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To my parents, brother,

My wife and my princess!

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AASLD	American Association for the Study of Liver Diseases
ADCC	Antibody-dependent cellular cytotoxicity
AFP	Alpha-1-fetoprotein
APC	Antigen Presenting Cells
BCLC	Barcelona Clinic Liver Cancer
BLI	Bioluminescence imaging
CID	Chemical inducer of death
CIKs	Cytokine-induced killer cella
CK18	Cytokeratin-18
CMV	CytoMegaloVirus
CT	Computed Tomography
CTLA4	Cytotoxic T lymphocyte antigen 4
CTLs	Cytotoxic T lymphocytes
CXCR	CXC-chemokine receptor
DAAs	Direct-Acting Antiviral agents
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DEN	Diethylnitrosamine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	DeoxyriboNucleic Acid
EASL	European Association for the Study of the Liver
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
ESs	Embryonic stem cells
EVR	Early Virological Response
FC	Flow Cytometer
FK506	Tacrolimus
FOXP3	Fork-head box P3
GAG	Glycosaminoglycan
GCV	Ganciclovir
GML	Genetically Modified Lymphocytes
GvHD	Graft versus Host Disease
GvL	Graft versus leukemia
HAV	Hepatitis A Virus
HBeAg	Hepatitis B e antigen
HBV	Hepatitis B Virus

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell culture system of HCV
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HS	Heparan sulfate
HSCc	Hematopoietic stem cells
HSCs	Hepatic stellate cells
HSCT	Hematopoietic stem cells transplantation
HSPG	Heparan sulphate proteoglycan
HSV-tk	Herpes simplex virus thymidine kinase
IFN-γ	Interferon gamma
Ig	ImmunoGlobulin
IH	Intrahepatic
IL	Interleukin
iNKT	Invariant Natural Killer T cells
IPT	Intraportal
IRE	Irreversible electroporation
IS	Intrasplenic
KIR	Killer-cell Immunoglobulin-like Receptors
LDL	Low-density lipoprotein
LSEC	Liver sinusoidal endothelial cell
MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatibility Complex
MIC	MHC class I-related molecule
MMPs	Metalloproteinases
MRI	Magnetic Resonance Imaging
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKG2D	Natural killer group 2 member D
NKs	Natural Killer cells
OKT3	Soluble CD3 monoclonal antibody
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1
PEG-IFN	Pegylated interferon-alpha
PEI	Percutaneous ethanol injection
PHH	Primary humain hepatocyte
PLCG	Polylactide-co-glycolide microspheres
PPH	Primary porcine hepatocyte
PRRs	Pattern-recognition receptors
RFA	Radio Frequency Ablation
RNA	RiboNucleic Acid
RNAi	RNA interference

RVR	Rapid Virological Response
SCID	Severe combined immunodeficiency
STAT	Signal Transducer and Activator of Transcription
SVR	Sustained Virological Response
TACE	TransArterial ChemoEmbolization
TCR	T-Cell Receptor
TGFβ	Transforming Growth Factor beta
TLRs	Toll-like receptors
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
VEGF	Vascular Endothelial Growth Factor
VSV	Vesicular Stomatitis Virus
α-SMA	Alpha-smooth muscle actin

INTRODUCTION

1.Liver

The terminology related to the liver often starts by hepat- from the Greek word for liver, hēpar (ἧπαρ, root hepat-, ἡπατ-). The liver is both the largest internal organ and the largest gland for survival in the human body and lies below the diaphragm in the abdominal-pelvic region of the abdomen. It has a wide range of functions including detoxification, metabolism of carbohydrates, proteins and lipids, production of several biochemicals necessary for the digestion and the regulation of immune responses. Lipids, peptides, carbohydrates and nutrients are transported from the gut to the liver through portal venous blood, and then pass through sinusoidal lining cells and finally are taken up and metabolized by hepatocytes.

1.1. Liver functions

The liver is a major vital organ since, in case of failure, there is no available solution to compensate for the liver functions long-term. Despite the improvement of medicine, replacement methods such as dialysis compensate, under specific conditions, for the liver function only for a short-term period, mostly due to the complex and vital functions of the liver.

Synthesis is a major function of the liver: as a large “factory”, the liver synthesizes daily a large part of amino acids, performs a major role in carbohydrate metabolism as well as in protein metabolism and also takes part in lipid metabolism such as cholesterol synthesis; the liver is responsible for the balance in the blood coagulation system through producing coagulation factors I (fibrinogen), II (prothrombin), V, VII, IX, X and XI, as well as protein C, protein S and antithrombin. To absorb vitamin K from diet, the body needs the liver to produce bile, an alkaline

compound required for emulsifying fats.

The liver is also involved in the reverse function of synthesis: catalysis, another vital function to ensure the homeostasis of the body. Its major breakdown substrates include insulin, hormones and bilirubin. It also converts ammonia to urea into the urea cycle. Importantly, the liver could break down or modify toxic substances as well as most medicinal products by a process called drug metabolism: the body can be poisoned when liver detoxification capabilities are exceeded.

Apart from the above functions, the liver has other important roles: the liver produces albumin, the major osmolar component of blood serum and stores a multitude of substances such as vitamin A, D, K, ions and glucose. The liver is responsible for immunological effects, such as immune responses against antigens that are metabolized in the liver.

1.2. Liver anatomy

1.2.1. Gross anatomy of liver

As the largest organ in the body, the liver is a soft, brown or reddish brown, triangular organ consisting of four lobes of unequal size and shape. In adult, a liver normally weighs 1.44 to 1.66 kg. In order to better understand the anatomy and the function of the liver, researchers usually divide the liver either into several anatomical units or into functional units, both types of units being called segments or lobes, depending on the authors.

Since 1654, when Francis Glisson put forward for the first time the concept of liver segment anatomy in his book “Anatomia hepatis”, the following surgeons or anatomists subsequently presented novel conceptions of liver anatomy in order to better understand the structure and function of liver, such as the German Rex and the British Cantlie, who suggested that the liver should be divided into two lobes by a line named Rex-Cantlie. After the 1950s, several researchers proposed to divide the liver

by sectors or segments, of which the classic and popular conception based on the artery and bile duct form, presented by Healey et al. (Healey and Schroy 1953; Healey, Schroy et al. 1953; Healey 1954), called the North American segmentation. Otherwise, the French segmentation, based on the anatomy of the portal vein and hepatic vein, was determined by Couinaud (Couinaud 1994), and Bismuth (Bismuth 1982). However, due to the improvement of liver anatomical techniques, medical imaging and computing methods, and because of differences between these methods, there is currently no unique conception of liver anatomy accepted by the entire world.

At present, most surgeons and anatomists accept the liver anatomy based on the opinion raised by Couinaud et al. (Couinaud 1994), through an anatomical analysis from almost 100 livers' portal vein (Couinaud 1953), hepatic artery (Couinaud 1954), and bile duct systems (Couinaud 1953; Couinaud 1954).

This description is based on the portal vein distribution within the liver: there are two livers (or hemilivers), right and left, both of them are divided into two sectors according to the secondary division of the branches of the portal vein (Figure.1). The right hemi-liver is divided into two sectors, each divided into two segments, the superior and the inferior segment (right posterior sector: segments n° 7+6; right anterior sector: segments n° 5+8). In the left hemiliver, the falciform ligament and the round ligament separate the median sector into two segments, n°3 and n°4. The left hemiliver is further completed with a unique lateral segment, n°2. The segment 1, between the portal vein bifurcation and the vena cava in the small central and posterior part of the liver, is not considered as being included in either the left or the right hemiliver. Therefore, there is a total of eight segments in the liver, numbered in an anti-clockwise manner from 1 to 8: four segments (n°5 to n°8) are in the right liver and three segments (n°2 to n°4) are in the left liver (Figure 1).

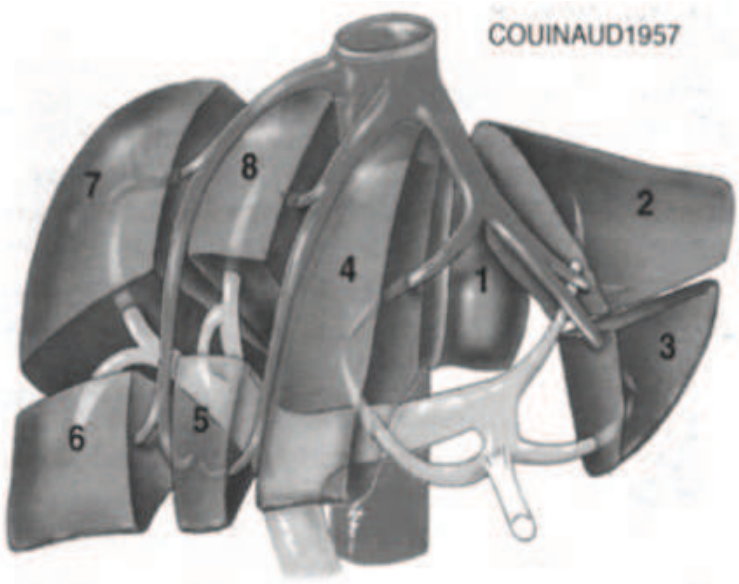


Figure 1. Anatomy of the liver according to Couinaud

Two hemilivers (right and left) are constituted by three sectors (the right posterior and the right anterior in the right hemiliver and the median sector in the left hemiliver) containing two segments each; right posterior sector: segment 6 and 7; right anterior sector: segments 5 and 8; left median sector: segments 3 and 4. The left hemi-liver is further constituted by the segment 2, while the segment 1 is neither included in the left nor in the right hemi-liver (Bismuth 2013).

In order to show the anatomical ways of liver resection, Bismuth (Bismuth, 1982) improved in 1982 Couinaud's description of the division of the liver, taking into account some abnormalities of the original description. As an example, he named the sectors of the right liver as "anterior" and "posterior" sectors instead of "anteromedial" and "posterolateral" sectors, that were named by Couinaud. Another useful example is about the left liver, one sector called the lateral sector by Couinaud has been substituted by segment 2, while another sector of the medial sector has been replaced by two segments (3 and 4); therefore, it keeps the numbers 3 and 4, knowing that these segments are in fact half-segments. Finally, the liver anatomy description is as follows (Figure. 2):

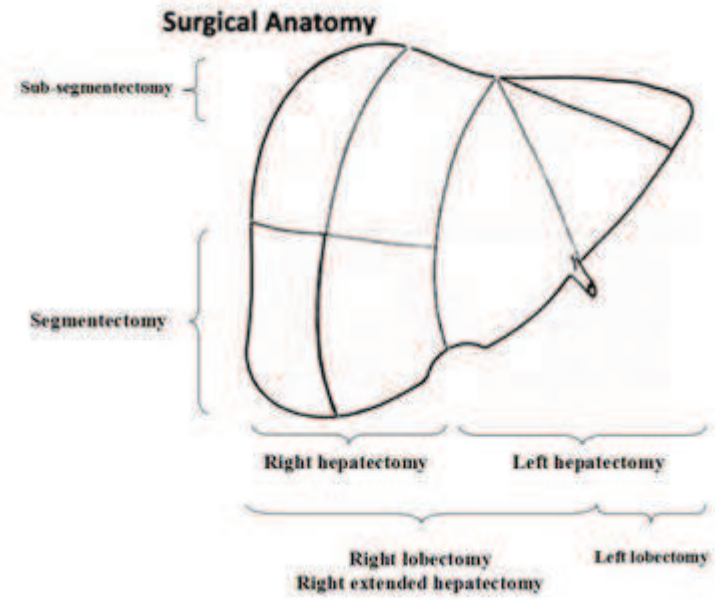


Figure 2. Anatomical surgery of the liver with definition of segmentectomies, hepatectomies and lobectomies

The description of surgical resection corresponds to surgical anatomy: Sub-segmentectomy indicates a partial resection of a segment; a complete resection of a segment is called segmentectomy. Right and left hepatectomy is defined as a resection of hemilivers. Right lobectomy means a right hepatectomy plus segment n°4 (right extended hepatectomy), while left lobectomy indicates only a resection of segments n°2 and 3 (Bismuth 2013).

There are also some other descriptions of liver anatomy (Takasaki, 1986; Strasbourg, 2005), but they do not represent the major conceptions because of their disadvantages: for example, although it individualizes the middle segment to facilitate the central hepatectomy, the Takasaki's description does not take into account the division of the left liver by the round ligament, which is an important anatomical and surgical landmark of the liver.

Conventionally, we refer to the description as the "classical anatomy" which is well defined and rightly called the "lobe" (Figure.3). However, "left lobe," has been banned since the 1950s because of the confusion between lobectomy and hepatectomy. The ancient term for liver resection was left or right lobectomy and has been now renamed left or right hepatectomy. Up to now, the word "lobectomy" is no longer used, while the best way to call the left lateral resection is to come back to the

classical anatomy and to speak about left lobectomy, indicating the resection of the true left lobe of the liver. (Bismuth 2013).

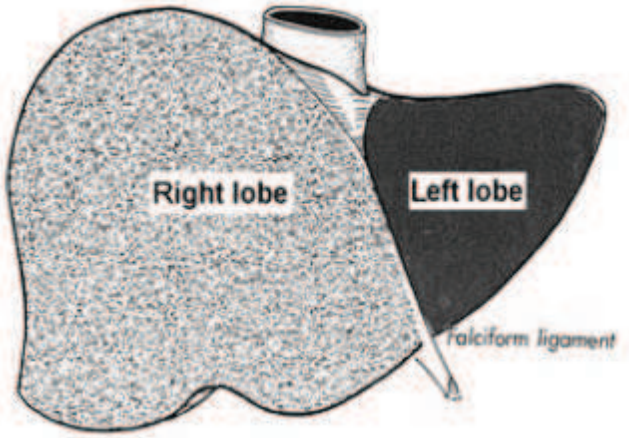


Figure 3. The morphologic anatomy of the liver.

The true lobe is defined as a morphologic anatomy of the liver which is divided into right lobe and left lobe by the falciform ligament and the round ligament (Bismuth 2013).

Taken together, the most useful and modern definition of the liver anatomy for most surgeons and hepatologists, considers that the liver consists of five distinct lobes: the right anterior, right posterior, left medial, left lateral and caudate lobe; the right hemi-liver is the largest lobe, about five to six times larger than the left, that is tapered; the caudate lobe is small and extends from the posterior side of the right lobe and wraps around the inferior vena cava (Figure. 4)

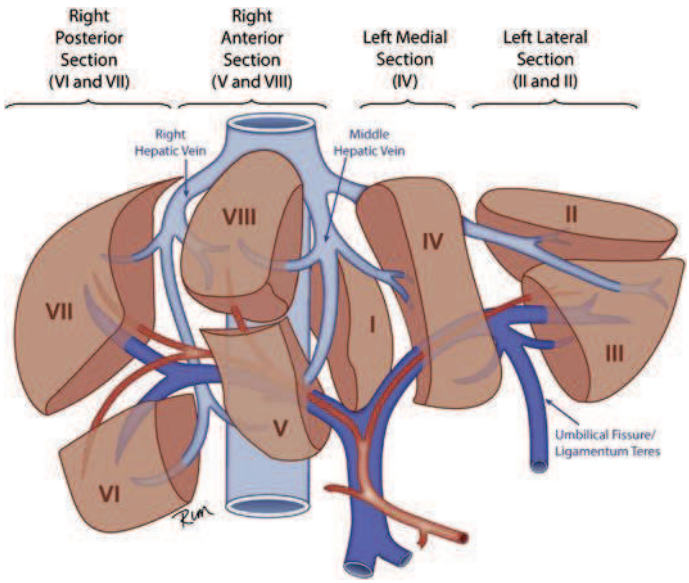


Figure 4. Segments numbering

There are eight liver segments. The numbering of the segments is in a clockwise manner. The segment 1 (caudate lobe) is located posteriorly and is not visible on a frontal view (Lopez-Terrada, Alaggio et al. 2014).

1.2.2. Bile Ducts

Bile duct is a special system that only belongs to the liver: according to its functional and morphological form, it is called the bile tree. Normally, the bile produced by hepatocytes drains into bile canaliculi (microscopic canals), then the countless bile canaliculi in the liver join together into increasingly larger bile ducts. Finally, these bile ducts join to form two large hepatic ducts, the left and right, which carry the bile respectively from the left and right lobes of the liver, in the area of hilus hepatic. These two hepatic ducts join to form the common hepatic duct that drains all the bile away from the liver. From here, the whole bile duct system is divided into intra- or extra-hepatic system. In the extra-hepatic duct, the common hepatic duct joins with the cystic duct from the gallbladder to form the common bile duct, carrying the bile. The diabasis of the common hepatic duct into the pancreas allows it to join with pancreatic duct, and then finally drains the bile and the pancreatic juice to the duodenum of the small intestine (Figure 5). Most of the bile produced by the liver is pushed back up into the cystic duct by peristalsis to arrive in the gallbladder for

storage, until it is needed for digestion.

Bile duct tree

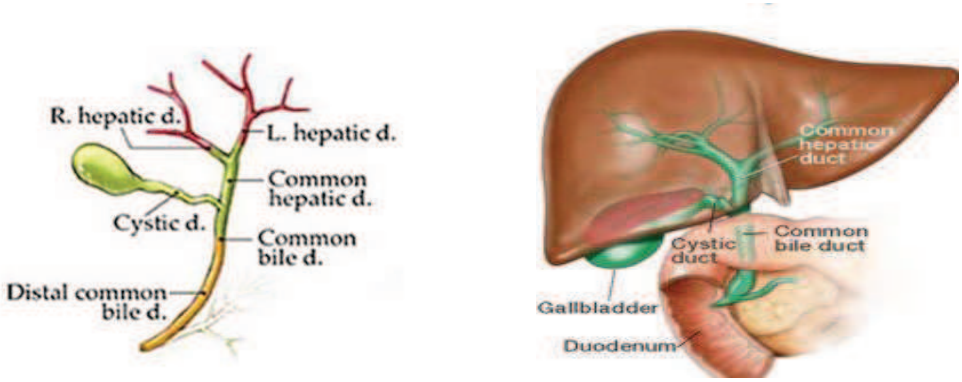


Figure 5. Bile ducts system

The biliary system consists of the organs and ducts (bile ducts, gallbladder, and associated structures). When the liver cells secrete bile, it is collected by the right and left hepatic ducts (intrahepatic duct), ultimately drains into the common hepatic duct (extrahepatic duct); The common hepatic duct then joins the cystic duct from the gallbladder to form the common bile duct, which runs from the liver to the duodenum (the first section of the small intestine) (according to http://www.cpmc.org/advanced/liver/patients/topics/bileduct-profile.html).

1.2.3. Blood Vessels

The blood supply system of the liver is unique among all organs of the body as it has two independent systems: the portal vein system and the hepatic artery system. Seventy-five percent of the blood entering the liver is venous blood coming from the hepatic portal vein (deoxygenated and nutrient-rich): the blood traveling to the spleen, stomach, pancreas, gallbladder, and intestines passes through capillaries in these organs and is collected in the hepatic portal vein. The remaining 25% of the blood supply to the liver is arterial blood coming from the hepatic artery (oxygenated). Terminal branches of the hepatic portal vein and hepatic artery mix together as they enter the liver. All the blood, now deoxygenated, detoxified, and containing normal (homeostatic) nutrient levels, exits the liver via the hepatic vein and returns to the heart (Figure 6).

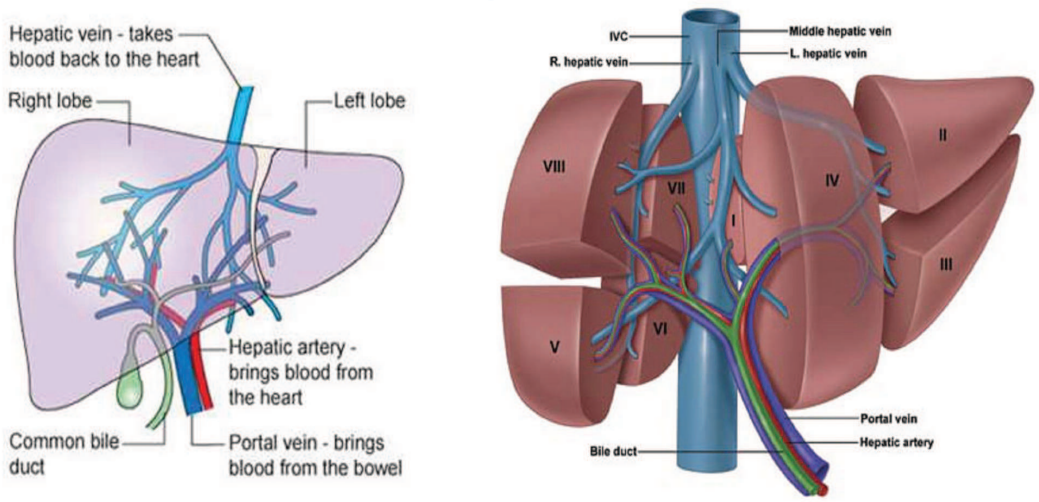


Figure 6. Blood supply in the liver

Liver's two separated sources of blood supply: portal vein system and hepatic artery system; the liver's blood collected by three hepatic veins to join the heart (according to http://www.cancerresearchuk.org/about-cancer/type/liver-cancer/about/the-liver).

1.2.4. Lobules

The liver contains two major types of cells: parenchymal and non-parenchymal cells. Parenchymal cells, commonly referred to as hepatocytes, occupy almost 80% of the liver volume, while the non-parenchymal cells occupy only 6.5% of its volume but constitute 40% of the total number of liver cells. Besides these two kinds of cells, the liver is constituted by other cells such as sinusoidal hepatic endothelial cells, Kupffer cells and hepatic stellate cells. An accurate function of the liver is decided partially by the structure and form of essential liver units, termed liver lobules. The internal structure of the liver is made of around 100,000 of these small hexagonal functional units. Each lobule consists of a central vein surrounded by six hepatic portal veins and six hepatic arteries. These small intra-hepatic blood vessels are connected by capillary-like tubes called sinusoids, a type of capillary similar to a fenestrated endothelium which extends from the portal veins and arteries to join the central vein. Each sinusoid passes through liver tissue containing two main cell types, the Kupffer cells and the hepatocytes. The Kupffer cells are a type of macrophage responsible for capturing and breaking down old, worn out red blood cells. The

hepatocytes are cuboidal epithelial cells that line the sinusoids, make up the majority of cells in the liver and contribute to most of the liver's functions – metabolism, storage, digestion, and bile production. Tiny bile collection vessels known as bile canaliculi run parallel to the sinusoids on the other side of the hepatocytes and drain into the bile ducts of the liver (Figure 7).

Lobular organization

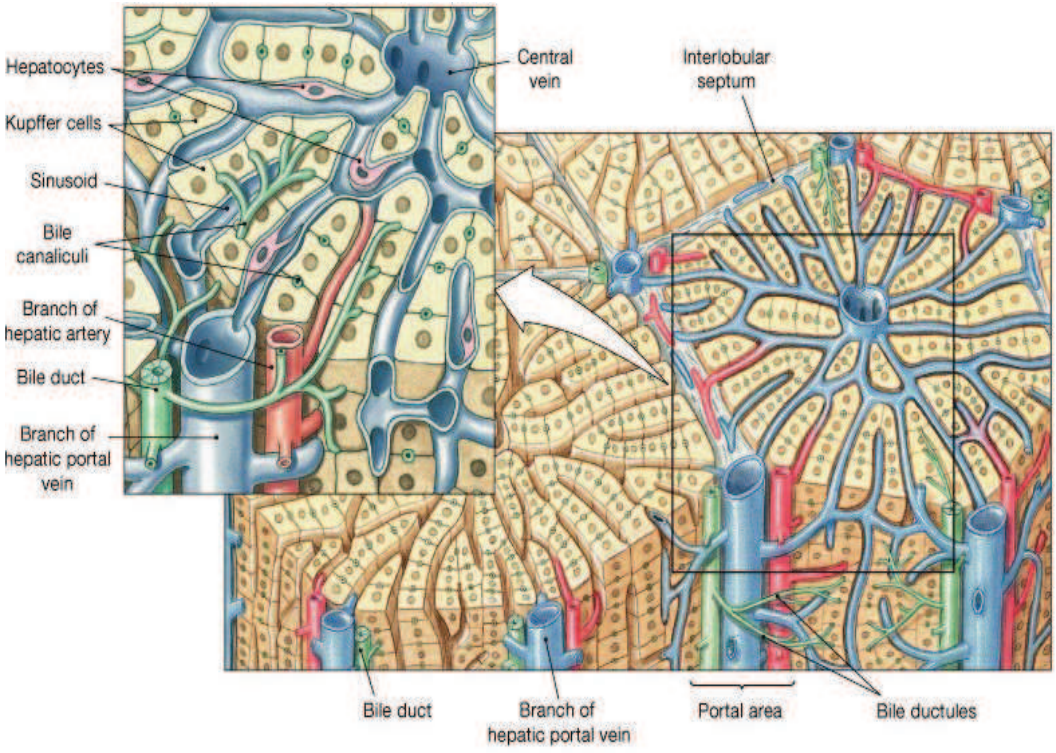


Figure 7: The structure of the liver's functional units or lobules.

Blood enters the lobules through branches of the portal vein and hepatic artery proper, then flows through sinusoids, finally exits the liver through hepatic veins (according to http://chronopause.com/chronopause.com/index.php/2012/02/14/the-effects-of-cryopreservation-on-the-cat-part-2/index.html).

1.3. Interaction between liver and pathogens

1.3.1. Liver microanatomy and pathogens entry pathways

Owing to the vital function of the liver, liver diseases are one of the severe menaces for public health. The main pathogens, such as parasites, bacteria and viruses, cause liver damage by inducing the injury of hepatocytes. Particularly, during the process of attacking, a lot of pathogens, including the hepatitis B and hepatitis C viruses, successfully escape T-cell mediated immunity, by presenting antigens in the liver instead of in lymphoid tissues (Protzer, Maini et al. 2012), then cause hepatic chronic damage through the induction of persistent inflammatory responses.

Normally, blood-borne pathogens are taken up by non-parenchymal cells, such as liver sinusoidal endothelial cells (LSECs) and Kupffer cells. Actually, there is no identified liver-specific receptor or hepatocyte-specific receptor molecules that mediate a pathogen-specific recognition and binding, suggesting that most of the hepatotropic pathogens recognize and bind to hepatocytes through broadly-expressed receptor molecules. Therefore, the hepatotropism of pathogens may be rather related to the functional properties of the liver and to its central role in metabolism, catabolism and blood detoxification (Figure 8).

