than the frequency of CD4<sup>+</sup> lymphocytes (17.4% ± 8.4%) (p<0.05). The frequency of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> lymphocytes observed in the liver was 5.9% ± 1.1% and 3.4% ± 1.8% respectively (**Fig.1B**). In the blood of the 8 HCV infected patients, the frequency of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells was 8.4% ± 6.1% and 2.8% ± 1.6% respectively (**Fig.1B**). Taken together, these results underline that, among CD4<sup>+</sup>CD25<sup>+</sup> cells of HCV infected patients, only about 50% express CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in the liver (54.5 ± 22.7%) or in blood (45.9 ± 21.8%) (**Table I**). It means that CD4<sup>+</sup>CD25<sup>+</sup> cells represent a larger cell population than that phenotyped as CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub>.

Concerning CD8<sup>+</sup> subset, less than 0.1% of CD8<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells (0.03% ± 0.01%) were observed in the blood of the 8 patients and no cell expressing the 3 markers i.e. CD8<sup>+</sup>, CD25<sup>+</sup> and FoxP3<sup>+</sup> was detected among the liver infiltrating lymphocytes.

In conclusion, in the liver of HCV patients, the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells represent around 50% of CD4<sup>+</sup>CD25<sup>+</sup> cells, these later cells corresponding to 6% of CD4<sup>+</sup> cells. As CD4<sup>+</sup> cells represent less than 20% of the lymphocytes (actually an average of 17.4%), CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells stand for less than 0.6% of the lymphocyte pool. (**Table I**).

#### **Immunohistochemical localization of intra-hepatic FoxP3<sup>+</sup> cells.**

To determine precisely where are localized the FoxP3 regulatory T cells, we completed our study by an immunohistochemical analysis in a group of 20 patients with chronic hepatitis C. The characteristics of these patients are presented in **Table (II)**. The procedure adopted for cell visualization and counting is detailed in material and methods and allowed to observe CD4, CD8 and FoxP3 single positive cells and CD4-FoxP3 and CD8-FoxP3 double positive cells.

Most FoxP3<sup>+</sup> cells were CD4<sup>+</sup> lymphocytes, showing a double immunostaining with a characteristic blue nuclear staining with anti-FoxP3 antibody, circled by a brown cytoplasmic membrane staining with anti-CD4 antibody (**Fig 2A,B**). However, few FoxP3<sup>+</sup> lymphocytes did not stain with anti-CD4 antibody and remained single FoxP3<sup>+</sup> lymphocytes (**Fig 2B**). In addition, double stainings with anti-CD8 and anti-FoxP3 antibodies confirmed that almost all FoxP3<sup>+</sup> cells were not CD8<sup>+</sup> lymphocytes, since FoxP3<sup>+</sup> cells with brown nuclear staining were not circled by blue cytoplasmic membrane staining with anti-CD8 antibody (**Fig 2C-F**). We saw only one or two CD8<sup>+</sup>/FoxP3<sup>+</sup> cells in all the liver tissue sections of the 20 analyzed patients (**Fig 2G,H**) and this corroborated our results obtained by flow cytometry analysis, suggesting that CD8<sup>+</sup>FoxP3<sup>+</sup> cells are a very rare in the liver and that most of FoxP3<sup>+</sup> cells co-expressed CD4. A lot of FoxP3<sup>+</sup> cells were observed in portal tracts and fibrous septa (**Fig 2C**), particularly when lymphoid aggregates were present (**Fig 2D**). They were also observed in parenchymatous areas where they preferentially localized in necrotic areas (piecemeal necrosis as well as lobular necrosis) in close contact with CD8<sup>+</sup> lymphocytes (**Fig 2E,F**),

suggesting contact dependent inhibition of the effector functions of CD8<sup>+</sup> cells by FoxP3<sup>+</sup> cells.

### **Relationship between intra-hepatic FoxP3<sup>+</sup> cells and CD8<sup>+</sup> cells in different histological areas.**

A mean 50 fields (20 to 105) were analyzed per liver biopsy at the magnification of 20X. CD8-FoxP3 double stainings allowed to evaluate CD4<sup>+</sup>FoxP3<sup>+</sup>/CD8<sup>+</sup> cell ratio and visualized localization of FoxP3 cells in different histological areas, particularly in fibrous areas (fibrous septa and portal tracts), in hepatic parenchyma and in the necro-inflammatory lesions (both piecemeal and lobular necroses). Statistical analysis showed a significant positive correlation between the total number of FoxP3<sup>+</sup> cells and the number of CD8<sup>+</sup> cells in fibrotic and parenchymatous areas ( $r= 0.505$ - $p=0.023$  and  $r= 0.555$ - $p=0.011$  respectively) (**Fig. 3 A,B**). These findings were supported by the quantitative RT-PCR data described in the next section. Moreover, the total number of FoxP3<sup>+</sup> cells correlated positively with the number of FoxP3<sup>+</sup> cells in the fibrotic tissue ( $r=0.923$ - $p<0.001$ ) (**Fig. 3 C**), in the parenchymatous areas ( $r=0.777$ - $p<0.001$ ) (**Fig. 3 D**) and in the necro-inflammatory lesions ( $r=0.833$ - $p<0.001$ ) (**Fig. 3 E**), illustrating an overall increase of FoxP3<sup>+</sup> cells in all hepatic areas. Interestingly, there was a strong correlation between CD8<sup>+</sup> and FoxP3<sup>+</sup> cells in parenchymatous areas ( $r=0.511$ - $p=0.021$ ), especially inside the necro-inflammatory lesions ( $r=0.695$ - $p=0.001$ ). It means that FoxP3<sup>+</sup> cells were mostly associated with CD8<sup>+</sup> cells. We found 22% of FoxP3<sup>+</sup> cells present in the necro-inflammatory foci were in contact with CD8<sup>+</sup> cells or apoptotic bodies, suggesting a crucial role of FoxP3<sup>+</sup> in these necro-inflammatory areas.

### **Detection and quantification of intrahepatic CD4, CD8, FoxP3 and IL-10 by quantitative RT-PCR.**

As mentioned above, the low number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub> did not permit to measure cytokine productions by flow cytometry or ELISA analysis. To overcome this difficulty, we performed an analysis of CD4, CD8, FoxP3, IL-10, TGFβ, IFNγ, and TNFα transcripts by quantitative RT-PCR. For this, we extended our analysis up to 47 patients. All the results were expressed as ratio of the number of target amplicons/number of HPRT amplicons. A significant positive correlation was observed among the expression levels of different cellular immune markers and cytokines. Principal Component Analysis (PCA) allowed to determine the presence of gene clusters and only the most relevant correlations are depicted in **Fig.4**. Briefly, positive correlations were observed between CD8 and FoxP3 ( $r=0.564-p=0.001$ ) (**Fig.4 A**), CD8 with IL-10 ( $r=0.405-p=0.022$ ) (**Fig.4 B**), TNFα with TGFβ ( $r=0.652-p=0.005$ ) (**Fig.4 C**) and TGFβ with IL-10 ( $r=0.688-p<0.001$ ) (**Fig.4 D**). Then, univariate and multivariate regression analyses were conducted for the correlated genes. Regression model [ $CD8 = 0.88 \cdot IL-10 - 0.01 \cdot FoxP3 + 0.04 \cdot IFN\gamma$  ( $r=0.810 - p<10^{-6}$ )] revealed that i) when the expression levels of IL-10 increased by 1 unit, the expression levels of CD8 increased by 0.88 units keeping the expression levels of FoxP3 and IFNγ as constants; ii) when the expression levels of FoxP3 increased by 1 unit, the expression levels of CD8 decreased by 0.01 units, keeping the expression levels of IL-10 and IFNγ as constants. In conclusion, regression analysis reveals that the expression levels of FoxP3 and IL-10 increase, accordingly to the expression levels of CD8, underlining that these 3 parameters are somehow linked together. Moreover, pro-inflammatory (TNF-α and IFN-γ) and anti-inflammatory (TGF-β and IL-10) cytokines are closely correlated.



### **Relationship between histological parameters and immune markers.**

We then analyzed the relationship between grades and stage according to the Metavir classification and immune markers. We found significant positive correlation between fibrosis stage and FoxP3<sup>+</sup> or CD8<sup>+</sup> cells in different histological areas of the liver. Most of significant correlations were between i) Metavir-F and FoxP3<sup>+</sup> cells in the necro-inflammatory lesions ( $r=0.469$ - $p=0.037$ ), and ii) Metavir-F and FoxP3<sup>+</sup> cells in the piecemeal necrosis, in contact with CD8<sup>+</sup> cells ( $r=0.510$ - $p=0.022$ ). Considering CD8, Metavir fibrosis stage was correlated, with the total number of CD8<sup>+</sup> cells ( $r=0.714$ - $p<0.001$ ), with CD8 cells in fibrotic areas ( $r=0.693$ - $p=0.001$ ), and finally with CD8 transcripts ( $r=0.481$ - $p=0.005$ ). As previously observed (5), Metavir activity grade was positively correlated with the expression levels of CD8 ( $r=0.547$ - $p=0.001$ ) and IL-10 ( $r=0.318$ - $p=0.040$ ).

Analysis of the relationship between the frequency of FoxP3<sup>+</sup> cells and the gravity of the necro-inflammatory score revealed changes in term of number of FoxP3<sup>+</sup> cells (**Fig. 5**). The numbers of FoxP3<sup>+</sup> cells were two fold higher in patients with Metavir  $\leq$  A2 (median range of number of FoxP3<sup>+</sup> cells: 2 vs 4), whereas they were slightly decreased in patients with Metavir  $\geq$  A2 (median range of number of FoxP3<sup>+</sup> cells: 4 vs 2) (**Fig. 5 A**). We confirm, as already reported (5, 28), that CD8<sup>+</sup> cells increase with the progression of inflammation (A1 to A2, median range: 67 vs 111 and A2 to A3: 111 vs 123) (**Fig.5 B**). On the other hand, we found a similar profile according to the Metavir stage with an increase of FoxP3<sup>+</sup> cells in patients with Metavir  $F \leq 3$  (F1 to F2, median range of number of FoxP3<sup>+</sup> cells: 1 vs 3 and F2 to F3: 3 vs 4) and, a decrease of FoxP3<sup>+</sup> cells in patients with Metavir  $F \geq 3$  (F3 to F4, median range of number of FoxP3<sup>+</sup> cells: 4 vs 2). Actually, the frequency of CD8<sup>+</sup> cells was highest in patients with cirrhosis (A2 to A3, median range of number of CD8<sup>+</sup> cells: 111 vs 123 and F3 to F4, median range: 121 vs 168) (**Fig.5 C, D**). Interestingly, the ratio of CD8<sup>+</sup>/FoxP3<sup>+</sup> cells remained similar in both moderate and mild fibrosis stages while this ratio differed

significantly in severe stages of the disease (F3 to F4, median range of cells : 8.65% vs 2.96% -  $z=2.324$ ,  $p=0.024$ ) (**Fig.5 E**).

It is worth to note that no correlation was found between the serum viral load and any immune marker included in our study at cellular and transcriptional levels.

## DISCUSSION

In chronic hepatitis C, HCV-specific T-cell responses are generally weak, narrowly focused and often dysfunctional. The current debate is to determine whether the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cell population, defining T<sub>regs</sub>, plays a decisive role in the immune dysfunction. Here, we focused our analysis on intrahepatic T<sub>regs</sub> with the aim to determine the frequency, the localization, and the roles of these cells in chronic hepatitis C. For this, we performed an analysis of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells on fresh liver biopsies by flow cytometry to detect presence and frequency of these two subsets. Then, we extended the analysis by immunohistochemistry in more patients to precise localization of CD4<sup>+</sup>FoxP3<sup>+</sup> cells and finally we completed this analysis by RT-PCR on frozen samples.

Previously, intra-hepatic regulatory T cells were mostly defined by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T lymphocytes (9) which did not give true picture of natural T<sub>regs</sub> defined by CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells in HCV patients. Our flow cytometry data show that about 50% of CD4<sup>+</sup>CD25<sup>+</sup> intra-hepatic T cells do not express FoxP3 marker, suggesting that the actual numbers of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub> are almost half of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. Taken together T<sub>regs</sub> represented small fractions of T cells in HCV infected liver.

To date, T<sub>regs</sub> of HCV patients were studied from PBMC samples, which were shown to be expanded *in vitro* after HCV peptides stimulation (23). Here, we confirm by flow cytometry, the very low frequency of CD8<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells in the blood as already published (23, 25, 29-31). We report for the first time, their scarcity in the liver of chronic hepatitis C patients. This suggests that CD8<sup>+</sup>FoxP3<sup>+</sup> T cells do not expand in the liver infected by HCV. This can be due to, either a weak level of stimulation by the presenting cells to induce FoxP3, or the lack of appropriate cytokines in the environmental tissue to permit the survival of these cells.

Our immunochemical studies indicate that CD4<sup>+</sup>FoxP3<sup>+</sup> cells infiltrate the hepatic lymphoid aggregates, portal or septal tracts and parenchymatous lobules or nodules, as already reported (22). In addition, we show that FoxP3<sup>+</sup> cells are found essentially in piecemeal necrosis and lobular necrosis, predominantly in contact with CD8<sup>+</sup> cells. It means that regulatory T cells within HCV infected livers have the direct access required to inhibit T cell cytotoxicity at the site of action. Furthermore, since such cells are localized in portal tracts, antiviral and non-specifically activated lymphocytes entering the liver are likely to encounter a FoxP3<sup>+</sup> cells at an early stage in the inflammatory process. FoxP3<sup>+</sup> cells inside necro-inflammatory lesions also correlate positively with CD8<sup>+</sup> cells, again confirming their plausible inhibitory role on the effector cells.

It is worth to note that the intra-hepatic FoxP3 or CD8 (transcripts or cells) are not correlated during the course of the disease with the viral load although the numbers of FoxP3<sup>+</sup> and CD8<sup>+</sup> cells increase.

The next step was to determine whether, the presence/proliferation of regulatory T cells and CD8 T cells was dependent on cytokines regulation. In our study, a significant positive correlation was found among the expression levels of four cytokines: IL-10, TGF- $\beta$ , TNF- $\alpha$  and IFN- $\gamma$ . All these cytokines are produced by different types of cells, but this technical approach does not allow determining what kind of cells actually produce cytokines. Secretion of TGF- $\beta$  in high concentration during chronic HCV infection is an important factor for the local survival and function of natural Treg cells (9, 32) but might also contribute to induction of fibrosis. IL-10 expression is high in the liver of hepatitis C virus-human immunodeficiency virus co-infected patients (33). *In vitro* studies demonstrated that both TGF- $\beta$  and IL-10 inhibited the proliferation, cytokine secretion and cytotoxicity of CD8<sup>+</sup> cells (9, 34). Here, extrapolating the results achieved in PBMC, a positive correlation found between the transcriptional levels of TGF- $\beta$  and IL-10 may have synergistic effects on each other in

HCV infected liver. They favour i) the maintenance of the FoxP3 cells pool and ii) the reduction of pro-inflammatory cytokine secretions. It is known that the intra-hepatic TNF- $\alpha$  production is induced and up-regulated in the liver by HCV NS3 (35). TNF- $\alpha$  comes at an early stage of the cascade of events leading to liver damage (36) and is involved in the healing process including fibrogenesis (37). As the majority of the patients included in our study have proven fibrosis (44/47), TNF- $\alpha$  expression might be induced and produced in the liver of the studied patients. It has been established that T<sub>regs</sub> inhibit the *in vitro* production of INF- $\gamma$  by core specific CD4<sup>+</sup> cells and HCV-specific CD8<sup>+</sup> T cells (11, 38). As in the liver, expression levels for TNF- $\alpha$  as well as for IFN- $\gamma$  display a correlation with those of IL-10 and of TGF- $\beta$ , one can argue that the infectious/inflammatory context favors both the production of anti-inflammatory (IL-10, TGF- $\beta$ ) and pro-inflammatory (TNF- $\alpha$ , IFN- $\gamma$ ) cytokines. We hypothesize that T<sub>regs</sub> inhibit partially the pool of intra-hepatic effector T cells and that some effectors cells produce IFN- $\gamma$ . That is supported by recent data showing that IFN- $\gamma$  is released rather by effector T cells in these infections (3, 5, 6). Whatever the balance between the four cytokines, our regression analysis points out that the expression levels of FoxP3, CD8 and IL-10 are positively co-dependent upon each other. This means that together, FoxP3, CD8 and IL-10 allow establishing equilibrium beneficial both to the host and to the virus. The host gains benefit from FoxP3 which contains the expansion of CD8<sup>+</sup> cells, thus preventing liver tissue damages. The virus is preserved through the suppression of CD8<sup>+</sup> cells by T<sub>regs</sub>.

It is important to note that the number of CD8<sup>+</sup> cells increases with the necro-inflammatory activity and fibrosis, whereas FoxP3<sup>+</sup> cells increase up to Metavir-A2 and Metavir-F3 but decrease after this grade and stage. Interestingly, FoxP3<sup>+</sup>/CD8<sup>+</sup> ratio is reduced significantly from stage F3 to F4. On the light of these observations, the most likely scenario is that, during the first step of the disease, FoxP3<sup>+</sup> cells modulate effector functions of lymphocytes whereas, during the severe stage when the tissue environment is altered, the

co-dependency between FoxP3<sup>+</sup> cells and effector cells is broken. It remains to be determined whether the loss of the FoxP3-CD8 interaction, beyond the stage A2/F3, results from an increase in the cytolytic function of CD8<sup>+</sup> cells, thus impacting on the expansion of liver damages. Therefore, facing the immune response and the equilibrium FoxP3-CD8 cells, the stage/grade A2/F3 appears to be a turning point in the course of the disease.

In conclusion, this study provides new insights into the distribution of regulatory T cells in the different histological areas within HCV infected liver, the regulation being essentially FoxP3-CD8 contact-dependent. More interestingly, during the course of the disease, no correlation was found between viral load and intra-hepatic T<sub>regs</sub> (transcripts or cells) suggesting that the essential role of T<sub>regs</sub> is to limit the CD8 cell pool to contain the damage of the liver. These findings are crucial for understanding the HCV immune pathogenesis and for immunotherapeutic treatments.

## **MATERIAL and METHODS**

### **Patients.**

Patients with chronic hepatitis C followed in the University Hospital, Grenoble were included in this study. Six liver biopsies and 8 blood samples were selected for flow cytometry analysis (**Table II**). The number of patients was then extended up to 20 for immunohistochemical studies, and up to 47 for quantifications of cytokines by RT-PCR. Their biological and physioclinical parameters were analyzed as previously described (39). Their main characteristics are shown in **Table II**. This study was approved by the local ethics committee. All patients gave informed consent prior to liver biopsy.

### **Cell preparation.**

Liver biopsies obtained by using 1.5 mm diameter disposable biopsy needle were divided into two parts, one for histo-pathological examination and the other for immunological analysis. Biopsies were processed and experimented as described previously (39-41).

### **Immunostaining and flow cytometric analysis.**

Immediately after separation on density gradient or mechanical disruption, peripheral blood and intrahepatic lymphocytes were stained as follow. Normal Rat Serum, permeabilization buffer, Fixation/Permeabilization buffer, Human anti-FoxP3-PE and rat IgG2a-PE antibodies were both provided by the Human/Non Human Primate Regulatory T Cells Staining Kit (BD Biosciences, Pont de Claix, France). Human anti-CD4 APC, human anti-CD8 FITC and human anti-CD25 PC7 were all provided by Beckman Coulter (Villepinte, France). First, one million of cells were incubated with anti-CD4 APC and anti-CD8 FITC for 30 min at 4°C in the dark. Then, according to manufacturer's instructions, cells were washing in cold PBS and resuspended in fixation/permeabilization buffer for 45 min at 4°C in the dark. Samples were then washed twice in permeabilization buffer. After elimination of the supernatant, normal Rat Serum was added in the residual volume for 15 min in the dark. Then, human anti-FoxP3-PE or Rat IgG2a-PE was added directly for 30 min at 4°C in the dark. Cells were then washed

twice with permeabilization buffer and resuspended in the adequate volume of PBS for flow Cytometry analysis.

All the samples were analyzed with a FACScan cytometer (Becton Dickinson) using CellQuest Pro Software for acquisition as described previously (40). Data analysis was performed with FCS Express V3 (DeNovo Software).

### **Immunohistochemical double labelling study**

Double immunoenzymatic labeling was performed manually (Sequenza station) on formalin-fixed, paraffin-embedded liver biopsies from 20 chronic HCV patients. Two methods of immunodetection were applied: streptavidin-biotin peroxidase (SBP) method with diaminobenzidine (DAB) as chromogen and streptavidin-biotin alkaline phosphatase (SBAP) method with fast blue (FB) as chromogen. Four microns thick consecutive tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. For CD8/FoxP3 double staining, antigen retrieval was done by heating slides in microwave for 10 min in 0.01M Citrate buffer (pH 6). Non specific protein binding were blocked by 30 min incubation with milk (diluted at 2% in PBS, pH 7.4). Slides were incubated with the primary mouse monoclonal anti-CD8 antibody (CD8, clone C8/144B, 1/100, Dako) for 12 h at 4°C then with the biotinylated secondary goat anti-mouse antibody (1/500, Jackson ImmunoResearch, reference 115-065-003) for 60 min at room temperature, followed by detection with SBAP complex (StreptABComplex/AP, 1/50, reference K391, Dako) and FB chromogen staining. For second FoxP3 staining, additional heat induced antigen retrieval was performed in Tris-citrate buffer with 0.02% saponin for 15 min in microwave followed by blocking of non specific protein binding, incubation with mouse monoclonal primary antibody (FoxP3, 1/20, clone 22510, Abcam), incubation with secondary biotinylated antibody (goat anti-mouse antibody, 1/500, Jackson ImmunoResearch), detection with SBP complex (StreptABComplex/HRP, 1/200, Dako, reference K377) and DAB chromogen staining. For



CD4/FoxP3 double staining, antigen retrieval was performed by heating slides in microwave for 15 min in Tris-Citrate buffer (pH 9). Following steps included blocking of non specific protein binding, incubation with primary mouse monoclonal antibody (CD4 clone 4B12, 1/75, Novocastra), endogenous peroxydase inhibition, incubation with secondary biotinylated goat anti-mouse antibody (1/500, Jackson ImmunoResearch, reference 115-065-003), detection with SBP complex (StreptABCComplex/HRP, 1/200, Dako, reference K377) and DAB chromogen staining. The second immunostaining for FoxP3 was performed with SBAP method and FB staining. All sections were stained in parallel with an unrelated primary antibody (mouse IgG, 2 µg/ml, Jackson ImmunoResearch) to provide a negative control.

Immunopositive cells were counted in all consecutive fields along the whole section of liver biopsy on a computer screen with the help of automatic software LUCIA (Laboratory Universal Computer Image Analysis, version 5.0, Laboratory Imaging, Prague, Czech Republic). The cell counts were performed by a single liver pathologist (NS) on serial sections obtained from the paraffin block used for histological Metavir scoring system evaluation. Several parameters were observed separately, including total number of CD8<sup>+</sup> and FoxP3<sup>+</sup> cells, localization of FoxP3<sup>+</sup> cells in the foci of piecemeal and lobular necrosis, their distribution in the fibrotic tissue (fibrous septa and portal tracts) and in the parenchymatous areas (lobules or nodules). The numbers of cells were expressed as per field observed at 20X magnification on the computer screen (1.002 mm<sup>2</sup>).

### **RT-PCR Analysis**

Total RNA was extracted from frozen liver biopsies (5-10mg) by homogenization of tissues in silica balls using MagnaLyser System (Roche Diagnostics, Meylan, France). After centrifugation, 600 µl of 70% ethanol was added in the aliquot. The mixture was filtered on a silica column and digested by DNase I for 15 min at room temperature in order to eliminate the residual DNA. Total RNA was precipitate in presence of ethanol. Single-stranded cDNA

was synthesized by using the Superscript II Reverse Transcriptase (Gibco BRL, Life Technologies) in a final volume of 20  $\mu$ l. Primer pairs and probes for quantification of CD4, CD8 $\beta$ , FoxP3, IL-10, IFN $\gamma$ , TGF $\beta$ , and TNF $\alpha$  genes were designed by using ProbeFinder Software for Human version 2.35 (Roche Diagnostics, Meylan, France) and were synthesized by Genset (Paris, France) **Table III**. Quantification of transcripts from liver samples was performed by using the LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche Diagnostics) and target sequences were detected with UPL (Universal ProbeLibrary, Roche Diagnostics). Briefly, each PCR reaction mixture contained: 1X of LightCycler<sup>®</sup> 480 Probes Master (Roche Diagnostics), 0.5  $\mu$ M of each primer, 0.1 $\mu$ M of appropriate probe and 2.5 $\mu$ L of cDNA preparation, in a final volume of 10 $\mu$ L. After an initial denaturation at 95°C for 10 min, the PCR reactions were cycled 35-40 times as follows; 10 s at 95°C, 15 s at 60°C (for all primers and probes) and 10 s at 72°C. Data were analyzed with the LightCycler<sup>®</sup> 480 Software. The detection format was set to “Mono Colour Hydrolysis Probe” and the Second Derivative Maximum Method was used for absolute quantification. The household gene used for normalization of the values was the Hypoxanthine Phosphoribosyl Transferase gene (HPRT). Results were expressed as the log of the number of amplicons ( $2^n - 2n$ , n = number of cycles) of the targeted transcripts.

### **Statistical methods.**

Statistical analysis was performed by using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). The non-parametric Wilcoxon and Mann-Whitney U-test were performed to compare quantitative variables. Within group comparisons were performed by using the 2-tailed Spearman's correlation coefficient. Statistical analysis was also performed by Principal Component Analysis (PCA) and linear univariate and multivariate regression analysis. P values less than 0.05 were considered significant.

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## FIGURE LEGENDS

**Fig.1. CD25<sup>+</sup>/FoxP3<sup>+</sup> Gating Strategy.** **A)** Intrahepatic lymphocytes were analyzed by flow cytometry among total cells and electronically separated in two subpopulations, CD4<sup>+</sup> and CD8<sup>+</sup>. **B)** On each CD4<sup>+</sup> or CD8<sup>+</sup> gate, the expression level of CD25<sup>+</sup> and FoxP3<sup>+</sup> was measured. **C)** On both gates, the level of double positives CD25<sup>+</sup> and FoxP3<sup>+</sup> was determined to isolate T<sub>regs</sub>. **D)** Average of each subpopulations CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> in the liver and in the blood.