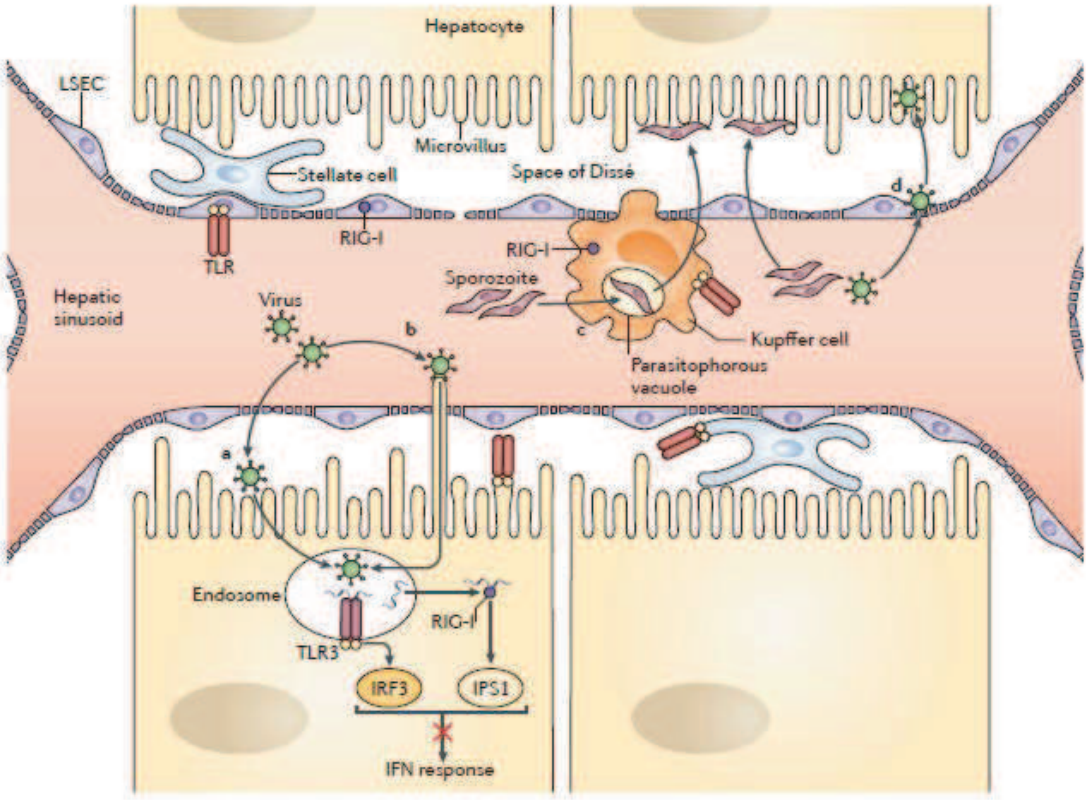


Figure 8. Liver microanatomy and pathogens entry pathways.

Sinusoidal cell populations (Kupffer cells, liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells) form a loose physical barrier between hepatocytes and the blood circulating within the sinusoids. Blood-borne pathogens may infect hepatocytes through direct contact with hepatocytes, either after passage through fenestrae **(a)** or by contacting microvilli that extend into the sinusoidal lumen **(b)**. Pathogens may also first exit the bloodstream by entering Kupffer cells **(c)** or LSECs **(d)** before infecting their final target cell, the hepatocyte (Protzer, Maini et al. 2012).

In LSECs, there is a sinusoidal barrier (fenestrae), whose diameter is up to 100nm, which prevent most of the pathogens from entering or binding to hepatocytes. Nutrients and blood-borne pathogens that induce liver damage could pass through this barrier by either the mechanical force, that allow some of them, such as chylomicrons, to squeeze through endothelial cell fenestrae (Wisse, Dezanger et al. 1985) (this force generated normally by cells flows through sinusoids), or by active transport to pass through LSECs, such as the delivery of IgA across mucosal cells or chemokines (such as the prototype chemokine IL-8 internalized by venular ECs abluminally and

transcytosed to the luminal surface) (Mostov 1994; Middleton, Neil et al. 1997). This suggests that the LSECs and Kupffer cells have broad receptor-mediated endocytic capacities (Smedsrod 2004). On the other hand, it is difficult to identify which pathway is specifically used by the pathogens, but there is more and more evidence showing that the transport properties of sinusoidal cells are usually used by the pathogens to increase the liver infection.

1.3.2. Innate immune defenses of the liver

As most organs in the body, the liver has its innate immune system to protect from the different pathogens (Figure 9). This process is initiated by the recognition of pathogens by a primitive part of the immune system: the pattern-recognition receptors (PRRs), expressed by cells of the innate immune system to identify pathogen-associated molecular patterns (PAMPs) associated with microbial pathogens or cellular stress, as well as damage-associated molecular patterns (DAMPs), which are associated with cell components released during cell damage. They are also called pathogen recognition receptors or primitive pattern recognition receptors because they evolved before other parts of the immune system, particularly before the adaptive immunity. The PRRs are classified according to their ligand specificity, function, localization and/or evolutionary relationships. On the basis of their function, PRRs may be divided into (i) endocytic PRRs, promoting the attachment, engulfment and destruction of microorganisms by phagocytes without relaying an intracellular signal, or (ii) signaling PRRs such as membrane-bound Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors. Interestingly, Toll-like receptors and cytosolic helicases are expressed not only on the Kupffer cells and hepatic DCs, bone marrow-derived immune cells, but also expressed on the hepatocytes, LSECs and hepatic stellate cells (Saito, Owen et al. 2008; Wang, Trippler et al. 2009; Wu, Meng et al. 2009; Kern, Popov et al. 2010; Ebert, Poeck et al. 2011), termed liver-resident cells. Moreover, the hepatic and splenic immune cells have the same capacity of

recognition of pathogens (Gao, Jeong et al. 2008), although there are small differences in PRR expression (Seki, De Minicis et al. 2007). Kupffer cells and LSECs are capable of sensing low concentrations of TLR ligands and then producing interleukin-6 (IL-6) and type I interferons (IFNs) (Wu, Lu et al. 2007; Kern, Popov et al. 2010). IL-6 promotes the hepatocytes to express a lot of innate effector molecules such as the acute-phase protein C-reactive protein (Baumann and Gauldie 1994). In parallel, type I IFNs have an important role to fight the pathogens, such as improving antigen presentation, increasing natural killer (NK) cell activity and inducing antiviral effects. On the other hand, the liver cells may be induced into a state of lipopolysaccharide (LPS) tolerance towards further pro-inflammation stimulation (Biswas and Lopez-Collazo 2009) when they are constantly stimulated by the TLR ligands. This may reduce the local immune effects of cytotoxic lymphocyte responses (Limmer, Ohl et al. 2000), by preventing the LSECs and hepatic DCs from maturing into immunogenic antigen-presenting cells (APCs)(de Creus, Abe et al. 2005; Kern, Popov et al. 2010).

There is a type of cytotoxic lymphocyte critical to the innate immune system in liver called NK cells, providing rapid responses to virally infected cells and respond to tumor formation. They play a critical role against the pathogens through either directly contact or secretion of different cytokines, such as IFN-γ and TNF-α. Moreover, they are able to modulate the function of other cells such as DCs, Kupffer cells, B cells and LSECs by secreting multiple cytokines. Interestingly, compared with the circulation, the frequency of NK cells is greatly enriched in the liver.

During the process of antibacterial defense, other innate immune cells, termed as invariant Natural Killer T cells (iNKT), have an important role (Lee, Moriarty et al. 2010). Moreover, non-classic innate immune cell populations (such as γδ T cells) may also contribute to the hepatic immune defense following infection.

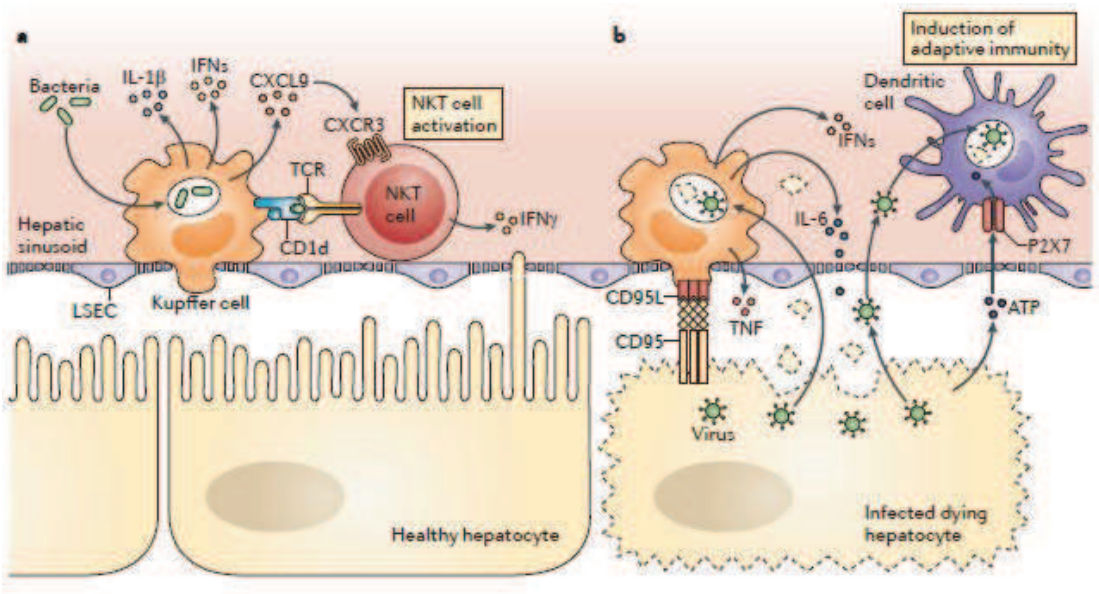


Figure 9. Host mechanisms involved in the clearance of pathogens in the liver.

(a) Sinusoidal cell populations, such as Kupffer cells, phagocytose circulating bacteria and crosstalk with natural killer T (NKT) cells to generate strong intravascular pathogen-specific immune responses. Inhibiting the access of pathogens to hepatocytes may have an important role in preventing the development of persistent hepatic infections. **(b)** The death of infected hepatocytes during viral replication may cause the activation of Kupffer cells or dendritic cells (DCs), which in turn promote the killing of other hepatocytes through CD95 and the release of pro-inflammatory mediators. Combinatorial stimulation by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), such as ATP, may allow immune-mediated control of established hepatic infections (Protzer, Maini et al. 2012).

1.3.3. Liver infection by parasites

The liver is a target organ that is often attacked by several parasites (Table 1). When the parasites are delivered into the body, most migrate rapidly into the lymphatic vessels as well as blood vessels and finally reach the liver through evading the clearance of phagocytic cells, followed by binding to Kupffer cell (Pradel and Frevert 2001). It has been suggested that Kupffer cells have a critical role during this process of hepatocytes infection (Ishino, Yano et al. 2004; Baer, Roosevelt et al. 2007) and

are used by the parasites to cross the sinusoidal barrier and therefore to infect hepatocytes (Mota, Hafalla et al. 2002). Therefore, besides using their innate migratory capacity to evade elimination of phagocytic cells, parasites also use host cells such as Kupffer cells to increase their efficiency at infecting hepatocytes.

Table 1 Clinically relevant pathogens that target the liver

Common name	Body parts affected	Diagnostic specimen	Source/ Transmission
Amoebiasis	Intestines, liver	Stool	Fecal-oral transmission of cyst
Echinococcosis - tapeworm	liver, lungs, kidney, spleen	Imaging of hydatid cysts in the liver	Intermediate host or definite host
Clonorchiasis	Gall bladder ducts, inflammation of liver		Ingestion of under prepared fresh water fish
Liver fluke Fasciolosis	Liver, gall bladder	Stool	freshwater snails
Chinese Liver Fluke	Bile duct		Consuming infected raw, slightly salted or frozen fish
Intestinal schistosomiasis	Intestine, liver, spleen, lungs,	Stool	Skin exposure to water contaminated with infected Biomphalariafresh water snails
Schistosomiasisby Schistosoma japonicum	Intestine, liver, spleen, lungs, skin	Stool	Skin exposure to water contaminated with infected Oncomelania sp. snails
Anisakiasis	Allergic reaction	Biopsy	Ingestion of raw fish, squid, cuttlefish, octopus
Roundworm -Baylisascariasis	Intestines, liver, lungs, brain		Stool from raccoons
Toxocariasis	Liver, brain, eyes	Blood, ocular examination	Unwashed food contamined with Toxocara eggs, undercooked livers of chicken
Trichinosis	Liver, intestines	Stool	Undercooked meat from an animal infected with the trichinella spiralis parasite
Clonorchiasis	Liver, intestines	Stool	Ingestion of raw or undercooked fish
Schistomiasis	Liver, intestines, spleen, lymph notes	Stool	Ingestion of raw
Plasmodium spp	Liver, intestines	Saliva of a biting female mosquito	Anopheles spp. mosquitoes

1.3.4. Liver interaction with bacteria

Most of the bacteria from the blood are enable to be cleared by the immune system before reaching the liver. However, liver is an organ targeted by some bacteria, such as mycobacteria and *Listeria* spp. When these bacteria are enable to escape the immune clearance, they can cause liver damage that is characterized by forming a "granuloma" (Popov, Abdullah et al. 2006; Egen, Rothfuchs et al. 2008), in which macrophages infected by mycobacteria secrete bacterial proteins, then cause the liver remodeling by inducing the expression of the matrix metalloproteinase 9 (Taylor, Hattle et al. 2006). Although these granulomas could separate normal tissue from the infecting bacteria (Popov, Abdullah et al. 2006), it has been suggested that granulomas also contribute to the dissemination of virulent bacteria once they form a "wall" (Davis and Ramakrishnan 2009) in the tissues. Therefore, it implies that granuloma probably offers a long-term survival environment for the bacteria by providing a specific anatomical compartment in the liver.

In a normal immune system body, most of the blood-borne bacteria are rapidly eliminated from the liver by its functional hepatic immune cells. After recognizing the ingested blood-borne bacteria, iNKT cells are attracted by Kupffer cells via a CXC-chemokine receptor 3 (CXCR3)-dependent process, then, induce an immune response in order to further prevent the bacterial infection (Lee, Moriarty et al. 2010). Thus, iNKT cells play a critical role against pathogens within the liver by recognizing microbial antigens and carrying out immune effector functions. This suggests that iNKT cells represent a bridge that connects innate and adaptive immunity (Taniguchi, Seino et al. 2003; Klugewitz, Adams et al. 2004). Apart from iNKT cells, the effector memory T cells as well as effector T cells in the liver also contribute to the induction of immune defense (Klugewitz, Adams et al. 2004; Keating, Yue et al. 2007; Polakos, Klein et al. 2007; Paust, Gill et al. 2010). Hence, one opinion about the clearance of bacteria in liver is that pathogen recognition and prevention from access to the hepatocytes are exerted in the hepatic sinusoidal compartment, which is functionally distinct from the parenchymal compartment, where it is more difficult to eliminate the

infection and it even facilitates the infection through the tolerogenic properties of the local microenvironment and organ-resident cell populations (Protzer, Maini et al. 2012).

1.3.5. Liver infection by hepatic viruses

Viruses primarily attacking the liver are called hepatitis viruses. Infection of hepatotropic viruses involves several processes that are different from parasite infection, owing to their inability to actively move. There are several types of hepatitis viruses including types A, B, C, D, E, and possibly G, the types of A, B, and C being the most common. All hepatitis viruses can cause acute hepatitis and types B and C can also cause chronic liver infection (Table 2). The biology of HCV will be detailed in the next chapter, due to its importance in our project.

Table 2 list of viral infection of the liver

Virus	Outcome of infection
HAV	**Resolved after protracted infection**
HBV	**May be cleared after protracted infection or persist for years**
HCV	**May be cleared after protracted infection or persist for years**
HDV	**May establish superinfection in combination with HBV infection**
HEV	**Resolved after acute infection**
EBC	**May infect the liver during systemic infection**
CMV	**May infect the liver during systemic infection**

1.3.5.1. Hepatitis A Virus (HAV)

Hepatitis A is a food-borne pathogen. Following ingestion, HAV enters the bloodstream through the epithelium of the oropharynx or intestine, then reaches its target, the liver. The receptor for HAV is a mucin-like class I integral-membrane glycoprotein that is ubiquitously expressed (Kaplan, Totsuka et al. 1996; Ashida and Hamada 1997; Feigelstock, Thompson et al. 1998). However, the HAV replicates only

within hepatocytes and Kupffer cells. HAV targets the liver through the physiological transport pathway of IgA in the enterohepatic circulation. After their production in the intestinal mucosa, HAV-specific IgA antibodies serve as carriers of the virus by binding to circulating HAV. However, because of the IgA-HAV complexes are probably able to clear before reaching hepatocytes, the *in vivo* relevance of the above process is still not clear.

Although HAV could infect several primate cells, at present there is no report to document a long-term persistent established infection in the liver (Gust and Feinstone 1990; Cuthbert 2001). Owing to the absence of adapted virus to culture, the pathogenesis of HAV infection as well as the virus clearance from the liver is still unknown.

1.3.5.2. Hepatitis B Virus (HBV).

Although many developments have been obtained about the behaviors of this virus, due to short available animal models, at present, it is still not clear how hepatotropic viruses target the liver *in vivo*. Most research about HBV is based on the cell culture systems and allows better understanding of the molecular mechanisms involved in HBV infection, such as the HBV entry (Gripon, Rumin et al. 2002). Only one receptor, sodium-taurocholate co-transporter polypeptide (NTCP), has been identified to date as a receptor for HBV (Yan, Zhong et al. 2012; Ni, Lempp et al. 2014) but some additional receptors or co-receptors may still be identified. In contrast to HAV, HBV is a major virus able to induce a chronic liver infection as well as several complications such as cirrhosis and liver cancer. Initial HBV infection is controlled in most patients but it is not completely eliminated, suggesting that HBV infection could reactivate under special conditions such as immunosuppression (Rehermann, Ferrari et al. 1996). Normally, HBV does not cause cytopathic effects and its infection outcome depends on host-viral interactions, i.e., the relationship between the host immune response and the virus determines the natural history of HBV infection. A complete immune response to HBV causes the greatest viral

clearance, but, in parallel, this complete response could also cause the worst liver injury (Jindal, Kumar et al. 2013), while a chronic hepatitis B infection is usually caused by an inefficient immune response.

In acute infection, there is an initial incubation phase (2 to 6 weeks), followed by acute hepatitis with high level of serum aminotransferases. Acute hepatitis could be resolved with normalization of liver function tests. Hepatitis B surface antigen (HBsAg) usually persists for a few months until it is cleared from serum. However, low levels of HBV persist in the liver for life, and HBV infection may reactivate during some condition such as under immunosuppression (Villeneuve 2005). The transition from acute to chronic infection signifies a failure of the immune response to eradicate the virus. The host immune responses determine the outcome of infection and the severity of liver injury.

In chronic infection, an initial immune tolerance phase is characterized by high HBV viral loads, combining with a positive hepatitis Be antigen (HBeAg), but amino-transferase levels are normal. This immune tolerance phase is analogous to the incubation period of acute infection, except that it may last for decades. It is followed by an immune competence phase where an immunologic response develops leading to hepatocyte necrosis. High HBV viral loads and HBeAg persist, but are accompanied by chronic hepatitis with abnormal amino-transferase levels and progressive liver damage, which can lead to cirrhosis and its complications.

1.4. Hepatitis C virus (HCV)

1.4.1. Virion structure and circulation in the host

The HCV has a diameter of 55 to 65 nm and is constituted by a nucleocapside surrounded by a lipid bilayer in which are anchored to the E1 and E2 envelope glycoproteins (Figure 10). The nucleocapsid is constituted by a capsid, with a diameter of 30 to 35 nm, formed by the core protein and contains the viral genome

which consists of a single-stranded RNA of positive polarity of 9.6Kb.

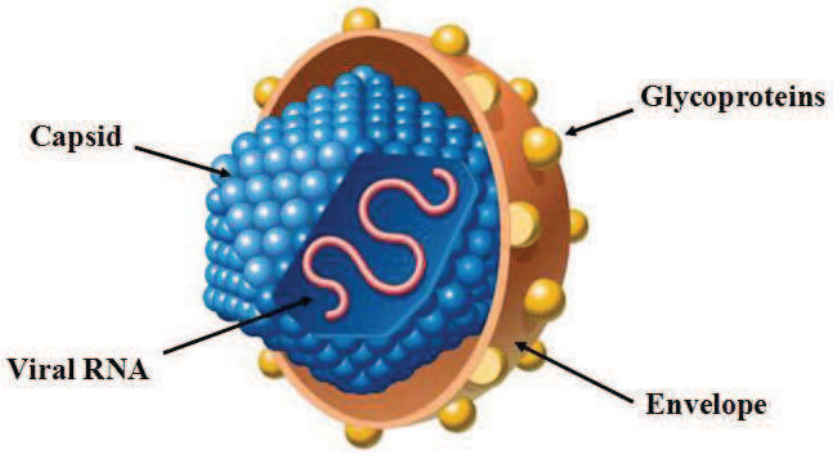


Figure 10. HCV structure

HCV is constituted by a nucleocapside surrounded by a lipid bilayer in which are anchored the E1 and E2 envelope glycoproteins.

(according to http://trialx.com/curebyte/2011/05/22/hcv-photos-and-related-clinical-trials/).

1.4.2. The viral cycle

The HCV has a tropism particularly restricted since the only target cells of the virus are hepatocytes. Other cell types such as B cells and dendritic cells may be the reservoirs of the virus but do not have the ability to support viral replication (Dustin and Rice 2007). Only humans and chimpanzees can be infected with HCV, which reflects the existence of species-specific host factors that are essential for infection at all stages of the cycle HCV viral.

The key steps in the viral life cycle are shown in Figure 11. After interaction with specific membrane receptors, the virus is endocytosed into the target cell and the viral RNA is released into the cytoplasm after uncoating. The translation and RNA replication take place in the endoplasmic reticulum, and the viral particles are assembled, matured in the Golgi apparatus prior to being leached out of the cell. The newly formed virions can then infect new target cells.

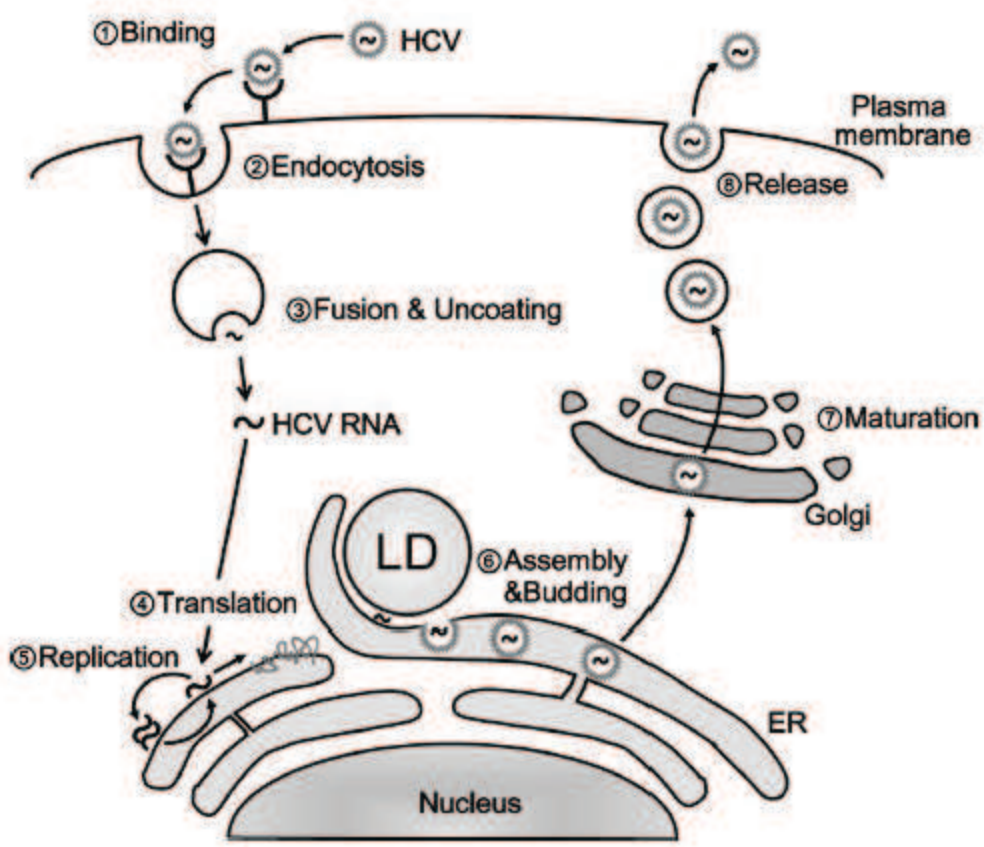


Figure 11. HCV Life Cycle

After attachment to the target cell **(1)**, HCV interacts with membrane receptors to be endocytosed **(2)**. After uncoating (3), the viral RNA is released into the cytoplasm and translated **(4)** and replicated in the ER **(5)** where it will be assembled **(6).** Maturation takes place in the Golgi apparatus **(7)**, then the newly formed exocytosed virion is released in the extracellular space **(8)** for the infection of new cells. GL, lipid droplet; ER, endoplasmic reticulum (Fukasawa 2010)

1.4.3. HCV pathogenesis

HCV infection leads to 10 to 25% cases of acute infection associated with spontaneous recovery. However, in most cases, the infection becomes chronic, and can lead to severe complications such as cirrhosis (20 to 30% of chronic carriers) and hepatocellular carcinoma (HCC). The disease progresses slowly, with a period of 20 to 30 years between infection and the onset of HCC (Pawlotsky 2004). The mechanisms involving the progression of liver damage are not completely understood. It is widely accepted that the virus does not have a direct cytopathic effect on

hepatocytes, except the phenomenon of accumulation of lipids in hepatocyte cells called steatosis. Lesions in the liver are mainly inflammatory, resulting from lymphocytic infiltration (Pawlotsky 2004).

The APCs (Antigen-Presenting Cells) in the liver, such as dendritic cells, migrate to the lymph node in order to activate CD4 + T cells and CD8 + T that induce strong anti-viral responses due to their production of IFN-γ and their cytotoxicity towards infected cells (Georgel et al. 2010). The death of the infected hepatocytes active myeloid dendritic cells to stimulate NK and NKT cells also produce large amounts of IFN-γ. This strong cellular response results in a spontaneous resolution of the infection within six months of the start of the infection. The virus-specific T lymphocytes can be found even 20 years after the resolution of the infection (Diepolder 2009). In the majority of cases (75 to 90%), HCV viral RNA persists beyond six months after the start of infection and is then called chronic hepatitis. In order to persist chronically, the virus has developed strategies for escape the immune response of the host. Dendiritc cells are altered by Core, E1, E2 and NS3 functions of HCV, resulting in a poor presentation of viral antigen to T cells, non-specific cytotoxicity of NK cells and IFN-γ production is inhibited by E2, suggesting that the HCV acts directly on the functionality of these cells. Although T lymphocytes infiltrate the liver profusely, they are unable to fight infection, especially because the virus is active in inhibiting cellular responses of the host. Indeed, the core protein interacts with complement receptor gC1q present on the surface of T lymphocytes intrahepatic, which induces overexpression of the molecule Programmed Death-1 (PD-1) to the surface of these cells. This overexpression inhibits the activation of T cells and induces a functional impairment, termed exhaustion, in terms of cytotoxicity, proliferation and proinflammatory cytokine production (Larrubia, Benito-Martinez et al. 2009).

The main complication of chronic infection with HCV is the development of fibrosis, which is directly related to chronic inflammation of the liver. Fibrosis is characterized by abnormal accumulation of extracellular matrix components in the liver parenchyma (Pawlotsky 2004). The synthesis of these components (collagen,

laminin, fibronectin, proteoglycans), called fibrogenesis, powered by myofibroblasts is generated due to the activation of hepatic stellate cells (Pawlotsky 2004). Then in 1 to 4% of patients, cirrhosis could lead to the development of HCC.

Based on the cell-culture system, researchers understand more and more about HCV entry, replication, and release. HCV envelope proteins E1 and E2 bind to the host cell lipoproteins, then form as a complex 'lipoviroparticles'(Bartenschlager, Penin et al. 2011). Lipid transport pathways may be involved in liver targeting by HCV. After combining with heparan sulphate proteoglycans, HCV bind to the low-density lipoprotein receptor, scavenger receptor B1 and CD81, further combine with Claudin-1 and Occludin in tight junctions before endocytosis(Pileri, Uematsu et al. 1998; Agnello, Abel et al. 1999; Scarselli, Ansuini et al. 2002; Evans, von Hahn et al. 2007; Ploss, Evans et al. 2009; Lupberger, Zeisel et al. 2011). Moreover, several novel receptors such as the epidermal growth factor receptor (EGFR), ephrin type A receptor 2 (EPHA2) (Lupberger, Zeisel et al. 2011) and NPC1-L1 (Sainz, Barretto et al. 2012) have been reported recently. However, all of these receptors are not specifically expressed by hepatocytes, suggesting that there are still more developments needed to be improved in order to clarify how liver targeting by HCV is achieved.

1.4.4. Interactions between the immune system and HCV

Compared to HAV and HBV, HCV has its own specific properties about the interactions with the host immune responses. HCV may be eliminated more rapidly than HAV because it induces earlier and stronger innate immune responses (Lanford, Feng et al. 2011; Veerapu, Raghuraman et al. 2011), but on the other hand, there are still more than 50% patients whose innate immune responses fail to clear HCV infection, suggesting that HCV can persist in the liver after a successful escape of immune clearance. This period may last several years without inducing clinical liver disease, or, in some patients, may induce cirrhosis and even cancer. Interestingly, the

outcome of IFN-alpha treatment for acute HCV infection is always better than the outcome of IFN-alpha treatment for chronic infection.

1.4.5. The role of CTLs in the host-virus interactions

Cytotoxic T lymphocytes have an important role during the immune response against the virus infection. They may produce several cytokines such as TNF-α and IFN-γ to protect the liver by limiting the replication of HCV in the hepatocytes (Guidotti and Chisari 2001). The efficacy of CTLs in controlling HCV infection is controlled by switches in virus-specific CTLs quantity or dysfunction, contributing in the establishment of chronic viral infection. For example, the susceptibility of CTLs to apoptosis is relative to the tolerogenic hepatic priming (Bowen, Zen et al. 2004), in which the BCL-2-interacting mediator of cell death (BIM)(Holz, Benseler et al. 2008) has the property to induce T cell death. As an apoptotic mediator that depletes virus-specific CTLs during the infection of HBV and HCV(Larrubia, Benito-Martinez et al. 2011; Schurich, Khanna et al. 2011), BIM is initiated by co-inhibitory signals such as cytotoxic T lymphocyte antigen 4 (CTLA4) or T cell-intrinsic transforming growth factorβ (TGFβ) (Tinoco, Alcalde et al. 2009; Schurich, Khanna et al. 2011). Induction of virus-specific CTLs' dysfunctions during the chronic infection (called exhaustion) have been observed in the high-dose viral infections (Wherry 2011). One cause of exhaustion of CTLs is due to the switches of balance between co-inhibitory signals and co-stimulatory signals. For example, programmed cell death protein 1 (PD1), known as a co-inhibitory molecule that prevents autoimmunity by regulating T cells reactivity (Iwai, Terawaki et al. 2003; Dong, Zhu et al. 2004; Isogawa, Furuichi et al. 2005), contributes in the T cell exhaustion during the chronic infection (Blackburn, Shin et al. 2009).

Compared with the circulating part, the liver has the highest quantity of specific CTLs in the cases of the HBV and HCV infection (He, Rehermann et al. 1999; Maini, Boni et al. 2000). However, most of the CTLs in the liver have higher levels of

expression of co-inhibitory receptors (Fisicaro, Valdatta et al. 2010; Klenerman and Thimme 2012). Moreover, because mutations of viral epitopes occur often during the chronic infection, CTLs lose their capacity to recognize their specific antigen, which could induce the expression level of inhibitory co-receptors (Bengsch, Seigel et al. 2010). However, the T-cell expression of PD1 is induced in association with other events and is not determined only by the antigen load. High levels of ligands for the co-inhibitory molecules are expressed in the liver, which contribute to intrahepatic T-cell tolerance. In the liver, Kupffer cells, stellate cells, LSECs and hepatocytes all express PD1 ligand 1 (PDL1) (Yu, Chen et al. 2004; Diehl, Schurich et al. 2008). Compared with the controls, PDL1 levels are always upregulated in hepatitis patients (Zhang, Zhang et al. 2008; Kassel, Cruise et al. 2009). Thus, *in vivo*, virus infection may induce a specific mechanism of T-cell exhaustion through co-inhibitory pathway to avoid effective antiviral immunity in the liver(Barber, Wherry et al. 2006)

A lack of CD4+ T cell contribute to the CTL exhaustion in the chronic infection and cancer(Wherry 2011). Dysfunctions of CD4+ T cell as well as of CTLs were observed during the chronic infection, which affected their immune response through inhibitory mechanisms(Raziorrouh, Ulsenheimer et al. 2011). Particularly, the numbers of fork-head box P3 (FOXP3)-expressing CD4+ regulatory T (Treg) cells, known as an extrinsic regulation of effector T cells, have been fund increasing in the livers of patients with HCV chronic infection, compared with the normal livers. These cells limit T cell responses during chronic infection, thereby minimizing T cell-dependent immunopathology. However, they upregulate PD1 expression that, by controlling STAT-5 phosphorylation, negatively regulates Tregs at sites of chronic inflammation (Franceschini, Paroli et al. 2009). Thus, PD1 overexpression by Tregs may inhibit the Treg-mediated prevention of excessive immune responses, thereby contributing to increase T cell-dependent immunopathology.

1.4.6. Treatments of HCV infection

Currently, the standard of care for chronic HCV infection is pegylated

interferon-alpha (PEG-IFN) -α, a stabilized form of IFN-α, associated with ribavirin, a nucleoside analogue. It is effective in only 50% of patients infected with genotype 1 and in 80% of those infected by genotypes 2 and 3.

Several responses to treatment can occur; following initiation of treatment, responding patients may be subject to two types of responses: Rapid Virological Response (RVR), characterized by complete elimination of the virus after four weeks of treatment, or Early Virological Response (EVR), characterized by a reduction in viral load of at least two log after 12 weeks of treatment and then becomes undetectable. End-of-Treatment Response (ETR) also leads to two types of responses: Sustained Virological Response (SVR) during which the viral load remains negative for six months or more and that leads a cure in 99% of cases or, in 1% of cases, in relapse resulting in a detectable viral load up to and including similar to those before treatment rates. Finally, a partial response is defined as a decrease in viral load of 2 log during treatment but which is still detectable after treatment, while the non-responder patients are characterized by a viral load any stable throughout the 24 or 48 weeks.

Recent advances in understanding the viral cycle allowed the identification of new therapeutic targets and development of drugs that act directly on the virus, called "DAAs" (Direct-Acting Antiviral agents)

1.4.7. Direct-Acting Antiviral agents

DAAs were developed to improve SVR rates, reduce adverse events, and improve adherence to therapy in HCV patients. These drugs target the different stages of virus development and replication. Several clinical trials investigating DAAs have yielded encouraging results that provide hope for patients with chronic HCV. DAAs can be classified into two main groups: first-generation and second-generation protease inhibitors.

1.4.7.1. First-generation protease inhibitors

Since 2011, two inhibitors of protease NS3/4A, preventing cleavage of the polyprotein, telaprevir and boceprevir, showed their ability to induce a significant decrease in viral load, but variants resistant to treatment emerge quickly (Sarrazin, Kieffer et al. 2007; Susser, Welsch et al. 2009). Mutations responsible for these resistances, at the catalytic site of NS3, were identified (Susser, Vermehren et al. 2011). Used in combination with PEG-IFN-α and ribavirin, telaprevir and boceprevir increased SVR rates in treatment-naïve or previously treated patients infected with genotype 1 (Hezode, Forestier et al. 2009) (Kwo, Lawitz et al. 2010). Interestingly, it was recently shown that in patients infected with genotype 1, the telaprevir limits the period of combined PEG-IFN-α and ribavirin treatment: SVR in 75% of patients were obtained after 12 weeks of triple therapy followed by 12 weeks PEG-IFN-α and ribavirin alone (without telaprevir group, 24 weeks PEG-IFN-α and ribavirin alone: 44% SVR) (Jacobson, McHutchison et al. 2011).

Consequently, current practice guidelines recommend a triple therapy regimen combining PEG-IFN-α, ribavirin, and telaprevir or boceprevir (2014). However, triple therapy with boceprevir or telaprevir has some drawbacks, including drug–drug interactions and viral resistance (Kamal 2014).

Although triple therapy has improved SVR rates, there are some limitations for this strategy: this regimen increases adverse events such as anemia and might require reduction of the ribavirin dose. Moreover, boceprevir and telaprevir are only effective against genotype 1 and are without antiviral activity against genotype 2, 3, or 4. Furthermore, triple therapy is ineffective in patients who have not responded to previous dual PEG-IFN/ribavirin therapy (Kamal 2014).

1.4.7.2. Second-generation protease inhibitors

Second-generation protease inhibitors, such as simeprevir, asunaprevir, and danoprevir, are currently developing in order to overcome the shortages of the

first-generation protease inhibitors in HCV genotypes 2, 3, and 4 and to minimize their adverse events (Asselah and Marcellin 2014). The safety profile of triple therapy including simeprevir was found to be comparable with that of combination PEG-IFN-α and ribavirin therapy (Kamal 2014).Triple therapy comprising danoprevir, PEG-IFNα-2a, and ribavirin was assessed in a clinical trial conducted in patients with chronic HCV genotype 1. SVR rates were higher in patients treated with danoprevir than in those on control group. 79% of patients in the group of danoprevir had a rapid virologic response, 96% among these had an SVR (Marcellin, Cooper et al. 2013).

In the DAUPHINE trial (ClinicalTrials.gov registry number NCT0122094), treatment-naïve patients with HCV genotype 1 or 4 were randomized to receive danoprevir plus PEG-IFN-α and ribavirin for 12 or 24 weeks; or PEG-IFN-α and ribavirin alone for 48 weeks. The result showed that the patients in the response-guided therapy arm with an extended RVR stopped all therapy at week 12; patients without an extended RVR continued all treatment to week 24. This suggests that the combination of danoprevir/ribavirin plus PEG-IFN-α and ribavirin was an effective and safe strategy in treatment-naïve patients with HCV genotype 1 or 4 infection (Everson, Cooper et al. 2014).

2.Hepatocellular carcinoma

2.1. Epidemiology and risk factors

Hepatocellular carcinoma (HCC) is a major health problem worldwide. It presents the sixth most common cancer (Jemal, Bray et al. 2011; El-Serag 2012), and the third most common cause of cancer-related death (Parkin, Bray et al. 2001). Around the world, more than 600 000 new cases are currently diagnosed per year.

Major risk factors of HCC have been well defined (Bosch, Ribes et al. 1999; Colombo 2003). The major predisposing factor of HCC is cirrhosis, as almost 80% of HCC develop on cirrhotic livers (Colombo 2003). There is common consent that

epidemiology of HCC is different from area to area: in Asia and Africa, hepatitis B virus infection is the most common risk factor (Liaw, Tai et al. 1986), with aflatoxin B1 intake from contaminated food (Sun, Lu et al. 1999), while in the West, hepatitis C virus infection is the main risk factor (Bruix, Barrera et al. 1989; Colombo, Defranchis et al. 1991; Bruno, Silini et al. 1997), together with alcoholic and haemochromatosis cirrhosis (Niederau, Fischer et al. 1985). The role of other carcinogenic agents, such as tobacco or genetic disease, is not clearly established. Therefore, the multifactorial causes of HCC are indicative of its complex molecular pathogenesis.

HCC is frequently developed from a chronic viral infection, which is reflected in developing countries as patients with chronic HBV infection (Zhou, Tang et al. 2001), while in developed countries, HBV infection was replaced by chronic HCV infection, particularly in older patients (Colombo, Defranchis et al. 1991). At present, 380 million individuals have hepatitis B virus infection in the world, and the carrier rate ranges from 10% to 20% in endemic areas. Compared with non HBV infection carriers, chronic HBV carriers have 100-fold relative risk in developing into HCC (Beasley, Lin et al. 1981). Aflatoxins are naturally occurring mycotoxins that are produced by Aspergillus flavus and Aspergillus parasiticus, species of fungi. High-level aflatoxin B exposure produces an acute hepatic necrosis, resulting later in cirrhosis, or carcinoma of the liver. It increases the neoplastic risk three-fold by inducing a mutation on codon 249 in a tumor suppressor gene - P_{53} (Sun, Lu et al. 1999). Different from developing countries, in developed countries HCC is frequently caused by the HCV infection (Bruix, Barrera et al. 1989; Colombo, de Franchis et al. 1991), which involves almost 170 million people, and excessive alcohol intake (Tsukuma, Hiyama et al. 1993).

However, because there is no available vaccination against HCV (Forns, Bukh et al. 2002), the prevention of HCV transmission is critical for decreasing the HCC incidence. In the patients with HCV infection, 40% of them have a sustained response to classic antiviral strategies, such as pegylated interferon and rivabirin (Manns, McHutchison et al. 2001). Non alcoholic steatohepatitis, some metabolic disorders

(hemochromatosis, hereditary alpha-1 antitrypsin deficiency ...) are also risk factors for HCC. The coexistence of all such factors further increases the risk of developing the disease (Table 3)(Figure 12).

Table 3 Age-standardlized incidence rates for HCC

Countries	Men	Women
Low resource		
Mongolia	**116.6**	**74.8**
Eastern Africa	**18.9**	**9.6**
South-Eastern Asia	**21.4**	**9**
Melanesia	**12.9**	**5**
Western Africa	**16.6**	**8**
Intermediate resource		
China	**37.4**	**13.7**
Caribbean	**6.3**	**4.4**
South Africa	**13.9**	**5.1**
Central Africa	**7.3**	**7**
Western Asia	**4.4**	**2.3**
Northern Asia	**7.5**	**2.5**
South America	**5.3**	**3.9**
South Central Asia	**3.4**	**1.6**
High resource		
Korea	**38.4**	**10.6**
Southern Euroope	**9.8**	**3.2**
Western Europe	**7.2**	**2.1**
Eastern Europe	**4.6**	**1.9**
Northern America	**6.8**	**2.2**
Australia/New Zealand	**5**	**2**
Norther Europe	**3.8**	**1.6**

Table 3: Age-standardized incidence rate for HCC worldwide. *All values expressed per 100,000 of the population in 2008 (data from GLOBOCAN 2008).

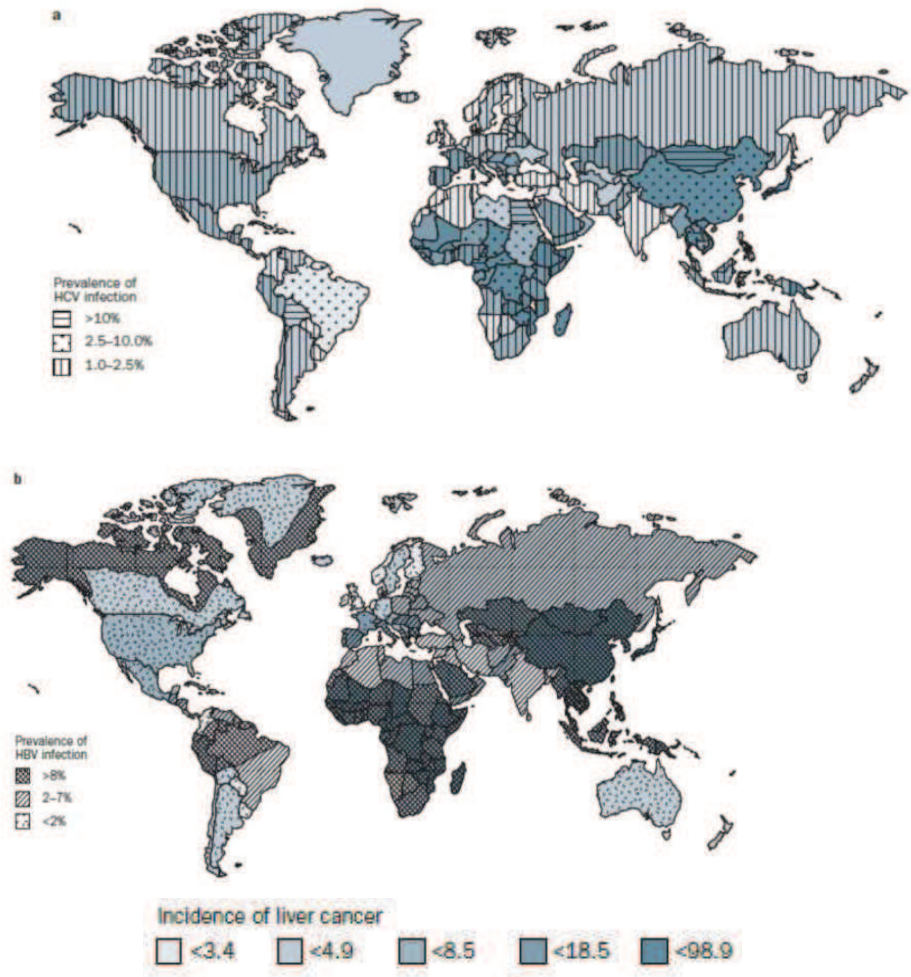


Figure 12 Global variations in age-adjusted incidence rates of liver cancer, prevalence of chronic HCV infection and chronic HBV infection.

Maps were generated using incidence rates of liver cancer from GLOBOCAN 2002; prevalence of chronic HBV infection from US Centers for Disease Control and Prevention; and prevalence of chronic HCV infection from WHO International Travel and Health (according to GLOBOCAN 2002)..

2.2. Diagnosis

2.2.1. Surveillance and diagnosis

Surveillance of the people who have one or multiple risk factors for HCC is important and may contribute to improving the outcomes of survival and decrease the HCC relevant mortality. Ultrasonography is a non-traumatic and high-sensitive

examination recommended every 6–12 months for specific individuals who have high risk of developing HCC (Table 4).

Table 4 Individuals for whom surveillance for HCC is recommended

Hepatitis B carriers	Individuals with cirrhosis
Those with a family history of HCC	Patients with hepatitis B
African individuals over age 20 years	Patients with hepatitis C
Asian men over age 40 years	Individuals with alcoholic liver disease
Asian women over age 50 years	Individuals with hereditary hemochromatosis
Patients with chronic hepatitis B and high viral load (HBV DNA >2,000 IU/ml)	Patients with primary biliary cirrhosis
Patients with persistently or intermittently increased alanine transaminase levels	Patients with cirrhosis of any other cause (prospective data to support this practice are still lacking)

Tumor markers are used primarily to monitor tumor development and the result of treatment. During the HCC surveillance in clinical practice, the serum tumor marker α-fetoprotein (also called alpha-1-fetoprotein, AFP), is frequently used in combination with ultrasonography. This combination of ultrasonography with AFP for HCC surveillance has shown a benefice in reducing the mortality of 37% in a randomized controlled trial, when compared with no surveillance group (Zhang, Yang et al. 2004). Therefore many hepatologists recommend that such kind of surveillance should be preformed every 6 months. A report showed that there is an equal outcome in terms of early diagnosis and 5-year survival when comparing annual surveillance with both AFP and ultrasonography with surveillance every 6 months (Trevisani, De Notariis et al. 2002), but considering this report was a retrospective study, it could not be considered sufficient enough to switch clinical practices.

2.2.2. Staging

For the cirrhotic patients who have a suspicious liver lesion revealed by the

surveillance ultrasonography, a further contrast-enhanced CT and MRI is a recommended examination. The American Association for the Study of Liver Diseases (AASLD) guidelines indicated that, in the cirrhotic patient a novel lesion of >1 cm diameter, if accompanied with arterial enhancement followed by portal venous washout on one dynamic imaging modality, might be diagnosed as HCC (Yang and Roberts 2010), while if the lesion is <1 cm in diameter, the patient should receive ultrasonography at intervals of 3 to 6 months for monitoring. In the cases where the liver lesion size shows no changes within a 2-year period, the examination schedule could return to a routine surveillance (Bruix and Sherman 2005). When a liver lesion of >1 cm has developed in a patient without underlying liver cirrhosis, or when the result of dynamic imaging modality is inconclusive, a liver biopsy is recommended.

In order to clarify and standardize the diagnosis as well as clinical practice, several countries and areas described staging systems of HCC, including the Barcelona Clinic Liver Cancer (BCLC) (Llovet, Bru et al. 1999), Cancer of the Liver Italian Program (ClIP), TNM (tumor, node and metastasis) (Lei, Chau et al. 2006), Okuda (Okuda, Ohtsuki et al. 1985), and Japanese Integrated staging score (JIs) systems (Kudo, Chung et al. 2003). To date, the BCLC system is accepted by the AASLD and the European Association for the Study of the Liver (EASL) and is the most broadly staging system used in clinical and routine practice, because its tumors staging is linked to the recommended therapy strategies (Table 5).

Table 5 Barcelona Clinic Liver Cancer (BCLC) staging classification (Llovet, Bru et al. 1999).

	Performance status	Tumor stage	Liver function
Stage A:			
A1 (very early HCC)	0	Single, <=2 cm	No portal hypertension and normal bilirubin level
A2 (early HCC)	0	Single, <=5 cm	No portal hypertension and normal bilirubin level
A3 (early HCC)	0	Single, <=5 cm	Portal hypertension and abnormal bilirubin level
A4 (early HCC)	0	<=3 tumors, all <3 cm	Child-Pugh A-B
Stage B: Intermediate HCC	0	Single, >5 cm or >3 tumors, >3 cm	Child-Pugh A-B
Stage C: Advanced HCC	1–2	Vascular invasion or extrahepatic spread	Child-Pugh A-B
Stage D: Terminal HCC	3–4	Any	Child-Pugh C

2.3. Treatment of HCC

HCC treatment has been divided into two categories: curative and palliative. Curative treatments of HCC attempt to induce a complete response and expect to improve survival in patients by using an effective procedure such as resection, liver transplantation or percutaneous ablation, while palliative treatments represent a set of healthcare that focuses on preventing and relieving the suffering of HCC patients. Although the aim of palliative treatments is not to cure, in some cases, the patient could obtain a good response and even a good survival.

Liver surgical resection and transplantation are optimal procedures that achieve the best results in well-selected patients, reflected by a 5-year survival ranging from 60% to 70% (Iwatsuki, Starzl et al. 1991; Bismuth, Majno et al. 1999; Fong, Sun et al. 1999; Takayama, Sekine et al. 2000; Jonas, Bechstein et al. 2001; Yao, Ferrell et al.

2001; Wayne, Lauwers et al. 2002). Therefore, they are the first option from an intention-to-treat perspective, although percutaneous treatments also provide good outcomes as 5-year survival of 40% to 50% (Livraghi, Giorgio et al. 1995; Rossi, DiStasi et al. 1996; Lencioni, Pinto et al. 1997; Buscarini, Buscarini et al. 2001), but still cannot reach a response rate comparable with surgical treatments. Liver transplantation is considered as the best treatment for selected patients because it might simultaneously cure tumor as well as the underlying cirrhosis.

For HCC patients who are in the advanced stage or end-stage, palliative treatments strategies are applied according to the individual cases. It includes the effectiveness of embolisation or chemoembolization (Lin, Liaw et al. 1988; Kawai, Okamura et al. 1992; Kawai, Tani et al. 1994; Bruix, Llovet et al. 1998; Pelletier, Ducreux et al. 1998; Llovet, Real et al. 2002; Lo, Ngan et al. 2002), arterial or systemic chemotherapy (Kawai, Tani et al. 1997), internal radiation with ^{131}I lipiodol (Raoul, Guyader et al. 1997), hormonal compounds (Liu, Fan et al. 2000; Villa, Ferretti et al. 2001; Chow, Tai et al. 2002), immunotherapy (Llovet, Sala et al. 2000), and others (Kouroumalis, Skordilis et al. 1998; Yuen, Poon et al. 2002). Survival advantages were identified with embolization or chemoembolization in well-selected candidates and, thus, constitute their standard treatment.

After all, based on the BCLC staging system, all the curative treatment strategies such as resection, liver transplantation, or percutaneous local ablative treatment, are recommended to be primarily procedure for stage A of HCC patients (asymptomatic patients with very early and early HCC). For the stage B, or intermediate, HCC patients, a primarily therapy strategy is TransArterial ChemoEmbolization (TACE). For the stage C, or advanced, HCC, characterized as invasive or extrahepatic tumors, chemotherapy with sorafenib is the only recommended treatment. This chemotherapy is recommended for patients in stage D, or terminal, HCC (Bruix and Sherman 2005; Forner, Reig et al. 2010). The purpose of this stage-by-stage treatment strategy based on BClC staging system is to obtain a survival benefit, particularly for patients with early-stage HCC (Farinati, Sergio et al. 2009).

2.3.1. Transarterial chemoembolization (TACE)

Since enriched blood supply is a basic condition for tumor growth and metastasis, also in HCC, the strategy of TACE consists in infusing into the hepatic artery, which supplies the tumor nutrition, a mixture of gel-foam particle with chemotherapy agents that will provide both the blocking of artery as well as chemotherapeutic effects to eliminate tumor cells. It is recommended in most HCC patients with unresectable tumor, but without vascular invasion or metastasis. TACE is the primary option that could improve the overall survival: when compared with symptomatic supportive care alone, it shows an advantage of 2-year overall survival ranging from 31% to 63%, versus 11% to 27% in the control groups (Llovet, Real et al. 2002; Lo, Ngan et al. 2002). Therefore, the TACE is suggested as an alternative option for the early-stage HCC patients when surgical procedure cannot be performed safely due to the tumor or patient conditions. In some cases of HCC, it is considered as an effective bridging treatment, or down-stage treatment before liver transplantation (Yao, Hirose et al. 2005; Alba, Valls et al. 2008). However, it is not recommended for the patient with poor liver function, as well as with portal vein invasion, in order to avoid acute liver decompensation following the treatment (Chan, Yuen et al. 2002).

2.3.2. Radio Frequency Ablation (RFA)

Percutaneous ablation combined with RFA is usually performed as a potentially curative treatment for HCC patients with a tumor size <3 cm, for whom surgical resection and liver transplantation is usually ineligible due to co-morbidity or liver dysfunction. Except the RFA, another clinical practice termed percutaneous ethanol injection (PEI) also has promising outcome in selected patient, who have small HCC tumor of <2 cm in diameter (Vakili, Pomposelli et al. 2009). However, randomized controlled trials have shown differences between PEI and RFA of about 3-year survival, overall survival, and the rate of complete tumor necrosis (Vilana, Bruix et al.

1992), with RFA being more effective than PEI. Furthermore, other randomized controlled trials have shown that for the early-stage HCCs, RFA has same outcome as surgical resection, while leading to lower complications (Chen, Li et al. 2006; Livraghi, Meloni et al. 2008; Lau and Lai 2009). (Table 6). At present, RFA is more frequently performed in high-resource countries (Yang and Roberts 2010).

Table 6 comparison of efficacy and safety or RFA and surgical resection for HCC

Parameter	Single tumor of ≤5 cm diameter*			Single tumor of ≤5 cm diameter*		
	RFA	Resection	P value	RFA	Resection	P value
Number of patients	71	90	-	51	54	-
Overall survival (%)	67.9(4 year)	64(4 year)	NS	87.1(3 year)	86.4(3 year)	0.81
Disease-free survival (%)	46.4(4 year)	51.6	NS	51.3(3 year)	82.4(3 year)	0.13
Treatment-related mortality (%)	0	1.1	NS	0	0	-
Treatment-related morbidity (%)‡	4.2	55.6	<0.05	7.8	11.1	0.74

*In these two randomized controlled trials, neither overall survival nor disease-free survival differed statistically between the two treatment groups. ‡Treatment-related morbidity was more common in the resection group in both trials, although the difference was statistically significant only in Chen and colleagues' study (Yang and Roberts 2010).

2.3.3. Surgical resection

Since the last century, surgical resection is considered as a potentially curative treatment for HCC. Following the development of surgical instruments and techniques, the outcomes of survival are more and more promising, accompanied with lower complication and mortality. Considering that 70% of the recurrence rate of 5-year is associated with the residual liver tissue in HCC patients (Imamura, Matsuyama et al. 2003), surgical resection candidates should be evaluated for their underlying liver function as well as tumor extent. In most Asian countries, the hepatic reserve is evaluated by the indocyanin green retention, while western countries prefer to test it through clinical signs of portal hypertension such as thrombocytopenia, varices and splenomegaly (Llovet, Fuster et al. 1999). MRI and CT scan are used for

detecting the tumor extent as well as designing preoperative operation plan. Common opinion is to not proceed to surgical resection for the HCC patients with associated vascular invasion or metastasis. However, for large tumors or HCC with multiple nodules, even having an elevated risk of postoperative recurrence, if the tumor is resectable and if a reasonable hepatic reserve exists, surgical resection is considered as a possible procedure for patients.