**Fig.2. CD4/FoxP3 or CD8/FoxP3 Double Immunohistochemical labelling** **A.** Double CD4/FoxP3 immunohistochemical labeling showing CD4<sup>+</sup>FoxP3<sup>+</sup> cells with blue nuclear Foxp3 staining and brown cytoplasmic CD4 staining, in contact with foci of parcellar necrosis (PN, arrow) and lobular necrosis (LN, asterisk). **B.** High magnification of CD4/FoxP3 double immunolabeling showing one blue FoxP3<sup>+</sup> cell without cytoplasmic CD4 staining (arrow). **C.** Double CD8/FoxP3 immunohistochemical labeling showing numerous separate brown foxP3<sup>+</sup> cells and blue CD8 lymphocytes, in a fibrous portal tract. **D.** FoxP3<sup>+</sup> cells were particularly numerous in fibrous areas when nodular lymphoid aggregates were present. **E.** Brown FoxP3<sup>+</sup> cells in contact with parcellar necro-inflammatory lesions (PN, arrow). **F.** Brown FoxP3<sup>+</sup> cells in contact with lobular necro-inflammatory lesions (LN, asterisk). **G-H.** High magnifications showing two putative double labeled CD8<sup>+</sup>FoxP3<sup>+</sup> lymphocytes in fibrous area (G) and in contact with lobular necrosis ( LN, asterisk).

**Fig.3. A-B)** Relationship between intra-hepatic total FoxP3<sup>+</sup> cells and CD8<sup>+</sup> cells in Fibrous Septa and in Parenchyma. **C-D)** Relationship between intra-hepatic total FoxP3<sup>+</sup> cells and FoxP3<sup>+</sup> cells in Fibrous Septa and in Parenchyma. **E)** Relationship between intra-hepatic total FoxP3<sup>+</sup> cells and FoxP3<sup>+</sup> cells in necrosis areas.

**Fig.4. Expression levels of four cytokines clusters.** Relationship between: **A)** CD8 and FoxP3 transcripts and **B)** CD8 and IL-10 transcripts; **C)** TGF- $\beta$  and TNF- $\alpha$  transcripts and **D)** TGF- $\beta$  and IL-10 transcripts.

**Fig.5. Relationship between frequency of FoxP3<sup>+</sup> cells or CD8<sup>+</sup> cells and gravity of the disease:** **A)** FoxP3<sup>+</sup> cells or **B)** CD8<sup>+</sup> cells and different stages of the Activity; **C)** FoxP3<sup>+</sup> cells or **D)** CD8<sup>+</sup> cells and different stages of the Fibrosis. **E)** Ratio FoxP3<sup>+</sup>/CD8<sup>+</sup> cells and different Activity or Fibrosis steps.

**Table I: Quantification of lymphocytes in the liver and the blood**

Sub populations of lymphocytes	Liver* (%)	Blood (%)
CD4 <sup>+</sup> / lymphocytes	17.4 ± 8.440.	1 ± 15.0
CD8 <sup>+</sup> / lymphocytes	32.5 ± 5.525.	6 ± 18.1
CD4 <sup>+</sup> CD25 <sup>+</sup> / CD4 <sup>+</sup>	6.0 ± 1.1	8.4 ± 6.1
CD4 <sup>+</sup> CD25 <sup>+</sup> FoxP3 <sup>+</sup> / CD4 <sup>+</sup> CD25 <sup>+</sup>	54.5 ± 22.7	45.9 ± 21.8
CD4 <sup>+</sup> CD25 <sup>+</sup> FoxP3 <sup>+</sup> / lymphocytes	0.6 ± 0.3	1.55 ± 0.8

\*Numbers indicate the % values of the mean and the standart deviation

**Table II. Demographic, biochemical and clinical characteristics in chronic HCV patients**

	<b>Hepatitis C patients studied by Histochemistry (n=20)</b>	<b>Hepatitis C patients Studied by RT/PCR (number of patients extended up to n=47)</b>
<i>Male, n(%)</i>	15 (75%)	32 (68%)
<i>Age (y)*</i>	44 (24-75)	43 (24-75)
<i>Serum Viral Load (logIU/mL)</i>	6.1± 6.1	6.1± 6.2
<i>Serum ALT (UI/mL)</i>	172.6 ± 105.5	130.1 ± 98.8
<b><u>Liver Histology**</u></b>		
<i>Metavir Activity Score, n(%)</i>		
0	0	0
1	4 (20%)	20 (43%)
2	9 (45%)	18 (38%)
3	7 (35%)	9 (19%)
<i>Metavir Fibrosis Score, n(%)</i>		
0	1 (5%)	7 (15%)
1	3 (15%)	12 (26%)
2	7 (35%)	14 (30%)
3	6 (30%)	10 (21%)
4	3 (15%)	4 (8%)

\* *Median (Range)*

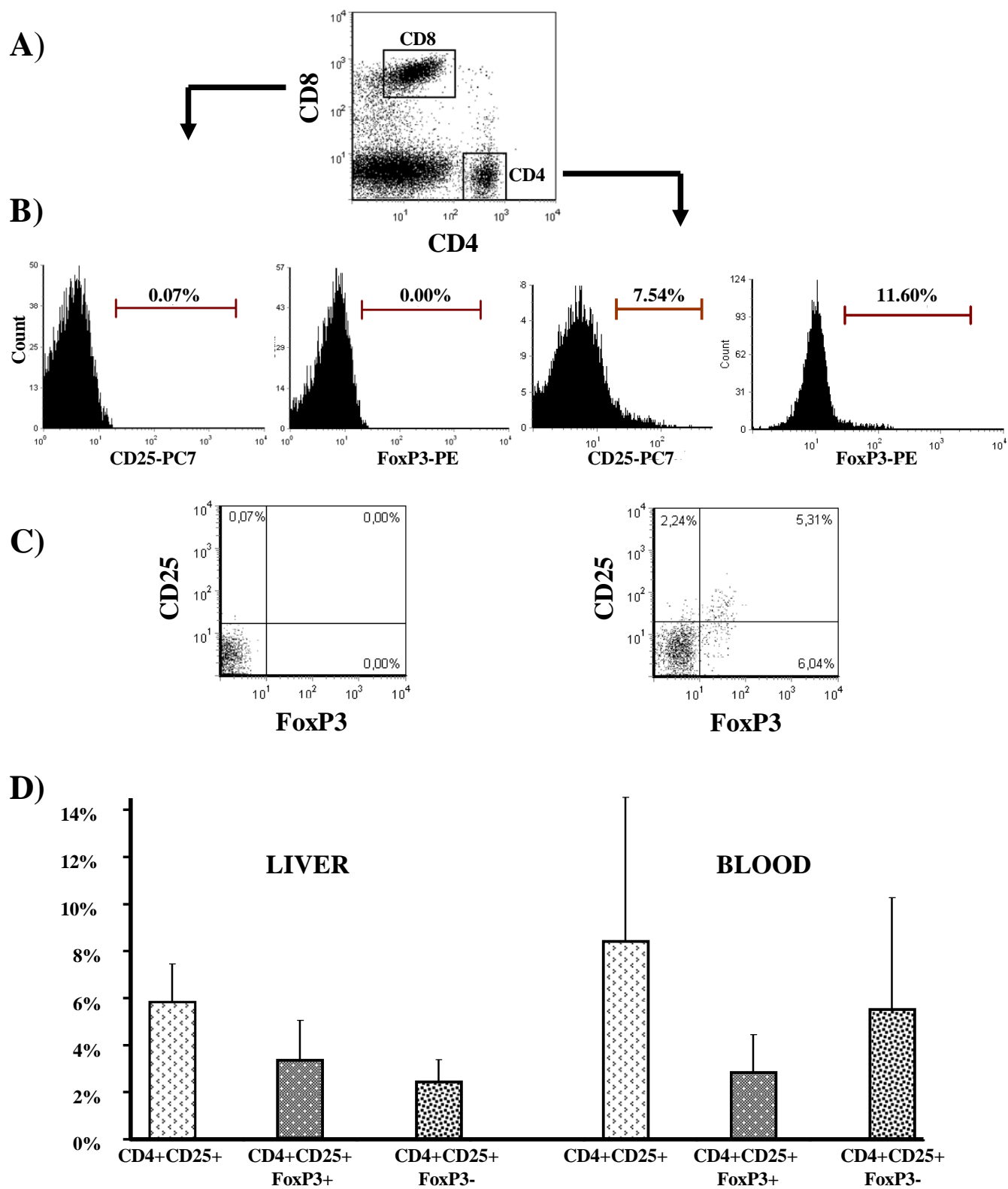
\*\* *Number of cases / total (%)*

*ALT: Alanine AminoTransferase*

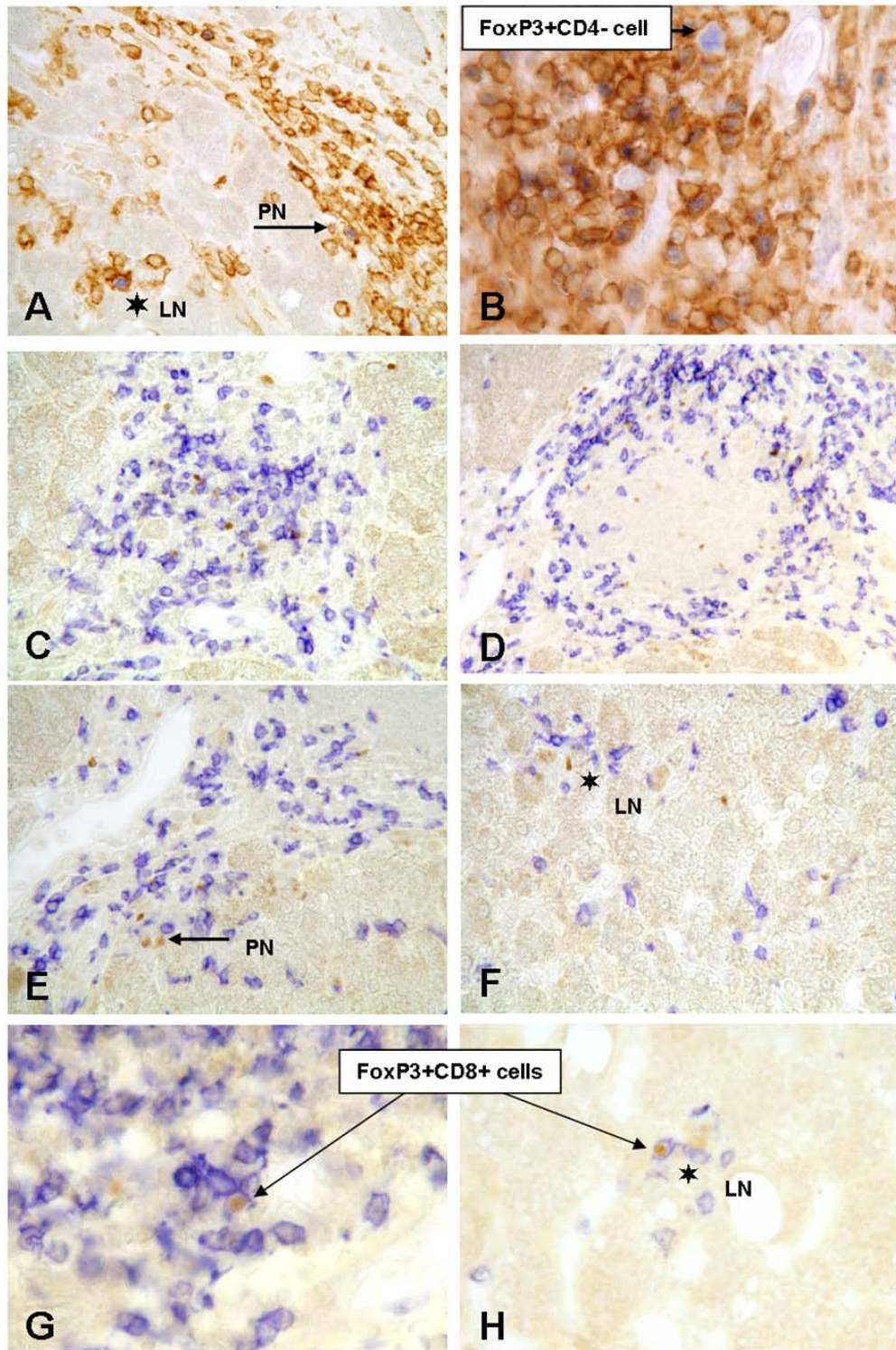
**Table III : Primers and Universal ProbeLibrary (UPL) sequences used in the LightCycler® 480 Real-Time PCR System**

<b>Gene</b>	<b>Primer sequences</b>	<b>Product length (nt)</b>	<b>UPL sequences</b>	<b>N° of UPL</b>
<b>CD4</b>	F agcgaaaacaggaagtgc R aaattttctggagctgagtg	72 nt	tggtgatg	<b>9</b>
<b>CD8 beta</b>	F caggccagagaccagaa R gaaaccagcagaaccaggac	84 nt	gcctgctg	<b>40</b>
<b>FoxP3</b>	F acctagcccagctcatc R tcattgagtgtccgctgct	65 nt	gctccaga	<b>50</b>
<b>IL-10</b>	F tgggggagaacctgaagac R acaggaagaatcgtgaca	62 nt	ggctgagg	<b>30</b>
<b>IFN gamma</b>	F ggcatttgaagaattgaaag R tttgatgctctggtcatctt	112 nt	cagagcca	<b>21</b>
<b>TGF beta</b>	F gcagcacgtggagctgta R cagccggtgctgagta	64 nt	ttcctggc	<b>72</b>
<b>TNF -alpha</b>	F cagcctcttctcctctgat R gccagagggtgattagaga	123 nt	cttctgcc	<b>29</b>
<b>HPRT</b>	F gaccagtcaacaggggacat R ggtcaattatatctccacaatcaag	95 nt	tggtggag	<b>22</b>

F, Forward primer - R, Reverse primer

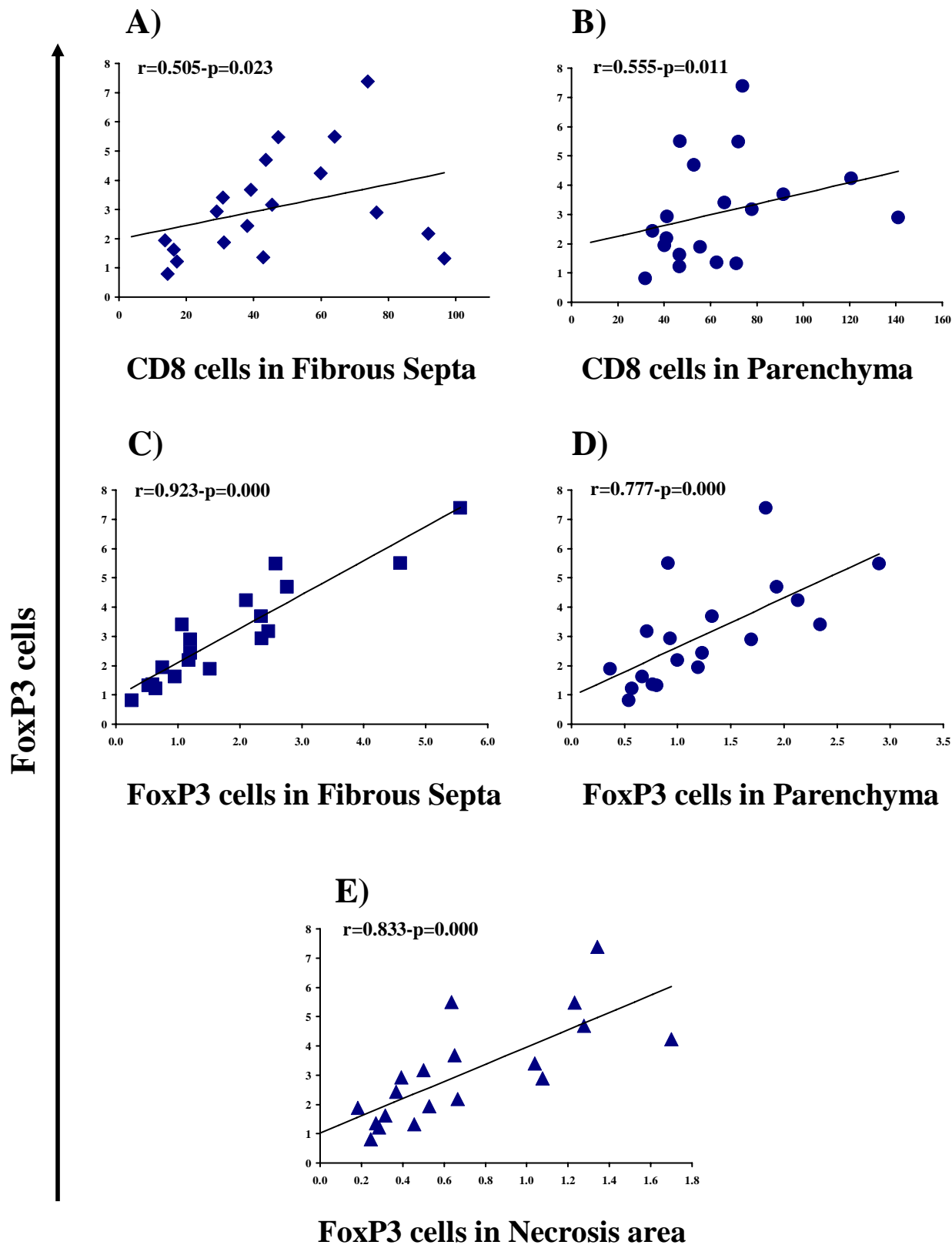


**Figure 1.**



**Figure 2**





**Figure 3.**

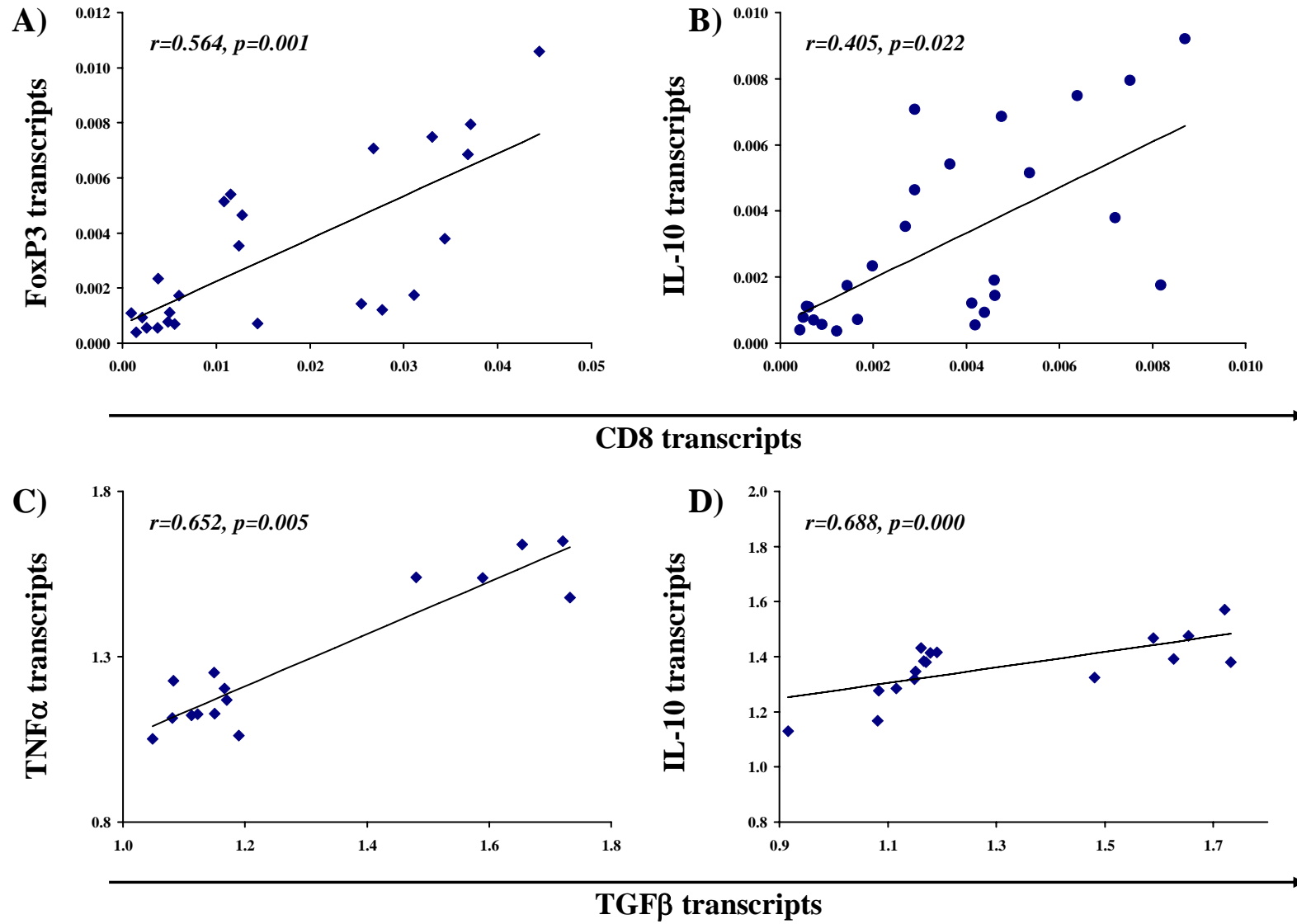


Figure 4.

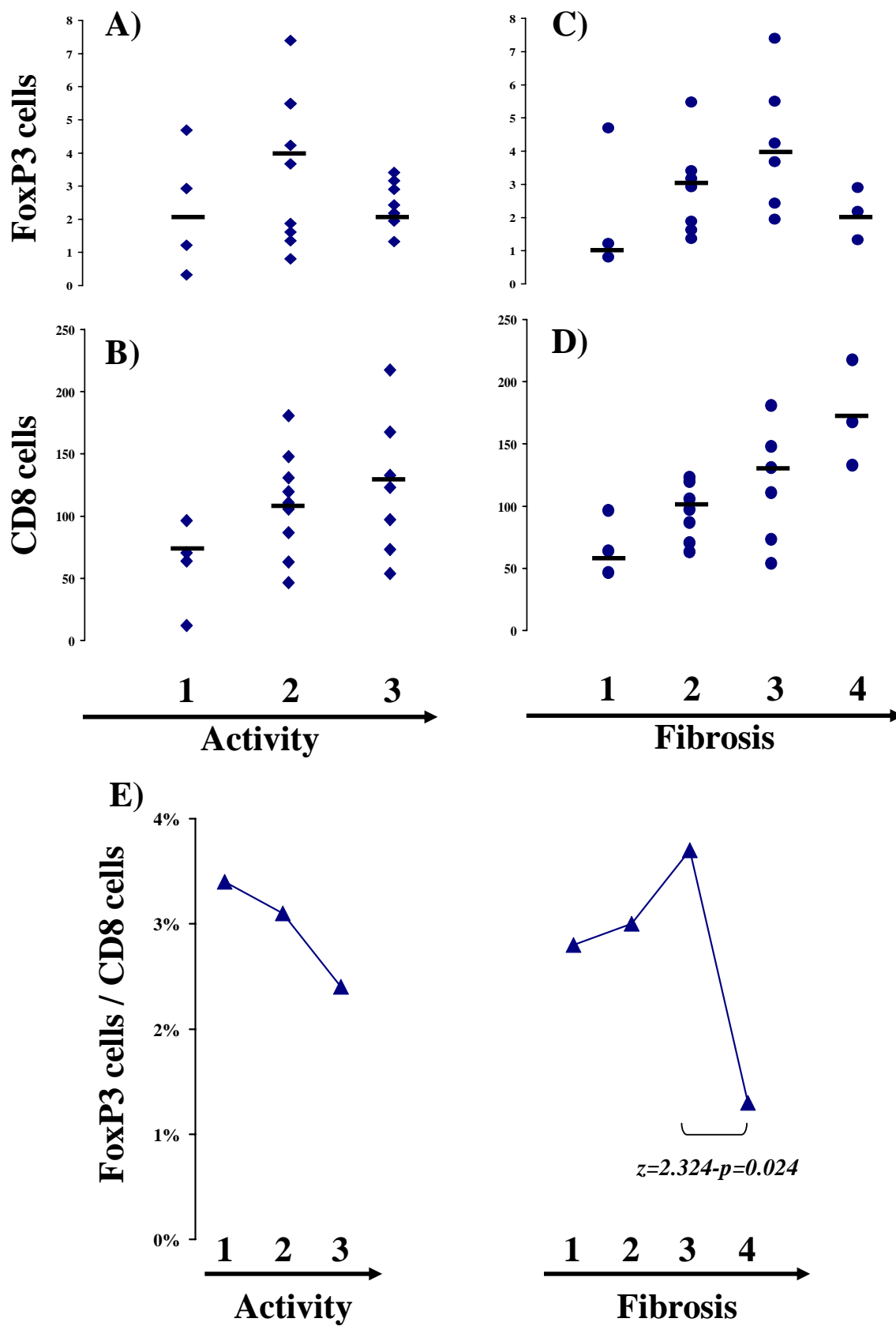


Figure 5.

# **APPENDIXES**

## **Article 4**

### **IRES complexity before IFN-alpha treatment and evolution of the viral load at the early stage of treatment in peripheral blood mononuclear cells from chronic hepatitis C patients**

Thelu, M. A., Ramzan, M., Dufeu-Duchesne, T., Leroy, V., Marche, P. & Zarski, J. P.  
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**Article 4**  
**IRES complexity before IFN- $\alpha$  treatment and evolution of the viral load at the early stage of treatment in peripheral blood mononuclear cells from chronic hepatitis C patients**

As HCC, associated with HCV infection, evolves after many years of chronic infection and is generally preceded after the development of cirrhosis, the role of chronic liver injury followed by regeneration, cirrhosis and the development of HCC has been the leading hypothesis for hepatocarcinogenesis. Increasing experimental evidence, however, raises the possibility that HCV might also contribute through more direct pathways in promoting malignant transformation of hepatocytes. Different viral proteins, notably core, NS3 and NS5A have been implicated in transformation and HCC development (Yoshida, Hanada et al. 2002; Kasprzak and Adamek 2008; Tanaka, Moriya et al. 2008). The synthesis of all these proteins is controlled by the internal ribosome entry site (IRES) at 5' end of HCV RNA molecule.