2.3.4. Transplantation

Since the first human liver transplant successfully performed in 1963 by Starzl surgical team, owing to the fact liver transplantation removes not only tumor, but also its underlying disease, responsible for cancer recurrence, orthotopic liver transplantation is considered as the definitive and most effective treatment for end-stage liver disease including HCC. However, this promising procedure is dependent upon the availability of liver donors. Thus, how to resolve this major difficulty is a critical challenge for hepatologists.

According to the Milano criteria that require a single tumor <5 cm in diameter or up to three lesions with the largest no more than 3 cm, HCC candidates may obtain a similar long-term survival rate after liver transplantation when compared with non-HCC recipients (Mazzaferro, Regalia et al. 1996). However, these strict criteria indicated that only a small part of HCC patients could benefit from this surgical strategy. Therefore, researchers have tried to find other criteria in order to enlarge the benefit scope without compromising the outcome, such as the University of California San Francisco criteria (Yao, Ferrell et al. 2001; D'Amico, Schwartz et al. 2009), that has been universally accepted by the United Network for Organ sharing (UNOS). Some HCC patients who even do not meet initially the Milan criteria may receive liver transplantation after effective local or loco-regional treatment (termed down-staging therapy) that has the aim to reduce the tumor size and tumor number (Yao, Hirose et al. 2005; Yao, Kerlan et al. 2008). Owing to the shortage of liver

donors, living donor liver transplantation has been propounded first in Asian countries. Despite its specific risk of morbidity and mortality for the donor, and a higher risk of HCC recurrence (5% to 15%) compared with deceased donor (20% to 30%), more and more surgeons and hepatologists have successfully implemented living donor liver transplantation worldwide (Trotter, Wachs et al. 2002; Vakili, Pomposelli et al. 2009).

It is still not clear why a higher HCC recurrence rate is associated with the living donor liver transplantation than with deceased donor liver transplantation, one possible reason being that in the case of deceased donor, the graft is allocated to the recipient with the "optimal" clinical and immunogenetic profile while living donor transplantation is usually an intrafamilial transplantation: since the graft is allocated to a relative, the immunogenetic compatibility or the clinical profile of the patient may not be as "optimal" as in the case of a deceased donor. Furthermore, the liver regeneration from a partial liver graft (in the case of a living donor) is dependent upon the hormonal environment that may possibly contribute to increasing the risk of tumor recurrence.

Although having some disadvantages such as a higher risk of recurrence, most countries and areas, especially in Asia, are currently performing living donor transplantation in order to overcome the global problem of limited availability of deceased donors.

2.3.5. Chemotherapy for HCC

Sorafenib, known as an oral multikinase inhibitor having a broad inhibitory profile of Raf kinase, PDGFR and VEGFR pathways, has been approved for the treatment for advanced HCC patients, who are at stage C of the BCLC classification: for patients with well-preserved liver function and extrahepatic spread or vascular invasion, Sorafenib treatment could increase patient survival from 7.9 months to 10.7 months (Bruix and Sherman 2011; 2012). In HBV-infected patients, it has an effect in

delaying tumor progression and improving survival rate (Yeo, Mok et al. 2005). On the other hand, the major side effect of the Sorafenib treatment includes diarrhea, hand–foot skin reaction and fatigue, which are manageable. Based on these promising outcomes, as depicted in the US and European clinical practice guidelines, Sorafenib is designed as one of the five recommended therapies for HCC (Villanueva, Hernandez-Gea et al. 2013), the other ones being resection, transplantation, radiofrequency ablation, and chemoembolization. The standard dose of Sorafenib is 400 mg, twice daily, that should be maintained at least until radiological progression, with periodic surveillance of adverse effects (Villanueva and Llovet 2011). Moreover, further research has shown that Sorafenib therapy could improve overall survival with tolerable adverse effect (Cheng, Kang et al. 2009). Nevertheless, these are also other undergoing trials that find the potential benefit of combining Sorafenib with other practices such as RFA or TACE.

2.4. Innovative treatments

In order to decrease HCC recurrence and mortality, new treatment strategies against HCC as well as HCC relevant disease are constantly developed. Adoptive immunotherapy is one of the innovative strategies for any type of malignancy including HCC. Its aim is to stimulate or restore the patient's innate or adaptive immune system by using different *in vitro* or *in vivo* procedures. Cytokine-induced killer (CIK) cells represent one of the promising adaptive immunotherapies which has been developed over the past two decades. These cells were generated from patients, by stimulating peripheral blood mononuclear cells (PBMCs) using a combination of several specific cytokines and antibodies (such as IFN-γ, Interleukin (IL-)1, IL-2 and monoclonal antibody against CD3), expanding the number of cells and, finally reinfusing them to the patients, in order to exert their immune function against pathogens and malignancies. At present, there is still not a standard protocol of CIK cell production but some parameters of induction are universal. For example, the

combination of IL-2 and CD3 antibody is critical for the expansion of CIKs (Ochoa, Gromo et al. 1987). CIK cells are known as a heterogeneous population of T cells, in which $CD3^+CD56^+$ double-positive cells, termed NK-like T cells, or NKT cells, are the major cytotoxic cells (Schmidt-Wolf, Lefterova et al. 1993; Lu and Negrin 1994; Sangiolo, Martinuzzi et al. 2008). CIK cells are responsible for a non-MHC-restricted, perforin-mediated cytotoxicity induced through specific cell-surface receptors - natural killer group 2 member D (NKG2D) (Verneris, Ito et al. 2001; Verneris, Karami et al. 2004), while the ligands of NKG2D such as MHC class I-related chain (MIC) A/B and UL-16 binding protein 1 – 4 are generally over-expressed in some solid or hematologic malignancies, making these tumor cells a target for CIKs (Groh, Rhinehart et al. 1999; Pende, Rivera et al. 2002). Recently, a report indicated that the clinical outcome of CIK strategy against tumor and the subsets of effector cells involved in the antitumor effect are different depending on the type of cancer, but a higher frequency, in the infused cell product, of $CD3^+CD56^+$ and/or $CD3^+CD8^+$ cells seems to be critical for obtaining an effective response against tumors (Pan, Wang et al. 2014).

Since the CIKs are a notable heterogeneous cell population, there is evidence that not only cytotoxic cell populations, but also regulatory T cells contribute to the CIKs effect. The purpose of antitumor response is to increase the specific cytotoxicity against the tumor with reducing the effect of immune inhibitors. Therefore a lot of cytokines have been added in the *in vitro* production protocol and shown several advantages to target the tumors: it comprises the IL-6 (Bettelli, Carrier et al. 2006; Lin, Wang et al. 2012), IL-12 (Zoll, Lefterova et al. 1998; Helms, Prescher et al. 2010), IL-15 (Rettinger, Kuci et al. 2012; Rettinger, Meyer et al. 2012; Tao, Chen et al. 2013; Wei, Wang et al. 2014), IL-21(Rajbhandary, Zhao et al. 2013; Cai, Chen et al. 2014).

2.4.1. Clinical studies on CIK cell therapy in HCC

2.4.1.1 TACE or RFA combined with CIKs

TACE and RFA are two major therapy strategies approved for the treatment of HCC, thus, one may wonder whether these two treatments may obtain better results if combined with CIKS. Up to now, clinical trials focusing on treating HCC through TACE or RFA combining with CIKS have shown some progress (Wang, Li et al. 2012): compared with the control group (TACE or RFA alone), additional autologous CIK cell transfusion following TACE/RFA group have obtained longer 1-, 3- and 5-year disease-free survival (DFS), although the OS is not significantly different between the two groups. Importantly, a retrospective analysis showed that in 85 patients, this combination strategy could significantly improve the 3-, 5- and 10-year OS rates (Huang, Li et al. 2013).

2.4.1.2 TACE or surgery combined with CIKs

Recently, a retrospective study has shown a promising outcome in OS rates when comparing the CIKs plus surgery with surgery alone in 410 HCC patients (Pan, Li et al. 2013). Interestingly, this analysis indicated that the patients with HCC may benefit from longer survival rate when they received more therapy cycles, while another study showed that when a standard therapy such as surgery or TACE followed by additional CIKs treatment, the 1-, 2- and 3- year OS rate are higher than without CIKs (Yu, Zhao et al. 2014).

Taken together, new adoptive immunotherapy strategies based on the CIKs have shown promising clinical outcomes when they are combined with other therapies. All these encouraging data support that CIK cell therapy may become an optimal option for patients with HCC in the future. Compared with conventional HCC treatment, CIK cell therapy has some advantages, for example, their relatively simple generation protocol, non-MHC-restricted cytotoxic capacity, they could increase survival as well

as decrease recurrence of HCCs without obvious side effects. However, on the other hand, up to now most of the clinical trials had only a low number of patients and they were frequently in advanced or end-stage status. Thus, more clinical trials should be conducted on a larger scale (Table 7).

Table 7: Clinical studies applying CIK cells for HCC

Ref.	Patients (n)	Application of CIK cells	Clinical response
Hao et al. [1]	**146**	**TACE (72) vs TACE + CIK cells (74)**	**Prolonging PFS, OS and TTP rate**
Zhao et al. [2]	**64**	**TACE + RFA (31) vs TACE + RFA + CIK cells (33)**	**29 patients of the TACE + RFA + CIK cells group were recurrence free during the 1-year follow-up**
Hui D et al. [3]	**127**	**Surgery + CIK (84) vs surgery (43) group**	**CIK may prevent recurrence/metastasis after radical resection of hepatocellular carcinoma**
Pan et al [4]	**410**	**Surgery; patients in the study group (n = 204) received at least four cycles of adjuvant CIK cell therapy**	**OS rates higher in study group than in control group ($p < 0.001$); survival of patients who received ≥ 8 cycles of CIK cells longer ($p < 0.05$)**
Yu et al [5]	**132**	**Surgery, TACE or BSC; patients in the study group (n = 66) received additional CIK cell therapy**	**1-, 2- and 3-year OS: 74.2/50.0%; 53.0/30.3%; 42.4/24.2% ($p \leq 0.005$)**

CIK: Cytokine-induced killer; **CR**: Complete response; **CT**: Chemotherapy; **HCC**: Hepatocellular carcinoma; **OS**: Overall survival; **PD**: Progressive disease; **PFS**: Progression-free survival; **PR**: Partial response; **QOL**: Quality of life; **RFA**: Radiofrequency ablation; **SD**: Stable disease; **TACE**: Transcatheter arterial chemoembolization; **TTP**: Time to progression. **BSC**: Best supportive care; **DFS**: Disease-free survival 1:(Hao, Lin et al. 2010)2:(Zhao, Wu et al. 2006),3:(Hui, Qiang et al. 2009)4:(Pan, Li et al. 2013);5:(Yu, Zhao et al. 2014)

3. Animal models of HCC

3.1. Background of HCC animal models

Developing HCC animal models that could provide the opportunity to mimic the process of liver carcinogenesis, study tumor-host interactions, perform drug screening and conduct preclinical therapeutic trials is crucial for both basic and clinical studies

of HCC. To date a unique, reproducible, standardized, affordable animal model recapitulating all steps of HCC pathogenesis (including fibrosis and cirrhosis) with genetic or cellular humanization and providing anatomical, physiological and size proximity to human does not exist. Most HCC research is based on the rodent models because of their breeding capacity, short lifespan and the unlimited options offered by genetic engineering.

3.2. Mouse models of HCC

3.2.1. Spontaneous models

Spontaneous liver tumor models have been described: inbred variant strains of wild-type mice are susceptible to spontaneously-induced liver tumors (Drinkwater, Evans et al. 1988) (Peychal, Bilger et al. 2009). However, some shortages such as long period to develop tumor, low penetrance and lack of synchronous kinetic have limited their wide use in HCC research. Thus, most HCC models are based on the use of chemicals or transgenic modifications, where genes considered as potentially relevant for HCC development are introduced or modified in the mouse genome.

3.2.2. Chemical carcinogenesis (DEN)

The carcinogenesis is a two-step process, comprising a first step (induction step) leading to the introduction of genomic alterations in target cells, followed by a second step (promotion step) of amplification of genomic alterations, leading to the transformation and expansion of tumor cells. Diethylnitrosamine (DEN) is a representative chemical carcinogen with the potential to cause tumors in various organs, including the liver. Since the 1960s, this genotoxic product has been used as an inducer to induce HCC in rat (Rajewsky, Dauber et al. 1966). Alternative HCC inducers can be carbon tetrachloride or, as recently reported, high fat diet (HFD)

feeding (Park, Lee et al. 2010). Up to now, DEN is still a chemical product widely used to induce HCC in animal models. Basically, DEN is a DNA alkylating promoting agent that forms mutagenic DNA adducts. Moreover, the bioactivation of DEN by cytochrome P450 can also damage DNA, lipids and proteins then lead to hepatocytes death by generating reactive oxygen species (ROS) (Qi, Chen et al. 2008). Indeed, this process of induced tumor, as well as the one observed in patients, usually evolves through a slow multistep sequence characterized by several cycles of necrosis and regeneration that promote transformation, the whole process being associated with the occurrence of genomic alterations (Thorgeirsson and Grisham 2002; Farazi and DePinho 2006). DEN acts as a carcinogen after metabolic activation in hepatocytes by cytochrome P450 family in younger mice (< 2 weeks) because the hepatocytes are actively proliferating, while when administered later, tumor induction needs to be combined with promoting agents such as phenobarbital (PB) or partial hepatectomy whose purpose is to stimulate the entry of hepatocytes in a proliferative state.

3.2.3. Hepatitis virus transgenic mice: HBV/HCV

Transgenic mouse models of HBV or HCV infection have reliably proven that viral genes could initiate or promote liver carcinogenesis. Since the mice are resistant to the HBV and HCV infection, this transgenic technology allows developing chronic carriers of HBV through integrating the viral DNA into the host genome (Babinet, Farza et al. 1985; Chisari, Pinkert et al. 1985). At present, the full HBV and every HBV gene have been integrated into the host genome to induce chronic carrier models. For HCV, the core protein alone or, in combination with envelope polyprotein, has been successfully integrated into transgenic mice (McGivern and Lemon 2011; Li, Tang et al. 2012). Based on these data, it has been shown that HBV or HCV can initiate and promote liver carcinogenesis. Only the HBx protein and large HBV envelope have a carcinogenic potential while, for HCV, the HCV core protein is the major factor related to hepatocarcinogenesis (Chisari, Klopchin et al. 1989; Kim,

Koike et al. 1991; Moriya, Fujie et al. 1998). It is considered that HBx induces HBV carcinogenesis through initiating a pre-neoplastic proliferative response that could facilitate further transformation events. It can activate both viral as well as cell genes in the nucleus and also stimulate signal transduction pathways in the cytoplasm (Bakiri and Wagner 2013). Moreover, HBx could increase the activity of multiple molecular pathways such as NFκ-B and Wnt/β-catenin, to switch cell proliferation, DNA stability and repair mechanisms as well as sensitize hepatocytes to chemical carcinogens (Li, Tang et al. 2012). For the HCV-related HCC, hepatic steatosis and oxidative stress are contributive to the hepatocarcinogenesis during "chronic HCV infection" in mouse models, in which the activity of transcription factors controlling metabolic gene expression of SREBP-1c, RXR and PPAR were interfered by the HCV core protein (McGivern and Lemon 2011).

3.2.4. Hepatic onco-mice

Onco-mice are an option concerning establishing mouse models of hepatocarcinogenesis by integrating directly known oncogenes into the liver. For example, mouse models expressing SV40 T-antigens, mutant *H-ras*, or c-myc under the control of alpha-1-antitrypsin or antithrombin III promoters may lead to neoplasia in the livers. (Sandgren, Quaife et al. 1989; Dubois, Bennoun et al. 1991). The first hepatocarcinogenesis mouse model was SV40 T transgenic mice in which the transgene induces the loss of p53 and Rb expression and leads to metastasis of the liver tumor to distant organs (Dubois, Bennoun et al. 1991). Subsequently, by using similar strategies, a lot of mouse models have been established to obtain liver-specific expression of oncogenes, growth factors or cell cycle proteins that are indicated as having an important roles in liver cancers (Conner, Lemmer et al. 2000; Cadoret, Ovejero et al. 2001; Wang, Ferrell et al. 2001).

These mouse models have some disadvantages. For example, since these mice were based on a random transgene integration, early onset as well as broad patterns of

transgene expression could be observed, limiting the development of the study of HCC pathogenesis. However, they have widely contributed to clarify the properties of HCC disease and liver physiology (Murakami, Sanderson et al. 1993; Shachaf, Kopelman et al. 2004; Tward, Jones et al. 2007). Particularly, some research involving the differences of molecular profiles between human and mouse HCC has found that there are large overlaps at the gene expression level. Interestingly, some reports even demonstrated that several mouse tumor features might be attributed to some subsets of human HCC and could even predict some prognosis of patients with HCC. By using a comparative functional genomics approach, the authors demonstrated that a temporal TGF-beta gene expression signature (early and late TGF-beta signatures) established in mouse primary hepatocytes successfully discriminated distinct subgroups of HCC. Kaplan-Meier plots and log-rank statistics indicated that the patients with a late TGF-beta signature showed significantly ($P < 0.005$) shortened mean survival time (16.2 +/- 5.3 months) compared to the patients with an early (60.7 +/- 16.1 months) TGF-beta signature (Lee, Chu et al. 2004; Coulouarn, Factor et al. 2008). Thus, these promising studies indicate that combining the onco-mice strategy with proteomic and microRNA analyses may highlight their importance in the development of HCC animal models (Pineau, Volinia et al. 2010; Ritorto and Borlak 2011).

3.2.5. Gene targeting technology opens new avenues and better GEMMs

Gene targeting is a novel technology based on the disruption (knock-out) or the modification (knock-in) of an allele in embryonic stem (ES) cells, focusing on the generation of precise mouse model for cancer research by accurately controlling the gene expression and mutation, which may sophistically reflect human disease and entail the functional exploration of carcinogenic events. The International Knockout Mouse Consortium (IKMC) (Bradley, Anastassiadis et al. 2012) and the International Mouse Phenotyping Consortium (IMPC) (Mallon, Iyer et al. 2012) were successfully

established in order to systematically determine gene function as well as establish disease models. The final purpose of this technology is to define the interactions among genes, cells, tissues and environment that contribute to cancer susceptibility, disease progression and potential response to therapeutic interventions.

3.2.6. Subcutaneous or orthotopic cell transplantation

Table 8 shows the cancer cell lines or tissues that have been successfully transplanted in mice or rats over the last three decades.

Table 8 Cells used in transplanted HCC models (Wu, Tang et al. 2009)

Cell line (human)	Origin	Characteristics	Refs
Xenograft			
Hep3B	Black male 8 year HBV	HCC well differentiated, AFP+	Knowles et al. ([1980]), *Aden et al. ([1979])*
Hep G2	Caucasian male 15 year	AFP+, Weakly tumorigenic in nude mice	Babinet et al. (1985), Busch et al.([1990]), *Zannis et al. ([1981])*
HLF	Male 68 year	HCC, AFP−	Jiao et al. (2002)
HuH-7	Asian male 57 year HBV	HCC well differentiated, AFP+	Takahashi et al. (2002)
SK-HEP-1	Caucasian male 52 year	Endothelial origin, AFP−	Fogh et al. ([1977]), Heffelfinger et al. *([1992])*
QGY-7703	Asian female 35 year	HCC, AFP+, aneuploid, doubling time: 20.5 h	Yan et al. (2004)
SMM-7721	Asian male	HCC, AFP+	Wang et al. (2001)
PLC/PRF/5	Black male 24 year HBV	HBsAg, HCC, AFP+	Monjardino and Crawford ([1979]), Skelly et al. *([1979])*
BEL-7402	Asian male	HCC undifferentiated, AFP+, doubling time: 20 h	Ling et al. *([2005,2006a, b])*
SNU-398	Asian male 42 year	HBV genomic RNA was not expressed, doubling time: 39 h	Park et al. (1995)
SNU-449	Asian male 52 year	HBV genomic RNA was not expressed, doubling time: 36 h	Park et al. (1995)

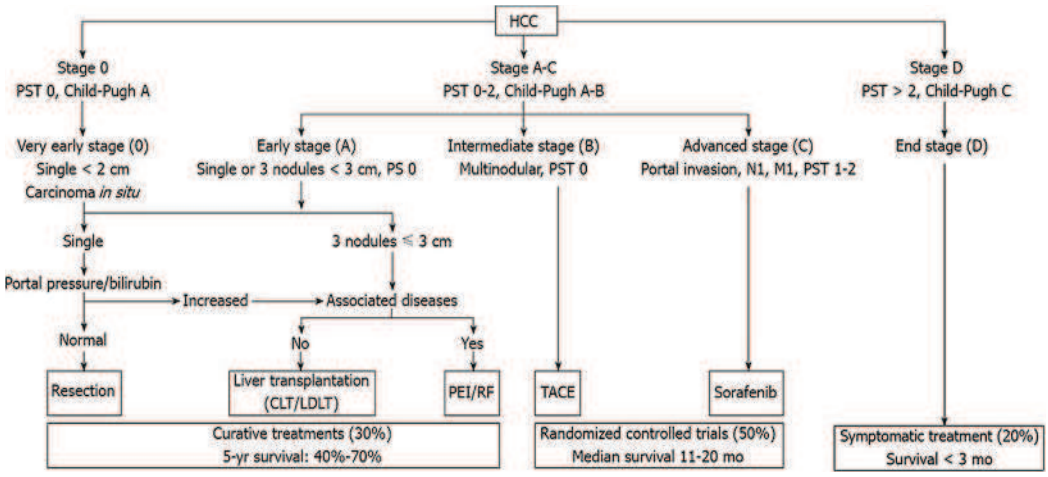
SNU-387	Asian female 41 year	HBV genomic RNA was not expressed, aneuploid; modal number = 67, doubling time: 61 h	Park et al. (1995)
SNU-423	Asian male 40 year	HBV genomic RNA was not expressed, aneuploid; modal number = 79, doubling time: 72 h	Park et al. (1995)
SNU-739,SNU-761, SNU-878, SNU-886	HCC patients infected with HBV	Their doubling times ranged from 20 to 29 h, HBx expression	Lee et al. (1999)
FHCC-98	Asian male 39 year	HCC, AFP−, CK+, cell DNA was tetraploid and the major chromosomes were triploid, doubling time: 21.4 h, tumorigenicity in nude mice was 100%	Lou et al. (2004)
H4-M	Asian male 50 year	HCC, cells were polygonal, with abundant cytoplasm. The nuclei were large and round with prominent nucleoli. Mitosis and giant tumor cells were readily apparent.	Wen et al. (2002)
BCLC-9		HCC, AFP−, HBV+	Armengol et al. (2004)
LCI-D20	Asian male 39 year HBV	HCC, AFP+	Sun et al. (1996)
MHCC97		AFP+, isolated from the LCI-D20 model	Tian et al. (1999)
MHCC97L,MHCC97H, HCCLM3, HCCLM6		Clones derived form MHCC97 with step increase in metastatic potential	Li et al. (2003, 2001,2004)
Syngenic			
Mouse			
Hepa1-6	C57L	AFP+	Darlington et al. (1980)
BNL	Balb/c	Derived from embryonic liver cells BNL CL.2 by carcinogen treatment	Tatsumi et al. (2001)
MH134	C3H/HeN	Carcinogen (carbon tetrachloride)	Yamashita et al. (2001)
Rat			
McA-RH7777	Buffalo rat female	AFP+, Morris hepatoma 7777 (N-2-fluorenylphtalamic acid)	Morris and Meranze (1974), Kulas et al. (1996), Schock et al. (1996)
MH-5123D	Buffalo rat	AFP+, Morris hepatoma 7777 (N-2-fluorenylphtalamic acid)	Ryall et al. (1984)

MH-3924A	ACI	Morris hepatoma 7777 (N-2-fluorenylphtalamic acid)	Calviello et al. (1998)
HTC (MH-7288C)	Buffalo rat male	Derived from the ascites of Morris hepatoma 7882. Carcinogen (N, N0-2,7 fluorenyle-bis 2, 2, 2-trifluoroacetamide)	Barnard et al. (1986)
H4-II-E	ACI. male	Derived from rat hepatoma Reuber H35	Benedict et al. (1973), Sassier and Bergeron (1977), Bradlaw and Casterline (1979)
H4TG	AxC male	Derived from H4-II-E-C3 rat hepatoma cell line	Haggerty et al. (1973)
AH-130	Wistar rat male	Yoshida ascites hepatoma, Carcinogen(30-methyl-4-dimethyl aminoazobenzene)	Carbó et al. (2000)
GP7 TB	Fischer 344 rat male	Derived from WB-F344 hepatic oval cells by carcinogen treatment (N-methyl-N0-6-nitro-N-nitrosoguanidine)	Lin et al. (2000)
C1, C2, C6, C5F, N1, L2	F344 rats male	All six cell lines proved to be tumorigenic in the injection site and C5F was highly metastatic to the lung. With injection into the tail vein, N1 and L2 formed frequent metastases in the lung as well as in lymph nodes	Ogawa et al. (2001)
JM1 and JM2	F344 rats	Cell strains produced by a carcinogen initiation, phenobarbital promotion protocol	Novicki et al. (1983)
Hca-F		For 17 generations, the percentages of metastasis of Hca-F cells remained between 80 and 100%	Li et al. (1998)

3.3. Choice of animal models for the evaluation of innovative HCC treatments

As mentioned above, the standard treatment of HCC includes curative options, such as surgical resection, liver transplantation and percutaneous ablation (Figure 13). However, most patients with HCC are not eligible for receiving the curative treatment owing to the advanced disease, they have a poor prognosis, with 1-year and 2-year survival of approximately 17.5% and 7.3% (Cabibbo, Enea et al. 2010). On the other

hand, for the patients who have received resection, the recurrence rate of HCC remains high, which leads to poor cure rates and poor long-term survival.



BCLC STAGING SYSTEM

BCLC Stage	PS	Tumor features	Liver Function	Treatment Options
A1	0	Single<5cm	No PH	Surgery, RFA
A2	0	Single<5cm	PH, normal bili	Surgery, RFA, transplant
A3	0	Single<5cm	PH, abnormal bili	RFA, transplant
A4	0	3 tumors<3cm	Not applicable	Transplant, TACE
B	0	Large multinodular	CP A-B	TACE
C	1-2	Vascular invasion or metastases	CP A-B	Sorafenib
D	3-4	Any	CP C	Supportive care

Figure 13. Barcelona Clinic Liver Cancer (BCLC) Staging and Treatment Strategy for advanced HCC.

PST: performance status test; **N**: lymph node, **M**: metastases (Llovet, Di Bisceglie et al. 2008; Yang, Lin et al. 2012); **Bili**=total bilirubin; **CP**=Child-Pugh class; **PH**=portal hypertension; **RFA**=radiofrequency ablation; **TACE**=transarterial chemoembolization(O'Neil and Venook 2007)

The palliative treatment of HCC includes chemotherapy (Sorafenib), and transarterial chemoembolization (TACE) (Wang, Shi et al. 2010; Zhong and Li 2010). In addition, there are other supplementary treatments of HCC such as percutaneous alcohol injection (Germani, Pleguezuelo et al. 2010) and adjuvant interferon therapy

(Shen, Hsu et al. 2010; Singal, Freeman et al. 2010). Various emerging options, such as gene-based treatments and targeted therapies, are under investigation as potential treatments for HCC. Based on these options, various animal models have been developed for investigating the effects of each of these therapies.

3.3.1. New chemotherapeutic agents

For a long time, subcutaneous implantation of several human hepatoma cells in animal models is a commonly used approach in order to develop the effect of new drugs. Although the validity of treatment through tumor xenografts to predict clinical practice has been doubted and criticized (Newell, Villanueva et al. 2008), human tumor xenografts present always a usefulness to assess the mechanism of cytostatic drugs and cytotoxic agents, for which the drugs target specific abnormalities that drive the malignant alternation (Kelland 2004; Newell, Villanueva et al. 2008). Researchers suggested that all xenograft trials should comply with a standard procedure to maximize their effects with minimizing the specific limitations of these models (Li, Tang et al. 2012).

By dint of subcutaneous xenograft models, several chemotherapeutic agents and their molecular mechanisms of action have been evaluated. For instance, the involvement of the Raf – mitogen-activated protein kinase – extracellular signal regulated kinase (Raf–MAPK–ERK) signaling pathway in the progression of HCC has been clarified through subcutaneous models in which drugs targeting this pathway were assessed using different primary HCC cell lines (Huynh, Soo et al. 2006), suggesting that the subcutaneous xenograft modes could be an effective solution to elucidate the mechanisms of molecular targeted drugs. By using a similar approach comparing small molecules alone or in combination, the same team also identified other signaling pathways as well as chemotherapeutic strategies, such as AZD6244 plus doxorubicin (Huynh, Chow et al. 2007), bevacizumab plus rapamycin (Huynh, Chow et al. 2008), brivanib alaninate (Huynh, Ngo et al. 2008), everolimus (Huynh,

Ngo et al. 2009), sunitinib (Huynh, Ngo et al. 2009), sorafenib plus rapamycin (Huynh, Ngo et al. 2009). All of these data successfully provide a proof-of-concept that link preclinical investigations to phase II clinical trials in HCC patients (Park, Finn et al. 2011).

However, if the purpose of experiment is to elucidate the tumor microenvironment and organ selectivity of HCC, subcutaneous xenograft models should be replaced by an orthotopic model, although the latter procedure is more challenging than subcutaneous xenograft models. Conventional orthotopic HCC models involve implanting directly the HCC cells or tumor fragment into the mouse livers. However, a major drawback of these techniques is the possibility of tumor seeding into the bloodstream or along with the needle track. Therefore the validity of trials of therapeutic efficacy is considerably compromised. Fortunately, important progress was been made since 1992, a biological material called Gelfoam® (Pharmacia & Upjohn Company, North Peapack, USA) has been used in experiment because of its two advantages: facilitating hemostasis and forming a pocket that prevents the leak of the injected tumor cells (Yang, Rescorla et al. 1992). At present, this procedure is widely used, as it allows a complete development of HCC: local growth, direct invasion of adjacent organs, ascites, and distant metastasis. Interestingly, compared with the conventional orthotopic models, this procedure significantly reduces the rate of early metastasis. Nevertheless, it also avoids artificial metastasis. By dint of these models, a lot of molecular targeted agents have been successfully evaluated (Yang, Rescorla et al. 1992), such as the sirolimus (mTOR inhibitor), gefitinib (vascular epidermal growth factor receptor (VEGFR) inhibitor) (Semela, Piguet et al. 2007; Piguet, Semela et al. 2008; Piguet, Saar et al. 2011). One group reported an orthotopic mouse model by transfecting a vector carrying the gene of the β-subunit of human choriogonadotropin (β-hCG) into HCC cells in order to reproduce the extensive liver disease which is associated with advanced HCC metastasis (Tang, Man et al. 2010).

3.3.2. New TACE modalities

Compared with several HCC animal models, the rat HCC animal models that have been reported by Yang *et al* (Yang, Rescorla et al. 1992) might be more available for the development of efficacy of TACE strategies for HCC because of their hypervascular property. Based on these models, other teams have developed different embolic agents such as polylactide-co-glycolide microspheres (PLCG), that present a more prolonged half-life as well as better tissue compatibility. Therefore, they could decrease the tumor growth rate when combining PLCG with TACE (Qian, Truebenbach et al. 2003). Meanwhile, these animal models have been used in order to improve the HCC therapy by combining TACE with other approaches, such as combination with immunotherapy and antiangiogenic therapy (Maataoui, Qian et al. 2005). Interestingly, the results showed that both above combination therapies based on TACE have a significant effect, delaying the tumor growth when compared with TACE therapy alone (Maataoui, Qian et al. 2005).

3.3.3. Investigational treatment modalities

3.3.3.1 Radiolabeled vesicles

Radiotherapy is a major strategy for most malignancies. However, in patients with HCC, the effect of radiotherapy is not as evident as other strategies such as surgical resection or TACE. A suitable HCC rat model has been successfully used in order to improve the efficacy of radiotherapy (Vanpouille-Box, Lacoeuille et al. 2011) through the use of lipid nanocapsules of 50nm in diameter loaded with Rhenium-188 (LNC(188)Re-SSS). This radiopharmaceutical carrier could easily penetrate into HCC tumor because of its nanometric size. In a rat model of DEN-induced, a significant median survival rate and reduced tumor size was obtained when internal irradiation with LNC(188)Re-SSS was obtained. Importantly, it did not cause obvious side effects. Thus, with this rat model, this report indicated that internal radiation is a

potential strategy for HCC treatment.

3.3.3.2 Gene therapy

Several animal models have also been used for developing gene therapy for HCC. By using Morris hepatoma 3924A (MH) cell line, which is a poorly differentiated and rapidly growing nodular HCC that can be implanted only in syngeneic ACI rats, Graepler *et al.* (Graepler, Verbeek et al. 2005) showed that combination of different targeted angiostatic gene therapy might be a potential treatment for HCCs. The results showed that combined expression of sFlt-1 and endostatin effectively suppresses HCC growth compared with the control groups. A lot of research using transfer of genes encoding, for instance, human plasminogen fragment containing five kringle regions (Plgk1-5) (Schmitz, Raskopf et al. 2007), or TNF-related apoptosis-inducing ligand (TRAIL) combined with cisplatin (Wang, Huang et al. 2010) have also provided evidence that gene therapy strategies might be potential strategies in terms of HCC treatment.

Interestingly, using an orthotopic rat model established by injecting syngeneic N1-S1 hepatoma cells, a report has introduced an innovative loco-regional therapy that involves delivery of intense electrical pulses to induce nanoscale cell membrane defects for liver-directed ablation, termed Irreversible electroporation (IRE) (Guo, Zhang et al. 2010). Serial MRI scans and histopathological analyses allowed to demonstrate that the IRE induced a reduction of HCC tumor size without major side effects, when compared with untreated groups. Thus IRE might be a safe and effective targeted treatment of liver tumors.

3.3.3.3 RNA interference

Up to now, more and more researchers focus on developing the potential curative therapeutic approach for HCC by using RNA interference (RNAi) (Romano, McCallus et al. 2006; Arbuthnot and Thompson 2008). Considering that

subcutaneous xenograft models do not reflect the true clinical situation, although they have investigated the efficiency of RNAi-mediated procedure in the prevention and therapy of HCC (Li, Fu et al. 2005; Cho-Rok, Yoo et al. 2006; Salvi, Arici et al. 2007), preclinical trials have tried to develop the RNAi strategy in an orthotopic animal model of HCC. An RNAi-based therapy using an antisense oligonucleotide against human telomerase reverse transcriptase (termed "Cantide") has been performed in an orthotopic mouse model (Lin, Tuo et al. 2005), in which nude mice have been xenografted with surgical specimen derived from HCC patients. The authors showed that the Cantide had a significant effect in reducing tumor weight, liver tumor relapse and reducing distant metastasis. Similar results have been obtained by using, in orthotopic HCC models, antisense oligonucleotides against surviving (Sun, Tang et al. 2006) and type I insulin-like growth factor receptor (Lin, Wang et al. 2007).

Taken together, these xenografts orthotopic animal models present several advantages compared with subcutaneous models: first, xenografted frozen tumor tissue can be persevered in liquid nitrogen, retaining the initial tumor microenvironment and biological behaviors; second, the design of xenograft animal model is more similar to the clinical situation, improving the study reliability; third, xenograft models reflect the major issues regarding the evaluation of clinical treatment including the evaluation of tumor size and occurrence of metastasis.

3.4. Porcine HCC models

However, the rodent model is limited in terms of preclinical application because of their difference considerably from humans in size, general physiology, anatomy and lifespan. For example, small size effectively limits the use for the evaluation in part of most human surgical procedures as well as the radio imaging. Moreover, clinical therapeutic agent from mouse model to human is confounded by uncertainties appropriate dose and tumor size (Adam, Rund et al. 2007).

Although mice and humans share some fundamental similarities, but some

research has demonstrated that there is the difference on cancer biology. For example, comparing with human cells, murine cells are more easily to transform *in vitro* (Hooper 1998; Rangarajan, Hong et al. 2004), requiring different genetic events for tumorigenesis (Kendall, Linardic et al. 2005). It suggested that the similar genetic lesion could produce the different disease phenotype because of the dissimilar protein interactions.

No single species is able to provide the accurate model for human disease, each of the species has advantages and disadvantages. Comparative analysis of gene expression data shows some abilities to identify the networks of expression and gene regulatory regions, also unraveling the complex interactions between genetic, environmental and lifestyle factors that influence disease pathology.

The porcine model shows many similarities to humans, such as body size, anatomy, and their physiological and pathophysiological responses (Flisikowska, Kind et al. 2013). At present, the porcine model has been widely used by the purpose to evaluate novel equipment and instruments for the human surgical procedure. On the other hand, the porcine model has also relatively long life, there is possibility to establish longitudinal studies in individual animals by mimicking the different condition of the human patient, such as tumor progression and remission, response, toxicity, and even, drug resistance by cancer cells (Flisikowska, Kind et al. 2013).

Because of the lack of porcine original tumors, researchers focused on generating a mimic structure closely to the human scale in order to develop the tumor therapeutic strategy, for example, a lot of chemical products, such as agarose, cellulose and glycerol have been injected into the porcine liver for a subject of studying ultrasound thermal ablation (N'Djin, Melodelima et al. 2007). Liquid plastic injected into pig kidney to mimic exophytic kidney tumor is also reported for the development of laparoscopic nephrectomy (Hidalgo, Belani et al. 2005). Importantly, some research teams have tried to induce the porcine tumors by introduction of chemical carcinogens (Li, Zhou et al. 2006). One year later, Adam and his team reported a porcine tumor model which generated by autologously transplanting primary porcine cells transduced with three retroviral vectors, each of them carrying two oncogenic

gens respectively, (Adam, Rund et al. 2007), it indicated that the changes necessary to convert porcine cells to a tumorigenic state resemble those required by humans more than those in mouse cells, suggesting that a similar process is involved (Flisikowska, Kind et al. 2013).

The first gene-targeted pigs for cancer were generated by adeno-associated virus mediated gene inactivation of BRCA1 (breast cancer associated gene 1) in fibroblasts (Luo, Li et al. 2011), while the use of this pig model is limited since the animals producing by nuclear transfer showed short survival cycle (< 18 days). Interestingly, another team has reported a viable gene-targeted 'oncopigs' by mutations in the adenomatous polyposis coli (APC) gene (APC) which is responsible for human familial adenomatous polyposis (FAP) (Flisikowska, Merkl et al. 2012), It suggested that it is possible to replicate a variety of cancers by combining and activating defined oncogenic mutations in chosen tissues. Currently, a study based on transgenic fibroblasts that induced to over-express two proto-oncogenes (porcine TGF-α and c-myc genes) specifically in hepatocytes. It suggested that the transgenic cell lines could induce liver cancer in porcine models following somatic cell nuclear transfer (Kim, Ahn et al. 2014)

All of these encouraging data indicated that establishing a mimic hua-+mn tumor model in porcine, or even artificial porcine tumor model is probably coming of age, cancer has not so far been a major focus of pig biotechnology, although, such procedures actually present some disadvantages of applications, labor intensive, ethical concerns and clearly do not provide fully representative models of human cancers.

4. Genetically Modified Lymphocytes (GML)

4.1. Alloreactivity

Alloreactivity is defined as the reactivity of the immune system of one individual

against cells from another individual. It is known as a major mechanism responsible for the graft rejection that is characterized by recipient cells reacting to and eliminating donor cells. Alloreactivity can also occur in the opposite direction, namely the donor lymphocytes that are present in the graft tissue may react toward the tissues or the cells of recipient. This reaction, termed Graft versus-Host Disease (GvHD), may occur when the recipient is highly immunocompromised, especially after allogeneic hematopoietic stem cells (HSC) transplantation (HSCT), but also after solid organ transplantations such as liver transplantation. On the other hand, alloreactivity has also been used to obtain some benefits in the treatment of hematological malignancies with HSCT. Indeed, clinical studies have shown that allogeneic HSCT is associated, in addition to the reconstitution of the immune system, with a very significant antileukemic activity by virtue of the presence of mature immune cells in the HSC graft. Alloreactivity presents a potential anti-tumor immunotherapeutic strategy mediated by T lymphocytes and NK cells, which involve different mechanisms of alloantigens recognition.

4.1.1. T-cell alloreactivity

As a major effector cells of adaptive immunity, T-cells continuously circulate through lymphoid organs to induce a rapid immune response when encountering an antigen. They are characterized by the expression of an antigen-specific T-cell receptor (T-Cell Receptor, TCR): a disulfide-linked membrane-anchored heterodimer normally consisted of the alpha (α) and beta (β) chains expressed as part of a complex with the invariant CD3 chain molecules (Figure 14). Both the TCR- α and TCR- β chains have (i) a variable region with three hypervariable domains, that constitute the binding sites of the antigen, (ii) a constant region, (iii) a transmembrane domain and –iv) a short cytoplasmic tail. Each TCR is complexed to CD3 that has a role in signal transduction. However, the T-cells are unable to recognize directly target cells such as infected or tumor cells: antigenic proteins must be processed and presented by

molecules present on the surface of APCs and target cells, the molecules of the Major Histocompatibility Complex (MHC).

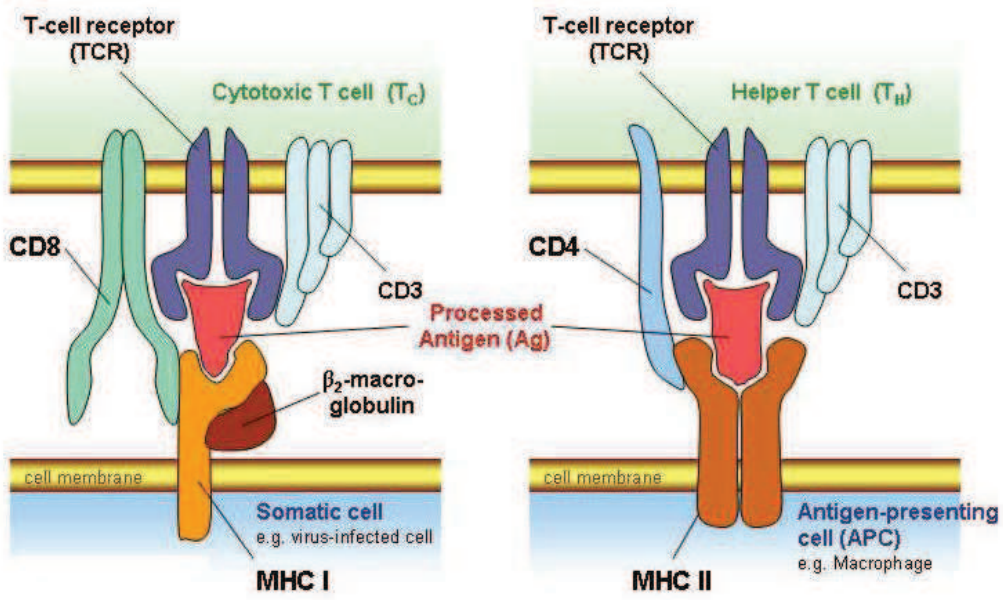


Figure 14: TCR-mediated interaction of T-cells with Ag/MHC complex

T-cell receptor binding to MHC-antigen complex: TCR Activation of naïve CD4 or CD8 T-cells requires two independent signals. Binding of T-cell receptor (TCR) to the antigen-HLA complex on the APC delivers a signal (signal 1) that can induce activation and expansion of T-cells when the costimulatory signal (signal 2) is given by binding of CD28 to B7 molecules (according to http://classroom.sdmesa.edu/eschmid/Chapter17-Zoo145.htm).

The T-cells are divided into two major populations, CD4+ and CD8+ T cells. The CD4+ T cells are called helper T-cells because they initiate the adaptive immune responses. They recognize peptides that are presented by the APCs, dendritic cells (DCs) and macrophages, via MHC class II molecules (MHC II). By contrast, the CD8+ T-cells, also termed cytotoxic T lymphocytes (CTLs), recognize specific peptides that are presented by MHC class I molecules (MHC-I) on the surface of the APC or abnormal cells such as infected or tumor cells. After this specific recognition, CD8+ T-cells promote the ability to destroy targets by producing cytotoxic granules (perforin/granzyme), expressing Fas-L and/or secreting TNF-alpha .

In general, a T-cell recognizes a specific antigenic peptide only when it is

presented by a self MHC molecule on the surface of an autologous APC (MHC-restricted antigen presentation); However, almost 1% to 10% of the T-cells have allogeneic reactivity, indicating that they can react with allo-MHC molecules, and thus can be activated. In the context of transplant rejection, three modes of allorecognition have been identified: direct, indirect and semi-direct (Jiang, Herrera et al. 2004) (Sanchez-Fueyo and Strom 2011) (Figure 15).

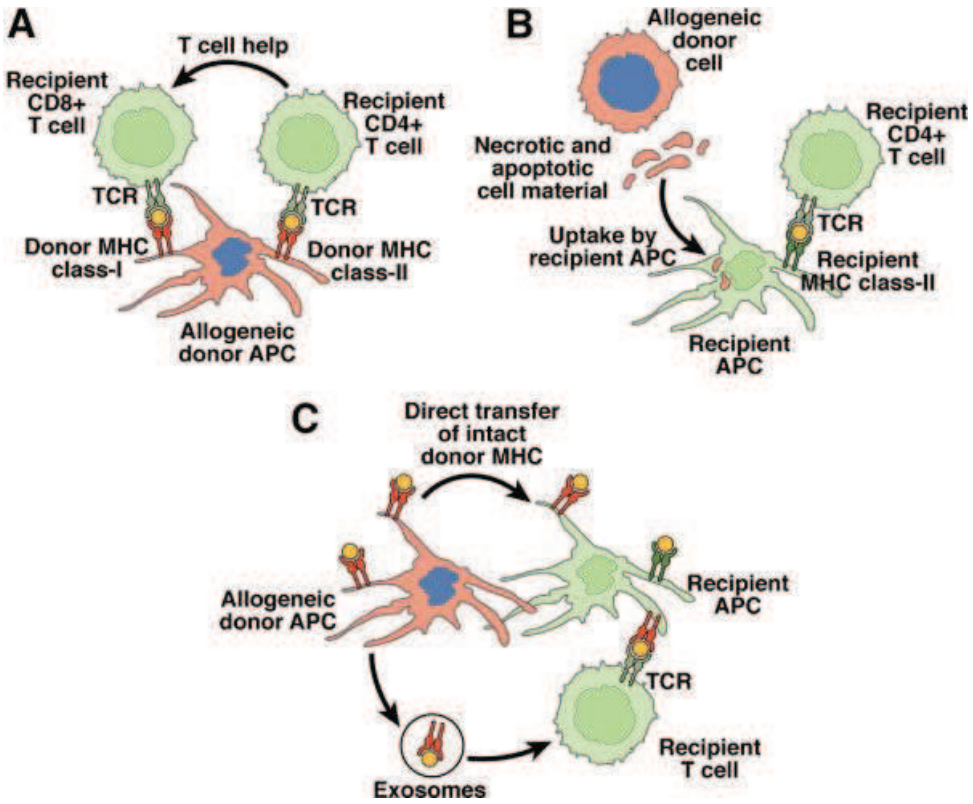


Figure 15. Pathways of allo-antigen presentation.

Three non-mutually exclusive pathways of allorecognition have been described: **(*A*)** In the direct pathway, recipient T-cells recognize intact allogeneic MHC molecules on the surface of donor APCs. The direct pathway is responsible for the large proportion of T-cells that have reactivity against allo-antigens due to cross-reactivity of the T-cell receptor (TCR) with self and foreign MHC molecules. **(*B*)** In the indirect pathway, recipient APCs trafficking through the allograft phagocytose allogeneic material shed by donor cells (mostly peptides derived from allogeneic MHC molecules) and present it to recipient T-cells on recipient MHC molecules. **(*C*)** In the semidirect pathway, recipient APCs acquire intact MHC molecules following direct contact with donor APCs and/or through fusion with donor APC-derived exosomes. These chimeric recipient APCs stimulate recipient T-cells through direct and indirect pathways (Sanchez-Fueyo and Strom 2011).

In the case of direct allorecognition (Figure 15A), donor allo-antigens are

presented by MHC class-I and MHC class-II molecules of the donor on the surface of donor's APCs and are directly recognized by recipient's CD4+ and CD8+ T-cells. This recognition occurs due to cross-reactivity of recipient's TCR between self and non-self MHC molecules.

As the recipient's APC circulate continuously in the body, they also pass through the grafted tissue where they capture allo-antigens (that are mostly peptides that are derived from MHC molecules of the donor) and present them to recipient's CD4+ T- cells by autologous MHC-II. This process is called indirect allorecognition (Figure 15B).

Finally, a third way of recognition was highlighted: the semi-direct allorecognition. In this case, the recipient's APC completely acquire the MHC molecules of the donor through either fusion of membranes between donor's and recipient's APCs, or fusion of donor APC-derived exosomes (containing donor MHC molecules) and recipient's APCs. Therefore, the recipient's APCs present the allo-antigens to recipient's T-cells via donor's MHC molecules (Figure 15 C).

4.1.2. NK-cell alloreactivity

NK cells are a type of cytotoxic lymphocyte critical to the innate immune system. They represent a population ranging from 1% to 10% in the blood lymphocytes and are known to differentiate and mature mainly in the bone marrow_where they then enter into the circulation. In the innate immune system, NK cells provide rapid responses to viral infections and to tumor formation. NK cells are unique in their ability to recognize stressed cells in the absence of antibodies and MHC, allowing for a much faster immune reaction. Thus, they are considered as one of the first lines of defense of the organism. They were named "natural killers" because of the initial notion that they do not require activation in order to kill cells that are missing "self" MHC class I molecules. At the phenotypic level, they are defined by membrane expression of CD56, CD16 and by the absence of neither T-cell markers (CD3, TCR)

nor B cell markers (CD19, B-Cell Receptor (BCR)).

Two distinct populations of NK cells were identified on the basis of the expression of CD56 and CD16. The majority of NK cells (approximately 90%) express low levels of CD56 ($CD56^{dim}$) and high levels of CD16 ($CD16^{high}$ or $CD16^{bright}$); by contrast, 10% of the NK cells express high CD56 ($CD56^{high}$ or $CD56^{bright}$) and no (CD16-) or low levels of CD16 ($CD16^{dim}$). The population of NK $CD56^{dim}CD16^{high}$ cells exerts a high level of cytotoxicity, while the population of $CD56^{bright}CD16^{low}$ NK cells show a regulatory role in the immune response by the production of pro- or anti-inflammatory cytokines (Cooper, Fehniger et al. 2001) (Figure 16).

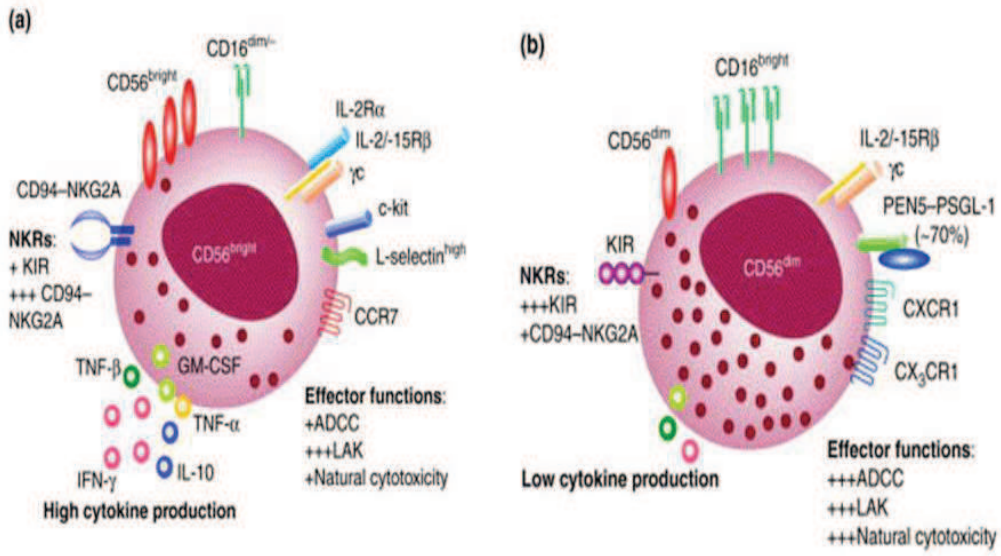


Figure 16. Schema of human natural killer (NK)-cell subsets.

(a) $CD56^{bright}$ NK cells produce high levels of cytokines following stimulation with monokines. This subset has low-density expression of CD16 and exhibits low natural cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC). This NK-cell subset expresses constitutively a number of cytokine and chemokine receptors, including the high-affinity interleukin-2 receptor (IL-2Rαβγ), is involved in trafficking to secondary lymph nodes. **(b)** By contrast, CD56dim NK cells produce low levels of NK-derived cytokines but are potent mediators of ADCC, LAK activity and natural cytotoxicity, and have a more granular morphology than CD56bright NK cells. These cells have distinct expression of cytokine (e.g. IL-2Rβγ) and chemokine receptors (according to https://www.pinterest.com/tormeypeter/nk-cells/).

The balance between inhibitory and activating signaling decides the NK cell killing effect. It is mediated by a lot of surface receptors that could recognize cognate

ligands on targets cells. A large number of inhibitory receptors such as Killer-cell Immunoglobulin-like Receptors (KIR), NKG2A/CD94, are expressed on NK cells, while the activating receptors such as Natural Cytotoxicity Receptor (NCR), NKG2D, CD244 are also identified on the surface of NK cells, both inhibitory and activating receptors having the ability to recognize specifically ligands (Figure 16). For example, KIR bind MHC I ligand group (Vilches and Parham 2002), while the CD94–NKG2A/B heterodimer recognizes HLA-E(Lee, Llano et al. 1998); among the activating receptors, NCRs including three molecules NKp46, NKp30, and NKp44 specifically bind to unknown ligands (Pogge von Strandmann, Simhadri et al. 2007; Baychelier, Sennepin et al. 2013) then induce signals of tumor cell lysis. If inhibitory receptors recognize a self HLA molecule on the surface of normal cells, the NK cell will receive a signal preventing cytotoxicity effect towards healthy normal cells, a phenomenon called self-tolerance. It has been proven that each NK cell of one individual has at least one inhibitory receptor specific for an autologous HLA molecule. By contrast, when the NK cell does not recognize any self HLA on the cell surface, it will initiate its cytotoxicity to eliminate the target cell, including autologous infected or tumor cells that under-express HLA molecules. Furthermore, the activating receptor may recognize stress molecules that are expressed by tumor cells, such as MHC class I-related molecules A / B (MICA / MICB), or viral proteins expressed by infected cells. It is known that the NK cells have not only lytic activity, but also produce different cytokines. Several reports showed that NK cells are also regulatory cells that could interact with dendritic cells, macrophages, T-cells and endothelial cells (Vivier, Tomasello et al. 2008).

4.2. Suicide gene therapy to GVHD

In order to differentiate and separate beneficial GvL effect from GvHD, one of the promising procedures is to genetically modify donor’s T-cells by integrating a suicide gene to improve their safety profile. Indeed, the use of genetic modification of

lymphocytes allows the generation of pharmacologically improved immune cells.

4.2.1. Rationale of suicide gene therapy

Suicide gene therapy initiated from the observation that in the same patient, the association of GvHD and GvT frequently occurs at the same time while presenting different kinetics. It was shown that a large part of the antitumor immunological capacity of donor lymphocytes depends on the alloreactive lymphocytes; however, these specific immune lymphocytes are also responsible for GvHD.