IRES is a nucleotide sequence that allows for translation initiation in the middle of an mRNA sequence in the process of protein synthesis. Usually, in eukaryotes, translation can only be initiated at the 5' end of the mRNA molecule, since 5'cap recognition is required for the assembly of the initiation complex. HCV IRES binds independently two components of eukaryotic translation initiation machinery, protein eIF3 and 40S small ribosome subunit. Moreover, it binds 40S in such a manner that AUG initiator codon is positioned in ribosomal P-site, thus no ribosomal scanning is required. Consequently scanning factors eIF1 and eIF4A are dispensable for HCV translation. Many other factors are so for required for mRNA binding and unwinding of 5'-UTR e.g. eIF4A, eIF4B and eIF4F. Initiator tRNA is brought then either by eIF2 or, in stress conditions when eIF2 is inactivated, by eIF5B, homologue of prokaryotic IF2 protein (Lytle, Wu et al. 2002).

IFN- $\alpha$  in combination with ribavirin is a proven treatment for HCV infection. However, the antiviral mechanisms by which IFN- $\alpha$  induces modification of immune responses in the infected cells is unknown. Here, a question arises whether IFN- $\alpha$  treatment makes HCV to change in IRES. In the following article, some results regarding IRES complexity before IFN- $\alpha$  treatment and with relation to viral load have been given in the lymphocytes of chronic HCV patients.

# IRES Complexity Before IFN-Alpha Treatment and Evolution of the Viral Load at the Early Stage of Treatment in Peripheral Blood Mononuclear Cells From Chronic Hepatitis C Patients

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At the early stage of treatment, IFN alpha-2a induces inhibition of HCV replication. The viral load reflects mainly the degradation rate of the viruses. However, differences in the behavior of the viral population depend on changes, which occurred in the HCV-IRES genome. In this study, cloning and sequencing strategies permitted the generation of a large number of IRES sequences from the PBMCs of 18 patients (5 women, 13 men) with chronic hepatitis C. The HCV IRES appeared to be highly conserved structurally. However, some variability was found between the different isolates obtained: 467 substitutions with a median of 7 variants/patients. No relationship was observed between pre-treatment IRES complexity and the viral load at the beginning. However, on review of the evolution of viral load in the PBMCs during the first 3 days of IFN alpha-2a treatment, patients could be classified into two groups: Group 1, in which the viral population continued to replicate and Group 2, in which the viral load decreased significantly ( $P=0.01727$ ). Positioning of the mutations on the predicted IRES secondary structure showed that the distribution of the mutations and their apparition frequency were different between the two groups. At the early stage of treatment, IFN alpha-2a was efficient in reducing the viral replication in a significant number of patients; mechanisms of response might affect the virus directly. However, pre-treatment genomic variations observed in the 5'NCR of HCV were not a parameter of a later response to antiviral therapy in chronic hepatitis C patients. (244) *J. Med. Virol.* 00:1–12, 2007.

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**KEY WORDS:** HCV-IRES; PBMCs; quasi-species; variants; viral load

## INTRODUCTION

Hepatitis C virus (HCV) is one of the major causative agent of chronic hepatitis, which can result in cirrhosis and ultimately in hepatocellular carcinoma. The cell's first-line of defense is the IFN-induced antiviral response [Pawlotsky, 2003b]. However, the antiviral mechanisms by which IFN- $\alpha$  induces modifications of specific immune responses and the establishment of non-specific immune antiviral state in the infected cells is unknown. It has been suggested that it may act at various stages of the viral cycle and was likely mediated by several biochemical pathways involving various proteins: 2'-5'-oligoadenylate synthetase (2–5 OAS), the Mx proteins and the double-stranded RNA-activated protein kinase RNA-dependent (dsRNA-PKR) [Giannelli et al., 1993; Pawlotsky et al., 1995, 1996; Samuel, 1998, 2001; Tan & Katze, 2001; He & Katze, 2002; Shimazaki et al., 2002; Taylor et al., 2005].

The basis of treatment of chronic hepatitis C is interferon- $\alpha$  (IFN- $\alpha$ ) which is used currently in combination with ribavirine, a molecule that potentiates its antiviral effects. Nevertheless, therapeutic failure is frequent and the outcome of antiviral treatment seems to depend on many factors. Indeed, many viruses possess strategies to evade the IFN responses and some virological factors are well known to play a key role in the failure of IFN treatment. Amongst them, it is known that the high rate of virus mutations leads to the generation of a mixture of closely related genomes, the quasispecies, which persist and evolved in the infected patients [Rumin et al., 1999; Soler et al., 2002; von

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Wagner et al., 2003; Pawlotsky, 2003a,c; Vuillermoz et al., 2004].

Similar to coding regions, the 5'Non-Coding Region (5'NCR) is distributed as a quasispecies. Previous studies have shown that this part of HCV genome might accumulate nucleotide substitutions, [Vizmanos et al., 1998] but it appears to be subjected to strong conservation constraints than the other regions of the HCV genome. This is probably due to the need for structural conservation of the mediated translational element: the Internal Ribosome Entry Site (IRES) located in the 5'NC region. This segment (nt 40–372), highly conserved between isolates [Soler et al., 2002], has the potential to form a stable secondary and tertiary structure. Changes in the secondary or tertiary structure of IRES as well as changes in the primary nucleotide sequence result in a decrease of efficiency of protein translation [Honda et al., 1999b; Lerat et al., 2000; Kieft et al., 2001; Collier et al., 2002; Laporte et al., 2003; Luo et al., 2003].

HCV is assumed to replicate in PBMCs on the basis of detection of HCV-RNA negative-strand by RT-PCR and in situ hybridization [Moldvay et al., 1994]. Interference with the cells of the immune system is a known mechanism by which viruses evade the host response and become infected chronically [Oldstone, 1997]. Infection of extrahepatic tissues is supported by the finding that HCV quasispecies composition differs according to the sample type, that is, liver, serum, or PBMCs [Fujii et al., 1996; Cabot et al., 1997; Maggi et al., 1997; Navas et al., 1998; Di Liberto et al., 2006]. However, the notion of infection in extrahepatic tissues by HCV is controversial. Indeed, detection of HCV RNA in the PBMCs might be due to the simple adsorption of viral particles.

The aim of this study was to generate a large number of IRES sequences from PBMCs of the patients with chronic hepatitis C, in order to assess whether quasispecies distribution can be evidenced within the IRES sequences found by using cloning and sequencing strategies. Then, the IRES complexity and its relationship with viral load before IFN alpha-2a treatment was investigated in order to determine if that relationship is

a prognostic factor for the response to the treatment. Finally, the relationship between IRES variability before IFN alpha-2a treatment and the evolution of viral load at the early stage of the therapy was investigated.

## PATIENTS, MATERIALS AND METHODS

### Patients

Eighteen chronic HCV-infected patients were enrolled in the University Hospital de Grenoble (France). All were included in a randomized clinical trial for testing the safety and the efficacy of high daily doses of interferon alpha-2a (Laroféron, Roche) in combination with Ribavirin. At the time of inclusion, each patient had HCV antibodies (ELISA 3), HCV RNA (PCR Monitor, Roche<sup>Q3</sup>) positive in serum, ALT serum levels more than the upper limit of normal, and the liver biopsy compatible with the diagnosis of chronic hepatitis C. Genotyping was carried out after standardized RT-PCR using biotinylated primers (Amplicor, Roche). Among the 18 HCV genotypes studied, a majority of patients were found to be infected with HCV genotype 1 [1a (n = 6)—1a/b (n = 1) and 1b (n = 7)], 1 patient with HCV genotype 2a/2c, 1 patient with genotype 3a, and 2 patients with genotype 4c/d. The main characteristics of patients are summarized in Table I.

### Treatment

Patients were randomized to receive an induction course of IFN alpha-2a (Laroféron, Roche) subcutaneously 18 MUI/d for 2 days followed by 9 MUI/d for 12 days. In addition, all patients received 3 MUI/d of IFN alpha-2a for 20 weeks followed by 3 MUI twice a week for 24 weeks. Ribavirin (1,000–1,200 mg) was given in association with IFN alpha-2a for 48 weeks to all patients.

Serum viral load was determined at the start of the treatment that is, D0, then D2, D3, D4, W2, W4, and W12.

TABLE I. Clinical and Virological Characteristics of the Patients at Entry

Patients N = 18		
Age (years) <sup>a</sup>		44.1 ± 7.9
Sex (no. of men)		13
Mode of transmission	Blood transfusion	4
	Intravenous drug use	7
	Others/unknown	3/5
	Activity/fibrosis	2.0 ± 0.8/2.0 ± 0.9
Histological Metavir index <sup>b</sup>		
Genotype	1a-1a/1b-1b-2c-3a-4c/d	5-1-8-1-1-2
ALAT level <sup>a</sup>		2.3 ± 1.8
Serum viral load (UI/ml) (log) <sup>a</sup>		6.2 ± 6.2
PBMCs viral load (UI/ml) (log) <sup>a</sup>		3.2 ± 3.2

<sup>a</sup>Mean ± one standard deviation.

<sup>b</sup>Median.



### Response to Therapy

Eighteen patients with chronic hepatitis C were selected strictly and paired according to age, sex, mode of infection, and duration of disease. Then they were separated into two groups according to their response to antiviral therapy. Patients with undetectable serum HCV RNA after 6-month follow-up were classified as sustained responders (SR=9/18). Patients with continuously positive HCV RNA in the serum were considered as non-responders (NR=9/18).

### Preparation of Serums and PBMCs

Peripheral venous blood was collected in EDTA-treated tubes. Serum samples were obtained by centrifugation (10 min–1,400 rpm –4°C) of the blood samples at the start of the treatment that is, D0. The HCV concentration was then measured in these samples by using the Amplicor Monitor Test Kit (Hoffmann<sup>Q4</sup>-La Roche AG).

The PBMCs were isolated by Ficoll density gradient centrifugation at the start of the treatment and 72 hr after the start of the treatment (D4) and were stored in liquid nitrogen in RPMI medium containing 10% DMSO (3 aliquots of  $>10^7$  cells for each patient).

### In Vivo Cells Culture

The frozen PBMCs pellets ( $>10^7$  cells) were incubated at 37°C for 10 min with 1 ml RPMI supplemented with 20% Human AB serum and the mixture was centrifuged for 10 min at 1,400 rpm. PBMCs adjusted to  $1 \times 10^6$  cells/well in PBS were plated in duplicate in 24-well plate and cultured at 37°C in a humidified atmosphere with 50 ml/L CO<sub>2</sub> for 30 min. Then samples were pooled in order to obtain  $2 \times 10^6$  cells.

In order to eliminate the extra cellular HCV possibly adsorbed on the mononuclear cells surface, an adapted assay was performed [Cribier et al., 1995]. Each sample was treated with trypsin (final concentration 0.05%) and EDTA (final concentration 0.02%) before incubation. Trypsin activity was then blocked by the addition of fetal calf serum (Invitrogen<sup>Q5</sup>) (final concentration 10%) and the pellets were washed in PBS. Before manual extraction, the pellet was resuspended in 100 µl of PBS followed by quantification with COBAS Amplicor.

### In Vitro Cells Culture

The frozen PBMCs pellets ( $>10^7$  cells) were defrosted in the same way as mentioned above (10 min with 1 ml RPMI supplemented with 20% Human AB serum—centrifuged for 10 min at 1,400 rpm. PBMCs adjusted to  $1 \times 10^6$  cells/well in PBS—plated in duplicate in 24-well plate). Then, they were cultured for 6 hr, at 37°C in a humidified atmosphere with 50 ml/L CO<sub>2</sub> in the absence or presence of IFN at a concentration of 100 UI/10<sup>6</sup> cells or presence of IFN (100 UI/10<sup>6</sup> cells) plus Ribavirin at a concentration of 600 ng/10<sup>6</sup> cells. Then the samples were pooled in order to obtain  $2 \times 10^6$  cells and underwent for the trypsin treatment before quantification of the viral load.

### Quantitation of HCV in the PBMCs

In order to adapt the standardized method for quantitation of HCV in the PBMCs (i.e., The COBAS AMPLICOR HCV MONITOR v 2.0 standard serum protocol provides manual HCV RNA extraction, uses an automated RT-PCR to co-amplify an internal RNA quantitation calibrator and the viral quantification was achieved by comparison to the internal RNA quantitation calibrator), the following assays were carried out: (i) determination of the optimal cell number for the assay by testing  $1 \times 10^6$ ,  $2 \times 10^6$ ,  $5 \times 10^6$  isolated PBMCs,  $2 \times 10^6$  cells were retained; (ii) the pellet was resuspended from 1 ml to 100 µl, which represented a 10-fold increment in the target RNA to be amplified and would consequently increase the assay sensibility. Taking these modifications under consideration, the formula calculated was:

$$\frac{\text{Adsorbance HCV} \times \text{dilution factor}}{\text{Adsorbance IQS} \times \text{dilution factor}} \times 200 \times 2 \times \text{number of cells}$$

The linearity of the assay range was between 50 and  $5.10^5$  RNA copies/ $2.10^6$  cells. The results regarding the viral load were expressed as log of multiples of UI/ml (= 2.7 Copies/ml).

### Detection of 5'NC Variants in PBMCs by Cloning and Sequencing

HCV-specific RNA was extracted from PBMCs (100 µl) using QIAamp viral RNA Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. The 5'NCR was amplified by RT/PCR by using the set of primers designed from the IRES region (*IRES forward* (nt 21–40): *gac act cca cca tga atc ac*, *IRES reverse* (nt 360–374): *gtt ttt ctt tga ggt tta gg*). PCR was carried out for 40 cycles (94°C-30 sec, 55°C-15 sec, 72°C-30 sec). The final PCR products of 354 bp were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed under UV light. These PCR products were purified with QIAquick PCR purification kit (Qiagen) as specified by the manufacturer and quantified by UV Spectrophotometry. Amplicons were ligated directly into pCR2.1-TOPO vector (Invitrogen, San Diego, CA), 1 min at room temperature. Recombinant plasmids were used to transform *Escherichia coli* competent cells (Invitrogen). Transformants were grown on Luria-Bertani Broth agar plates containing ampicillin (100 mg/ml) and incubated overnight at 37°C. More than 24 independently isolated cDNA clones from PCR products were selected. Plasmid DNAs containing 5'NCR sequences were extracted by the alkaline lysis method with the Concert Rapid Plasmid Purification System (Life technologies, GIBCO BRL products, Gaithersburg, MD). A total of 352/456 clones were quantified by spectrophotometry and sequenced bidirectionally by the ABI PRISM automated sequencer by using M13 universal primers. Electrophoresis data obtained by the automatic sequencer were analyzed by using the sequences navigator BLAST search. Artefactual quasiespecies could result

from polymerase-derived errors introduced during amplification or from selection during amplification and cloning procedures. A thermostable polymerase with proof-reading functions (BRL Life Technologies) was used in order to minimize these artefacts. Moreover, if incorporation errors from polymerase were implicated, an increment in mutations from single mutant to multiple mutants should have been observed, which was not the case. These two points argued in favor of the reliability of our sequence data. Sequences were aligned and compared with the sequence prototypes or the nearest prototype (PBIL, IBCP, Lyon). Sequence alignments were reviewed manually; mutation sites were identified by the CLUSTAL alignment multiple software and edited prior to phylogenetic analysis. Sequence gaps were excluded from the analysis. The non-random distribution of the observed substitutions was incompatible with artefacts related to errors made by DNA polymerase during PCR reaction. In some patients, the HCV-IRESs appeared chimeric, with successive blocks of sequence reminiscent of different genotypes. This may suggest multiple recombination events.

### Phylogenetic Tree

Viral sequences were aligned by using CLUSTAL X software version 1.8. Neighbor joining analyses were performed by using DNADIST and NEIGHBOR in the PHYLIP package, version 3.572, with pairwise distances estimated by using Kimamura two parameters distances. We analyzed the robustness of different branches by bootstrapping (103 replicates). Tree was visualized with Tree View v32.

### Statistical Analysis

Results are presented as means  $\pm$  standard error of the mean or as percentages. Differences between the two groups were assessed by Spearman's correlation and coefficient test, Kruskal–Wallis test and Wilcoxon test. Factors with  $P < 0.05$  were considered as significant.

## RESULTS

### Quasispecies Distribution of the HCV-5'NCR in the PBMCs of Patients

The PBMCs from 18 patients (5 women, 13 men) with chronic HCV disease were collected before treatment initiation. Quantitation of HCV in the PBMCs was assayed according to adaptive modification of the standard method. A fragment of 341 nt-long 5'Non-coding region (5'NCR) including the IRES was analyzed. After cloning in *Escherichia coli*, an average of 19 clones from each patient was sequenced and each IRES sequence was aligned with the reference sequence strands: HCV-J4 for genotype 1b [Okamoto et al., 1992], HCV NZL1 for genotype 3a [Okamoto et al., 1993] and HCV-S83 or HCV CH-563 for genotype 2 or with the closest prototype reference strains after BLAST research. Although the 5'NC region appeared to be

highly conserved structurally; a quasispecies distribution was found between the different isolates obtained. Indeed, 467 substitutions distributed all along the IRES were included in 131 variants ( $>7$  variants/patients). These variants were characterized by the presence of both major variants; without any nucleotidic changes, accounting for 1/3 of the clones studied and, the minor variants; accounting for 2/3 of the clones which undergo changes.

### Location of the Mutations on the Predicted Secondary and Tertiary Structures of HCV-IRES

The observed 467 mutations were then positioned on the predicted IRES secondary structure [Honda et al., 1999b] (Fig. 1) and out of the 341 nucleotides forming the IRES sequence, only 102 nucleotidic sites underwent mutations. It means that the nucleotidic change observed on a particular site will be found on the numerous other clones of the same patient. These particular sites, gathering numerous substitutions ( $>2\%$  of mutations), were called "clustering position". Alignment of sequences confirmed that these clustering sites did not result by cross contaminations. Indeed, in each patient, numerous sequences presented both clustering positions and minor mutations and these minor mutations were different among isolates of the same patient. Nine clustering positions gathering 3/4 (70%—332/467) of the total mutations were observed. These nine clustering positions were evident for nucleotidic sites 78, 104, 107 in domain II, gathered 35.4% of the changes (166/467); sites 183, 204, 206 in domain IIIb gathered 15.4% of the mutations (72/467); position 243 in domain IIIc and 259 in domain IIIId gathered 4.5% (21/467) and 2.5% of the mutations (12/467) respectively. In the smaller stem-loop IV; 13.1% (61/467) of mutations on position 340 were found. Out of the 467 mutations, only one mutation (U to C) on nt 343 inside the AUG-4 initiator codon was observed. As the paired regions without compensation were very important for the stability of the structure, a majority of mutations occurred in the paired regions were such that maintained the base-pairing. Out of the 467 changes observed in the viral population, only a minority (21.5%) would be expected to result in a loss of base-pairing, that is, an alteration of the IRES structure. Changes on some positions were transferred in a concomitant way at numerous clones (29%). They acted on positions 78-104-206 and 259 and on positions 107, 183, and 340. Finally, the GGG triplet between nucleotides 266 and 268 of the hexanucleotide apical loop of stem loop III was conserved in all of the observed sequences except the clones found in two patients for which two mutations of nt 267 (G to C) and two deletions of nt G in position 268 have been found.

### Relationship Between Pre-Treatment IRES Complexity and the Viral Load

As the global viral population infecting PBMCs was considered at the outset, the viral load was on average of

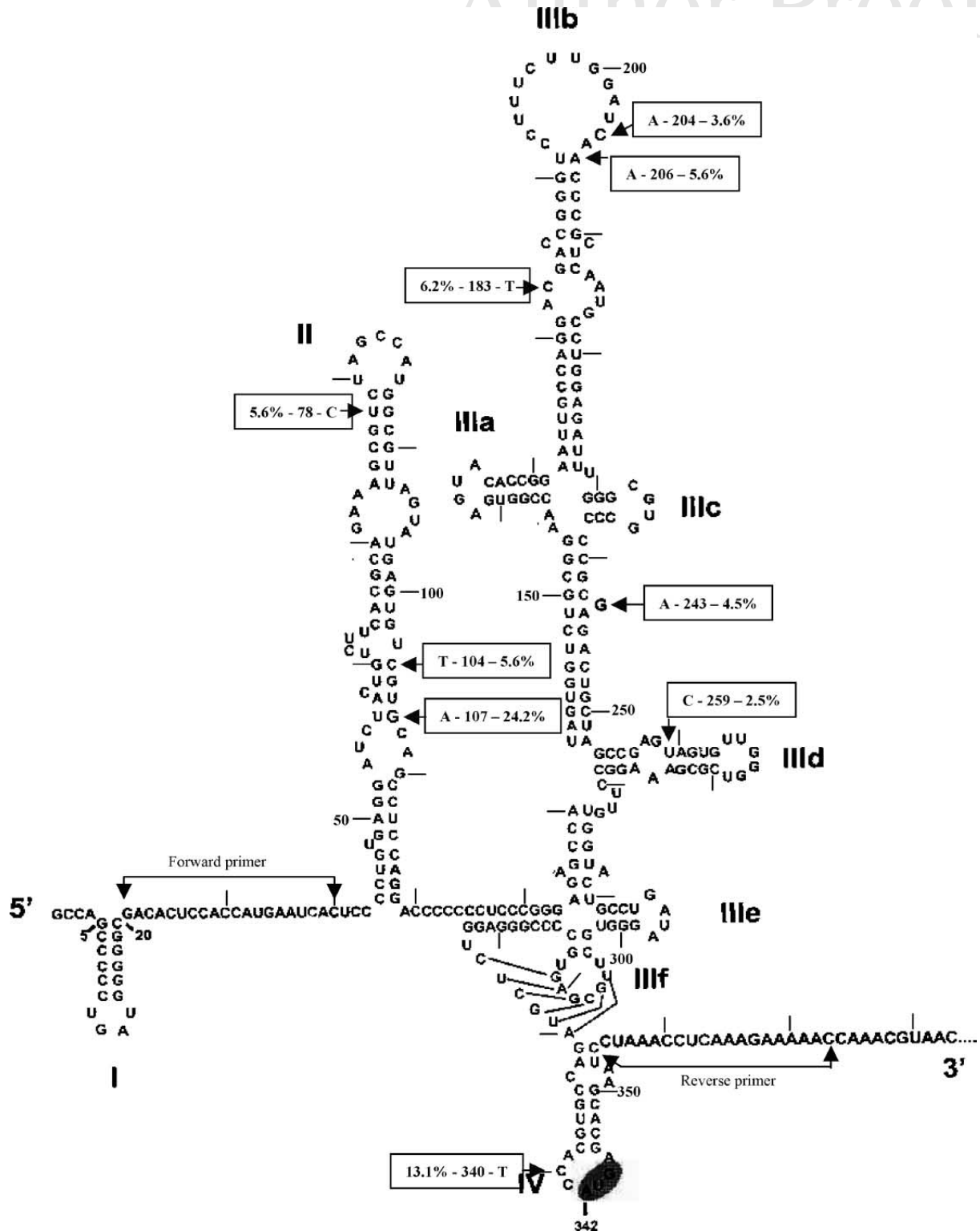


Fig. 1. Predicted secondary and tertiary RNA structures within the 5'NCR of the type 1b virus HCV-J4 [Okamoto et al., 1992]. The AUG codon at nt 342 in stem-loop IV is highlighted. Major structural domains are labelled I, II, III, and IV. The set of primers used are positioned on the predicted secondary structure [forward (nt 21-40), reverse (360-374)]. Principal mutations positioned on the predicted IRES secondary and tertiary RNA structure of IRES observed among viral population infecting patients. Apparition frequencies appears in italics.

1,591 ± 1,637 UI/ml ( $3.2 \log_{10} \pm 3.2$  UI/ml), whereas, the number of viral genomes in the serum was on average of 1,660 712 ± 1 617 850 UI/ml ( $6.2 \log_{10} \pm 6.2$  UI/ml).

No relationship was observed between pre-treatment IRES variability and the viral load at Day 0 (data not shown).

### Evolution of Viral Load at the Early Stage of Treatment

During the first 3 days of treatment with IFN alpha-2a, the viral load in PBMCs decreased by 50.3% ( $2.9 \log_{10} \pm 3.1$  vs.  $3.2 \log_{10} \pm 3.2$  UI/ml;  $P = 0.1341$ ) and by 88% ( $5.3 \log_{10} \pm 5.3$  vs.  $6.2 \log_{10} \pm 6.2$  UI/ml;  $P = 0.0001322$ ) in the serum. A decrease occurred significantly in the serum, whereas, a decreasing tendency was noted in the PBMCs.

In parallel, the study of the evolution of viral load in ex vivo 6 hr cultured cells showed that these values decreased by 94.5% ( $1.9 \log_{10} \pm 2.2$  vs.  $3.2 \log_{10} \pm 3.2$  UI/ml;  $P = 0.00003642$ ) when the cells were treated with IFN alpha-2a alone and by 94.2% ( $2.0 \log_{10} \pm 2.3$  vs.  $3.2 \log_{10} \pm 3.2$  UI/ml;  $P = 0.00001015$ ) when the cells were treated with IFN alpha-2a plus ribavirin.

Decrease of viral load in PBMCs was more important in ex vivo experiment than in in vivo treatment. Addition of ribavirin did not affect the ex vivo response. No relationship was observed between pre-treatment IRES variability and the viral load at the early stage of the treatment.

The evolution of viral load during the first three days of treatment was considered and the patients might be classified into two groups:

- Group 1: included 1/3 of the patients ( $n = 6$ ), whose viral load in the PBMCs did not decrease during the therapy (increasing by 104%) ( $3.2 \log_{10} \pm 3.3$  vs.  $2.9 \log_{10} \pm 3.0$  UI/ml;  $P = 0.4848$ ). In the serum, a significant decrease of viral load by 82% was noted ( $5.4 \log_{10} \pm 6.1$  vs.  $6.2 \log_{10} \pm 6.3$  UI/ml;  $P = 0.09307$ ). As the later response to the treatment (more than 6 months) was considered, it should be notice that four out of six patients included in this group were non-responders.
- In parallel, a significant decrease by 83% of the viral load occurred in the ex vivo study ( $2.1 \log_{10} \pm 2.3$  vs.  $2.9 \log_{10} \pm 3.0$  UI/ml;  $P = 0.1797$ ) when the cells were cultured with IFN alpha-2a for 6 hr. Obviously, in the ex vivo experiment, viral resistance to treatment has disappeared.
- Group 2: This group included the remaining 2/3 of the patients ( $n = 12$ ) with a significant decrease of the viral load by 77% in the PBMCs ( $2.7 \log_{10} \pm 2.7$  vs.  $3.3 \log_{10} \pm 3.2$  UI/ml;  $P = 0.01727$ ) and by 90% ( $5.2 \log_{10} \pm 5.3$  vs.  $6.2 \log_{10} \pm 6.1$  UI/ml;  $P = 0.000656$ ) in the serum during the first 3 days of the therapy. However, the later response to treatment (more than 6 months) has shown that

3 out of 12 patients of this group were non-responders.