Suicide gene therapy has a great advantage in this approach: first, suicide gene can be transferred into T lymphocytes with a wide range of antigenic reactivity, including the antitumor as well as alloreactive cells; second, following the suicide gene transfer, all the T-cells permanently express the suicide gene and then show the sensitivity to its correspondent prodrug. In case of GvHD occurrence, a specific compound can activate the suicide gene system, then induce a selective elimination of transferred T-cells (Bonini, Ferrari et al. 1997). These results were a landmark allowing for the specific modulation of the donor-derived T-cells' alloreactivity without use of immunosuppressive drugs which might interfere with the natural process of post-transplant immune reconstitution (Oliveira, Greco et al. 2012).

4.2.2. Clinical experience with the TK suicide gene

Among the different suicide genes that have been designed, the herpes simplex virus thymidine kinase (HSV-tk) gene is certainly the most extensively tested in experimental and clinical trials. It has been developed to control alloreactivity of alloreactive lymphocytes in HSCT based on the enzymatic activity of the thymidine kinase of herpes simplex virus type 1 (HSV-tk). Donor lymphocytes are gene-modified prior to the transplantation and are infused either together with a T-cell-depleted donor HSC graft or several weeks after HSCT. The GML should provide their beneficial effects (GvL, prevention of graft rejection, anti-infectious

effects…) and only in the case of adverse events (GvHD), the suicide gene will be activated by infusion of its prodrug, the ganciclovir (GCV), a thymidine nucleoside analog used for treating herpes infection. Indeed, the HSV-TK suicide machinery activation relies on the administration GCV that is phosphorylated in its active compound, the GCV monophosphate, only in transduced cells. The GCV monophosphate is then di- and tri-phosphorylated by cellular kinases of the GML, where it can ultimately act as a guanosine tri-phosphate analog that inhibits DNA chain elongation and then induce cell death in proliferating cells, while resting transduced lymphocytes or untransduced cells are spared. Up to now, more than 120 patients have been treated with HSV-tk GMLs in phase I-II clinical trials (Tiberghien 2001; Burt, Drobyski et al. 2003; Fehse, Ayuk et al. 2004), that showed the efficacy (in terms of level of GML depletion and control of GvHD) and safety (in terms of lack of severe side effects) of this strategy, without documented side effects related to the HSV-tk gene transfer procedure.

However, some limitations for this system have been identified. For example, approximately 10% of the retroviral particles underwent deletions in the initial (wild type-derived) HSV-tk transgene, causing resistance to GCV(Garin, Garrett et al. 2001); this limitation was overcome by introducing point mutations in the HSV-tk transgene (Garin, Garrett et al. 2001). Another limitation of the HSV-tk system comes from its viral origin, that could cause development of an immune response against the transgenic product (Riddell, Elliott et al. 1996; Berger, Flowers et al. 2006).

In order to overcome the immunogenicity of the HSV-TK system, an alternative suicide gene has been developed, based on a chimeric gene incorporating the death domain of caspase-9 (iCasp9) and a FK506 binding domain allowing for the conditional dimerization and activation of the iCasp9. The administration of a highly specific chemical dimerizer instead of GCV is able to rapidly induce cell apoptosis in all transduced cells. Importantly, this process is independent to the proliferation state of GML. The *in vitro* and *in vivo* trials have demonstrated that the depletion of iCasp9+ cells is more rapid and complete than HSV-tk+ GML (Leboeuf, Mailly et al. 2014). The efficacy of iCasp9 suicide gene in controlling GVHD was tested in a phase

I/II clinical trial. *Ex vivo* allodepleted iCasp9+ transduced cells were infused to patients after haploidentical HSCT and the suicide gene machinery proved to be effective in controlling grade I-II acute GvHD (Di Stasi, Tey et al. 2011).

THESIS PROJECT

1. Development of animal models of HCC

Available animal models are critical for basic research and preclinical experiments. The ideal animal model should show all the properties of HCC including pathogenesis, etiology, interaction between host and tumors, yet up to now, no animal model presents all these properties. Thus, different animal models are used in various domains according to the purpose of experiments. Therefore, one goal of my thesis is to establish a well-defined murine model for the *in vivo* evaluation of HCC. Meanwhile, based on this xenograft HCC model, we will evaluate innovative HCC therapy strategies.

Based on previous research, we tried to establish different xenograft HCC animal models through different procedures by using two kinds of mouse models. There are different methods to establish orthotopic HCC mouse models, but three procedures are used for most research: intrahepatic injection, intrasplenic injection and intraportal vein injection. However, there is still no available standard procedure. Therefore, one project of my thesis is (i) to compare the per-operatory and post-operatory mortality among the three methods; (ii) using *in vivo* bioluminescence imaging (BLI) and magnetic resonance imaging (MRI) to identify the usefulness of different mouse models and (iii) to clarify the relationship between the BLI with MRI during the development the kinetic of HCC *in vivo*. For this purpose, severe combined immunodeficiency (SCID-bg) and NMRI-nu mice were used.

On the other hand, considering that pigs are closer to humans than mice in terms of size and physiology (especially liver metabolism), a second aim in this project was to establish an orthotopic porcine HCC model. To this aim, our approach was based on the *ex vivo* transduction of autologous primary porcine hepatocytes (PPH) with three lentiviral vectors containing each two oncogenes as well as one reporter gene. In

order to improve the safety of the procedure, we aimed at performing this transduction in a closed culture system, using a safety device allowing to protect both the experiment and manipulators. The final step of the approach was supposed to be the infusion of these transduced PPH back to the donor pig in order to establish an orthotopic HCC porcine model.

2.Development of innovative immunotherapies against HCC and HCV infection

Alloreactivity is described as a very powerful tool for tumor immunotherapy, particularly for the treatment of hematologic malignancies. Gene-modified lymphocytes were used clinically for over 15 years, their efficacy and safety have been demonstrated in the context of hematopoietic stem cells. Moreover, the CIK cells used in autologous situation have a significant anti-tumor activity in HCC patients in combination with current treatments.

Owing to the severity of HCC and HCV infection, the moderate effectiveness of current treatments and the number of patients steadily increasing, the development of new therapeutic strategies is crucial. Indeed, liver transplantation is the optimal treatment of choice for HCC, but is limited in part by a shortage of organs, and by the extremely rapid and systematic graft reinfection with HCV in patients with chronically viral infection. The second part of my thesis consisted in developing an approach of adoptive immunotherapy of HCC and HCV infection.

Our objective is to provide the proof of concept that allogeneic GML expressing a suicide gene as a safety control system, can provide an antitumor effect toward HCC cells and an antiviral effect toward HCV-infected cells. Such SGML may be produced as “ready-for-use” cell therapy products for adjuvant treatment of HCC, possibly in combination with current treatments, and also for the prevention of reinfection graft by HCV in liver transplanted patients.

MATERIAL AND METHODS

1. Cell lines

1.1. Culture

Human adherent cells (HeLa, Huh7, Huh7.5.1, HepG2, PLC-PRF-5, and SKHep1) were cultured in Dulbecco's Modified Eagle Medium (DMEM, PAA Laboratories) supplemented with 10% FCS, 1% non-essential amino acid (GIBCO ®) and 10µg/ml gentamycin (GIBCO ®). Human cells in suspension (B-EBV, K562) were cultured in RPMI-1640 supplemented with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin. The cells were passaged every three or four days.

1.2. Production of cell lines expressing luciferase

Human HeLa cells and Huh7 stably expressing the luciferase been produced (HeLa-Luc, Huh7-Luc) after transduction with a pCLNCX vector encoding luciferase (Dr Lorang, NIH, Bethesda, MD, USA) by centrifugation for 3h (1000g, 32 ° C). They were subsequently cloned by limit dilution at 10, 3 and 1 cell(s) per well (96 well plate) and each clone was tested for luciferase expression. The most strongly expressing luciferase clone was selected, and kept in culture as well as cryopreserved.

1.3. Flow Cytometry

1.3.1. Antibodies used

Table 9 shows the antibodies used for phenotypic characterization of GML and cell lines used in functional tests.

Antigen	Fluorochrome	Supplier	Application
CD34	PE	Becton Dickinson (BD), San Diego,CA, USA	Purity of GML after cell sorting
CD19	APC	BD	Purity of GML after cell sorting
CD3	Pacific blue	BD	Identifying subpopulation of GML CD3+CD56-(T cells), CD3-CD56+ (NK cells)and CD3+CD56+ (NK like T cells)
CD56	APC	BD	
CD4	AF700	BD	Identifying CD4+ LC
CD8	APC-Cy7	BD	Identifying CD8+ LC
CD107a	PE	BD	Cytotoxicity test by identification of degranulation marker CD107a

1.3.2. Membrane immunostaining

The cells were washed, centrifuged and incubated for 20 min at 4 ° C. in the presence of the antibodies described above. The cells were washed with PBS at 4 ° C and then fixed in PBS 2% v/v formaldehyde. The immunostainings were analyzed using a Flow Cytometer LSRII, 4 lasers, 16 colors (Becton Dickinson, Le Pont de Claix, France) and Diva software (Becton Dickinson).

2.Immunodeficient mouse models

Two kinds of immunodeficient mice were used: C.B-17 Severe combined immunodeficient-beige (SCID-bg) mice and NMRI-nu (Nude) mice. SCID-bg mice (Taconic, Bomholtvej, Denmark) are inbred mutant mice that are severely deficient in B and T lymphocytes; they generally lack detectable immunoglobulin and have impaired NK cell immunity. The mutation on chromosome 16 appears to impair the recombination of antigen receptor genes and thereby causes an arrest in the early development of B and T lineage-committed cells. SCID-bg mice readily support

normal lymphocyte differentiation and can be reconstituted with normal lymphocytes from other mice and even partially reconstituted with human lymphocytes. They support the growth of allogeneic and xenogeneic tumors. NMRI-Foxn1nu/Foxn1nu (or NMRI-nu) mice (Janvier Labs, Le Genest Saint Isle, France) are outbred mutant mice that have the particularity to be albinos, naked and immunodeficient. The autosomal recessive mutation in the Foxn1 gene (fork head box N1), located on chromosome 11, causes a total or partial aplasia of the thymus, responsible for the T-cell deficiency of the immune system (Figure 17). NK cells and B lymphocytes remain functional but humoral responses are impaired due to a lack of T-B cooperation.

Figure 17. Immunodeficient mice used for the studies.

A: Severe combined immunodeficiency-beige (SCID-bg) mouse; **B**: NMRI-Foxn1 nu/Foxn1nu (NMRI-nu) mouse.

Animal experimentations received the approval of the local ethical committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg, approvals n°AL/34/41/02/13, and AL/33/40/02/13). All the procedures were performed under general anesthesia by inhalation of isoflurane, the concentration being maintained under 3%.

2.1. Orthotopic HCC injection

2.1.1. Intraportal vein injection of Huh-7-luc cells

Mice were placed in a supine position on the procedure table, an abdominal midline incision was made from the sternum downward for 2-3 cm, the two layers

(cutaneous and muscular) of the abdominal cavity were opened, the upper abdomen was exposed with two retractors towards the lateral sides of the abdomen, the liver was retracted upward by using a retractor, meanwhile, the intestines and stomach were lightly pulled out of the abdominal cavity downward in order to expose the portal vein. Huh-7-luc cells ($1x10^6$ cells in 50 µl) were injected slowly via a 30-gauge needle in the middle of the portal vein, the needle tip was gently pushed into the vein from the injection site upward 3-4mm in order to decrease the possibility of cell leaking from the injection point. The process of injection was performed in one minute in which the successful injection was indicated by the fact that a transparent bleb of the cells could be seen through the liver capsule. Subsequently the vein was pressured with a 5x5mm Surgicel piece (Johnson & Johnson Health Care Systems Inc, U.S) for three minutes when removing the syringe. The intestines were put in place and the muscular and cuteaneous layers were closed with a 3-0 string suture (Polysorb, CONVIDIEN, Tyco Healthcare,U.S) (Figure 18).

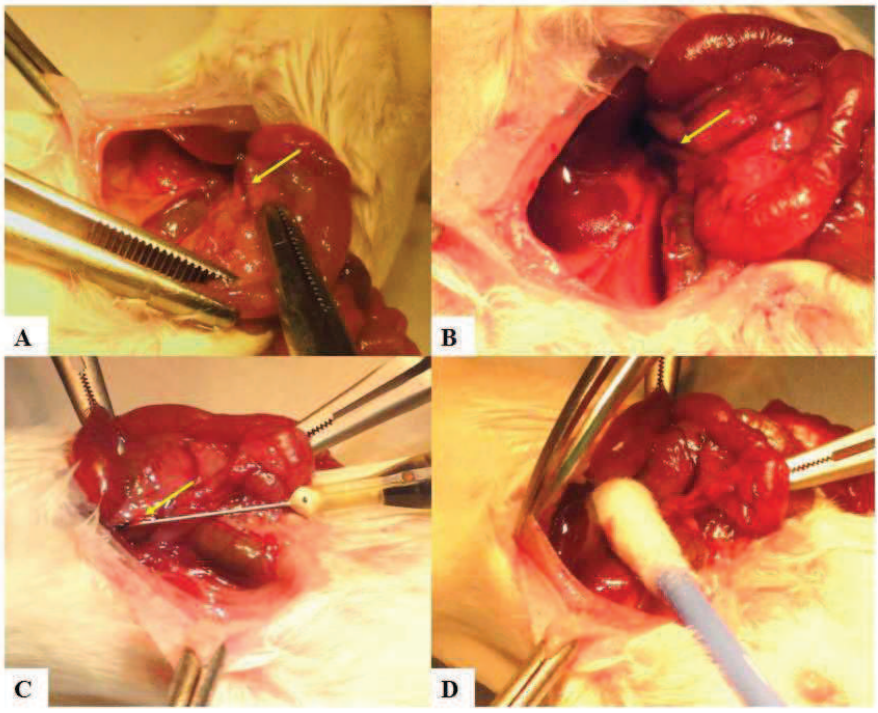


Figure 18. Intraportal injection

A, B: Exposure of the portal vein,**C**: Injection of Huh7-luc cells; **D**: Compression on the injection point when removing the syringe, The yellow arrow indicates the portal vein.

2.1.2. Intrahepatic injection

Mice were placed in a supine position on the procedure table, an abdominal midline incision was made from the sternum downward for 1.5 cm, the two layers (cutaneous and muscular) of the abdominal cavity were opened, the lower left lobe of the liver was exposed with one retractor which took the peritoneum towards the left lateral side. A piece of 5x5mm Tachosil (Baxter Healthcare Corporation, U.S) was pierced by the needle of a syringe of 29-gauge previously filled with $1x10^6$ Huh-7 cells in 50 µl. Cells were injected at a 30-degree angle into the liver, so that a transparent bleb of cells could be seen through the liver capsule. After injection, the piece of Tachosil was immediately left in place on the injection site, and a slight pressure was applied for one minute to prevent bleeding as well as leaking of cells. The muscular and cuteaneous layers of the abdomen were closed with a 3-0 string suture (Tyco Healthcare,Waltham, CT) (Figure 19).

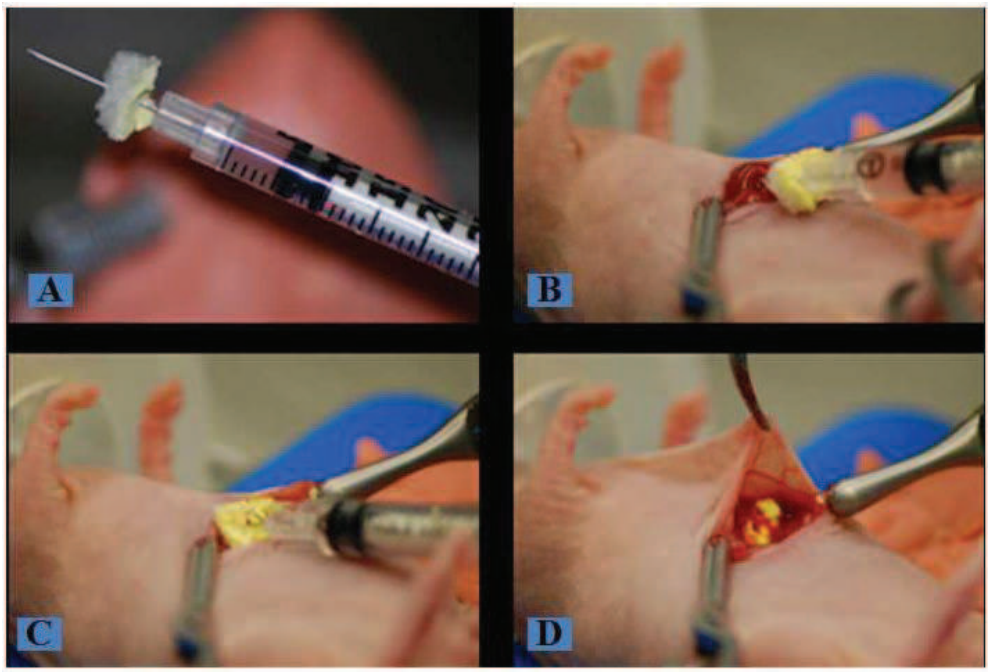


Figure 19 Intrahepatic injection

A: A piece of TachoSil is passed through a syringe; **B**: Cells are injected into the left lobe of the liver; **C**: The piece of TachoSilis left in place on the injection site when the syringe is removed; **D:** Mouse after transplantation of cells.

2.1.3. Intrasplenic injection

Mice were swabbed with alcohol before performing a left lateral incision of abdomen (10-15mm). The spleen was identified and carefully pulled through the incision to sit outside the mouse. $1x10^6$ Huh7-luc cells suspended in 50µl medium culture were injected with a syringe of 29-gauge within 60s, the site of injection was chosen in the inferior marge of spleen with up-towards the axis. The site of injection was pressured with a tampon for 30s. Finally, the cutaneous layer of abdomen was closed with staples.

For all three injection procedures, the skin of the mice was sterilized with Betadin and ethanol before and after surgical procedure. At the end of each manipulation, mice were placed on a heat pad and injected subcutaneously with Buprenorphine analgesic (0.05mg/kg) before awaking and becoming mobile, then were returned to their regular cage once awaken.

2.2. *In vivo* imaging

2.2.1. Bioluminescence imaging

The luciferase activity of Huh-7-Luc cells was measured at the indicated time points after transplantation by bioluminescence imaging (BLI) using an IVIS 50 camera (Caliper Lifesciences, Roissy, France) after intraperitoneal injection of 100µl d-luciferin (20 mg/ml; Caliper Lifesciences). Repeated acquisition was performed for one minute, starting from the time of luciferin injection and during all the period of light emission, until a decrease of the signal was observed, as previously described (Inoue, Kiryu et al. 2010). Tumor cell bioluminescence, analyzed with Living Image 3.1 software (Caliper Lifesciences), was expressed as photons/second/cm^2/steradian (p/s/cm^2/sr) at the time point after luciferin injection leading to the highest bioluminescence value. As previously reported (Inoue, Kiryu et al. 2010), the timing

of imaging after d-luciferin injection affects the longitudinal assessment of tumor growth using *in vivo* bioluminescence imaging, this optimal time point was different from one tumor to another and from a time post-transplantation to another but was usually obtained <20 minutes after luciferin injection. Relative tumor growth was obtained by calculating the ratio of bioluminescence at the indicated day to the bioluminescence at D0.

2.2.2. Fluorescence imaging

The *in vivo* fluorescence signal was detected at the indicated time after epi-illumination with an IVIS 50 camera using a 675nm excitation filter and ICG (810-875nm) emission filter. The fluorescence emission was expressed as average of efficiency (% emission radiance - p/s/cm²/sr - to illumination irradiance - µw/cm²).

2.2.3. Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed with an Aera 1.5 Tesla MRI (Siemens, Erlangen, Germany). The signal was enhanced with a loop antenna (Siemens). Fat saturation T2 sequences (TR:2540 ms, TE: 69 ms) were acquired in the axial and coronal plans in a 50 mm x 50 mm field of view, with a 192 x 192 matrice and 1 mm thickness (voxels' size: 0.3x0.3x1 mm in the XYZ axes).

2.3. Histological analysis

For hematoxilin-eosin staining and immunostaining, the organs (liver, spleen lung, intestine, kidneys) were embedded in TissueTek and immediately frozen on dry ice prior to sectioning (10 microns) and stained as previously described.[5] Tissue sections were incubated with 1/25-diluted anti-human cytokeratin-18 (hCK18) mAb

(M7010, clone DC10; Dako France, Les Ulis, France) overnight at 4°C. Following washing, immunostained cells were detected using the peroxidase Vectastain Elite ABC kits (Vector Laboratories, Clinisciences, Nanterre, France) and counterstained with eosin.

3. Isolation and transduction of primary porcine hepatocytes

3.1. Plasmids

In order to select the best candidate recipient plasmid encoding the luciferase to combine with the oncogens plasmid, three candidates plasmids, pHIV-Luc (Addgene, Cambridge, MA), pLenti CMV V5-LUC Blast (Addgene) and pHIV-Luc Zs Green (Addgene) were tested *in vitro* in Huh 7.5.1 cells, primary human hepatocytes and PPH.

The retroviral vectors encoding a single transgene, pBabepuro-cyclinD1, pBabehygro-CDK4^{R24C}, pBabeblasto-c-Myc^{T58A}, pBabeneo-p53^{DD}, pBabepuro-flag H-Ras^{G12V} and pBabepuro-flag hTERT, have been described previously (Hamad, Elconin et al. 2002; Yeh, Cunningham et al. 2004). Plasmid 11128, pbabe-hTERT+p53^{DD}, was generated by cloning FLAG-hTERT cDNA with flanking EcoRI-SalI restriction sites added by PCR into the same sites of the polylinker of pBABEpuro or pBABEblast and by excising the selectable marker from the Hind III/ClaI sites and ligating into the same site p53^{DD} cDNA in which Hind III/ClaI sites were again added by PCR. Add gene has sequenced a portion of this plasmid for verification; Plasmid 11129, pbabe-cyclinD1+CDK4^{R24C}, was generated by cloning cyclinD1 cDNA with flanking EcoRI-SalI restriction sites added by PCR into the same sites of the polylinker of pBABEpuro or pBABEblast and by excising the selectable marker from the Hind III/ClaI sites and ligating into the same site CDK4^{R24C} cDNA in which Hind III/ClaI sites were again added by PCR. Add gene has sequenced a portion of this plasmid for verification; Plasmid 11130,

pbabe-c-myc^{T58A}+HRas^{G12V}, was generated by cloning c-Myc^{T58A} cDNA with flanking BamHI-EcoRI restriction sites added by PCR into the same sites of the polylinker of pBABEpuro or pBABEblast and by excising the selectable marker from the HindIII/ClaI sites and ligating into the same site H-Ras^{G12V} cDNA in which HindIII/ClaI sites were again added by PCR.

The inserts hTERT + p53^{DD}, cyclinD1 + CDK^{4R24C} and c-myc^{T58A} + hRas^{G21V} were amplified by PCR from 11128, 11129 and 11130 donor vectors, respectively. The sequencing was carried out for the three inserts in the donor vector and it was checked that in each group of inserts, there is a SV40 promoter between the oncogene 1 and the oncogene 2, permitting the expression of the second insert. The cloning of the three inserts was performed in the pLenti CMV V5-LUC Blast acceptor plasmid between the Xma I and KpnI sites. Amplification of each construct was performed by DNA midi-preparation.

The nomenclature of the different plasmids was defined as: the plasmids for production of retroviral vectors, used as donor plasmids, were named 11128, 11129 and 11130 (according to Addgene's identification), while the plasmids used for the production of lentiviral vectors encoding the oncogenes combined with the luciferase and their corresponding lentiviral vectors were named 11128 or 28, 11129 or 29 and 11130 or 30, respectively.

3.2. Primary Porcine hepatocytes isolation

Primary porcine hepatocytes were isolated from liver resection pieces obtained from the IRCAD (Research Institute against Digestive Cancer, Strasbourg, France) as described by Borlak et al. (Borlak, Hock et al. 2003).

The liver specimens (< 100 g) were immediately transferred in ice-cold physiologic saline solution to the laboratory. Vessels visible on the cut surface were cannulated and the perfusion was done with 500 ml of EGTA-containing HEPES

buffer (Roche, Germany) at pH 7.4 and 37°C, followed by perfusion with 500 ml HEPES buffer. Thereafter, a collagenase IV (Hofmey Rock , Basel, Switzerland) perfusion was performed with 250 ml of HEPES buffer containing collagenase IV and calcium chloride dihydrate at 37° C. The collagenase IV perfusate was recirculated. Following perfusion, the liver capsule was carefully removed and the cells were liberated by gentle shaking of the liver specimen in ice-cold buffer containing Hanks buffered salt HEPES and bovine serum albumin. The resulting cell suspension was filtered through a nylon mesh and washed three times in buffer at 4° C. Viability of the hepatocytes was assessed by the Trypan blue exclusion assay and cells were counted followed by either manipulate immediately or frozen at -80°C in the culture medium supplemented with 10% DMSO.

3.3. Production of lentiviral supernatants in a culture system closed

A closed culture system was developed using 25 cm² CliniCell devices (Mabio International, Tourcoing, France) and connecting devices (Becton Dickinson) as described in figure 20, 21. The CliniCell is a compact cell culture cassette consisting of two culture surfaces fixed on a rigid frame creating a sterile cell culture chamber with a connectivity based on the standard Luer Lock connections. The culture surfaces are flat, transparent and gas permeable. The CliniCell is produced according to the Good Manufacturing Practices recommendations. This system was used both for the production of lentiviral supernatants and for the transduction of primary hepatocytes.

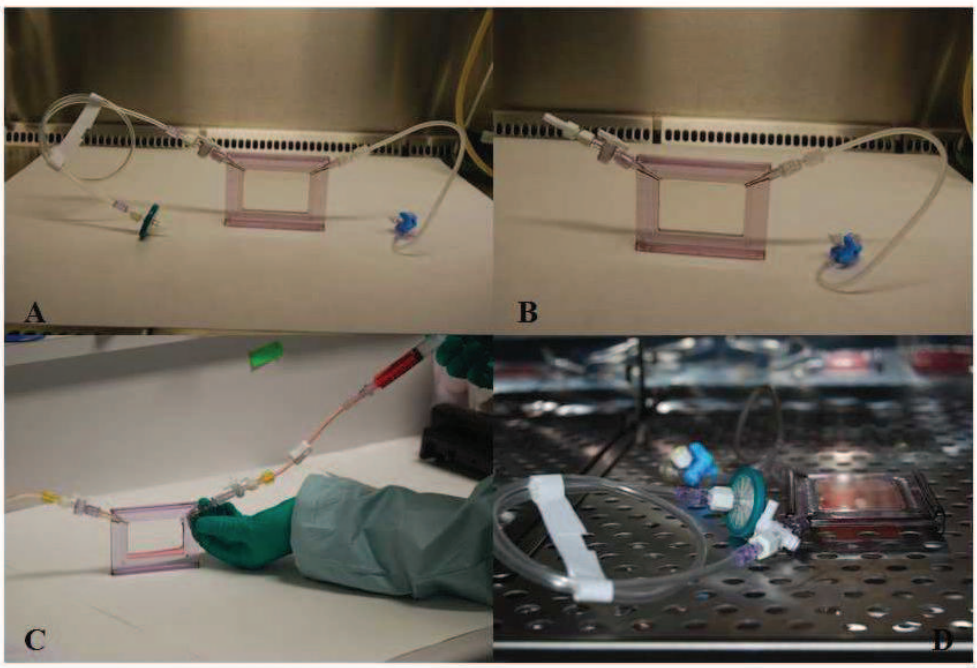


Figure 20. Closed culture system

A: Connect the CliniCell with a switch (right) and a filter (left); **B**: Close the CliniCell at both sides for protecting; **C**: Inject the culture medium, lentiviral vector or cell suspension into the CliniCell via a syringe; **D**: Put the system containing the medium (cells or viral supernatants) into the incubator.