- In addition, during the first 6 hr of cultured cells with IFN alpha-2a, viral load decreased by 97% ( $1.8 \log_{10} \pm 2.1$  vs.  $3.3 \log_{10} \pm 3.2$  UI/ml;  $P = 0.0001955$ ). It was noticed that even in this group, the viral resistance mechanisms might have disappeared in the cultured cells but the differences between the evolution of viral load values in in vivo and ex vivo experiments were less notable (77% vs. 97%).
- Briefly, (i) in vivo: viral load decreased in the serum of both groups (decreasing 82% and 90% respectively), but the evolution of viral load in the PBMCs of both groups was different: increasing by 104% in group 1 and decreasing by 77% in group 2; (ii) ex vivo: as significant decreases of viral load in PBMCs occurred in both groups, however, addition of IFN alpha-2a in the cultured PBMCs of group 1 was less effective than in the group 2 (83% vs. 97%).

### Analysis of Relationship Between Viral Load and IRES Complexity in Both Groups

Comparison between the average PBMCs viral load from group 1 and 2 at the beginning of therapy showed no significant difference ( $2.9 \log_{10} \pm 3.0$  vs.  $3.3 \log_{10} \pm 3.2$  UI/ml;  $P = 0.09182$ ). However, in group 1, the corresponding viral population gathered 37% of the total quasispecies (49/131). The 303 mutations (303/467—65%) observed in these variants were mainly (254/303—84%) distributed on seven clustering sites 78, 104, 107, 183, 206, 243, and 340 (Fig. 2); in group 2, the corresponding viral population included 63% of the variants (82/131) which concentrated 164 mutations (164/467—35%). Out of these, 81/164 (49%) mutations, which composed of these quasispecies appeared on six clustering sites such as 78, 104, 107, 204, 206, and 259, whereas, changes on site 183, 243, and 340 were undetectable (Fig. 3).

It was noted that in group 1, less variant was observed than in group 2, but it gathered more changes (303 and 164 respectively;  $P = 0.001045$ ). Significant difference between group 1 and group 2 for the number of clustering substitutions (254 vs. 81 clustering mutations;  $P = 0.0006438$ ) was observed. In both groups, mutations were distributed all along the IRES genome; however in group 1, majority of them were concentrated on few nucleotidic sites.

Some positions (78, 104, 107, and 206) were retrieved in both groups but mutations on these sites were encountered more frequently in group 1 than in group 2 (Figs 2 and 3): (i) in stem-loop II, U to C at nt 78 (4.3% vs. 1.5%), C to U at nt 104 (4.3% vs. 1.3%), G to A at nt 107 (17.6% vs. 6.6%); (ii) in stem-loop III, nucleotide C insertion at 206 (4.3% vs. 1.3%). In addition, mutations C to U (6.2%) at position 183 in domain III, G to A (4.5%) at nt 243 in stem-loop IIIc and C to U (13.1%) at position 340 in stem-loop IV, occurred



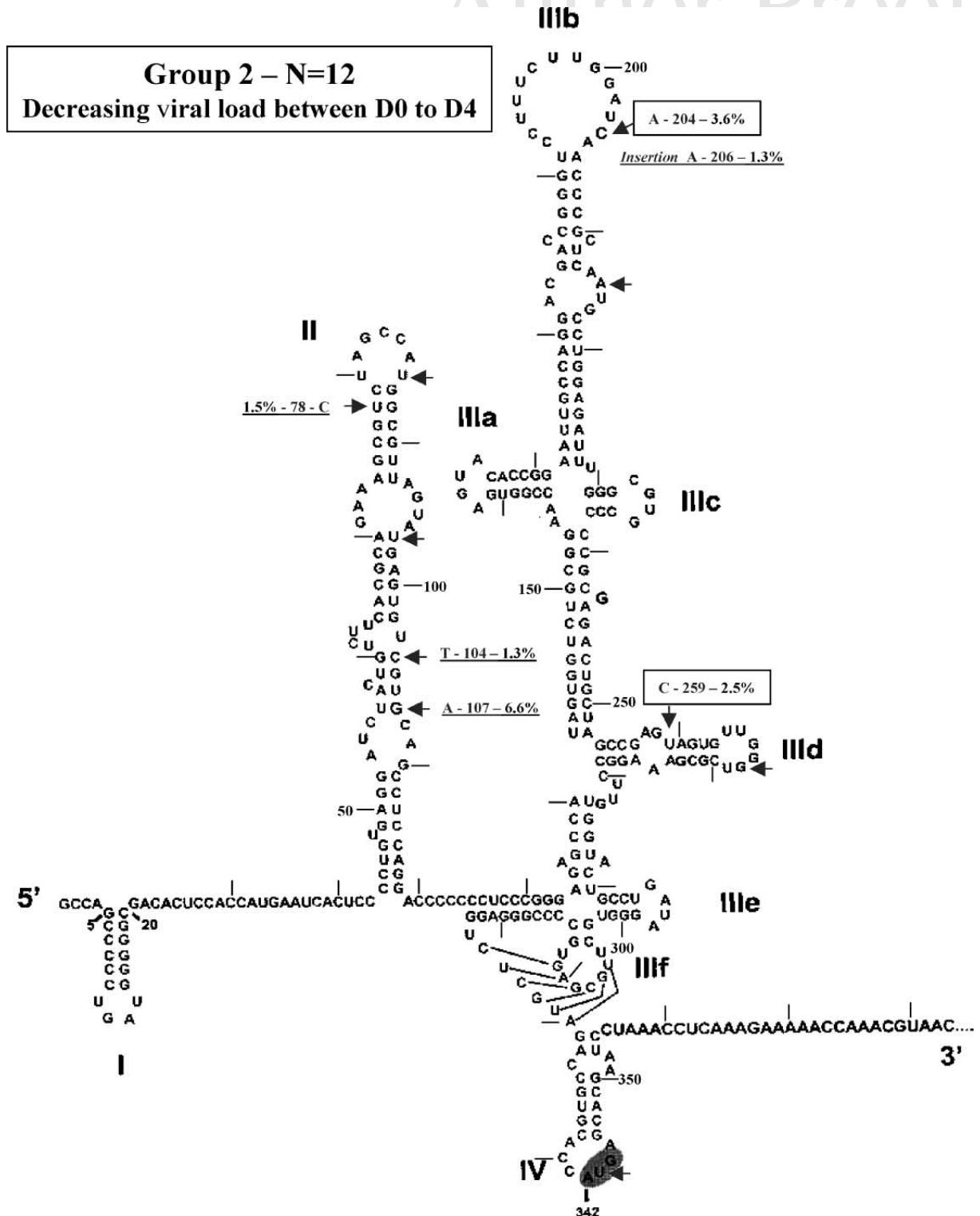


Fig. 3. Principal mutations positioned on the predicted IRES secondary and tertiary RNA structure of IRES observed in the patients with a significant decrease of viral load during the therapy. Apparition frequencies appears in italics. The arrows specify the sites, which are changed in a minor way.

located in domain II, start at positions 85–87 (AUG-1) and 96-98 (AUG-2). Mutation on nt 87 (G to C) inside AUG-1, on positions 96 (A to G) and 97 (U to C) inside AUG-2 were encountered only in viral population from

group 1. In the smaller bulge IV, mutations at nt 340 and 350, flanking both sides of the AUG-4 initiator codon were found. Upstream mutations frequency of this initiator codon was present only in group 1. In addition,



one mutation was observed at nt 343 inside the AUG-4 initiator codon in group 2.

## DISCUSSION

The presence of negative-strand RNA in PBMCs from infected patients has been demonstrated [Moldvay et al., 1994; Lanford et al., 1995; Laskus et al., 2000; Lerat et al., 2000; Ducoulombier et al., 2004] which suggests the existence of a potentially important extrahepatic site of HCV replication [Roque Afonso et al., 1999; Bain et al., 2001; Laporte et al., 2003; Roque-Afonso et al., 2005]. Many viruses possess strategies for evading or limiting the interferon-induced antiviral response and lead to progression of a disease. HCV-IRES variability is one of the mechanisms by which the virus might escape the treatment. Indeed, strong selection pressure against any mutations in this region alters the structure of the HCV-IRES [Soler et al., 2002]. Moreover, previous works [Gale et al., 1998a,b; Odreman-Macchioli et al., 2000, 2001] have demonstrated that correct RNA-protein interactions involving the HCV-IRES are highly dependent on both the primary nucleotide sequence and its ability to form complex of secondary and tertiary RNA structures. Recently, Dash et al. [2005] have showed that IFN-alpha, -beta, and gamma inhibit replication of HCV in a cell culture model by directly inhibiting the internal translation site of HCV-IRES.

Clustering sites observed in HCV-IRES sequences were mainly located in domains II (sites 78, 104, 107–35%), III (sites 183, 204, 206, 243, 259–22%) and IV (site 340–13%) (Fig. 1) and mutations on positions 107, 204, and 243 might reflect a viral phenotype that was particularly well adapted for replication in the lymphoid cells with a specifically enhanced HCV translation in these cells [Nakajima et al., 1996; Maggi et al., 1997; Shimizu et al., 1997; Navas et al., 1998; Lerat et al., 2000]. In spite of that, no significant difference in viral load (reflecting enhance of translational activity) was related to these nucleotidic sites in this study. These results agreed with previous observations [Yamamoto et al., 1997]: sequence variability of HCV-IRES was not appearing to correlate with any difference in HCV-RNA concentration in the PBMCs, which reflects the level of HCV replication. Obviously, the viral population has established an equilibrium between the quasispecies and immune constraints.

In parallel with this quasispecies characterization, viral load of patients who carried these variants was studied. Before treatment, viral load in the serum was 3 log greater than in the PBMCs (6.2 log vs. 3.2 log). During the first 3 days of in vivo treatment, viral load decreased more significantly in the serum than in the PBMCs (88% vs. 50%). These results agreed with investigators who indicated that human lymphoid cells were permissive for replication of HCV but at low-level [Shimizu et al., 1993, 1997], nevertheless, the HCV-RNA level in the serum remained high (5.3 log vs. 6.2 log). Obviously, IFN treatment has an impact on the

circulating viral particles, but the intracellular viruses seemed to be affected in a less significant manner. Then the evolution of in vivo viral load during the first 3 days of therapy was observed, and the results allowed the patients to be classified into two groups. These two groups behaved differently according to the in vivo or ex vivo context. Indeed, during in vivo treatment, viral load was affected by both mechanisms: one by which the viral population escape from the treatment and continued to replicate and another by which IFN alpha-2a has an impact on the viral genome. In ex vivo treatment, HCV might not replicate and might not evade the treatment or the immune pressure, as the viral load decreased significantly. These results suggested that at an early stage of the treatment, mechanisms of response affected directly the capacity of the virus to replicate, whereas, the adaptive immunological response did not occur yet. As viral replication depends on the IRES integrity, it might be suggest that IRES complexity play a key role in this phenomenon.

Previous results [Laporte et al., 2000] have demonstrated that IRES activity was independent of the number of additional mutations detected but that the nucleotide location which played the most important role for IRES efficiency. So location and frequency of the clustering mutations observed before therapy were investigated in order to understand the behaviour of the viral population.

The HCV-IRES domain II induces conformational changes on the ribosome which have been implicated in the decoding process [Kalliampakou et al., 2002]. The introduction of mutations in this domain can cause a moderate or total loss of translational ability [Kalliampakou et al., 2002; Kim et al., 2003]. In this study, three clustering sites (78-104-107) located in base paired region on the stem-loop II gathered numerous changes (35.4%). These mutations were higher in patients among whom virus continued to replicate than in patients with decrease of viral population. Viral load which reflect the ability of HCV to replicate was not affected by these mutations. However, the importance of stem-loop II for IRES function has been discussed controversially and the changes that occurred in the base-paired regions have not been demonstrated as affecting the translational activity of HCV-IRES [Rijnbrand et al., 1995; Reynolds et al., 1996; Honda et al., 1999a; Odreman-Macchioli et al., 2001; Kalliampakou et al., 2002; Da Rocha Gomes et al., 2004].

The apical stem-loop IIIb has been shown to bind initiation factor eIF3. Disruption of the secondary structure of this region results in the loss of both eIF3 binding and IRES activity [Odreman-Macchioli et al., 2001; Collier et al., 2002]. In this study, changes in position 183, located in the stem loop IIIb, were often associated with the changes in positions 107 and 340 in group 1. These latest mutations observed only in the group of patients whose viral load did not change could lead to stabilisation of the IRES structure. In the same way, insertion in the site 206 was associated with

mutations in position 204 in group 1 and, might likely contribute to stabilise the IRES complexity.

The correct secondary structure of IIIId has also been suggested to be important for the binding of ribosomal protein S9. Indeed, mutations within the IIIId stem-loop abolish IRES activity [Kieft et al., 2002]. In this study, the mutated site with the most frequent changes located on the stem-loop IIIId, was in position 259 found only in the patients with decreasing viral population. Consequently, it is suggested that the mutation encountered in this region did not allow the three-dimensional structure to be conserved between isolates of varying primary sequence and could impair viral replication. Finally, the changes observed in position 243 (likely important for maintain the IRES structure) encountered only in the patients with stabilization or increase of viral load, might serve the virus to escape from the treatment, whereas, mutations in site 259 could expose the virus to IFN action.

The mutations observed at positions 340 and 350, flanking both sides of the AUG-4 initiator codon, inside the smaller stem-loop IV which modulated the translation during virus replication [Collier et al., 2002], were only found in the patients with increasing viral population. This result was agreed with Honda et al. [1996] who have demonstrated that mutations which destabilize the stem-loop IV restored translation to normal.

Study of the later response to treatment showed that even in group 2 with a significant decrease of the viral load at the start, some patients did not respond to the therapy at the later stage. IFN alpha-2a induced inhibition affected definitively the virus only in ex vivo replication. In contrast, in the group in which the virus continued to replicate during the first 3 days of treatment, some individuals respond in the later stage of therapy. These results suggest that, at the early stage of therapy, the virus alone was the key of the response, and that the HCV complexity influenced the response to treatment. In contrast, during treatment, other mechanisms implying host immune defences were set up. These observations agreed with those of von Wagner et al. [2003] who concluded that although quasispecies evolution during IFN therapy is common, it occurs after a wide range of time intervals after initiation of therapy. However in some cases, at the early stage of treatment, escape mechanisms were set up quickly, reflecting a fast adaptation of the virus to its environment.

Obviously, some viral population are more sensitive to therapy than others but these mechanisms remain to be explored. Although, IFN alpha-2a was efficient in reducing the viral replication in a significant number of cases at the early stage of therapy, non-responsiveness to IFN cannot be explained exclusively by the changes in quasispecies. Consequently, pre-treatment genomic variations observed in the 5'NCR of HCV are not parameters of later response to antiviral therapy in chronic hepatitis C patients.

Several genomes and/or viral protein functions and their interactions with numerous host cell functions are

likely involved in HCV resistance to the non-specific antiviral action of IFN alpha-2a in the infected cells.

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## **Article 5**

### **Characterization of circulating dendritic cells in melanoma, role of CCR6 in PDC recruitment to the tumor site**

Julie Charles, Jérémy Di Domizio, Dimitri Salameire, Nathalie Bendriss-Vermare, Ramzan Muhammad, Joël Plumas, Marie-Thérèse Leccia and Laurence Chaperot

Submitted article

**Article 5**  
**Characterization of circulating dendritic cells in melanoma, role of CCR6 in PDC recruitment to the tumor site**

Dendritic Cells (DCs) are the immune cells whose main function is to process antigen material and present it on the surface to other cells of the immune system. They can be divided as Myeloid Dendritic Cells (MDC) and Plasmacytoid Dendritic Cells (PDC). MDC are most similar to monocytes and their main function is antigen presentation while PDC look like plasma cells and produce high amounts of IFN- $\alpha$  and were known as IPC (interferon-producing cells). The interaction between tumor cells and the host immune system are complex, involving a multitude of cell types and mediators. Induction of effective tumor immunity can be viewed as a three-step process that includes firstly, appropriate presentation of tumor-associated antigens (TAA), secondly, selection and activation of TAA-specific T cells as well as non-Ag-specific effectors and, lastly, homing of TAA-specific T cells to the tumor site and effective elimination of malignant cells expressing TAA (Sogn 1998). DCs are the crucial cells providing the necessary components for initiating and developing effective cell-mediated immune responses. Dendritic cells, located in most tissues of the body, capture and process Ags, which are then displayed as MHC-peptide complexes on the DC surface. Essential co-stimulatory molecules are upregulated on DCs as they migrate to secondary lymphoid organs (the spleen and lymph nodes) where they liaise with naïve T cells, inducing the activation and proliferation of Ag specific CTLs (Timmerman and Levy 1999). Escape from immune surveillance is believed to be a fundamental biological feature of malignant disease in man. Defects in immune response in patients with a variety of tumors and in tumor-bearing animals have been well documented (Saththaporn and Eremin 2001). A key element in the induction of the anti-cancer immune response, namely TAA presentation to T cells in a tumor-bearing host, has been poorly documented and inadequately studied.

In the following article, we found that the frequencies of circulating DC subsets were equivalent in healthy controls and melanoma patients. In melanoma patients both PDC and MDC displayed an immature phenotype and can have a potential to mature after appropriate stimulation. Moreover, CCR6 expression by the circulating PDC in melanoma patients was higher than normal control while no difference was observed in the expression levels of CCR6 on MDC in the two groups. CCL20/MIP-3-alpha was found to produce in the tumor microenvironment which might be involved in the recruitment of CCR<sup>+</sup> PCD in the melanoma tumors.

**Title:**

Characterization of circulating dendritic cells in melanoma, role of CCR6 in PDC recruitment to the tumor site.

**Running Title** : Circulating DC in melanoma

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## Abstract

Dendritic cells are central cells in the development of antitumor immune responses, but the number and function of these cells can be altered during the course of various kinds of cancers. In this study, we investigated the presence, phenotype, and functionality of circulating myeloid dendritic cells (MDC) and plasmacytoid dendritic cells (PDC) in newly diagnosed melanoma patients. Flow cytometric analysis showed that the frequencies of PDC and MDC were equivalent in melanoma patients as compared with normal subjects. Both circulating DC subsets were immature, but upon *ex vivo* stimulation with R848 (a synthetic TLR7 and TLR8 ligand) they efficiently up-regulated their expression of costimulatory molecules. We also analyzed the expression of chemokine receptors by circulating PDC and MDC, and found that DC from melanoma patients and controls displayed similar pattern of expression of CXCR3, CXCR4, CCR7, and CCR10. Strikingly, PDC from melanoma patients expressed higher levels of CCR6 than control PDC, suggesting that this receptor could play a role in PDC recruitment to the tumor. This hypothesis was supported by our further data showing that CCR6-expressing PDC were present in melanoma primary lesions, and that CCL20/MIP3-alpha was produced in melanoma tumors. Altogether, these results suggest that PDC and MDC are fully functional in melanoma patients at the time of diagnosis, and that CCL20/MIP3-alpha may participate to their recruitment from the blood to the tumor.

## Introduction

During the past decades, there has been a huge increase in the incidence of cutaneous malignant melanoma among all the caucasian populations as well as in the number of death due to this cancer throughout the world. Overall 5-year survival is superior to 95% for the patients with localized tumors of less than 0.75 mm in depth while it drops below 40% for localized melanomas with Breslow index superior to 4 mm. Such a poor outcome highlights the need to further develop new therapeutic strategies, among which immunotherapy is a promising one.

Melanoma cells are sensitive to lysis by cytotoxic T lymphocytes (CTL) because they express immunogenic tumor-associated antigens (TAA) such as Melan A, gp100, tyrosinase and Mage3 (1) and this property provides basis for vaccine development. Besides vaccination with TAA (with or without dendritic cells) (2) and innovative treatments based on adoptive transfer of antitumor CTL (3), the most common immunotherapy used in melanoma is the injection of Interferon (IFN) alpha. Therapeutic efficiency of IFN-alpha relies both on its direct antitumor effect and its ability to enhance the immune responses. IFN-alpha can be proposed as an adjuvant for the treatment of malignant melanoma but its use remains controversial. However, the latest study by the European Organization for Research and Treatment of Cancer (EORTC) shows that the adjuvant therapy with pegylated IFN-alpha-2b has a beneficial effect on recurrence free-survival in melanoma patients especially in patients at stage IIIa (4). Another way to take advantage of Type I IFNs antitumor effects could be to induce their endogenous production by plasmacytoid dendritic cells (PDC) which represent a major source of IFN-alpha *in vivo*. Indeed, these cells secrete huge amounts of IFN-alpha upon recognition of ssRNA and ssDNA viruses by Toll-like receptor (TLR)7 and TLR9 respectively (5, 6). PDC are usually detected in the peripheral blood (7) and lymphoid organs, (8) in the normal humans while in pathological situations such as lupus or psoriasis they have been found in the inflamed skin producing type I IFNs (9, 10). Besides producing IFN-alpha, activated PDC also express costimulatory molecules and secrete other cytokines and chemokines, such as CXCL10/IP-10, thus participating in the recruitment of effector cells of the innate and adaptive immunity (11, 12). Moreover, PDC like MDC are able to prime Th1, Th2, and regulatory T cells (13, 14) and can also efficiently stimulate CD8+ T lymphocytes upon appropriate activation (15).

In the context of cancer immunotherapy, these properties suggest that it could be relevant to target PDC in order to induce an endogenous secretion of Type-I IFNs as well as an activation

of innate and adaptive immunity. Among the new adjuvants developed for cancer treatment, CpG ODN and imiquimod are known to activate PDC via TLR9 and TLR7 respectively, demonstrating antitumor effects in clinical trials (16, 17). Moreover, we and others have demonstrated that activated PDC can acquire cytotoxic functions by expressing the death-inducing ligand TRAIL on their surface (18, 19). This killer activity may participate in the antitumor effect of therapeutic TLR7 or TLR9 agonists. Indeed, the few studies analyzing the immunological effects of these therapies have shown PDC activation (20-22), local secretion of IFN-alpha (23) and TRAIL expression at the treated site (19). The most impressive clinical results have been obtained in the superficial basal cell carcinoma with complete histological clearance of the treated tumors in almost 80 % of the cases after topical treatment with imiquimod (24). In the pilot studies performed in melanoma, such treatment has resulted only in limited regression or stabilization of lentigo malignant melanoma (25) or cutaneous melanoma metastasis (26, 27). However, in order to improve the efficiency of these new TLR agonists-based treatments in melanoma, it is necessary to improve the knowledge of DC functionality and ability to respond to TLR ligands in this cancer.

The purpose of this study is to characterize circulating MDC and PDC in melanoma patients and to evaluate whether they are altered or not either quantitatively or qualitatively. We demonstrate the ability of MDC and PDC isolated from melanoma patient's blood to mature upon TLR7/8 triggering in vitro. We also describe the expression of chemokine receptors by circulating DC, showing that CCR6 expressed at higher level on PDC from melanoma patients at the time of diagnosis compared to healthy individuals could participate to their migration to the tumor site where CCL20/MIP3-alpha was produced.



## **Materials and methods**

### ***Patients***

71 patients (43 men and 28 women) diagnosed as cutaneous melanoma were enrolled in the study. Fresh blood samples were obtained from 43 patients in the 3 weeks following the initial diagnosis of melanoma before the wide local excision of the primary lesion and any complementary surgical or medical treatment. 33 patients were at stage I to II according to the American Joint Committee on Cancer classification (28), 9 patients were in stage III (according to lymph node analysis) and one patient was at stage IV (pulmonary metastasis were present at the time of initial staging). Fresh blood samples of 22 healthy controls were also studied. 19 confirmed primary cutaneous melanoma samples were screened for immunohistochemical analysis. Tumor supernatants were obtained from 12 fresh metastatic tumor samples. The mean age of the patients at sampling time was 58 years (ranging from 21 to 87). The written informed consent was obtained from each subject.

### ***Antibodies and flow cytometry***

Immunophenotyping was performed by flow cytometry on a FACScan or FACScalibur (BD Biosciences, Mountain View, CA, USA), using direct and indirect labeling. Monoclonal antibodies (mAbs) conjugated with biotin, FITC, PE, PECy-5 or APC were used. Anti-lineage Cocktail 1 (Lin1, a mix of CD3, CD14, CD16, CD19, CD20 and CD56), -CD11c, and -CD123 (9F5) antibodies were obtained from Becton Dickinson (San Jose, CA USA). Anti-BDCA2 (AC144) and anti-BDCA-4 (AD5-17F6) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). PE-conjugated Control IgG1 (679.1Mc7), anti-HLA-DR (B8.12.2), streptavidin, CD40 (HIT2), CD45RO (UCHL1), CD62L (Dreg46), CD80 (MAB104), CD83 (HB15A), CD86 (HA5.2B7), Goat F(ab')<sub>2</sub> Fragment anti-mouse IgM, and purified IgM (GC323) were obtained from Immunotech (Marseille, France). Goat F(ab')<sub>2</sub> anti-rabbit IgG antibodies (GAR) was purchased from CALTAG Laboratories (USA), PE-conjugated-anti-CCR6 (11A9), anti-CXCR3 (1C6), anti-CXCR4 (12G6) and purified anti-CCR7 (2H4) antibodies were purchased from Pharmingen (San Diego, CA USA). Purified anti-CCR10 antibody was from IMGENEX Corporation (San Diego, USA).

### ***Enumeration and phenotyping of dendritic cells.***

Blood dendritic cells were identified by multicolour staining performed on whole blood. Briefly, 200µL heparinized blood was used for each staining tube. Cells were incubated for 20

min at room temperature with FITC-Lin1, APC-CD11c, and biotinylated-anti-BDCA4, washed, then further incubated for 20 min with PeCy-5-streptavidin. Red cell lysis was performed by the addition of 2 mL of FACSLysing solution (Becton Dickinson) for 10 min according to manufacturer's instructions. After washing with PBS, cells were acquired by flow cytometry. At least  $2 \times 10^5$  PBMC were acquired according to the forward light scatter (FSC) versus side light scatter (SSC) profile. Among Lin<sup>neg</sup> cells, MDC were recognized as CD11c<sup>+</sup> BDCA4<sup>neg</sup> cells whereas PDC were CD11c<sup>neg</sup> BDCA4<sup>+</sup> and their respective percentages among PBMC were determined.

For surface marker analysis of costimulatory molecules or chemokine receptor expression, PE-conjugated mAbs were added to the previously described cocktail that allowed the differential analysis of PDC and MDC phenotype.

### ***Activation of PBMCs***

Whole blood samples (2ml) from healthy donors and melanoma patients were cultured for 24 h with or without 1  $\mu\text{g/mL}$  of R848 (TLR7/8 ligand), (Invivogen, San Diego, CA USA). Blood samples were then handled as previously described and immunostained for the following activation markers: CD40, CD80, CD83, CD86 or CCR7. Each marker was associated with PE-labeling and the cocktail of FITC-Lin, biotinylated-BDCA-4, APC-CD11c, followed by labeling with PeCy-5-streptavidin. At least  $2 \cdot 10^5$  PBMC were acquired by flow cytometry. Among Lin<sup>neg</sup> cells, MDC were recognized as CD11c<sup>+</sup> cells whereas PDC were CD11c<sup>neg</sup> BDCA4<sup>+</sup> and their respective expression of activation markers was determined.

### ***Immunohistochemistry***

Alcohol-formalin-acetic acid (AFA)-fixed and paraffin embedded surgical specimens from 13 cases of confirmed melanoma were cut at 3  $\mu\text{m}$ , deparaffinized, and pretreated in Tris citrate Buffer (pH=8, 100°C, 30min). A double immuno-staining was performed. In brief, non-specific sites were blocked by donkey serum (2%, 30 min, room temperature). The first anti-BDCA2 antibody (Clone 104C12, Dendritics, Dardilly, France) was incubated at room temperature overnight at the dilution of 1:25. The sections were incubated with biotinylated-donkey anti-mouse IgG for 60 min, then treated with peroxidase-conjugated avidin-biotin complex (ZYMED Laboratories, Histostain Plus Bulk Kit, Invitrogen Corporation, Carlsbad, CA, USA) and followed by immunodetection with diaminobenzidine (DAB). After heating antigen retrieval with citrate buffer (pH=6, 30 min, 100°C), the sections were sequentially

incubated with the second anti-CCR6 antibody (Clone 53103, R&D System, Mineapolis, MN, USA) at the dilution of 1:200 for overnight at room temperature, followed by secondary staining with biotinylated-donkey anti-mouse IgG for 60 min, peroxydase–conjugated avidin-biotin complex and revealed by Histogreen Peroxydase Substrate (Vector laboratories, Burlingame, CA, USA). Control included the omission of the first primary antibody in the second immunolabelling step.

Cryopreserved surgical specimens of primary cutaneous melanoma (6 samples) were used to evaluate CCL20/MIP3-alpha expression. Frozen 8 µm tissue sections were i) fixed with acetone and 4% paraformaldehyde, ii) preprocessed with 0.3% H<sub>2</sub>O<sub>2</sub> (Sigma) followed by an avidin and biotin blocking step (Blocking kit, Vector Laboratories), iii) incubated with blocking rabbit serum for 30 min, iv) stained with polyclonal goat anti-MIP-3a/CCL20 IgG (R&D Systems) for 1 h, and v) revealed by biotinylated rabbit anti-goat IgG (Vector Laboratories) and Extravidin-Peroxydase (Sigma) with the AEC substrate (Vector Laboratories).

### ***Measurement of CCL20/MIP3-alpha chemokine concentrations***

Melanoma supernatants were obtained from 12 cutaneous or nodal metastasis. In brief, fresh tumor samples were dilacerated and digested in collagenase (Roche Diagnostics GmbH, Mannheim Germany). The cell suspension was seeded at the concentration of  $1 \times 10^6$  cells per ml. Then, the supernatant was recovered after 24 h. The concentrations of MIP3-alpha were measured by ELISA (R&D Systems).

### ***Statistical analysis***

The Mann-Whitney U test was used for the comparisons between independent groups.

## Results

### **The frequency of circulating PDC and MDC is intact in melanoma patients**

Circulating MDC or PDC numbers are modified in various kinds of pathologies. We first asked whether it could be the case in melanoma at the time of diagnosis. We analyzed the frequency of the two main subsets of blood DC in 22 healthy donors and 43 melanoma patients. A multicolour staining of MDC and PDC by flow cytometry allows the identification and counting of both DC subsets among the peripheral blood mononuclear cells (PBMC) (Fig.1A). After gating to exclude debris and most polynuclear cells (Fig 1A, left dot plot), a selection of lineage (CD3, CD14, CD16, CD19, CD20 and CD56) negative ( $lin^{neg}$ ) cells was performed (Fig 1A, middle dot plot). Among these  $lin^{neg}$  cells, PDC and MDC were identified as  $BDCA4^+CD11c^-$  and  $BDCA4^+CD11c^+$  cells respectively (Fig 1A, right dot plot) and their respective percentages in PBMC were determined. We checked that both subsets of DC expressed high levels of HLA-DR molecules and PDC were  $BDCA2^+$  and  $CD123^{hi}$  whereas MDC were  $BDCA2^-$  and  $CD123^{low}$  (Fig 1B). As shown in figure 1C, the mean percentage of circulating MDC and PDC in the control individuals was  $0.49\pm 0.18\%$ , and  $0.30\pm 0.17\%$  respectively. Among PBMC from melanoma patients, MDC represent  $0.50\pm 0.23\%$  and PDC  $0.29\pm 0.20\%$ . Altogether, these data show that the frequencies of circulating DC subsets are equivalent in healthy controls and melanoma patients.

### **Blood PDC and MDC display an immature phenotype in melanoma patients**

In order to determine whether the quality of DC could be altered in the context of melanoma, a detailed phenotypic analysis of circulating MDC and PDC was then performed in the melanoma patients and healthy controls. A typical expression of costimulatory molecules by MDC and PDC is shown in the figure 2A (for a control healthy volunteer). Both subsets of DC expressed neither CD80 nor CD83 whereas they both expressed CD40 and CD86 and these later markers were expressed at a higher level by MDC than PDC (Fig 2B). Moreover, both subsets expressed high levels of CD62L (data not shown). There were no obvious differences in costimulatory molecules expression by circulating DC between healthy controls and melanoma patients at the time of diagnosis. As in healthy subjects, both PDC and MDC displayed an immature phenotype in the blood from melanoma patients.

## **The maturation potential of circulating DC is maintained in melanoma patients**

In order to determine whether the ability of immature DC to mature in melanoma patients remains intact, original cell culture experiments were performed by adding R848 in the whole blood samples to activate both PDC and MDC *via* TLR7 and TLR8, respectively. After 24-hour incubation, we evaluated the up-regulation of molecules associated with DC maturation on both MDC and PDC by multicolor flow cytometry. As illustrated in figure 3, both MDC and PDC highly up-regulated their expression of CD40, CD80, CD83, CD86, and CCR7 after activation with R848. Moreover, table I shows that MDC and PDC from melanoma patients and healthy controls matured to the same extent upon activation by TLR-7/8 ligand, indicating that circulating DC retain their ability to mature in melanoma.

## **Blood PDC from melanoma patients express CCR6, a skin homing receptor**

To further characterize DC in the context of melanoma and try to understand their recruitment in the tumor site, we analyzed their chemokine receptors expression profile. As illustrated in figure 4A (for a healthy donor), both MDC and PDC expressed high levels of CXCR4 and intermediate levels of CCR10. As already described, PDC expressed high levels of CCR7 and low levels of CXCR3 whereas MDC were CCR7<sup>low</sup> and CXCR3<sup>hi</sup>. As shown in figure 4B, there were no differences between circulating MDC and PDC regarding CXCR3, CXCR4, CCR7, and CCR10 expression between the healthy controls and melanoma patients.

Strikingly, while analyzing CCR6 expression, we found that circulating PDC from some melanoma patients expressed higher levels of CCR6 as compared to PDC from healthy donors (Fig 5A). To go further, we increased the number of melanoma patients in our study for CCR6 expression by PDC in the two defined groups. As shown in figure 5B, significantly higher CCR6 levels ( $p < 0.005$ , Mann-Whitney U test) were detected on circulating PDC from melanoma patients (mean:  $34 \pm 21\%$  of positive cell) as compared to healthy controls ( $16 \pm 8\%$ ). CCR6 was expressed by more than 40% of circulating PDC in 15 out of 41 melanoma patients (36%) which was never the case in the control group. In contrast, no difference was observed in the expression levels of CCR6 on MDC in the two groups ( $52 \pm 17\%$  and  $55 \pm 18\%$ ). These results suggest that CCR6 is specifically up-regulated on circulating PDC in melanoma patients.

## **CCR6<sup>+</sup> PDC infiltrate melanoma tumors where CCL20/MIP3-alpha is produced by melanoma cells**

We investigated by immunohistochemistry whether CCR6<sup>+</sup> PDC and CCL20/MIP3-alpha producing cells could be detected in the primary melanoma tumors. By using BDCA2 a specific marker for PDC, we detected PDC in 4 cases (30%) out of 13. BDCA2<sup>+</sup> PDC were located close to the tumor within the peritumoral leukocyte infiltrate, representing 2 to 5% of these cells (data not shown). In 2 of these cases, we identified BDCA2 positive cells co-expressing CCR6 (representative immunohistochemical data shown in fig 6A, see arrows). Subsequently, we investigated the expression of the chemokine CCL20/MIP3-alpha on cryosections from primary melanomas specimens. MIP3-alpha was produced by melanoma cells in 5 out of 6 analyzed samples (representative staining Fig 6B). Moreover, we found MIP3-alpha in 7 out of 12 fresh melanoma metastasis supernatants (Fig 6C). Altogether these data suggest that CCL20/MIP-3-alpha is produced in the tumor microenvironment and might be involved in the recruitment of CCR6<sup>+</sup> PDC in the melanoma tumors.

## Discussion

Dendritic cells are central cells in the development of antitumor T cell responses. However, the relative contribution of PDC and MDC to antitumor immune responses is still a matter of debate. New cancer immunotherapeutic strategies targeting DC *in vivo* are currently under development, but their efficacy will directly rely on the availability of functional dendritic cells. This study focused on phenotype and functionality of the circulating dendritic cell in melanoma patients.

Equivalent frequencies of circulating MDC and PDC were found in the healthy controls and melanoma patients, contrarily to what has been described in other pathologies including cancers. Indeed, a decrease in the blood MDC and PDC frequencies has been described during the course of various kinds of haematological malignancies (29-32). In these cases low DC counts might be due to an alteration of “dendritopoiesis” during the disease progression. Elsewhere, in advanced solid tumors (33) such as breast cancers (34) or hepatocellular carcinomas (35), reduced frequencies of circulating DC or their absolute numbers have also been reported. In these advanced cancers, it has been suggested that decreased DC numbers are due to systemic tumor-induced immunosuppression. Our results show that the frequencies of circulating PDC and MDC are not affected in melanoma patients, suggesting that these cells could be targeted to stimulate immune responses in these patients.

We next analyzed the functionality of circulating DC in melanoma patients. Indeed, abnormalities in the maturation of MDC have been found in many kinds of cancers (review (36)) but very few data is available regarding the functionality of PDC. By analyzing the expression of costimulatory molecules, we found that the circulating MDC and PDC from melanoma patients were very similar to that of healthy donors and were immature. Furthermore, upon *in vitro* activation with R848, a TLR7/8 agonist, both MDC and PDC from melanoma patients matured as efficiently as MDC and PDC from healthy volunteers. These data show that the circulating DC in melanoma patients have retained their ability to respond to TLR-ligands which is encouraging for the development of alternative treatments based on TLR7 or TLR9 agonists. Although these molecules are still early in their clinical development (16, 17), they seem efficient regarding skin cancers. We have recently demonstrated that PDC produce IFN-alpha and express TRAIL upon activation (18), suggesting that they may participate in the efficacy of imiquimod. In the basal cell carcinomas treated by topical application of imiquimod, it has been shown that PDC are recruited in the tumor site (19, 23)

along with other immune cells. The mechanisms leading to this recruitment are still poorly understood.

MDC are constitutively recruited into the skin epithelium *via* CCR6 while PDC are not found in this peripheral tissue under steady state conditions. However, PDC accumulate in the cutaneous lesions of systemic lupus erythematosus (10) or psoriasis (9) where they secrete IFN- $\alpha$ . The recruitment of PDC in such inflammatory contexts has been suggested to depend on CXCR3 in synergy with CXCR4 (37, 38) or on ChemR23 (39). PDC have also been found in the infiltrate of various kinds of tumors including carcinomas of the head and neck (40), breast (41), lung (42), and ovarian cancers (43, 44). Our observation confirms the presence of tumor-infiltrating PDC in melanoma (45, 46) and we confirmed here their presence. It has been proposed that melanoma cells attract PDC via SDF-1/CXCL12 (43, 44, 46). We have analyzed the expression of chemokine receptors by the circulating PDC and as expected PDC expressed CXCR4 that could be involved in their recruitment to tumor site. Strikingly, circulating PDC from melanoma patients expressed higher levels of CCR6 as compared to healthy volunteers and PDC expressing CCR6 were detected in primary tumors. CCL20/MIP3- $\alpha$ , a ligand for CCR6, is expressed by keratinocytes in the skin and also by melanoma cells (47-49) (Figure 6), suggesting that CCR6/CCL20 could participate in the migration of PDC from the blood to the tumor. We thus propose that this CCR6-MIP3 $\alpha$  interaction could constitute a new way of PDC recruitment to primary melanoma. Interestingly, we have already described the expression of CCR6 on leukemic PDC (LPDC), a kind of malignancy that frequently develops into the skin (50). The question of the induction of CCR6 on PDC has to be further investigated. In this context, Bendriss-Vermare *et al.* recently demonstrate that IL-3 is able to up-regulate CCR6 expression on primary human PDC (submitted manuscript). It remains to determine whether the mechanism responsible for CCR6 expression by blood PDC from melanoma patients involves IL-3 or other factors that could be produced either by the tumor or by other cells during the development of melanoma. In the context of melanoma, TLR7 or TLR9 agonist mono-therapies have been used (20, 21, 51). Other studies have also tested topical application of imiquimod (R837) (52) or subcutaneous injection of PF-3512676 (CpG 7909) (53) as adjuvant for vaccination to improve T cell responses. Altogether, our data encourage the development of such therapeutic strategies based on the activation of DC to induce antitumor immune responses. Indeed, we demonstrate here that both MDC and PDC are available, functional, and mobilizable in the early phases of melanoma development.



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**Table I. R848-induced activation and maturation of PDC and MDC in melanoma patients and healthy donors<sup>a</sup>.**

		<b>Controls (N=7)</b>		<b>Melanoma patients (N=12)</b>	
		<b>PDC</b>	<b>MDC</b>	<b>PDC</b>	<b>MDC</b>
<b>CD40</b>	<b>T0</b>	86 ( 8)	67 (13)	77 (10)	71 (13)
	<b>24h medium</b>	79 ( 3)	94 ( 2)	75 (20)	97 ( 4)
	<b>24h R848</b>	94 ( 9)	98 ( 3)	87 (13)	96 (12)
<b>CD80</b>	<b>T0</b>	6 ( 6)	5 ( 3)	7 ( 5)	9 ( 5)
	<b>24h medium</b>	14 (11)	39 (11)	22 (11)	37 (16)
	<b>24h R848</b>	72 (18)	89 (10)	65 (19)	75 (18)
<b>CD83</b>	<b>T0</b>	8 ( 9)	5 ( 4)	7 ( 2)	7 ( 2)
	<b>24h medium</b>	14 (11)	46 (19)	22 ( 9)	31 (16)
	<b>24h R848</b>	77 (19)	86 (10)	63 (16)	65 (19)
<b>CD86</b>	<b>T0</b>	56 (16)	96 ( 2)	53 (16)	83 ( 5)
	<b>24h medium</b>	43 (17)	95 ( 6)	33 ( 9)	71 (24)
	<b>24h R848</b>	70 (22)	98 ( 2)	72 (13)	95 (14)
<b>CCR7</b>	<b>T0</b>	74 (23)	26 (24)	65 (22)	36 ( 6)
	<b>24h medium</b>	86 ( 4)	74 ( 5)	68 (25)	53 (14)
	<b>24h R848</b>	98 ( 3)	72 (29)	81 (24)	77 ( 9)

a- Whole blood cells were cultured in the presence of R848 to activate both MDC and PDC. The mean (standard deviation) percentage of positive cells for each marker was analyzed for MDC and PDC in melanoma patients and controls.

## Figure legends

### ***Figure 1. Frequencies of circulating PDC and MDC in melanoma patients and healthy donors are equivalent.***

Dendritic cell subsets were identified and quantified by flow cytometry. A) Identification of MDC (CD11c<sup>+</sup> BDCA4<sup>neg</sup>) and PDC (CD11c<sup>-</sup> BDCA4<sup>+</sup>) (right dot plot) within whole blood mononuclear (left dot plot) lineage negative (middle dot plot) cells. Numbers indicate the percentages of PDC and MDC within peripheral blood mononuclear cells. B) Representative phenotype of MDC and PDC taken from one of the control healthy volunteer. C) The frequencies of MDC (left panel) and PDC (right panel) in the peripheral blood of melanoma patients and healthy controls are shown. (Horizontal bars represent mean percentages).

### ***Figure 2. Circulating dendritic cells from melanoma patients display an immature phenotype***

A) Representative comparative phenotype of MDC (CD11c<sup>+</sup>, upper row of dot plots) and PDC (BDCA4<sup>+</sup>, lower row of dot plots) from one of the healthy control. B) Expression of the activation/maturation markers by MDC and PDC from melanoma patients (black bars, n=34) and healthy controls (white bars, n=15). Mean percentages and standard deviation are shown.

### ***Figure 3. Activation profiles of circulating MDC and PDC in response to TLR7/8 ligand.***

Evaluation of activated MDC (lin<sup>neg</sup>, CD11c<sup>+</sup>) and PDC (lin<sup>neg</sup>, BDCA2/BDCA4<sup>+</sup>, CD11c<sup>-</sup>) after 24 hours culture of whole blood with R848. The expression of CD40, CD80, CD83, CD86, and CCR7 is increased on both DC subsets after culture with R848 (dark grey) as compared to control culture (light grey). Representative graph was taken from a healthy control.

### ***Figure 4. Expression of chemokine receptors by circulating dendritic cells from melanoma patients.***

A) Representative expression of chemokine receptors by MDC (CD11c<sup>+</sup>, higher dot plots) and PDC (BDCA4<sup>+</sup>, lower dot plots) (taken from one of the healthy donor). B) Expression of chemokine receptors CXCR3, CXCR4, CCR7 and CCR10 by MDC and PDC from melanoma



patients (black bars, n=16) and healthy controls (white bars, n=14). Mean percentages and standard deviation are shown.

**Figure 5. Expression of CCR6 by PDC from melanoma patients**

A) Representative CCR6 expression for MDC (CD11c<sup>+</sup>, upper row of dot plots) and PDC (BDCA4<sup>+</sup>, lower row of dot plots) from two melanoma patients (Mel D1 and Mel D2, middle and right dot plots) and a healthy donor (control, left). B) Frequencies of MDC (left panel) and PDC (right panel) expressing CCR6 were determined by flow cytometry in the peripheral blood from melanoma patients and healthy donors. Horizontal bars represent the mean percentage of positive cells.

**Figure 6. Detection of CCR6-expressing PDC in melanoma microenvironment and of CCL20/MIP3-alpha production by melanoma cells.**

A) BDCA-2<sup>+</sup> PDC (brown) can be seen in the peritumoral area among the infiltrating leucocytes in melanoma (original magnification: x20). High magnification of the 3 insets show CCR6-expressing PDC (green) (original magnification: x64). B) Sections of frozen melanoma tumors stained with anti-CCL20/MIP3-alpha (red) showing its production in the tumor (2 representative sections of 5 positive tumors out of 6 analyzed) (original magnification: x20). C) Measurement of MIP3-alpha secretion in 12 supernatants of melanoma metastasis taken after 24 hours of culture.

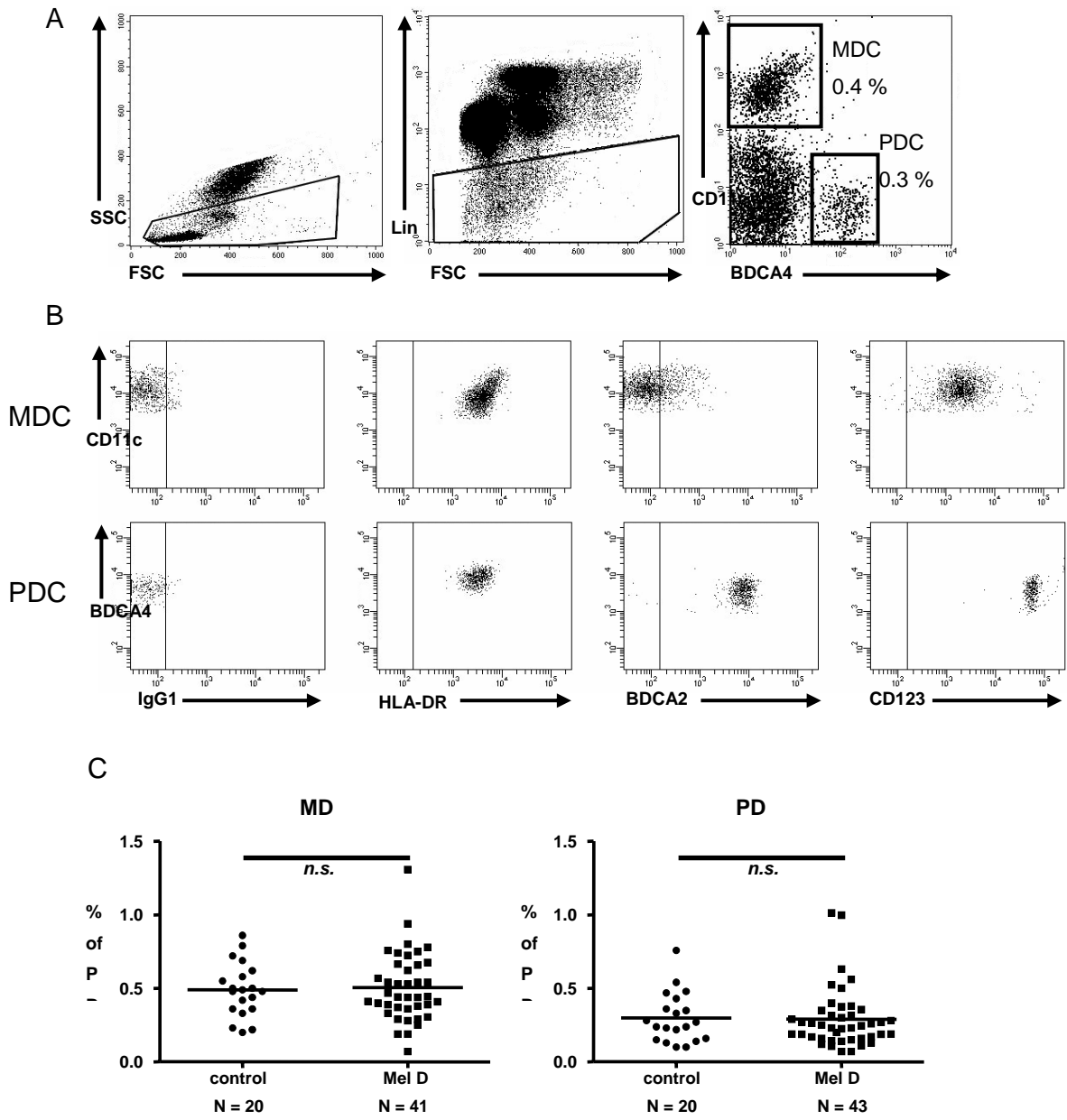
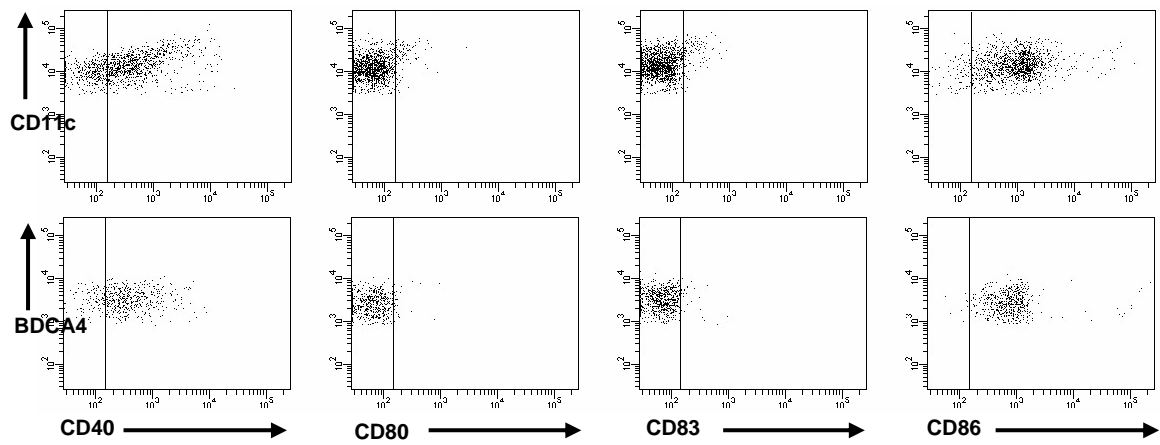