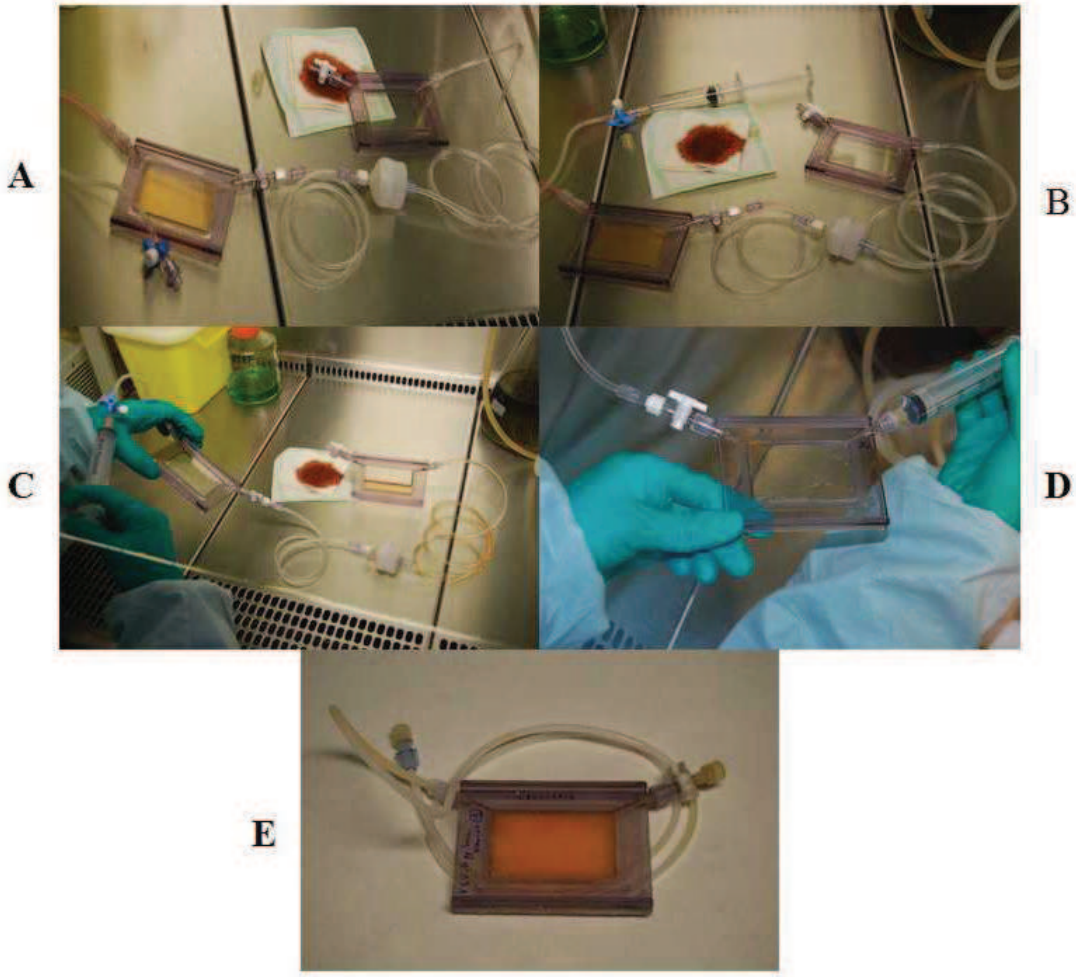


Figure 21. Detail of manipulation of system

A, B, C: Connect two CliniCell systems without exposure to environment in order to recover the retroviral vector (= culture supernatant) from the cell culture; **D**: Cells can remain adherent to both sides of the CliniCell after removing the medium; **E**. The CliniCell containing transduced cells or lentiviral supernatants can be frozen at -80°C.

To test the efficiency and safety of this device, we produced the lentiviral supernatants of three VSV-G-pseudotyped lentiviral vectors encoding two oncogenes and luciferase as well as a control lentiviral vector encoding only luciferase. Briefly, at Day 0, $10x10^6$ 293T cells suspend with 8 ml DMEM supplemented with 10% FCV, 0.5% gentamicin and 1% NEAA (Non-Essential Amino Acid, GIBCO®, Invitrogen, Cergy Pontoise, France) were injected into the CliniCell. Four hours later, after identifying that all the cells were adherent to one side of device, the medium was removed, then the same cell number was reinjected into the CliniCell that was put in the incubator on the other side. Therefore adherent cells could be obtained at both

sides of the device. At day 1, three plasmid mixtures were prepared containing the HIV-gag-pol plasmid (8.1 µg), the VSV-Env plasmid (2.7 µg) and one of the three plasmids encoding two oncogenes (8.1 µg) or control plasmid encoding only luciferase in a final volume of 10 ml culture medium. The mixture was then injected into the device after removing the ancient culture medium and the culture was continued in the same conditions as before. At day 2, the medium was changed with 10 ml culture medium of 293T cells. Finally, the culture supernatants containing the lentiviral vectors were recovered by transfer into another CliniCell device (both devices being separated by a 0.45 µm filter to prevent recovery of cell debris) at day 3 and replaced by fresh culture medium for a second run of supernatant recovery at day 4. The lentiviral vectors were frozen in the CliniCell devices at – 80°C until use.

In parallel, we produced the lentivirals by using an "open" system (as conventional protocol), the unique difference between "closed" and "open" was defined by replacing the CliniCell closed culture system by Petri dishes of $10cm^2$.

3.4. Lentiviral transduction of Huh7.5.1 cells and primary porcine hepatocytes in culture system closed

Fresh or frozen Huh7.5.1 cells or PPH were transduced with lentiviral vectors in the CliniCell device. Briefly, $10x10^6$ cells were suspended in 10 ml culture medium, Williams medium supplemented with 10% FCS, then injected into a CliniCell device that was incubated on one side at 37°C, 5% CO2 for four hours to assure the adherence of cells to one cell culture surface, then changing the medium by inject also the same number of cells in 10 ml culture medium and incubating the device on the other side to allow for the adherence on the other cell culture surface. Four hours later, the culture medium was removed and replaced by the lentiviral vector. The cells were cultured for 24h (from D0 to D1), then the transduced cells were washed once with 10 ml PBS and cultured for 48h (from D1 to D3) in culture medium. At D3, the cells were digested with 1 ml trypsin for one minute, harvested and used for experiments.

To test the efficacy of lentiviral transduction, 50000 transduced cells per each lentiviral transduction were plated into a well of 96 wells plates (in triplicates), centrifuged five minutes at 1250 rpm. The supernatants were removed and 50 µl lysis tampon were added. Three minutes later, 25 µl luciferin were added and 25 µl mixture were transfetred to an untransparent plate to test the expression of luciferase. The same number of normal (non-transduced) Huh 7.5.1 cells or PPH were assessed in parallel as a negative control group.

To investigate the efficacy of the gene transfer with three lentiviral vectors, PPH were transduced either individually with one of the three lentiviral vectors or with a combination of two of the three lentiviral vectors. Additionally, two groups that combined all of three lentiviral vectors were assessed, with the difference that in one group, the three lentiviral vectors were added at the same time (3.3 ml of each vector, instead of 10 ml) while in another groups, each lentiviral vector was added individually (10 ml per vector, one vector per day) until three days. A control group of VSV-G-pseudotyped lentiviral vector encoding only the luciferase reporter gene and a control group of non-transduced PPH were performed in parallel (Table 10).

Table 10 Experimental groups

Group number	plasmid numbers
Group A	28
Group B	29
Group C	30
Group D	28+29
Group E	28+30
Group F	29+30
Group G	28+29+30
Group H	28(d1)+29(d2)30(d3)
Group I	VSV-Gpp-luc
Group J	Non-transduced PPH

Table 10. Experimental groups for the transduction of PPH with three lentiviral vectors

28: $p53^{DD}$+hTERT; 29: cyclin D1+CDK^{4R12C}; 30: $HRas^{G12V}$+ c-myc

3.5. *In vivo* and *in vitro* evaluation of oncogenes expression

3.5.1. *In vivo* evaluation

The transduced PPH were harvested at day 3 after transduction, counted and suspended in culture medium at 1x 10^6/ 50µl, then injected subcutaneously in both side of NMRI-nu mice and monitored by BLI twice per week. Alternatively, PPH transduced according to the protocol of group G or control PPH transduced with VSV-Gpp-luc vector (group I) were intrasplenically injected in hepatodeficient and immunodeficient Alb-uPA/SCID-bg mice (see below, paragraph 5.1 ‘’Orthotopic xenogeneic model’’) and monitored weekly for growth by BLI.

3.5.2. *In vitro* evaluation

Western-blots (10 and 20 µg total protein/lane, for PPH and cell lines, respectively) were performed to identify the expression of all six oncogenes. Primary antibodies were as follows: monoclonal mouse anti-human p53 (clone DO-1, BioLegend), monoclonal mouse anti-human CyclinD1 (clone CD1.1, ABR), monoclonal rabbit anti-human CDK4 (clone D9G3E, Cell Signaling Technology), monoclonal rabbit anti-human c-myc (clone D84C12, Cell Signaling Technology) and polyclonal rabbit anti-human TERT (Osenses) were purchased from Ozyme (St Quentin en Yvelines, France), rabbit anti-human c-ras was purchased from Becton Dickinson Biosciences. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system with exposure of X-ray film. The images were analyzed by Quantity One (Bio-Rad).

4. Genetically modified lymphocytes (GML)

4.1. Production of retroviral supernatant

The packaging cell line PG34/TK was used for the production of the retroviral supernatant required for the production of GML. This cell line is derived from the transduction of the PG13 cell line with the vector MP71-T34FT, has the ability to produce retroviral particles encoding a fusion protein of the herpes simplex virus thymidine kinase (HSV-tk) and the truncated form human CD34 (Figure 22)(Fehse, Kustikova et al. 2002). Alternatively, we also used the SFG.iCasp9.2A.ΔCD19 retroviral vector (provided by Pr M.K. Brenner, Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX) encoding the human CD19 and iCasp9(Di Stasi, Tey et al. 2011) (Figure 23). This vector was also produced by the PG13-derived PG13/iCasp9 packaging cell line. After thawing, cells were cultured in RPMI 1640 (Invitrogen, Cergy Pontoise, France) containing 10% fetal calf serum (FCS, PAN Biotech GmbH, Aidenbach, Germany). Three days later, the cells were incubated overnight with RPMI 1640 but without serum, these deprivations leading to cellular stress and increased production of retroviral particles. Then, the supernatant was harvested, filtered on 0,22 µm filters and frozen at -80°C while the packaging cells were cultured in the presence of RPMI 10% FCS for 8 h. Again, the medium was replaced by serum-free medium for 12 hours; the supernatant production was carried out through the same protocol for two weeks. Each batch of supernatant was then tested by transduction of HeLa cells: HeLa cells were centrifuged for three hours (1000g, 32 ° C) in retroviral supernatant supplemented with protamine sulfate (5g/ml). Transduction efficiency, defined as the percentage of transduced cells (CD34+ or CD19+), was verified by membrane labeling of HeLa cells with a CD34 or CD19 antibody coupled to the fluorochrome PE-Cy7. The analysis was done by flow cytometry.

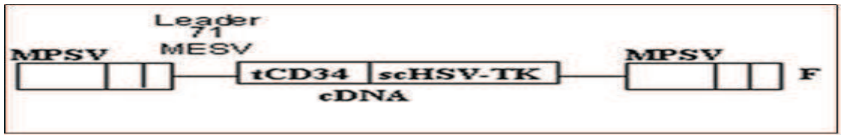


Figure 22. Map of the retroviral vector encoding both tCD34 and scHSVtk (Fehse, Kustikova et al. 2002).

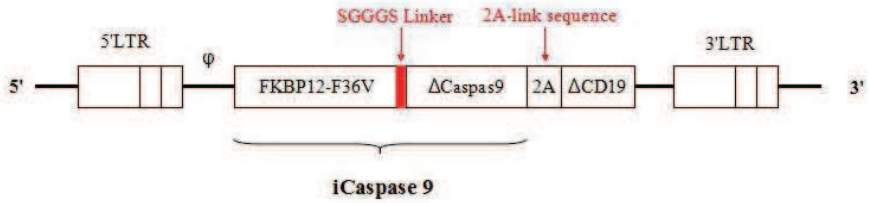


Figure 23. The structure of the iCaspase9.2A.ΔCD19

Bicistronic transgene (i.e., with two cistrons, the loci responsible for generating a protein), comprising the iCasp9 sequence, with truncated CD19 (ΔCD19) serving as the selectable marker. The sequence cassette is then incorporated into the SFG retroviral vector(Di Stasi, Tey et al. 2011).

4.2. Activation, transduction and expansion of PBMC

All the blood samples were from healthy donors (Etablissement Français du Sang -EFS- Alsace, Strasbourg, France), peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation, washed and cultured at a concentration of 1.10^6 cells/ml in RPMI-1640 supplemented with 10% normal human serum (NHS) (EFS Bourgogne / Franche-Comté, Besançon, France). They were then activated by 10ng/ml soluble CD3 monoclonal antibody (OKT3, Jenssen-Cilag Levallois-Perret, France, 10ng/ml) and cultured in the presence of 500 IU/ml recombinant human interleukin-2 (IL-2) (Proleukin ™, Novartis Pharma, Dorval, Quebec). Three days later, the cells were transduced with the retroviral supernatant PG34/TK, or PG13/iCasp9. They were centrifuged for three hours (1000g, 32 ° C) in retroviral supernatant supplemented with IL-2 (1000 IU/ml) and protamine sulfate (5g/ml), then returned to culture in RPMI supplemented with 10% NHS + IL-2 (500 IU/ml). Two or

three days later, the transduced cells (CD34+ or CD19+) were selected by immunomagnetic sorting which allowing obtaining purified Genetically Modified Lymphocytes (GML). The cells were then cultured in RPMI 10% NHS supplemented with 500 IU/ml IL-2 until D14 (Figure. 24). GML sensitivity to ganciclovir (GCV) or chemical inducer of death (CID) was checked by cell counting after culturing in the presence or absence GCV (1 µg/ml) or CID (10 nM) for one week.

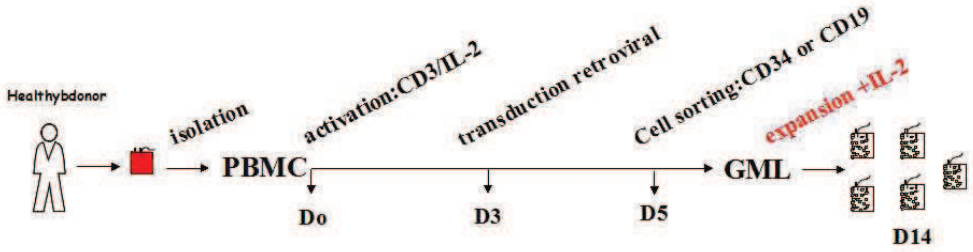


Figure 24. PBMC were isolated from healthy donor.

Activated by CD3 antibody and IL-2(D0), transduction with retroviral at D3, the cells transduced were selection by immunomagnetic sorting at D5, then the GML were cultured until D14.

In parallel, PBMC were activated and cultured for 14 days without transduction and selection, identified as control (Co) cells, were used as control group in various tests. Where indicated, the CD3 monoclonal antibody was replaced with CD3/CD28 antibodies co-immobilized on beads (Invitrogen, Cergy Pontoise, France; one ball to 1.10^6 cells) and IL-2 were replaced by IL-7 (20ng/ml; Cytheris, Issy-les-Moulineaux, France).

4.3. Purification of transduced cells

Two or three days after retroviral transduction, CD34+ or CD19+ cells were isolated by using a CD34 or CD19 Microbeads kit (Miltenyi Biotec SAS, Paris, France) according to the protocol provided: cells were washed and labeled with CD34 or CD19 beads for 30 minutes at 4°C. After washing, they were resuspended in PBS supplemented with 0.5% NHS, filtered and then placed on LS columns (Miltenyi

Biotec) fixed on a magnet, or sorted in an automated manner via an autoMACS (Miltenyi Biotec). Two passes of successive column were performed to improve the purity of the positive fraction. The cells were then centrifuged and resuspended in the culture medium as described above, for their expansion until D14.

5.*In vivo* evaluation of GML

5.1. Orthotopic xenogeneic model

SCID-bg mice were injected by intrasplenic with 1.10^6 cells of Huh7-Luc, allowing the cells to migrate in the liver. Bioluminescence was determined four days later as described above. Only mice showing location of Huh7-Luc in the liver were selected for the experiment and were injected iv with 200×10^6 of GML in 100µL PBS (D0) and were injected daily ip with IL-2 (10^6IU/ml). The luciferase activity was measured three and seven days after injection of GML as previously described.

The Alb-uPA/SCID-bg mice are SCID-bg mice that are transgenic for the ‘’Urokinase-like Plasminogen Activator’’ (uPA) gene under the control of the albumin promoter (Alb-uPA). This allows the expression of the transgene only in the liver. The uPA gene induces severe liver toxicity from birth. The degeneration of murine hepatocytes then enables the transplantation of primary murine of xenogeneic hepatocytes such as PHH, after intrasplenic injection of $0.5\text{-}1x10^6$ PHH at the age of three to five weeks. The primary hepatocytes repopulate then the liver and can replace up to 90% of murine hepatocytes within one to two months. In the case of PHH transplantation, this allows to support an HBV or HCV infection (Mercer, Schiller et al. 2001) The PHH engraftment is monitored by quantification of human serum albumin in the murine serum samples, using a human serum albumin-specific ELISA. Evaluation of PHH repopulation was also evaluated by immunohistochemistry of the liver, after staining with anti-hCK18 staining, as reported above.

5.2. Prevention of alloimmunization against GML

To investigate whether the immunosuppression induced by *in vivo* CsA interferes with alloreactivity of GML, the splenocytes of FvB-Luc mouse stably expressing luciferase were grown in RPMI containing 10% FCS 5µg/mL of Concanavalin A (ConA), 500U/mL of IL-2 and 50µM of β -mercaptoethanol for 14 days. Ten million of these cells were IP injected into Balb/c immunocompetent mice that received either PBS or CsA (50mg/kg) daily as well as IL-2 (10^6 IU/ kg). The presence of the injected cells was observed by measuring bioluminescence *in vivo* seven days after injection, as described above. Meanwhile, the grade of GvHD was blindly determined twice a week, on the basis of five parameters including weight, integrity of the skin, hair texture, activity and animals posture (Cooke, Kobzik et al. 1996).

6.*In vitro* evaluation of the antiviral activity of GML

6.1. Replicon model

Cells of Huh7.5.1 were electroporated (5 pulses, 99µsec, 820V) with a HCV replicon (genotype 2a isolate JFH1) deleted of E1/E2 envelope proteins and encoding luciferase (luc-JFH1△E1E2). Cells were co-cultured with GML as ratio of effector: target (E: T) ranging from 2:1 to 0,125:1, either separately ("Transwells"= lack of contact between effector and target) or jointly in the same well (= presence of contact between the effector and target). Cells of Huh7.5.1 were put only in the presence of IFN-γ (100ng/ml) as a positive control inhibition of viral replication. Three days later, the viable GML and dead Huh7.5.1 cells, that are non-adherent, were removed by washing and viable adherent Huh7.5.1 cells were lysed. Adding the substrate of luciferin was used to measure the luciferase activity, which reflects viral replication in the cells of Huh7.5.1. To identify which soluble factor is involved in the anti-viral activity of GML, blocking anti-IFN-α (Abcam, Paris, France), anti-IFN-β (Abcam),

anti-IFN-γ (eBioscience) or an isotype antibody (eBioscience) were added to a final concentration of 5μg/ml during the co-incubation of GML and target cells co-cultured at an E: T ratio of 0.5:1 or in transwell cultures at an E: T ratio of 2:1. Also, the anti-viral activity of CD56-fractions (T cells) and CD56 + (NK and NK-like T cells) was tested at different E: C after performing an immunomagnetic sorting of GML as described above. Finally, because of the context of immunosuppression in which we plan to use GML, their resistance to calcineurin inhibitors was tested. Thus, the GML and Huh7.5.1 cells were co-cultured at an E: T ratio of 0.5:1 in the presence of varying concentrations of cyclosporine A (CsA) and FK506 (Tacrolimus).

6.2. HCVcc model

The cell culture system of HCV (HCVcc) for the production of infectious virus particles was also used. Huh7.5.1 cells were infected with recombinant HCVcc Jc-1 expressing luciferase, and GML were added at E: C of 0.5:1 to 2:1, at different days post-infection. Three days after the addition of GML the Huh7.5.1 cells were lysed and assayed for luciferase activity. Huh7.5.1 cells viability was assessed by labeling with crystal violet as described above. The anti-viral activity of GML activated by CD3/CD28, CD56- / CD56 + fractions and the resistance of GML to calcineurin inhibitors FK506 and CsA were also tested in this system at E: C of 0.5:1 or 2:1.

RESULTS

1.Evaluation of three modes of cell injection to establish orthotopic and xenogeneic HCC mouse models

To optimize the orthotopic tumor (localized in the liver) models, three ways of Huh7-Luc cells injection were tested: intrasplenic (IS), intraportal (IPT) and intrahepatic (IH). We tried to compare these modes regarding the peroperative as well as postoperative mortality, the proportion of tumors effectively established in the liver and the correlation between BLI and tumor growth, evaluated by MRI or CT-scan. These results may allow us to choose the most suitable model in order to perform preclinical research aimed at evaluating innovative therapies, based on the use of nanovectors or adoptive cell therapies.

1.1. Per-operative and post-operative mortality

We defined the per-operative death as a death occurring the same day as the surgical procedure (i.e. during the few hours of the follow-up period of the same day) and the post-operative death as all death events recorded at D1 and D2 after the procedure (including the events that occurred during the first night after the surgical procedure). The study was performed on NMRI-nu and SCID-bg mice: a majority of NMRI-nu mice were used for the evaluation IH injection while a majority of SCID-bg mice were used for the evaluation of IS and IPT injections (Figure 25). The data obtained with NMRI-nu and SCID-bg mice have been merged in the results presented below but the detailed results are shown in the table in annex 1.

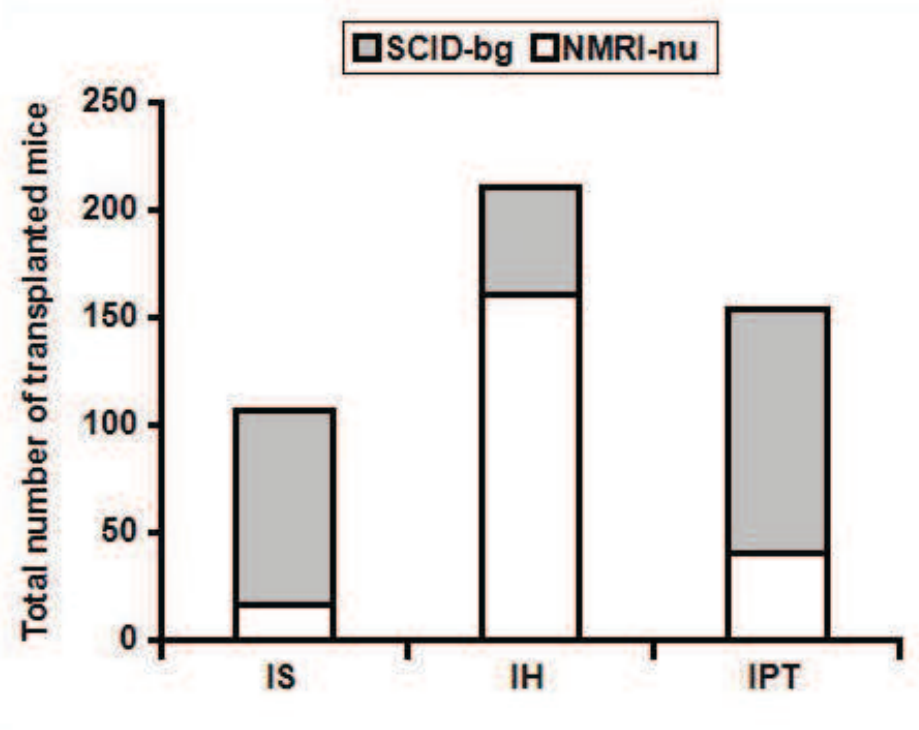


Figure 25. Total number mice of the two groups used in the experiments.

IS: intrasplenic injection group, **IH**: intrahepatic injection group, **IPT**: intraportal vein injection group.

Comparing the per-operative mortality of the three groups, the per-operative mortality is higher in the IPT group than in the IS and IH groups (16%, 6% and 3% respectively, $p<0.05$ for both comparisons; chi-square test) (Figure 26), probably because the IPT injection is technically more difficult and since the duration of anesthesia is longer than for the two other groups. Importantly, a lot of mice death occurred postoperatively in the IPT group, compared with the two other groups: the postoperative mortality was significantly higher in the IPT group than in the IS and IH groups (25%, 3% and 2% respectively, $p<0.001$; chi-square test), while there is no difference between the IS group and the IH group (3% and 2%, $p>0.05$; chi-square test) (Figure 26).

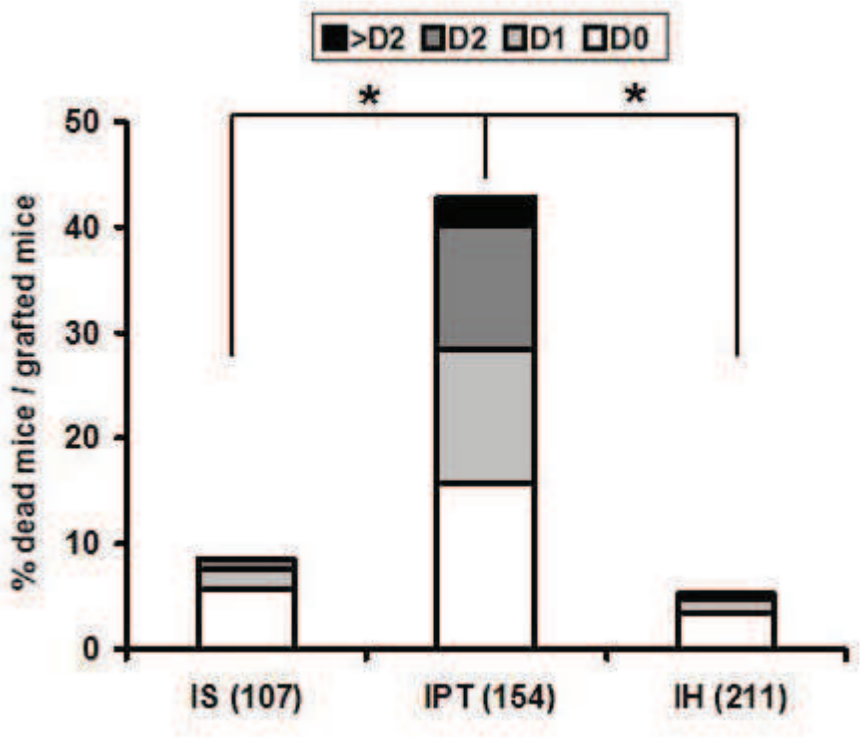


Figure 26. Comparison of the post-operative mortality according to the mode of injection of Huh-7-Luc cells

IS=intrasplenic injection, **IH**=intrahepatic injection, **IPT**=intraportal injection, The percentage of mice that died at D0, D1, D2 or later was calculated as (number of mice dead at Dx/number of living mice at Dx) x100. The number in brackets in the X axis indicates the total number of surgical procedures at D0. D0 is considered as per-operative period, D1,D2, or >D2 are considered as post-operative period. *: $p<0.05$ for the entire period (D0 to D>2) (chi-square test)

1.2. Evaluation of tumor location in three modes of cell injection

For all the experimental mice that survived the surgical procedure, the evaluation of tumor growth was investigated *in vivo* by BLI, performed at the indicated time point: D1, D7, D14, and D21. Thus, 92%, 57% and 95% of transplanted mice (n=98/107, 88/154 and 200/211 mice) in the IS, IPT and IH groups, respectively, were evaluated for tumor engraftment (Figure 27).