Figure 1

A



B

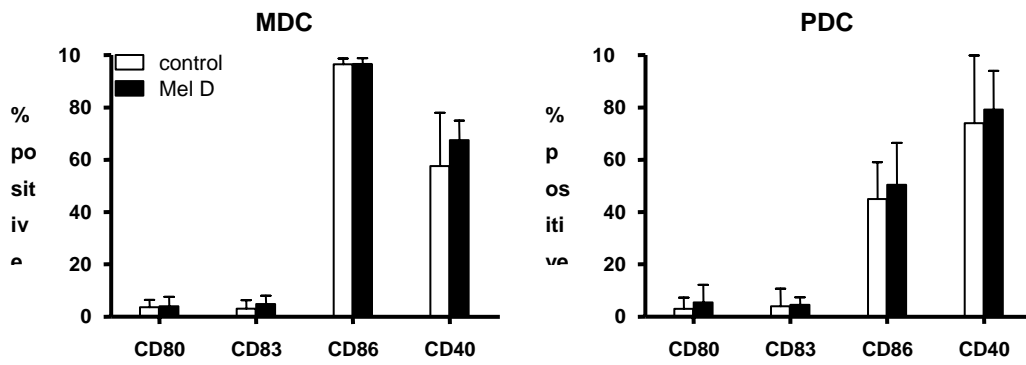


Figure. 2

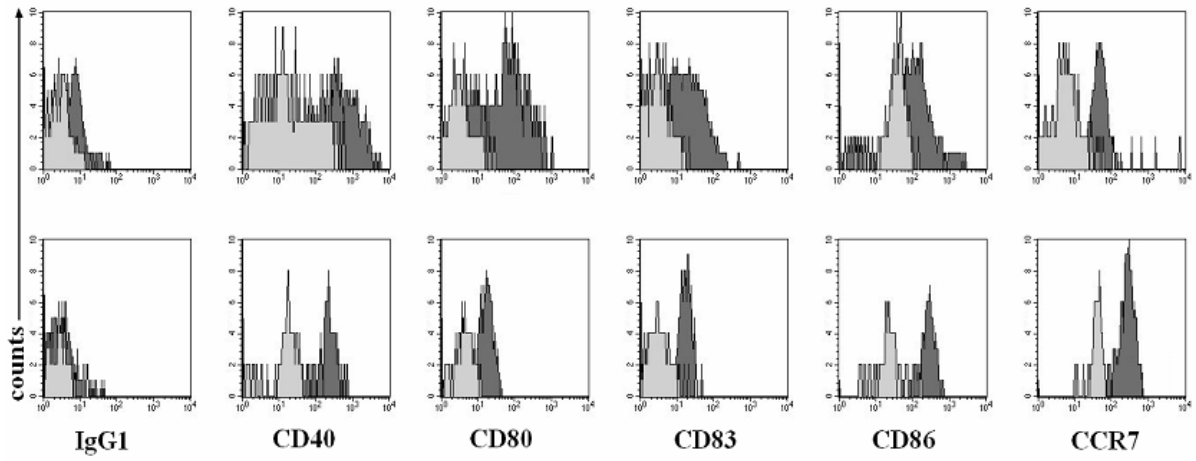


Figure 3

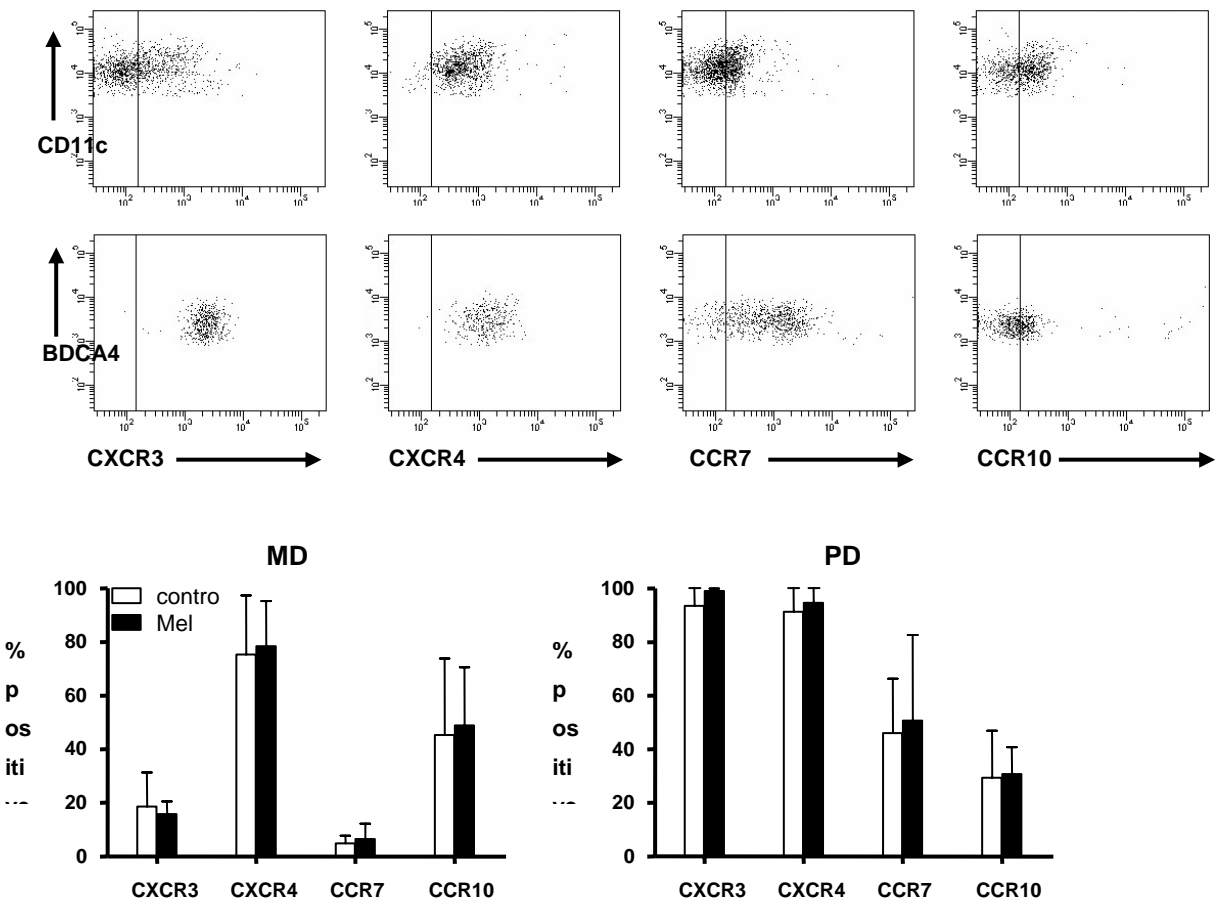


Figure 4

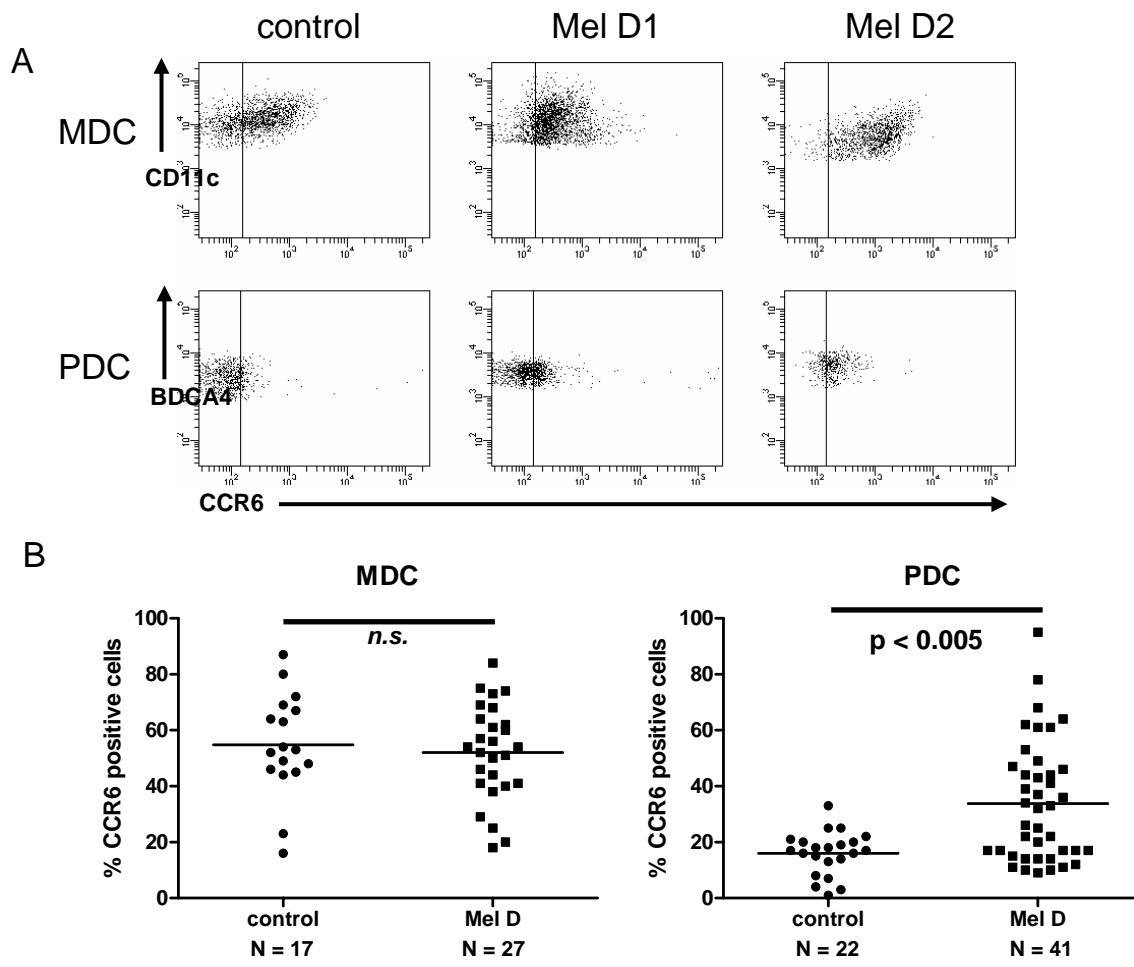


Figure 5

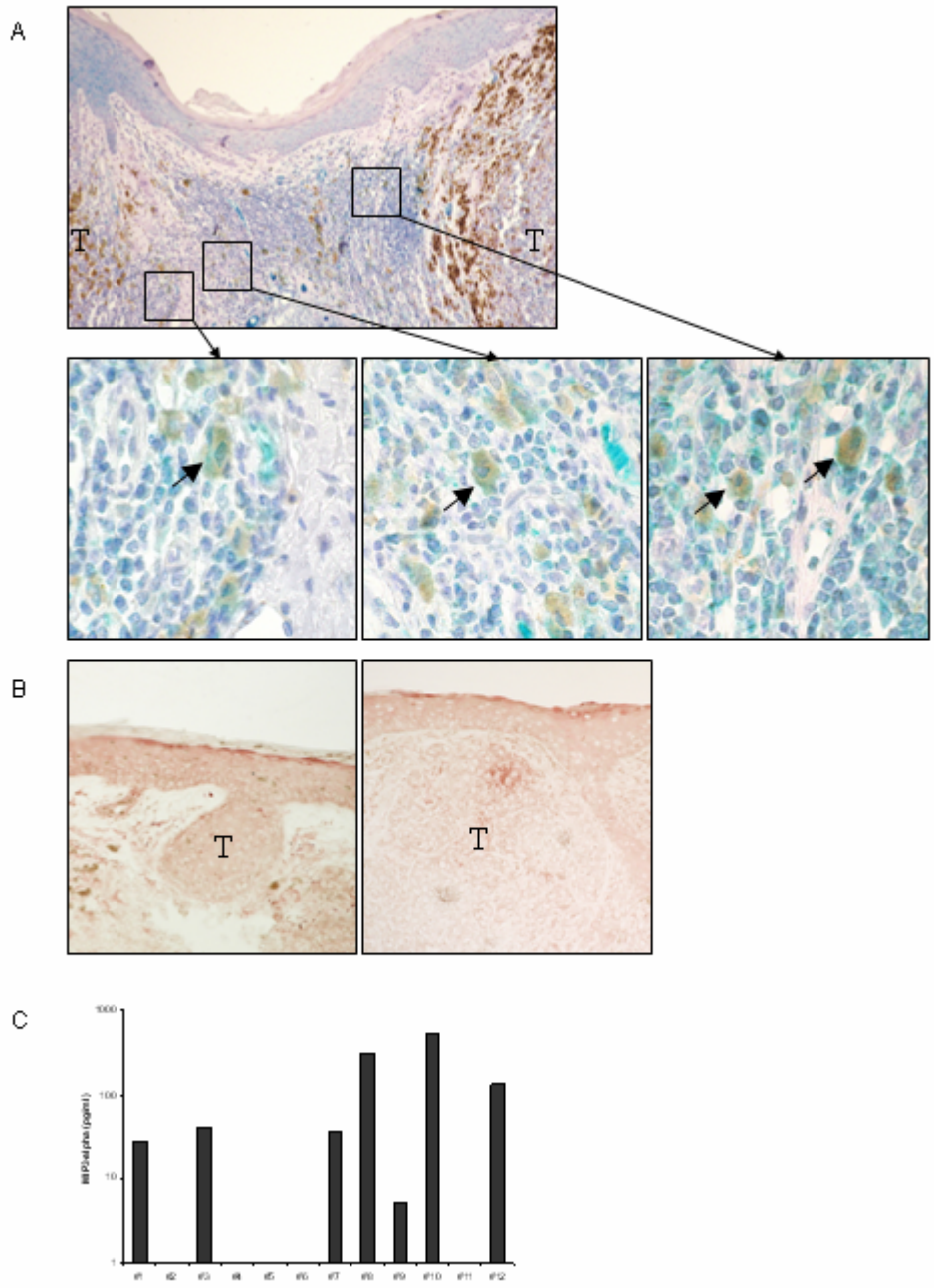


Figure 6

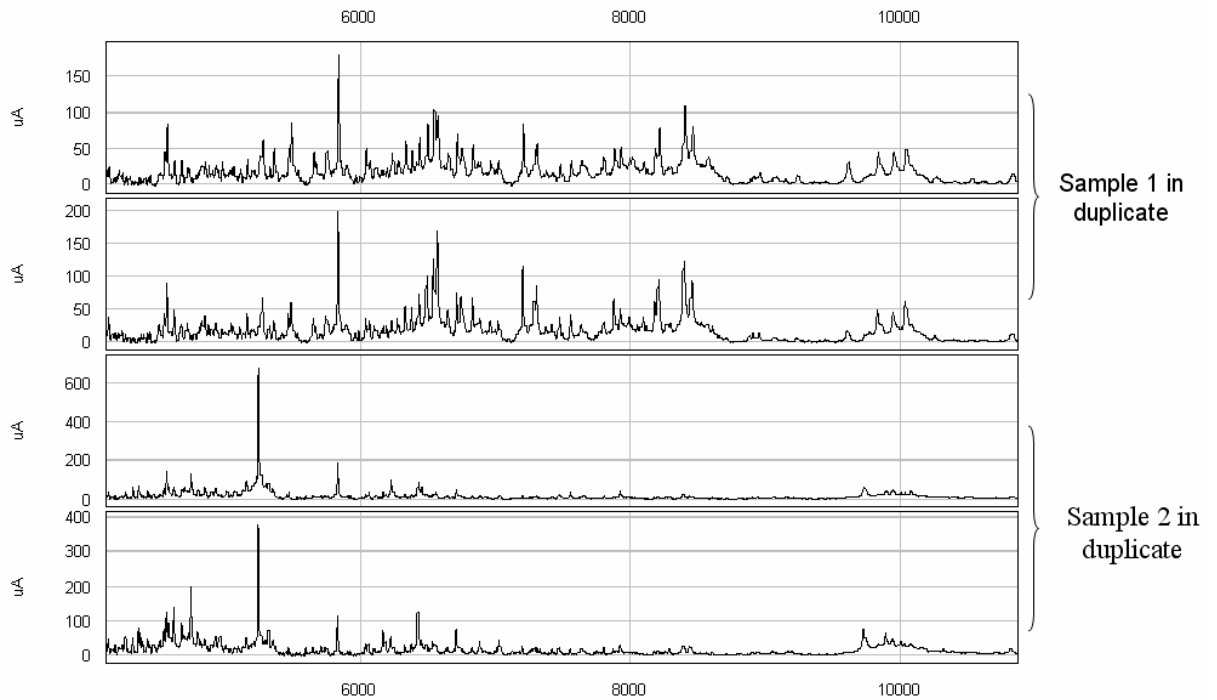
## **Evaluations of new markers for HCC by SELDI-TOF MS (Surface-Enhanced Laser Desorption Ionization Time-Of-Flight Mass Spectrometry).**

In light of the poor prognosis in advanced HCC and to improve patient survival significantly, development of new markers for HCC is mandatory. Up till now, HCC diagnosis has been relied on several tools combining imaging techniques, measurement of serum AFP and other markers including tumor associated antigens (Szklaruk, Silverman et al. 2003; Bhosale, Szklaruk et al. 2006; Looi, Nakayasu et al. 2008). It is need of the hour to identify the new markers, for diagnostic, therapeutic and for tumor specific immune response, in HCC patients. Development of new technologies for isolation of biomarkers in the cancer diseases currently is in progress. The recent development of proteomic array technology, including serum protein profiling, coupling ProteinChip Array with surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS; SELDI-TOF MS ProteinChip technology, Ciphergen Biosystems, Fremont, CA), provides a potentially powerful tool for the discovery of new markers, as demonstrated in human immunodeficiency virus infection and in patients with prostate, pancreatic, or ovarian malignancies (Ardekani, Liotta et al. 2002; Issaq, Veenstra et al. 2002; Koopmann, Zhang et al. 2004). This procedure results in the identification of protein profiles composed of isolated or clustered peaks characterized by molecular weight, enabling the discrimination between different pathological conditions with high sensitivity and significant reproducibility.

Mostly, SELDI-TOF MS techniques have been carried out by using serum or soluble proteins. Some other protocols like direct analysis of fresh frozen sections (Moroz, Gillette et al. 1999; Stoeckli, Chaurand et al. 2001), individual cell or cluster of cells isolated by laser-capture microdissection or contact blotting of a tissue on a membrane target (Chaurand, Stoeckli et al. 1999) have also been employed. We first time tried this technique on the frozen liver tissues and got very promising results. We used two types of tissues, one from the tumor and other from the non- involved liver outside the tumor and got different imaging profile on the spectra of SELDI-TOF MS. Here, we represent some results obtain from four liver and four tumor tissues from HCC patients. Two patients had HCC with alcoholic origin (HCC-OH) and other two had HCC with HCV (HCC-HCV).

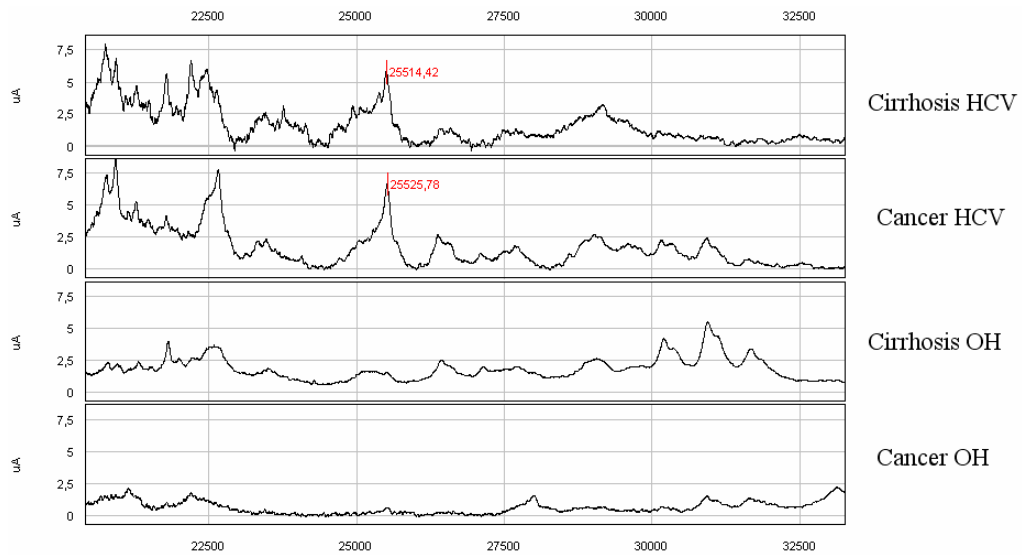






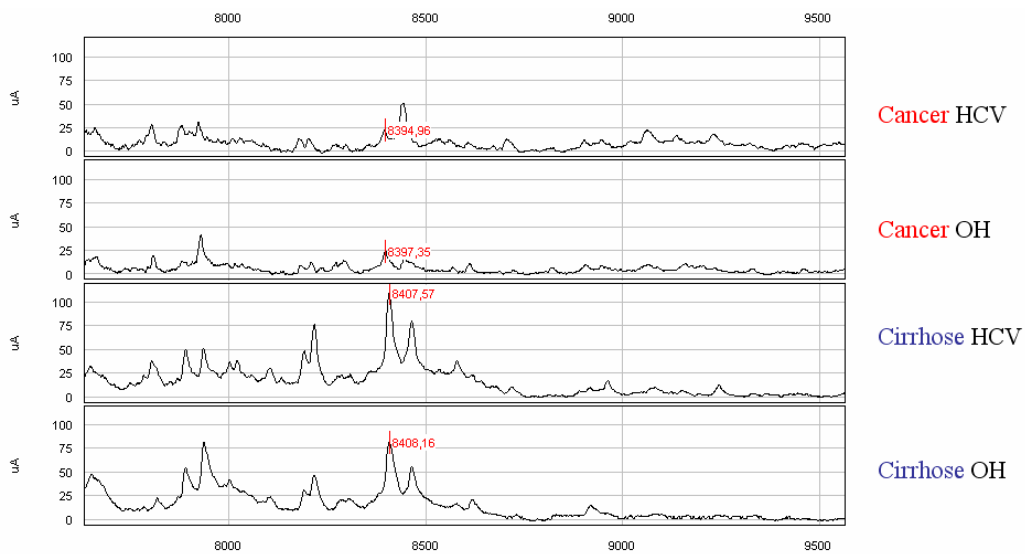
**Fig. 15. SELDI-TOF MS protein profile** (from 4000 to 10,000 mass-to-charge ratio). It is obtain from the two HCC samples, one from HCC-HCV and other from HCC-OH. The protein profile peaks clearly different in between these two samples (sample 1 = HCC-HCV; sample 2 = HCC-OH).

- i. *Feasibility of the experiment:* The protein profile spectra of the two tumor samples of HCC i.e. HCC-HCV and HCC-OH shown in the figures above clearly show that proteins of the two tumors have different protein profiles (Fig 14, 15). This data represents the feasibility of the experiment on liver tissue samples. This could be useful for the identification of new biomarkers for tumor with different pathological origin.
- ii. *Comparison between two different types of tissues within same HCC group:* In the figure 15, we can find some difference and similarities in the two protein profiles from cirrhosis versus cancer in HCC-HCV and HCC-OH groups. We found 42 peaks with significant p values in the non-involved cirrhotic liver versus tumor samples from HCC-HCV and HCC-OH groups. This indicates the differences and similarities in the proteins profile present in the two types of tissues from the same patient.



**Fig. 16.** SELDI-TOF MS protein profile of non-involved cirrhotic liver and cancer with HCV and alcohol. (from 20,000 to 32,500 mass-to-charge ratio obtain on CM10 chip).

iii. *Comparison between two similar types of tissues in two HCC group:* In the figure 16, we have comparison between the protein profiles of tumoral samples versus non-involved cirrhotic liver samples from the same group. We found on average 25 peaks with significant p values in the non-involved cirrhotic liver versus tumor samples from HCC-HCV and HCC-OH groups, indicating the possibilities of differences and similarities between the same kind of tissues but with different pathological origins.



**Fig. 17.** SELDI-TOF MS protein profile of the two types of tumors and non-involved cirrhotic liver. (from 7,500 to 9,500 mass-to-charge ratio obtain on CM10 chip).

Overall, we first time confirmed the feasibility of the liver protein profiling by SELDI-TOF MS. Our initial data were very promising and interesting. Work is going on the liver and tumor samples of 12 HCC-HCV, 12 HCC-OH and 15 cirrhotic patients with HCV. We are very hopeful for finding some new markers with diagnostic or therapeutic potential in the near future.

### **The new tumor suppressor genes ING (INhibitor of Growth): ING2 and HCC.**

Tumor suppressor genes are defined as a class of genes encoding proteins that negatively regulate the cell proliferation. They are divided into 2 classes: the “caretakers” of the genome (or Type I), they usually are DNA repair genes, which protect the genome from mutations (XPB, MSH2, etc.), and the gatekeepers (or Type II), which prevent cancer through direct control of cell growth (p53, p16, etc.) (Kinzler and Vogelstein 1998). Thus, restoration of missing gatekeeper functions to cancer cells leads to suppression of the neoplastic growth; whereas restoration of caretaker functions will not affect their growth. Tumor suppressor genes inactivation by loss or mutation is a critical step for the development of tumors (Ythier, Larrieu et al. 2008).

ING (INhibitor of Growth) gene family is recently discovered tumor suppressor genes. Five members of the ING gene family, ING1 to ING5, have been discovered up till now. All ING genes were mapped on independent chromosomes and interestingly, all ING genes are located on the subtelomeric region of their respective chromosomes except ING3. ING proteins are involved in chromatin remodeling, growth regulation, senescence, apoptosis, DNA repair and in cell migration (Gong, Suzuki et al. 2005; Bienz 2006; Russell, Berardi et al. 2006).

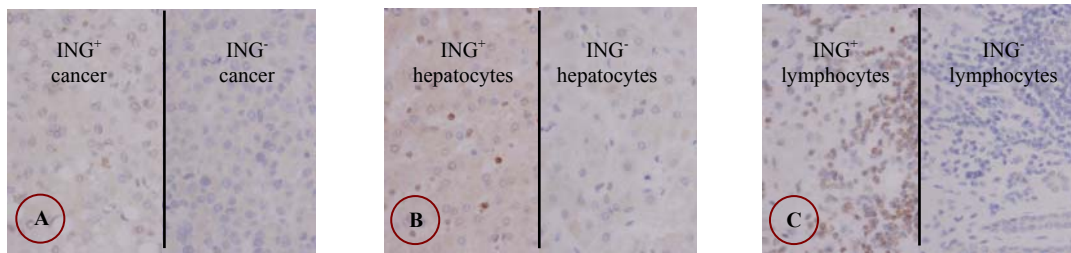
The inhibitor of growth (ING) family member 2 (ING2), as a candidate tumor suppressor isolated in 1998, is an important member of the ING family which encode a series of proteins as important cofactors of p53, associated with cell cycle progression, apoptosis and DNA repair (Shimada, Saito et al. 1998; Doyon, Cayrou et al. 2006). ING2 encodes a protein whose C-terminus contains a highly conserved plant homeodomain (PHD) zinc-finger that binds to histone-3 trimethylated at lysine 4 and acts as nuclear phosphoinositide receptors to active gene repression and regulate DNA damage (Gozani, Karuman et al. 2003; Pena, Davrazou et al. 2006; Shi, Hong et al. 2006). Its N-terminus contains a leucine zipper-like (LZL) motif critical for the proper functioning of ING2 in DNA repair, apoptosis, and chromatin remodeling after UV irradiation (Wang, Wang et al. 2006). ING2 proteins are mainly

localized in the nucleus, also with a diffuse distribution in the cytoplasm (Gozani, Karuman et al. 2003). It has been demonstrated that the expression of ING2 not only negatively regulates cell growth through induction of apoptosis and G1-phase cell-cycle arrest in a p53-dependent manner but also is involved in p53-mediated replicative senescence (Nagashima, Shiseki et al. 2001; Pedoux, Sengupta et al. 2005).

The molecular pathogenesis of HCC seems to involve multiple genetic aberrations in the molecular control of hepatocyte proliferation, differentiation, and the maintenance of genomic integrity. This process is influenced by the cumulative activation and inactivation of oncogenes, tumor suppressor genes, and other genes. Clarification of the genes involved in the development of HCC will contribute to our understanding of mechanisms of hepatocarcinogenesis. ING2 gene is in a frequent loss of heterozygosity (LOH) region in HCC (Bando, Nagai et al. 1999; Bluteau, Beaudoin et al. 2002), and has been implicated as a potential tumor suppressor gene in a few human malignancies (Lu, Dai et al. 2006; Okano, Gemma et al. 2006). Furthermore, ING2 shares the structural similarities with p33ING1b which may play an important role as a tumor suppressor gene in HCC (Ohgi, Masaki et al. 2002; Zhu, Lin et al. 2005), however, the involvement of ING2 in HCC remains largely unclear. A very recent study suggests that ING2 is involved in the progression of HCC (Zhang, Pan et al. 2008), therefore it is considered to be a candidate tumor suppressor gene and its significantly decreased expression in HCC may lead to an unfavorable prognosis.

We also tested ING2 staining by immunohistochemistry on HCC tumor and non-tumor tissues and found the following interesting results.

- i. ING2 functioned very well on almost all the samples tested, indicating the feasibility of the technique and the functioning of the antibody.
- ii. ING2 staining was found mostly in the cytoplasm, however some nuclear staining was also observed in the hepatocytes outside the tumor.
- iii. Inside the tumor, ING2 staining was very weak as compared to the outside of the tumor in non-cancerous tissues.
- iv. Interestingly, we also found that the nucleus of many infiltrated immune cells were well stain with ING2, indicating some of the role played by this gene in the tumor immunity. In fact, we are interested to find some role played by ING2 in the tumor immunity of HCC.



**Fig. 18. Different areas of HCC liver stain with ING antibodies and with non-specific peptide as a control. A.** Staining inside the tumor showing very few cells positive for ING. **B.** Staining outside the tumor showing some nucleus of hepatocytes positive with ING and also the cytoplasm of the hepatocytes is also positive for ING as compared to ING negative control. **C.** Staining inside the lymphocytes aggregates showing many lymphocytes nuclei positive with ING as compared to ING negative control.

Further work is in progress with a large cohort of the patients and we are also planning to identify the ING2 positive immune cells by double staining of CD3, CD4, CD8 and CD20 etc with ING2 and also by flow cytometry.

#### **Development of cell bank.**

Most of the studies on intrahepatic living immune cells are based on the liver biopsies. The problem with the used of liver biopsies is that one can get a small population of intrahepatic lymphocytes because the size of biopsies are generally very small. So, it is difficult to analyze the rare immune cells population, specially, regulatory T cells, dendritic cells and even NK, NKT cells. To launch functional experiment by isolation of certain specific types of immune cells is even more difficult.

To overcome these difficulties we decided to develop a cell bank based on resected tissues after tumor resection in 2005. Up till now, in the four years, we have  $1087 \times 10^6$  liver infiltrating lymphocytes,  $1184 \times 10^6$  tumor infiltrating lymphocytes,  $331 \times 10^6$  blood lymphocytes,  $1252 \times 10^6$  hepatocytes and  $1691 \times 10^6$  tumor cells from 31 patients of HCC with different etiology. Details of these cells are represented in table 6.

**Table 6. Patient wise record of cell bank showing different types of cells and their numbers.**

Patient name	No. Blood	No. Tissues	etiology	date	Blood	Tissues	Blood cells/Tube	No. Of Tubes	Tumor cells/tube	No. Of Tubes	Cirrhosis NL/Tube	No. Of Tubes
1 LO B	45574.0 H1	05B03787	HCC+HCV	10/10/2005	Yes	Yes	3.6X10 <sup>6</sup> cells	3	3.6X10 <sup>6</sup> cells	1	4X10 <sup>6</sup> cells	3
2 CAR-s	55991.7 H1		HCC+drug induced	17/10/2005	Yes	No	5X10 <sup>6</sup> cells	5				
3 CUO	73491.5H1		HCC+OH	24/10/2005	Yes	No	4.3X10 <sup>6</sup> cells	3				
4 SAM		05B04212	HCC+HCV	08/11/2005	No	Yes			17X10 <sup>6</sup> cells	10	27X10 <sup>6</sup> cells	10
5 CAR-f	90072.0H1		HCC+HCV+HBV	09/11/2005	Yes	No	3.5X10 <sup>6</sup> cells	1				
6 TET	79832.8	05B4248	HCC+HCV	10/11/2005	Yes	Yes	5X10 <sup>6</sup> cells	2	21X10 <sup>6</sup> cells	10	10.5X10 <sup>6</sup> cells	10
7 HAL		05B4414	HCC+HCV metas	22/11/2005	No	Yes			12.5X10 <sup>6</sup> cells	10	8.5X10 <sup>6</sup> cells	10
8 BIN	03035.0 H1	05B4616	HASH	06/12/2005	Yes	Yes	6X10 <sup>6</sup> cells	4	7X10 <sup>6</sup> cells	5	10X10 <sup>6</sup> cells	10
9 POR		06B0352	HCC+HCV	23/01/2006	No	Yes			16X10 <sup>6</sup> cells	10	12X10 <sup>6</sup> cells	10
10 WAK	61370.1 H1	06B0597	HCC+HBV	03/02/2006	Yes	Yes	10X10 <sup>6</sup> cells	2	4.3X10 <sup>6</sup> cells	5	3.6X10 <sup>6</sup> cells	10
11 RAV	74373.0	06B0681	HCC-OH	10/02/2006	Yes	Yes	5X10 <sup>6</sup> cells	3	16X10 <sup>6</sup> cells	10	7X10 <sup>6</sup> cells	10
12 SIL	61186.9 H1	06B1868	HCC+HCV	27/04/2006	Yes	Yes	4X10 <sup>6</sup> cells	1	5.7X10 <sup>6</sup> cells	5	20X10 <sup>6</sup> cells	5
13 IAN	81068.3 H1	06B2024	HCC+HCV	10/05/2006	Yes	Yes	5X10 <sup>6</sup> cells	2	7X10 <sup>6</sup> cells	2	25X10 <sup>6</sup> cells	10
14 FRA	43862.6 H1		HCC-OH	27/06/2006	Yes	No	3X10 <sup>6</sup> cells	4				
15 SAN	83085.4H1	06B4915	HCC+OH+HBV	28/11/2006	Yes	Yes	10X10 <sup>6</sup> cells	5	12X10 <sup>6</sup> cells	10	12X10 <sup>6</sup> cells	5
16 BOM		07B495	HBV? cholangiocarci	31/01/2007	No	Yes			4X10 <sup>6</sup> cells	4	4X10 <sup>6</sup> cells	4
17 MAH	91318.5	07B0716	HCC-OH	13/02/2007	Yes	Yes	2X10 <sup>6</sup> cells	1	4X10 <sup>6</sup> cells	3	11X10 <sup>6</sup> cells	5
18 FAL		?	HCC+HCV	16/02/2007	No	Yes			20X10 <sup>6</sup> cells	10	11X10 <sup>6</sup> cells	10
19 COR	98801.6 H1	07B0918	e+petit foyer de HCC	27/02/2007	Yes	Yes	6X10 <sup>6</sup> cells	3	11X10 <sup>6</sup> cells	5	25X10 <sup>6</sup> cells	10
20 SER	23108.9H1	?	denome+hyperplasi	28/03/2007	Yes	Yes	2.5X10 <sup>6</sup> cells	1	8.5X10 <sup>6</sup> cells	2	10X10 <sup>6</sup> cells	3
21 PAL	62798.6	07B1991	C+? (origine inconnu	27/04/2007	Yes	Yes	6X10 <sup>6</sup> cells	2	7X10 <sup>6</sup> cells	4	24X10 <sup>6</sup> cells	5
22 POU	51319.9	07B3180	HCC+OH	11/07/2007	Yes	Yes	6X10 <sup>6</sup> cells	2	50X10 <sup>6</sup> cells	10	18X10 <sup>6</sup> cells	5
23 GHO	79447.4	07B03475	HCC foie sain	31/07/2007	Yes	Yes	4X10 <sup>6</sup> cells	4	28X10 <sup>6</sup> cells	5	20X10 <sup>6</sup> cells	5
24 SOU	12628.1	08B1204	?	07/03/2008	yes	yes	5.5X10 <sup>6</sup> cells	2	42X10 <sup>6</sup> cells	8	35X10 <sup>6</sup> cells	5
25 ROC		08B1252	?	11/03/2008	no	yes			2X10 <sup>6</sup> cells	3	4.6X10 <sup>6</sup> cells	6
26 CAR	13335.6	08BXXXX	?	12/03/2008	yes	yes	4X10 <sup>6</sup> cells	4	4X10 <sup>6</sup> cells	5	6X10 <sup>6</sup> cells	5
27 REP	16559	08B1314	?	13/03/2008	yes	yes	1X10 <sup>6</sup> cells	1	3.5X10 <sup>6</sup> cells	4	7X10 <sup>6</sup> cells	4
28 GIR	38518.8	08B1614	?	01/04/2008	yes	yes	4X10 <sup>6</sup> cells	3	60X10 <sup>6</sup> cells	5	8X10 <sup>6</sup> cells	5
29 JUL	38293	08B1748	?	08/04/2008	yes	yes	4.5X10 <sup>6</sup> cells	3	13X10 <sup>6</sup> cells	5	10X10 <sup>6</sup> cells	5
30 MED	93900.9	08B2452	?	21/05/2008	yes	yes	7X10 <sup>6</sup> cells	2	14X10 <sup>6</sup> cells	5	7.5X10 <sup>6</sup> cells	2
31 MEL	33690	08B4431	HCC-HCV	23/09/2008	yes	yes	4X10 <sup>6</sup> cells	3	8X10 <sup>6</sup> cells	5	15X10 <sup>6</sup> cells	10

We have plans to use these cells for functional experiments on T<sub>reg</sub> and NK cells and also use them in SELDI-TOF MS to find some new immunomarkers in HCC patients. We hope that with the help of this cell bank we can able to explore some new mysteries of this complex disease of HCC.

**DISCUSSION  
AND  
PERSPECTIVE**

## General discussion and perspective

### Discussion.

Continued tumor growth has been observed despite the presence of a large numbers of lymphocytic infiltrations including tumor-specific T cells within and surroundings of the tumors, suggesting a failure of immune control. Many mechanisms have been proposed for an attenuated immune response to the tumors; these include failure of antigen processing, inadequate costimulation, functional impairment of cytotoxic cells and direct suppression of effector cells by regulatory T cells (Chouaib, Asselin-Paturel et al. 1997; Zerbini, Pilli et al. 2004; Unitt, Rushbrook et al. 2005). Our results showed that when hepatocellular carcinoma developed in chronic inflammatory liver disease, a large number of immune cells have been infiltrated in the tumoral as well as peri-tumoral cirrhotic liver. However, as partially reported by others (Chen, Lee et al. 2007), both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, NK and NKT cells,  $\gamma\delta$  T cells, FoxP3<sup>+</sup> regulatory T and dendritic cells and/or macrophages (CD123<sup>+</sup> cells) are many folds higher in the non tumoral areas than tumoral areas, suggesting that along with other factors previously proposed (Chouaib, Asselin-Paturel et al. 1997; Zerbini, Pilli et al. 2004; Unitt, Rushbrook et al. 2005), most of the immune cells fail to enter into the tumors.

It has been observed that a major clinical risk factor for HCC development is liver cirrhosis and 70–90% of all HCCs develop in a cirrhotic liver (Benvegnu, Noventa et al. 2001; Levrero 2006). In our study, almost all the patients were at cirrhotic level with different profile of liver infiltrating lymphocytes. Our data demonstrated that most of the infiltrating lymphocytes (e.g. CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup>, FoxP3<sup>+</sup>, TCR $\gamma\delta$ <sup>+</sup>, and CD123<sup>+</sup> cells) are significantly higher in the cirrhosis with viral hepatitis C and cancer (HCC-HCV group). It is noteworthy that this difference was evident in the portal tracts, lymphoid aggregates and fibrous septa level, while, in between the parenchymatous nodules of the two types of cirrhosis i.e. HCC-HCV and HCC-OH, such difference was almost absent. However, as reported by others, we also observed more lymphoid follicles/aggregates in HCC-HCV cirrhosis and Cirrhosis C than HCC-OH cirrhosis (Bianchi, Desmet et al. 1987; Lefkowitz, Schiff et al. 1993; Murakami, Shimizu et al. 1999). So, it seems that lymphoid aggregates are the characteristic features of HCV related cirrhosis and the difference of infiltrating lymphocytes in the cirrhotic liver with HCV infection might be due to the presence of HCV. Moreover, when we compare the infiltrating lymphocytes in the cirrhosis with HCV and with HCC (Outside the tumor of HCC-



HCV group) with that of cirrhosis with HCV but without cancer (Cirrhosis C), many types of infiltrating lymphocytes (e.g. CD3<sup>+</sup>, CD4<sup>+</sup>, ~CD8<sup>+</sup> cells) are significantly high in the cirrhosis HCC-HCV as compared to Cirrhosis C group. The higher number of infiltrating lymphocytes in the cirrhotic liver in HCC-HCV group as compared to Cirrhosis C group could be due to the presence of HCC in the former. Overall, our results showed that the order of the number of infiltrating lymphocytes are lowest in cirrhosis HCC-OH than Cirrhosis C and highest in HCC-HCV. It seems that the high numbers of infiltrating lymphocytes in Cirrhosis C may be due to presence of HCV and the highest number of these immune cells in the cirrhosis HCC-HCV might be due to the presence of both HCV and HCC.

*NK and/or NKT cells in chronic viral hepatitis and in HCC:*

Human NK cells are classified into two populations according to the intensity of CD56 surface expression, as CD56<sup>dim</sup> make up ~90% of circulating NK cells, whereas CD56<sup>bright</sup> comprises the remaining 10%. In chronic hepatitis, we found that the pool of NK cells in the liver from HCV<sup>+</sup> patients was low at any stage of the disease as compared to HBV<sup>+</sup> patients. We observed differential localization of intra hepatic CD56<sup>+</sup> cells in the different histological areas in both viral infections. We demonstrated clearly that the liver infiltrating lymphocytes (CD56<sup>-</sup> cells) tend to increase from low necroinflammatory activity to high necroinflammatory activity but strikingly, we did not detect any increase in CD56<sup>+</sup> cells in both groups as the disease progress. Generally, we observed an increasing trend of CD56<sup>+</sup> cells with the progression of disease towards outside of the necroinflammatory lesions in HCV infection, whereas, they remain constant in HBV infection.

In chronic HCV infection, as reported by others, our data confirm a significant reduction in NK cell frequency and a quantitative imbalance of the CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets within the total NK cells population (Meier, Owen et al. 2005; Golden-Mason, Madrigal-Estebas et al. 2008). On the other hand, we did not observe either decrease of CD56<sup>+</sup> cells nor difference in CD3<sup>-</sup>CD56<sup>bright</sup>/CD3<sup>-</sup>CD56<sup>dim</sup> ratio in HBV<sup>+</sup> patients, this suggests that the proinflammatory environment is not sufficient to explain that altered CD3<sup>-</sup>CD56<sup>dim</sup>/CD3<sup>-</sup>CD56<sup>bright</sup> ratio in HCV<sup>+</sup> group. The clear difference in NK cell frequencies and the expression of differential CD3<sup>-</sup>CD56<sup>dim</sup>/CD3<sup>-</sup>CD56<sup>bright</sup> ratio in the two viral infections, strongly supports that HCV itself may be able to alter significantly the activation, proliferation and cytotoxicity of NK cells (Pileri, Uematsu et al. 1998; Crotta, Stilla et al. 2002; Tseng and Klimpel 2002). NK cell activation may be impaired and/or prevented by cell surface

inhibitory receptors expressed by NK cells (Long, Burshtyn et al. 1997). We did not observe any significant change in the type 1 transmembrane proteins i.e. both CD158a,h<sup>+</sup> and CD158b,j<sup>+</sup> cells in the blood of HCV<sup>+</sup> and HBV<sup>+</sup> patients as depicted by others in the patients of chronic HCV and hepatocellular carcinoma (Yuen and Norris 2001; Golden-Mason, Madrigal-Estebas et al. 2008). We report a strong alteration in the frequencies of CD3<sup>-</sup>CD56<sup>+</sup>NK cells expressing CD158a,h<sup>+</sup> or CD158b,j<sup>+</sup> in the liver of HCV<sup>+</sup> patients but not in HBV<sup>+</sup> patients. To date, the role of these subsets of NK cells in the liver has not been clearly determined. As CD3<sup>-</sup>CD56<sup>+</sup>CD158a,h<sup>+</sup> and CD158b,j<sup>+</sup> are more expressed in CD3<sup>-</sup>CD56<sup>dim</sup> than in CD3<sup>-</sup>CD56<sup>bright</sup> (Cooper, Fehniger et al. 2001; Golden-Mason, Madrigal-Estebas et al. 2008), the decrease of these subsets in the liver of HCV<sup>+</sup> patients, again underlines that HCV may interfere with the activation or differentiation of CD3<sup>-</sup>CD56<sup>dim</sup> cells subset in the liver which is generally more cytotoxic. In the type 2 transmembrane proteins, no significant change in the frequencies of blood and liver infiltrating NKG2A<sup>+</sup> cells were detected in HCV<sup>+</sup> patients, while, they are significantly reduced in the blood and liver compartments of HBV<sup>+</sup> patients. Taken together, our results show that both viruses interfere differentially on the NK cells pool: HCV targets type 1 transmembrane proteins whereas HBV targets type 2 transmembrane proteins. Here we found a negative correlation between CD3<sup>-</sup>CD56<sup>+</sup>NKG2A<sup>+</sup> cells and viral load in HCV<sup>+</sup> and HBV<sup>+</sup> patients. To pin point this correlation, we observed that the viral load was correlated negatively with CD3<sup>-</sup>CD56<sup>dim</sup>NKG2A<sup>+</sup> cells subset in both infections, suggesting that antiviral property of NK cells might be restricted to NKG2A expressing CD3<sup>-</sup>CD56<sup>dim</sup> cells subset.

As for as CD56<sup>+</sup> cells in HCC is concern, we noted a significant lower numbers of CD56<sup>+</sup> cells in HCC-HCV and in HCC-OH than Cirrhosis C but they do not vary in both types of HCCs. These results suggest that HCC lowers the frequency of CD56<sup>+</sup> liver infiltrating lymphocytes independent of the presence or absence of the virus.

#### *Regulatory T cells (T<sub>reg</sub>) in chronic HCV infections and in HCC:*

Increased Foxp3<sup>+</sup> T-lymphocytes in different cancer including HCC and in chronic hepatitis C have been demonstrated and their presence is a predictor of reduced survival (Liyanage, Moore et al. 2002; Sugimoto, Ikeda et al. 2003; Cabrera, Tu et al. 2004; Curiel, Coukos et al. 2004; Sasaki, Tanaka et al. 2008). In chronic HCV infections, HCV-specific T-cell responses are generally weak, narrowly focused and often dysfunctional. Previously, intrahepatic regulatory T cells were mostly defined by CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells (Cabrera, Tu et al.

2004) which do not give true picture of natural regulatory T cells define by FoxP3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells in HCV patients. Our flow cytometric data show that about 50% of CD4<sup>+</sup>CD25<sup>+</sup> intrahepatic T cells do not express FoxP3 marker, suggesting that the actual numbers of FoxP3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells are almost half of CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells as already determined. Our immunohistological findings indicate that FoxP3<sup>+</sup> cells infiltrate the hepatic lymph nodes, portal tracts and in the parenchymatous nodules as indicated by others (Ward, Fox et al. 2007). As the mechanism for inhibition of T cell proliferation by T<sub>reg</sub> during HCV infection has been shown to be cell-contact dependent (Boettler, Spangenberg et al. 2005; Rushbrook, Ward et al. 2005), strikingly, more frequent FoxP3<sup>+</sup> cells were found in the necrotic lesions (both peicemeal and lobular necrosis) predominantly in contact with CD8<sup>+</sup> cells indicating that regulatory T cells within HCV infected livers have the direct access required to inhibit T cell cytotoxicity at the site of action. Furthermore, since such cells are localized in portal tracts, antiviral and non-specifically activated lymphocytes entering the liver are likely to encounter a FoxP3<sup>+</sup> cell at a relatively early stage in the transit. FoxP3<sup>+</sup> cells inside the necroinflammatory lesion also correlate positively with CD4 expression and CD8<sup>+</sup> cells, again confirms the inhibitory role of these cells not only for CD8<sup>+</sup> cells but also for CD4<sup>+</sup> cells. In brief, the regulation is essentially FoxP3-CD8 contact-dependent and probably non antigen-specific because no correlation has been found between the intra-hepatic FoxP3 or CD8 (transcripts or cells) and viral load.

In HCC patients, our data demonstrated that FoxP3<sup>+</sup>/CD4<sup>+</sup> cells ratio is significantly higher in cirrhosis HCC-HCV as compared to the cirrhosis HCC-OH. Increased regulatory T cells correlate with CD8<sup>+</sup> T-cell impairment and poor survival in hepatocellular carcinoma patients (Unitt, Marshall et al. 2006; Yang, Yamagiwa et al. 2006; Fu, Xu et al. 2007). Moreover, the site of action of CD8<sup>+</sup> cytotoxic T cells is also nodular parenchyma where they kill virus infected or transformed hepatocytes. Interestingly, we observed that Foxp3<sup>+</sup> cells are significantly higher in the nodular parenchyma of cirrhosis HCC-HCV where they have a positive correlation with CD8<sup>+</sup> cells, suggesting that FoxP3<sup>+</sup> regulatory T cells might inhibit CD8<sup>+</sup> cytotoxic T cells at the site of action perhaps in the necrotic lesions as shown in our results in the liver of HCV infected patients.

### *Cytokines and chemokines in chronic HCV infections and in HCC:*

In the context of cytokines and their role in HCV infected liver, we found a significant positive correlation among the expression levels of IL-10, TGF- $\beta$ , TNF- $\alpha$  and IFN- $\gamma$ . For instance, TGF- $\beta$  is often produced in high concentration during chronic HCV infections and is an important factor for the local survival and function of natural T<sub>reg</sub> cells (Green, Gorelik et al. 2003; Cabrera, Tu et al. 2004; Bolacchi, Sinistro et al. 2006). consequently, IL-10 expression is high in the liver of chronic HCV patients (Gonzalez, Zhang et al. 2008; Kaplan, Ikeda et al. 2008) and is produced by many cell types in the liver including natural regulatory T cells (Cabrera, Tu et al. 2004), HCV core specific CD4<sup>+</sup> cells (MacDonald, Duffy et al. 2002) and intrahepatic dysfunctional CD8<sup>+</sup> T cells (Accapezzato, Francavilla et al. 2004; Abel, Sene et al. 2006). Here, a positive correlation found between the expressional levels of TGF- $\beta$  and IL-10 may have synergistic effects on each other in HCV infected liver. It has been shown that the expression levels of TNF- $\alpha$  in the liver of chronic hepatitis C are increased (McGuinness, Painter et al. 2000) and up-regulated by HCV NS3 (Hassan, Selimovic et al. 2007). In addition, it is also involved in the healing process including fibrogenesis (Migita, Maeda et al. 2006). As the liver expressional levels of TNF- $\alpha$  as well as IFN- $\gamma$  have correlation with IL-10 and TGF- $\beta$ . So, one can argue that the infectious/inflammation context favor both the production of anti-inflammatory (IL-10 & TGF- $\beta$ ) and pro-inflammatory (TNF- $\alpha$  & IFN- $\gamma$ ) cytokines. We hypothesize here that FoxP3<sup>+</sup> T<sub>regs</sub> may inhibit partially the pool of intra-hepatic effector T cells and the rest continue to produce IFN $\gamma$ , supported by recent data that IFN $\gamma$  is rather released by effector T cells in these infections (Wedemeyer, He et al. 2002; Leroy, Vigan et al. 2003; Spangenberg, Viazov et al. 2005). Moreover, our regression analysis showed that the expression levels of FoxP3, CD8 and IL-10 are positively co-dependent upon each other, suggesting that IL-10 might play some important role in the interaction between T<sub>regs</sub> and CD8<sup>+</sup> T cells in HCV infected liver.

In HCC, we observed significant low expression levels of IFN- $\gamma$ , perforine and CD8 $\alpha$  in the cirrhosis HCC-HCV than in cirrhosis HCC-OH, where, CD8 $\alpha$  positively correlated with both IFN- $\gamma$  and perforine in cirrhosis HCC-OH. This represents a very interesting picture showing, that a high numbers of infiltrating lymphocytes present in cirrhosis HCC-HCV are less active than low numbers of infiltrating lymphocytes present in cirrhosis HCC-OH. Moreover, this functional attenuation might be at the expression levels of CD8 $\alpha$  among these infiltrate. We also found low expression levels of IL-6 and TGF- $\beta$  in cirrhosis HCC-HCV as compared to

Cirrhosis C, indicating a counterbalance in between pro-inflammatory and anti-inflammatory cytokines in HCV cirrhosis, again reinforcing our data presented above.

*HCC micro-environments and tumor infiltrating lymphocytes:*

Inside the tumor, our data represent a very interesting profile of the tumor infiltrating lymphocytes (TILs). There is no difference in between the numbers of TILs in the tumors of both HCC-HCV and HCC-OH. Moreover, there is also no difference in the expression levels of most of the immunomarkers including in our study except CD8 $\alpha$  which is significantly higher in HCC-OH tumor. This indicates that the tumor microenvironment behave similarly towards the immune response regardless of presence or absence of the virus. The role of T-regulatory cells in anti-tumor immunity is very crucial (Gallimore and Sakaguchi 2002) and they inhibit the cytotoxic functions of CD8 $^{+}$  lymphocytes (Unitt, Marshall et al. 2006; Yang, Yamagiwa et al. 2006; Fu, Xu et al. 2007). Here, we again found a positive correlation not only between intratumoral FoxP3 $^{+}$  cells and CD8 $^{+}$  cells but also between the intratumoral expression levels of FoxP3 and CD8 $\alpha$  and CD8 $\beta$ . It is noteworthy that this correlation is stronger with CD8 $\alpha$  than CD8 $\beta$ , indicating, that the regulatory functions of FoxP3 $^{+}$  T cells are stronger towards CD8 $\alpha$  expressing cytotoxic cells. In addition, the expression levels of FoxP3 also correlate with the expression levels of IFN- $\gamma$  in these tumors, suggesting another counterbalance of pro-inflammatory immune response by regulatory cells. Still another negative correlation has been found in between the expression levels of FoxP3 and perforine in these tumors, confirming the anti-effector role of FoxP3 in the tumor microenvironments. Our data also represents some interesting positive correlation in HCC tumors, such as, CD3 $^{+}$  cells with CD20 $^{+}$  cells, CD4 $^{+}$  cells with CD8 $^{+}$  cells and CD56 $^{+}$  cells with  $\gamma\delta$ T cells, showing a certain type of synergy in these different types of immune cells in the tumor microenvironments. The expression levels of CD8 $\alpha$  are high in HCC-OH tumor. To further investigate their role in these tumors, we found that their expression levels are correlated with the expression levels of RANTES as well as with perforine and FoxP3. In summary, it seems that RANTES may attract preferentially CD8 $\alpha$  cells which might be more cytotoxic but counterbalance by FoxP3 $^{+}$  cells in the microenvironments of HCC.

*Tumor aggressivity:*

Among the histological parameters, steatosis is more likely to be associated with chronic hepatitis C and one of the important risk factor for HCC (Lefkowitz, Schiff et al. 1993; Wu,

Tian et al. 2008). We observed a positive correlation between steatosis and IFN- $\gamma$  in the exterior of the tumor in HCC-HCV patients, suggesting that increase in IFN- $\gamma$  could be the cause or consequence of steatosis or vice versa. A positive correlation between the tumor size with age has been found in both groups, while, it is also positively correlated with the expression levels of FoxP3 in the tumor of HCC-HCV group, suggesting that age in general and the presence of regulatory T cells in particular are the negative regulators of the tumor immunity. A certain degrees of tumor necrosis have been identified by the pathologists in routine which is expressed as percentage. We observed a significant high percentage of tumor necrosis in HCC-OH group than HCC-HCV group. Interestingly, our data show inverse correlations among percentage of necrosis and age, CD4<sup>+</sup>, CD20<sup>+</sup> cells and also with the expression levels of RANTES in the tumors of both groups. We concluded from these results that the reduction of intratumoral immunity is evident with the passage of age. In addition, RANTES may attract different populations of the immune cells including CD4<sup>+</sup> and CD20<sup>+</sup> cells but somehow they are more regulatory in nature than cytotoxic in the tumor microenvironments. This hypothesis is supported by the notion that a large numbers of CD4<sup>+</sup> cells are regulatory T cells which can directly suppress B cell Ig response (Lim, Hillsamer et al. 2004; Lim, Hillsamer et al. 2005). Ki67 labeling index is another parameter which represents tumor progression (Quaglia, McStay et al. 2006). We showed that it correlates positively with the serum levels of AFP, ALT and PT in both groups indicating that high serum levels of certain clinical parameters are the indicators of tumor progression. As it has already been reported that HCV and its certain proteins are directly or indirectly involved in HCC development (Levrero 2006). Here, we also found a strong positive correlation between Ki67 labeling index and viremia, suggesting that HCV might be involved in the tumor aggressivity. Interestingly, we also found an inverse correlation between Ki67 labeling index and the expression levels of intratumoral perforine, demonstrating that attenuated immune response favors tumor growth.

#### *Tumor recurrence:*

Tumor recurrence following liver resection for HCC is common and a major cause of death from this disease (Regimbeau, Abdalla et al. 2004). A multiple risk factors e.g. AFP, AST, tumor size, presence of multiple tumors, portal invasion, HCV, steatosis, cirrhosis and histological activity have been reported in HCC patients (Kubo, Hirohashi et al. 2000; Peng, Chen et al. 2004; Portolani, Coniglio et al. 2006; Takuma, Nouso et al. 2007; Kubo,

Takemura et al. 2008). We have also found that tumor recurrence is significantly high in HCC-HCV group as compared to HCC-OH groups, confirming that HCV is a risk factor for tumor recurrence in these patients. Interestingly, we found that CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup> and FoxP3<sup>+</sup> cells in general and CD8<sup>+</sup> cells particularly in HCC-HCV group are significantly high outside the tumor in recurrent patients than non-recurrent patients, suggesting that the high numbers of liver infiltrating cells outside the tumor are not beneficial but are related to tumor recurrence, in contrast, high number of tumor infiltrating cells has been reported to link with better prognosis in HCC patients (Wada, Nakashima et al. 1998). Recently, it has been reported that a high number of tumor infiltrating regulatory T cells is an independent predictive factor of tumor recurrence after hepatic resection for HCC (Sasaki, Tanaka et al. 2008). We have also confirmed here that a significant high FoxP3/CD4 cell ratio is present inside the tumors of recurrent patients than non-recurrent patients. Tumoral and peritumoral macrophages have been thought to involve in the tumor progression, metastasis and recurrence in many type of cancer including HCC (Pollard 2004; Mantovani, Allavena et al. 2008; Zhu, Zhang et al. 2008). Moreover, plasmacytoid dendritic cells (pDCs) were also thought to be correlated with adverse outcome in breast cancer (Treilleux, Blay et al. 2004). Interestingly, we also found a high numbers of CD123<sup>+</sup> cells in recurrent patients than non-recurrent patients, indicating that tumoral pDCs/macrophages are also involved in tumor recurrence in HCC patients. In one of our studies in melanoma patients, we observed that circulating pDC and mDC are immature and have potential to become mature on appropriate stimulation. Moreover, immature pDC are attracted by CCL20/MIP3- $\alpha$  in the tumors in these patients. Plasmacytoid DCs associated with ovarian cancer induced CD8<sup>+</sup>FOXP3<sup>+</sup>IL-10<sup>+</sup> regulatory T cells (Zou, Machelon et al. 2001; Wei, Kryczek et al. 2005), and dysfunctional myeloid DCs also induced IL-10<sup>+</sup> regulatory T cells *in vitro* (Jonuleit, Schmitt et al. 2000) and *in vivo* in patients with cancer (Dhodapkar, Steinman et al. 2001; Chakraborty, Chattopadhyay et al. 2004). So, in HCC tumors, DC might be immature, induce regulatory cells which intern attenuate tumoral immunity and perhaps tumor recurrence. Taking together, these results shows that greater is the infiltrating cells outside the tumor, particularly regulatory T cells and CD8<sup>+</sup> T cells, greater will be the recurrence, while, a high number of regulatory T cells and pDCs/macrophages cells inside the tumor also seems to be associated with tumor recurrence in these patients.

### *Clinical parameters & HCC:*

Serum levels of ALT, AFP, and bilirubin are the indicators of liver injury and regeneration and their levels were reported high during HCC (Barazani, Hiatt et al. 2007; Rodriguez-Diaz, Rosas-Camargo et al. 2007; Wu, Tian et al. 2008). In our study, we found a positive correlation between ALT and CD3<sup>+</sup> cells of both groups, while, it correlates with CD4<sup>+</sup> cells in HCC-OH group and TCR $\gamma\delta$ <sup>+</sup> cells in HCC-HCV group. Notably all these correlations were restricted to the parenchymatous nodules and no correlation have been found in the periportal and fibrous septal areas. It has been reported that cancer specific CD4<sup>+</sup> cytotoxic T lymphocytes exist in HCC of both human and murine (Nakao, Sata et al. 1997; Homma, Komita et al. 2005) and these cells might exist outside the tumor which make an increase in ALT levels by killing occasional transformed cells outside the tumors. Moreover,  $\gamma\delta$ T cells seem to be very important in HCV related HCC immunosurveillance. Metavir activity score represents the disease gravity and the activeness of the immunity. Our results showed a regular increase in CD3<sup>+</sup>, CD4<sup>+</sup>, CD20<sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> cells in the parenchymatous nodules with an increase in Metavir activity score in HCC-HCV group, again indicating the cytotoxic role of CD4<sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> cells along with CD20<sup>+</sup> cells. Here, we also found that FoxP3<sup>+</sup> cells in the tumors of both HCC groups were inversely correlated with the serum bilirubin, indicating the regulatory T cells lessen the liver damage up to certain extents.

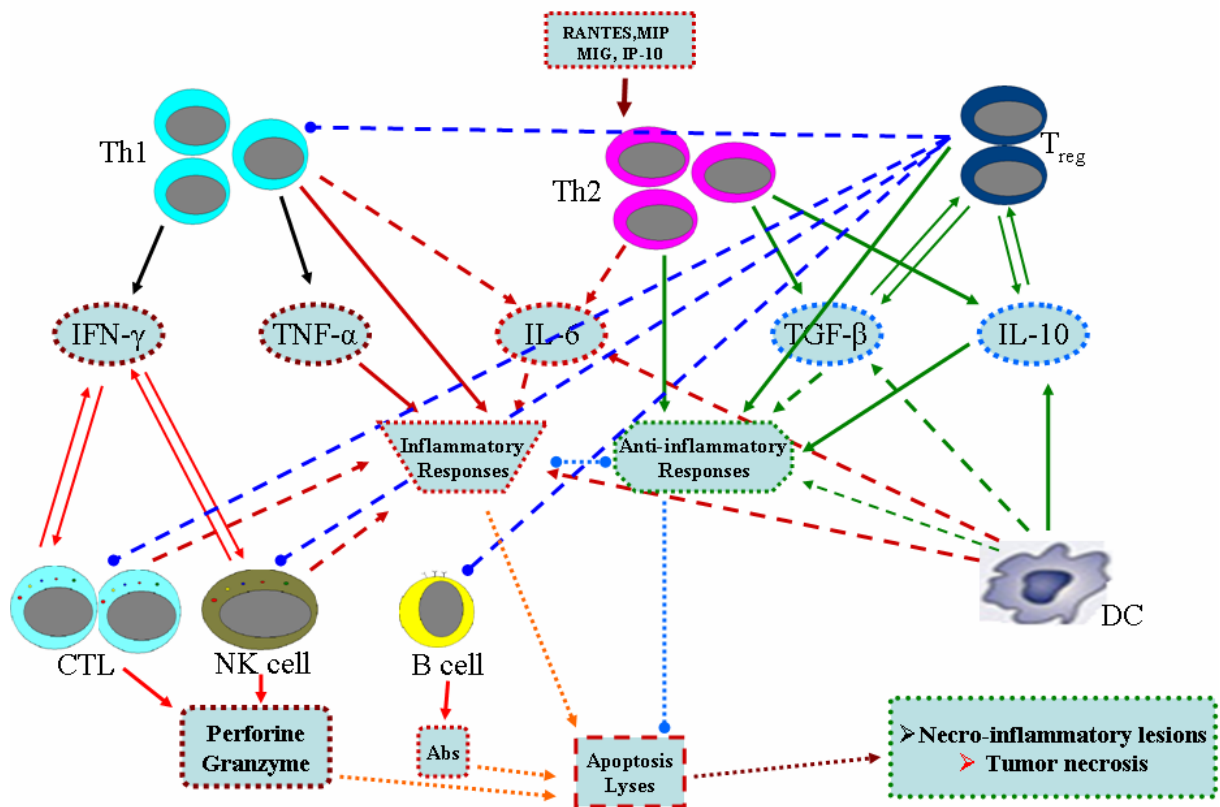


## Conclusion.

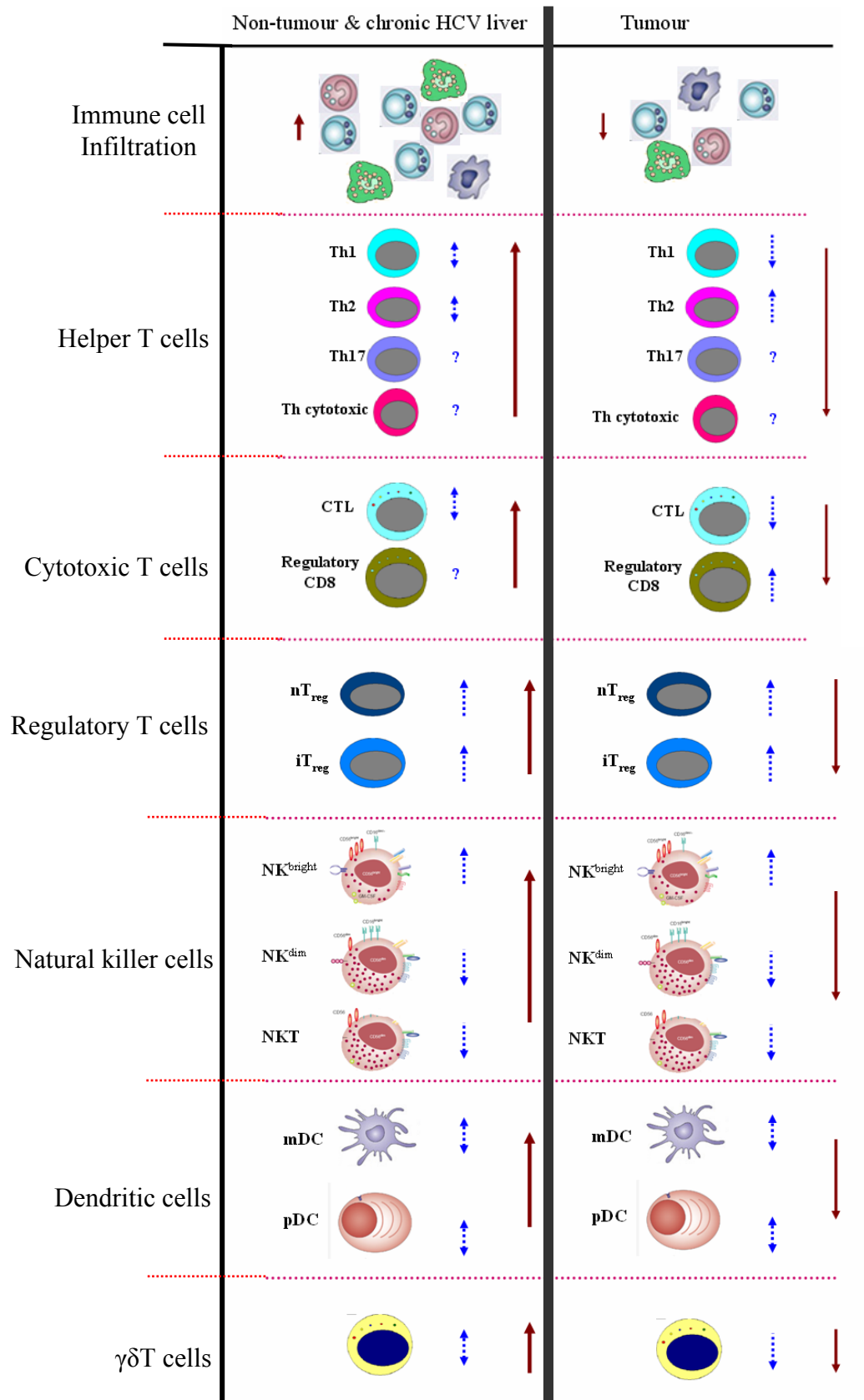
In summary, our data pointed out the following conclusions.

- i. In HCC patients, a very high numbers of infiltrating lymphocytes outside the tumors seems to be attenuated in their antitumoral function and are more regulatory which are associated with tumor recurrence and poor prognosis.
- ii. The tumor infiltrating lymphocytes are low in number as compared to outside of the tumors and also looking dysfunctional. Moreover, Moreover, regulatory T cells and pDCs/macrophages seem to be associated with tumor recurrence.
- iii. NK cells in chronic HCV and HCC patients are low in number, could be regulatory in nature and might favor fibrogenesis. In addition,  $CD3^- CD56^{dim}NKG2A^+$  subset seem to be very important for virus control and might be used as therapeutic target to control liver cirrhosis and hepatocellular carcinoma in the future.
- iv.  $FoxP3^+$  regulatory T cells in chronic HCV liver seems to involved in the suppression of cytolytic T cells in necroinflammatory lesions and might also be contributing in fibrogenesis and in the alterations of cytokine profile.
- v. Dendritic cells are immature in the cancer patients and may contribute towards the attenuated immune response and tumor recurrence in cancer patients.
- vi.  $CD8^+$  cells outside the tumors in HCC patients are not beneficial for the patients. They might be involved in the hepatocarcinogenesis and tumor recurrence in these patients.

We tried to explain our results in the figures 19 and 20 on the following pages.



**Fig. 19. Interactions among immune cells, cytokines and chemokines in the liver and tumoral microenvironments.** Chemokines like RANTES, MIP3- $\alpha$  and MCP attract immune cells to the liver and tumor. Th1 cells along with CTL and NK cells produce pro-inflammatory cytokines like IFN- $\gamma$ , TNF- $\alpha$  and IL-6 and promote lysis/apoptosis of transformed or virus infected cells by peforine, granzymes or Ab, preferentially, outside the tumor which result to produce necro-inflammatory lesions and increase ALT levels in blood, followed by liver regeneration result to increase AFP in blood. In response, Th2 cells, T<sub>reg</sub> and DCs (likely to be immature in cancer patients) produce anti-inflammatory cytokines like TGF- $\beta$ , IL-10 or/and interact directly (like T<sub>reg</sub>) with effector cells to reduce liver damage. The net result is an attenuated immunity in HCC patients which favors tumor progression, metastasis and recurrence. **Abbreviations.** Th, helper T lymphocytes; T<sub>reg</sub>, regulatory T lymphocytes; CTL, cytotoxic T lymphocytes; NK, natural killer cells; DC, dendritic cells; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; TGF, transforming growth factor, Ab, antibody.



**Fig. 20. Comparative immune cells population in the non-involved (or chronic HCV) liver and tumor in HCC patients.** Maroon arrows show our results obtained from HCC and chronic HCV patients while blue arrows represent suspected or literature supported results. Upward directed arrows mean increase in number/activity and downward arrows mean decrease in number/activity. Question marks mean results not confirmed.

## Perspectives.

This work provides global picture of immune cells, cytokines and chemokines in HCC and viral hepatitis patients. It also represents special insight of NK cell sub-populations and interaction between CD8<sup>+</sup> cells and regulatory T cells in chronic HCV patients. In spite of the presence of many types of regulatory cells (as reported in the introduction) and anti-inflammatory cytokines and chemokines, indicators of liver damage and repair (ALT, AFP) represent some cytolytic activities by effector cells in the liver of these patients. Among T helper lymphocytes (CD4<sup>+</sup>), one part seems to be functions as regulatory cells (like Th2, natural and induced T<sub>reg</sub>) in the liver of HCC patients and other part appears to act as pro-inflammatory and effector functions (Th1, Th17 & CD4<sup>+</sup> cytotoxic cells). This hypothesis is partially supported by our results from chronic HCV and HCC patients in which both pro- and anti-inflammatory cytokines have high expressional levels in the liver.

Among other effector cells, CD8<sup>+</sup> cells are very high in numbers in the liver of these patients. A part of these cells is reported to function as regulatory cells like IL-10 producing CD8<sup>+</sup> regulatory cells. NK and NKT cells are very important in the viral hepatitis and HCC, but, their numbers reduce very much in these diseases. Moreover, CD56<sup>bright</sup> NK cells which are more regulatory are significantly high than cytotoxic CD56<sup>dim</sup> NK cells.  $\gamma\delta$ T cells are also very low in numbers in these diseases. Antigen presenting cells like dendritic cells (DCs) are the key player in effective immune response. Both mDC and pDC are immature and promote regulatory functions in cancer patients. Generally, true cytotoxic effector cells are rare, having regulatory populations or having defective functions in chronic HCV and HCC patients. Contrarily, regulatory T cells (including all types) are more functional, active and having no functional defects in these patients.

A sufficient number of effector immune cells are present outside of the tumor. We have to mobilize these cells to enter inside the tumor and also activate them to produce effective anti-tumoral immune response. For this, we should have a complete knowledge (activation, proliferation, etc) of these immune cells in the liver. It is difficult to study these immune cells in the liver because of insufficient material (biopsies). We are planning to launch our functional experiments on these cells by utilizing our cells bank developed from HCC patients. Moreover, work on new immune and tumor markers is in progress on liver and tumor samples by SELDI-TOF MS. We are very hopeful to find some novel insights regarding tumor immunity which will help in the management of this fatal disease.

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### Abstract

We compared immunological and clinicopathological features in tumoral and cirrhotic areas of 12 HCV related hepatocellular carcinomas (HCC-HCV), 12 alcohol related HCC (HCC-OH) and 23 HCV related cirrhosis (Cirrhosis C). Liver infiltrating cells (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup>, CD56<sup>+</sup>, TCRγδ<sup>+</sup>, FoxP3<sup>+</sup> and CD123<sup>+</sup>) were significantly higher (p<0.05) in the cirrhotic areas as compared to the tumoral areas in both cancerous groups. Comparisons between cancerous cirrroses showed that CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup>, FoxP3<sup>+</sup>, CD123<sup>+</sup> cells and FoxP3/CD4 cell ratio were significantly higher (p<0.05) in cirrhosis HCC-HCV than cirrhosis HCC-OH. Expression levels of IFN-γ, CD8α and perforin were significantly lower (p<0.05) in cirrhosis HCC-HCV than cirrhosis HCC-OH. CD3<sup>+</sup>, CD4<sup>+</sup>, CD20<sup>+</sup> and CD8<sup>+</sup> cells were significantly higher (p<0.05) in cirrhosis HCC-HCV than Cirrhosis C, but CD56<sup>+</sup> cells was lower (p=0.067) in cirrhosis HCC-HCV. No difference was observed inside the tumours of both HCC groups at cellular and mRNA expression levels, except, CD8α which was significantly lower (p<0.05) in HCC-HCV group. Intra-tumoral FoxP3<sup>+</sup> cells correlated with CD4<sup>+</sup> and CD8<sup>+</sup> cells and tumoral expression levels of FoxP3 correlated with CD8α, CD8β, IFN-γ, while they inversely correlated with tumoral expression levels of perforin in all HCC patients. Positive correlations of CD4<sup>+</sup> cells with CD8<sup>+</sup> cells and perforin with IFN-γ and RANTES have also been observed. Tumour size was correlated with FoxP3 expression in HCC-HCV group. Tumour necrosis was significantly higher (p<0.05) in HCC-OH than HCC-HCV group and was inversely correlated with the expression levels of RANTES in both groups. Ki67 labelling index was correlated with AFP and viremia but inversely correlated with tumoral perforin. Tumour recurrence was significantly higher (p<0.05) in HCC-HCV than HCC-OH group. In recurrent patient, CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup> and FoxP3<sup>+</sup> cells were significantly higher (p<0.05) in the cirrhotic areas, while CD123<sup>+</sup> cells and FoxP3<sup>+</sup>/CD4<sup>+</sup> cell ratio were significantly higher (p<0.05) in the tumoral areas. Overall, high number of infiltrating immune cells outside and inside of the tumours seems to be less active and associated with tumour recurrence and poor prognosis.

### Résumé

Nous avons comparé les paramètres immunologiques et clinico-pathologiques dans des régions tumorales et cirrhotiques de 12 patients atteints de carcinomes hépatocellulaires (HCC) induits par VHC (HCC-HCV), 12 patients HCC induits par l'alcoolisme (HCC-OH) et 23 patients HCV cirrhotiques sans cancer (cirrhose C). Les cellules infiltrant le foie (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup>, CD56<sup>+</sup>, TCRγδ<sup>+</sup>, FoxP3<sup>+</sup> et CD123<sup>+</sup>) étaient significativement plus nombreuses (p<0.05) dans les zones cirrhotiques par rapport aux zones tumorales dans les deux groupes HCC. Les comparaisons entre les cirrroses cancéreuses ont montré que la fréquence des cellules CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup>, FoxP3<sup>+</sup>, CD123<sup>+</sup> ainsi que le rapport des cellules FoxP3/CD4 étaient sensiblement plus élevés (p<0.05) dans la cirrhose HCC-HCV que la cirrhose HCC-OH. Les niveaux d'expression d'IFN-γ, de CD8α et de perforine étaient sensiblement inférieurs (p<0.05) dans la cirrhose HCC-HCV que dans la cirrhose HCC-OH. Les cellules CD3<sup>+</sup>, CD4<sup>+</sup>, CD20<sup>+</sup> et CD8<sup>+</sup> étaient sensiblement plus nombreuses (p<0.05) dans la cirrhose HCC-HCV que dans la cirrhose C. En revanche, la fréquence des cellules CD56<sup>+</sup> était inférieure (p=0.067) dans la cirrhose HCC-HCV. Nous n'avons pas observé de différence à l'intérieur des tumeurs des 2 groupes HCC aux niveaux cellulaires et transformationnels à l'exception de l'expression de CD8α qui était sensiblement moins élevée (p<0.05) dans le groupe de HCC-HCV. La fréquence des cellules intra-tumorales FoxP3<sup>+</sup> est corrélée avec celle des cellules CD4<sup>+</sup> et CD8<sup>+</sup>. Les niveaux tumoraux d'expression de FoxP3 sont corrélés avec ceux de CD8α, CD8β, de l'IFN-γ alors que la corrélation était inverse avec les niveaux d'expression tumoraux de la perforine chez tous les patients HCC. On a également observé des corrélations positives entre la fréquence des cellules CD4<sup>+</sup> et celles des cellules CD8<sup>+</sup>, entre celle de la perforine et celles de l'IFN-γ et de RANTES. La taille des tumeurs est corrélée avec l'expression de FoxP3 chez tous les patients du groupe HCC-HCV. La nécrose tumorale était sensiblement plus importante (p<0.05) dans le groupe HCC-OH que dans le groupe de HCC-HCV et a été inversement corrélée avec les niveaux d'expression de RANTES dans les deux groupes. L'index d'agressivité tumorale, Ki67 a été corrélé avec AFP et la virémie mais inversement avec le niveau d'expression tumoral de perforine. La récurrence tumorale était sensiblement plus fréquente (p<0.05) dans le groupe HCC-HCV que le groupe de HCC-OH. Chez les patients récurrents, le nombre de cellules CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup> et FoxP3<sup>+</sup> étaient sensiblement plus élevé (p<0.05) dans les zones cirrhotique, alors que le nombre de cellules CD123<sup>+</sup> et le rapport FoxP3<sup>+</sup>/CD4<sup>+</sup> étaient sensiblement plus importants (p<0.05) dans les secteurs tumoraux. Dans leur ensemble, ces données indiquent que plus le nombre de cellules immunitaires infiltrant la tumeur et son environnement direct est élevé, moins elle sont actives et plus les risques de récurrence sont élevés, ce qui influence fortement le pronostic vital du patient.