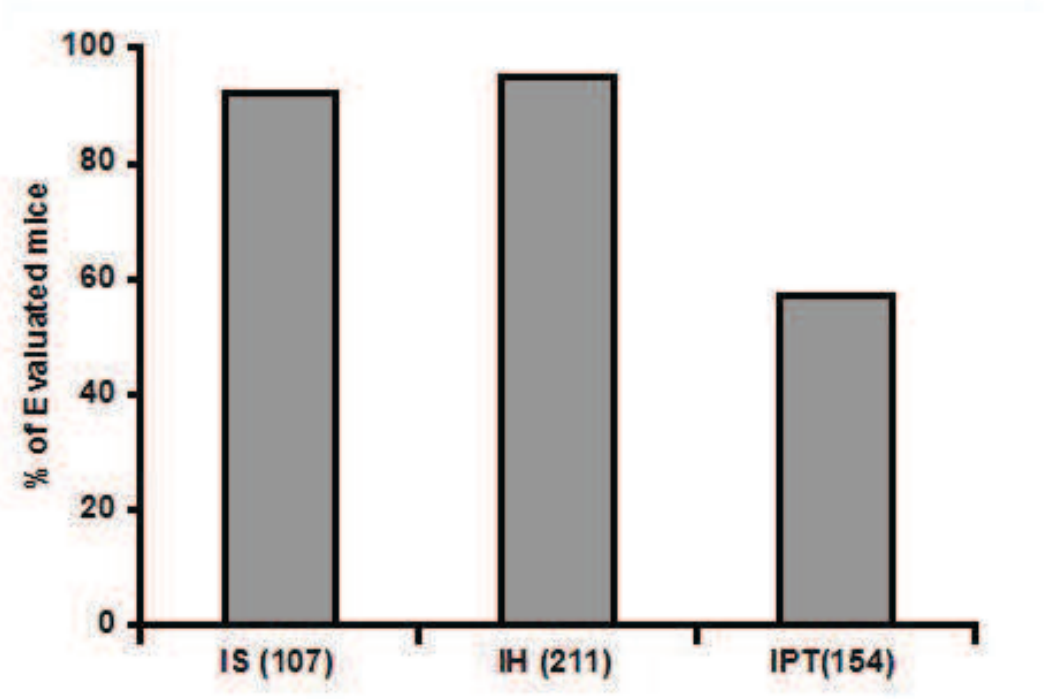


Figure 27. Percentage of mice evaluated for tumor growth among the total number of transplanted mice. IS=intrasplenic injection, IPT=intraportal vein injection, IH=intrahepatic injection. The number in brackets indicates the total number of transplanted mice .

Importantly, we observed that the proportion of mice with tumor engraftment (whatever the location of the tumor, i.e., in and/or outside the liver) among the evaluated mice was the highest in the IH group (86%), followed by the IS (71%) and the IPT (69%) groups (Figure 28).

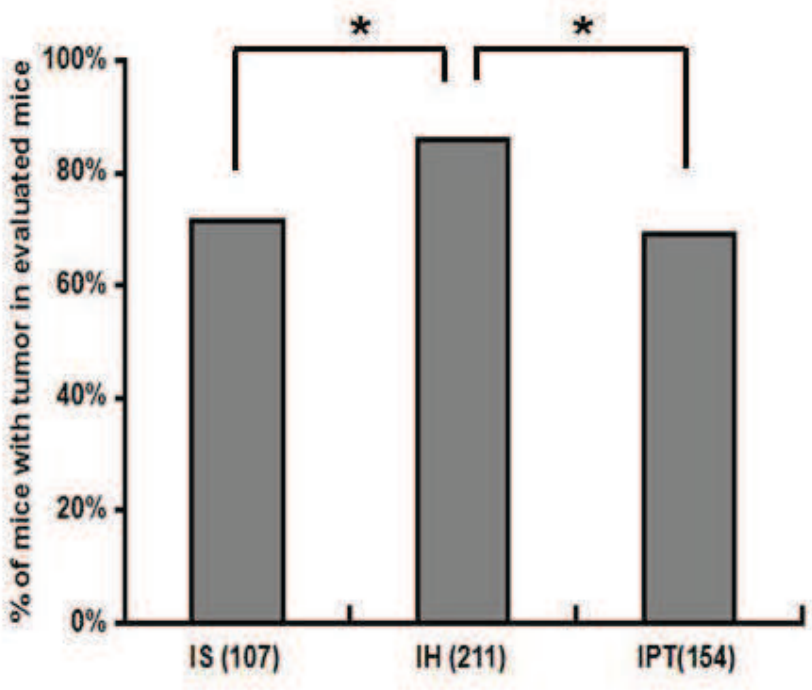


Figure 28. Rate of tumor engraftment in the three groups.

The data are expressed as the percent of mice with tumor among the evaluated mice (the number of evaluated mice is indicated in brackets). *: $p<0.05$ for IH vs IS and for IH vs IPT (chi-square test)

However, this analysis was a rough estimate. Indeed, when regarding the mice with tumors located exclusively in the liver, we found that, among the evaluated mice, the frequency of mice with orthotopic tumor location was significantly higher ($p<0.05$; chi-square test for IH vs IS and for IH vs IPT groups) in the IH group than in the IS and IPT groups (55% vs 35% vs 34% in the IH, IS and IPT groups, respectively, Figure 29A) and there is no significant difference between the IS and IPT groups. Even if we take into account the incidence of death (i.e., the percent of mice with orthotopic tumor among all transplanted mice, Figure 29B), the frequency of mice with tumor located exclusively in the liver remains significantly higher ($p<0.05$; chi-square test for IH vs IS and for IH vs IPT groups) in the IH group than in the IS and IPT groups (52% vs 32% vs 19% in the IH, IS and IPT groups, respectively, Figure 29B), but the IS injection provides also significantly ($p<0.05$; chi-square test

for IS vs IPT groups) higher orthotopic tumor location than the IPT injection. Altogether, these data indicate that the IH injection could provide a larger quantity of mice with HCC located in the liver than the two other procedures, associated with a lower mortality. Therefore, in the future experiments, we will choose the intrahepatic injection as the basic procedure (Figure 30).

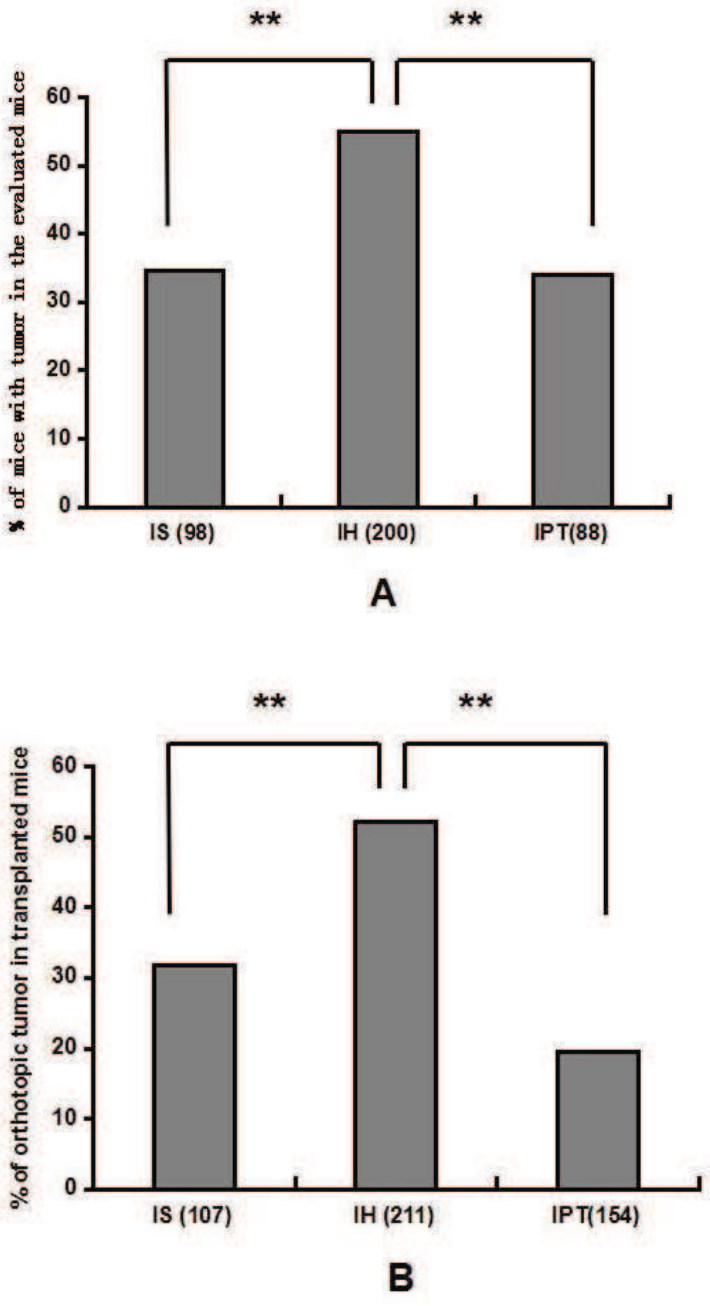


Figure 29, Percentage of mice with tumors located in the liver

A: Percentage of mice with tumor among evaluated mice (i.e. living mice).

B: Percentage of mice with tumor among total transplanted mice (including living & dead

mice)

*: $p<0.05$ (chi-square test)

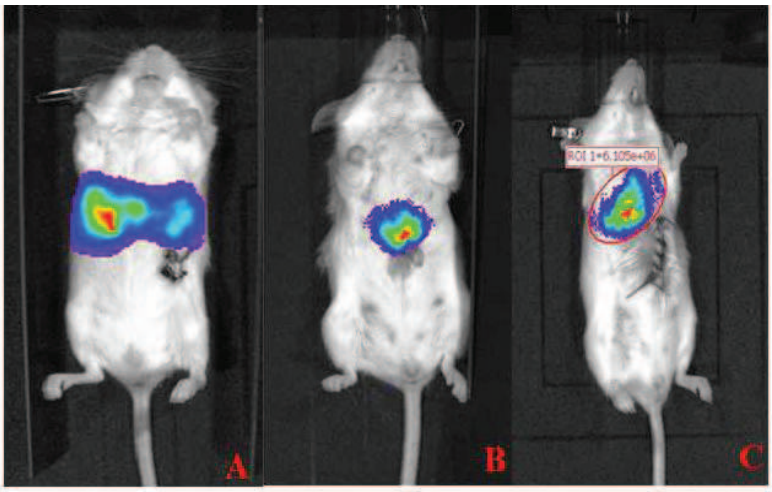


Figure 30, Representative mice transplanted according to the three modes of injection, showing orthotopic HCC tumors evaluated by BLI

A: intrasplenic injection; B:intrahepatic injection; C:intraportal vein injection

1.3. Correlation between the BLI with MRI

The BLI technique provides only a one-dimensional picture, which could be enough for some experiments such as tumor evaluation in subcutaneous models. However, if we want to improve the accuracy in orthotopic HCC models, particularly in order to investigate the efficiency of innovate treatments for HCC, a more precise evaluation of the tumor location is required. Based on our experience (Figure 31), the location of some tumors, considered as orthotopic HCC according to the BLI analysis, turned out to be incorrect when we performed an autopsy. All of three modes of injection, there is a possibility that some tumors were outside the liver rather than in the liver and were located, for example, under the incision (Figure 31 B), around the portal vein (Figure 31 B), or rarely, around the stomach (Figure 31 B). Thus, we tried to establish a link between BLI with other three-dimensional technic - MRI, to precisely locate the orthotopic HCC tumor.

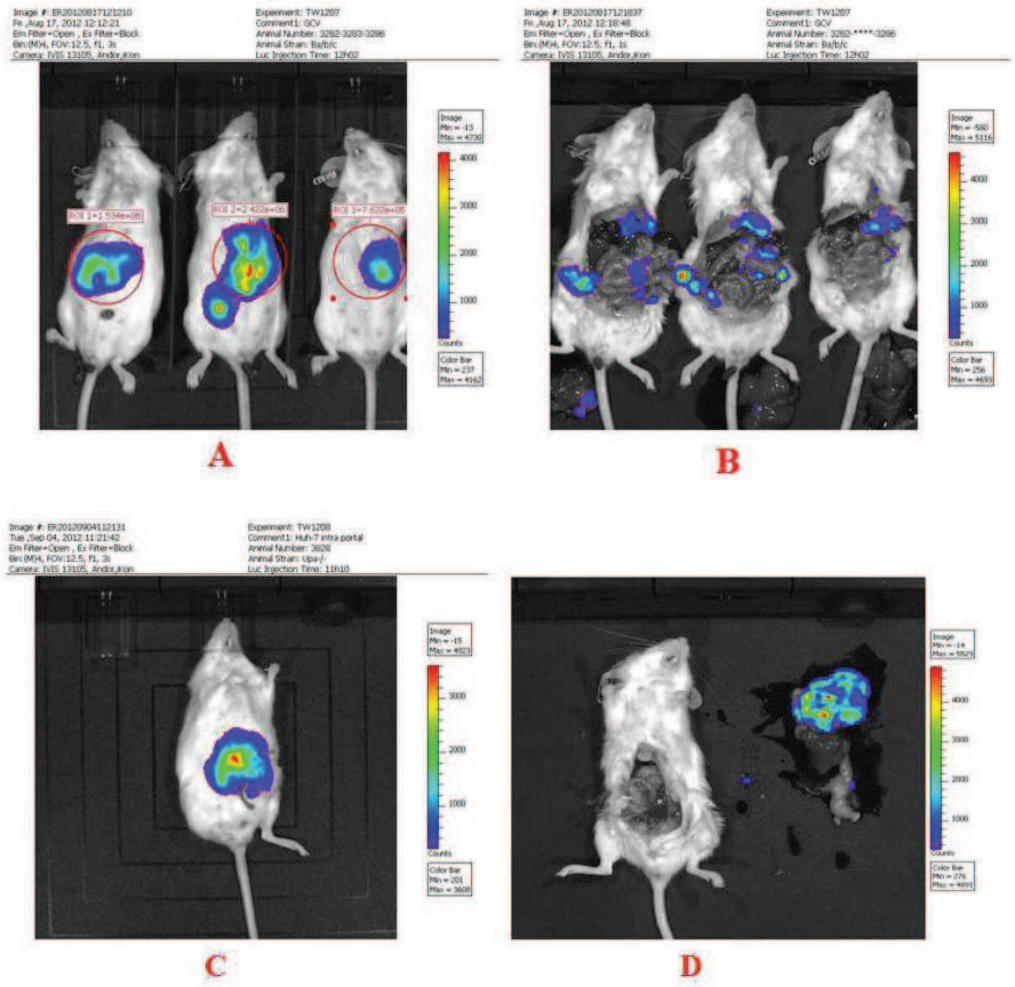


Figure 31. Tumor location

A: BLI showed the tumor was located in the liver; **B**: the same group mice analyzed by autopsy, most tumors were located in the incision. **C D** showed the same mouse, the tumor is completely in the liver.

1.4. Kinetic of tumor growth

First, we tried to characterize the kinetic of tumor growth, 14 mice were injected intrahepatically with Huh-7-Luc cells. Tumor engraftment was obtained in 13/14 mice, with intra-hepatic tumor in all 13 mice and up to five nodules in the liver (Figure. 32). Additional extra-hepatic tumors were observed in 5/13 mice. The tumor growth was monitored in parallel by BLI and MRI from d4 to d20 post-injection, as shown in Figure. 33A, B for one representative mouse. *In vivo* BLI was more sensitive than MRI, since tumor cells were detectable more frequently by BLI than by MRI (Table

11) but, in tumor-detectable mice, the kinetics of tumor growth evaluated by MRI and BLI were superimposable (Figure. 33). Furthermore, a positive correlation was observed between the luciferase activity, quantified by BLI and tumor size, quantified by MRI (Figure. 34). This allowed to establish an approximate correspondence between luciferase activity and tumor size (Table 12) and prompted us to propose to our ethical committee the value of 10^8 p/s/cm²/sr as the endpoint value for animal experimentation in this model.

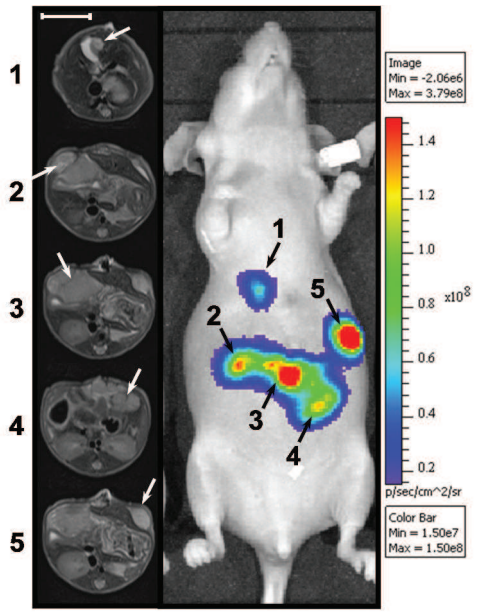


Figure 32. MRI and BLI of mouse transplanted through IH:
Multiple tumor centers showed in MRI according to BLI

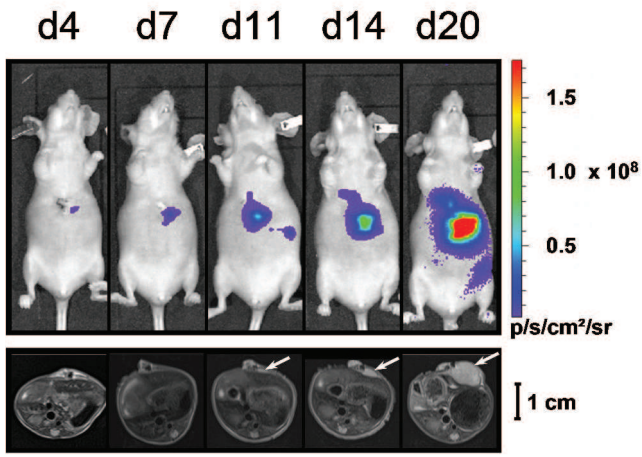


Figure 33. Correlation of MRI and BLI in different time
A: BLI showed tumor growth from d4 to d20 (up), while MRI showed in the same time the location of tumor (down); B: the expression of luciferase of BLI according to the tumor size in MRI

Table 11: Number of mice with tumors detectable by BLI or MRI

	D4	D7	D11	D14	D20
BLI	11/14 (78.6%)	12 (85.7%)	12 (85.7%)	13 (92.9%)	13 (92.9%)
MRI	1/14 (7.1%)	4/14 (28.6%)	7 (50.0%)	9 (64.3%)	11 (78.6%)

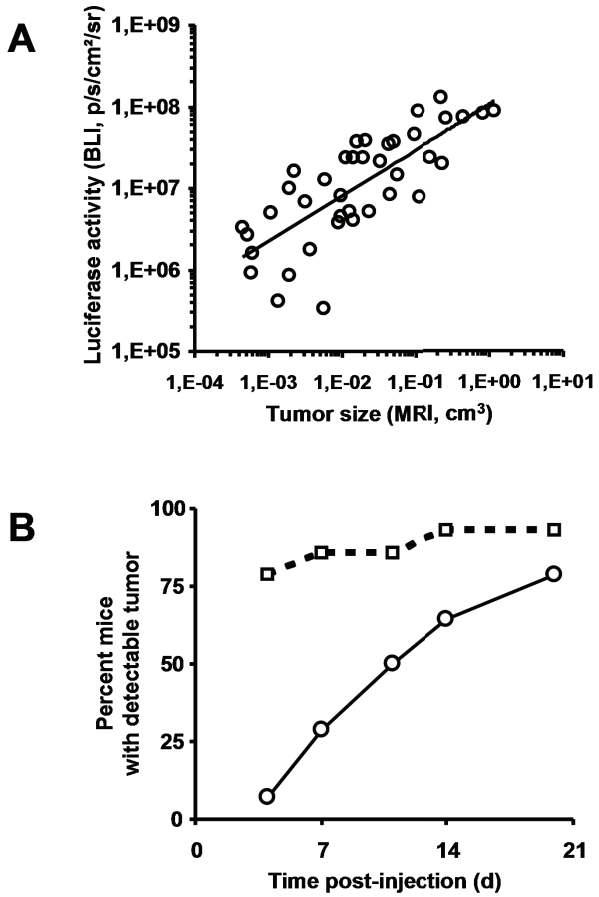


Figure 34. Correlation between BLI and MRI

A: correlation of luciferase expression detected by BLI and tumor size measured by MRI; **B** tumor detectable by MRI and BLI at different time indicated.

Table 12: Correspondence between luciferase activity of Huh-7-Luc tumors, evaluated by BLI, and tumor size evaluated by MRI

Bioluminescence activity (BLI; p/s/cm²/sr)	10^5	10^6	10^7	10^8	10^9
Tumor size (MRI; mm³)	< 1	1	15	160	1860

The study presented in this chapter is the object of a manuscript in preparation, intended for ‘’Disease Models and Mechanisms”, for which I am first author.

2.Development of an orthotopic HCC model in a large animal by autologous transplantation of *ex vivo*-transformed primary porcine hepatocytes

2.1.Rationale

In humans, the enforced expression of viral proteins that disrupt the tumor suppressors p53 and Rb and activate the proto-oncoprotein c-Myc, in conjunction with the mammalian oncogenic protein Ras and the hTERT telomerase catalytic subunit could convert the normal somatic cells to a tumorigenic state (Hahn, Counter et al. 1999; O'Hayer and Counter 2006)). They all reflect the alterations commonly found in human cancers (Bos 1989; Weinberg 1991; Nesbit, Tersak et al. 1999; Shay and Wright 2002). Importantly, it has been proven that expression of mammalian proteins can achieve the same features (Kendall, Adam et al. 2006): inactivation of p53 and Rb pathways by expression of a dominant-negative p53 protein ($p53^{DD}$) and an activated cyclin-dependent kinase (CDK)/cyclin complex (cyclin D1/$CDK4^{R24C}$) in conjunction with activation of c-Myc, Ras and hTERT pathways via expression of oncogenic forms of c-Myc (c-Myc^{T58A}) and H-Ras (H-Ras^{G12V}) and hTERT, could drive different human cells into a tumorigenic state. Recently, a porcine fibroblast tumorigenic conversion has been reported by using the above oncogenes combination (Adam, Rund et al. 2007), suggesting that normal porcine cells could be genetically engineered to recapitulate changes commonly found in human cancers, and when returned back to the host (isogenic) porcine, could form tumors. Therefore, it may be possible to similarly drive normal porcine hepatocytes to tumorigenic state by the enforced expression of the same mammalian proteins.

Thus, we designed to establish a porcine orthotopic HCC model via these oncogenes through the following steps: (1) Choice of a better candidate recipient plasmid which has capacity for the cloning of different oncogenes combining with a reporter gene, such as luciferase; (2) Cloning of the different oncogenes in the recipient plasmid, realized by a contract research organization (R&D Biotech, Besançon, France); (3) Production of lentiviral vectors in a closed culture system, in order to protect the manipulators without affecting the efficacy of lentiviral vectors production; (4) Lentiviral-mediated transduction of PPH in a closed culture system; (5) *In vitro* evaluation of oncogenes expression, identifying the expression of oncogenes in PPH transduced in open or closed culture system; (6) *In vivo* evaluation of transduced PPH, in order to observe the transformation process *in vivo* in a subcutaneous model as well as in an orthotopic mouse model and finally (7) perform these experiments back to porcine models (autologous transplantation of *ex vivo*-transformed PPH).

2.2. Choice of a recipient plasmid for the cloning of oncogenes

The first step of the project was to choose the best recipient plasmid that could ensure the efficient expression of oncogenes in a recipient plasmid encoding luciferase gene, characterized by high rate of transduction as well as of expression in targeted cells. Therefore, we compared three candidates of recipient plasmids: pHIV-Luc, pLenti CMV V5-LUC Blast and pHIV-Luc Zs Green, all expressing the luciferase. These three candidate plasmids were combined with VSV-g-envelop and HIV-gag-pol plasmids to produce three candidate lentiviral vectors, used to transduce Huh7.5.1 cells, PHH and PPH. Our results show that VSV-G pseudotyped lentiviral particles (VSV-Gpp) produced using the pLenti CMV V5-LUC Blast candidate plasmid reproducibly reached the highest transduction efficiency (evaluated by the luciferase activity

in transduced cells) in Huh7.5.1 cells, PHH and PPH (Figure. 35). Importantly, we found that the PPH showed also a similar, or even higher, efficiency to be transduced than the two other cell types (Figure 35). Therefore, the pLenti CMV V5-LUC Blast plasmid was chosen as a recipient plasmid to express the candidate oncogenes.

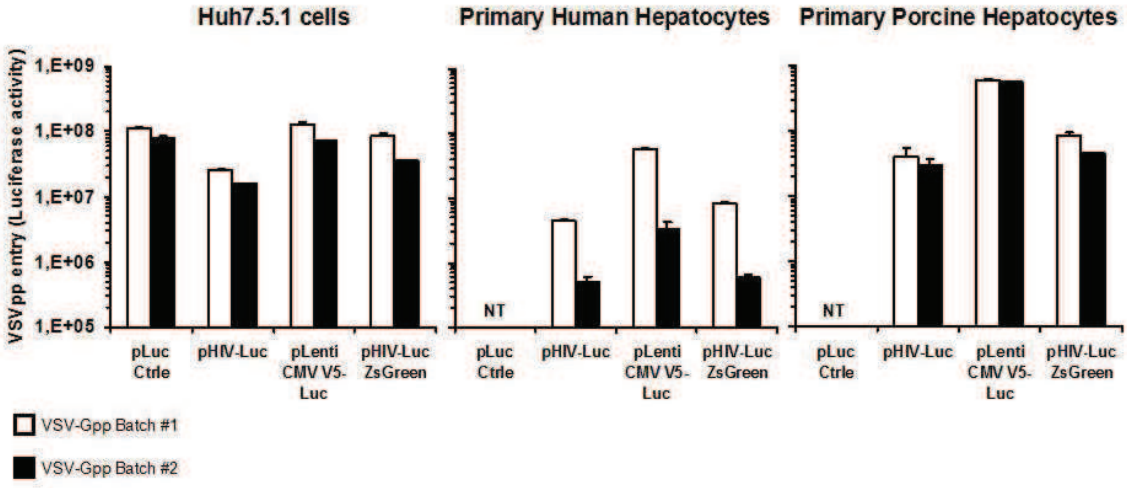


Figure 35. Choice of the best candidate recipient plasmid for the production of VSV-Gpp lentiviral vectors

VSV-Gpp lentiviral vectors were produced using a VSV-G-Env plasmid, a HIV-gag-pol plasmid and either the pHIV-luc or the pLenti CMV V5-Luc or the pHIV-Luc Zs Green plasmid as candidate recipient plasmid. The efficacy of VSV-pp entry is indicated by the expression of luciferase in the different target cells (Huh7.5.1, PHH and PPH). A control plasmid routinely used in the laboratory was used as a positive control (pLuc Ctrle) for the transduction of Huh7.5.1 cells. NT: not tested.

2.3. Production of lentiviral vectors in a closed culture system

After cloning of the oncogenes in the recipient plasmid (performed by a Contract research organization), three batches of lentiviral vectors were produced: the lentiviral vector 28 encoding $p53^{DD}$ and hTERT, the lentiviral vector 29 encoding the cyclin D1 and $CDK4^{R12C}$ and the lentiviral vector 30 encoding $HRas^{G12V}$ and c-myc. Huh 7.5.1

cells were transduced separately with the 28, 29 and 30 lentiviral vectors while non transduced Huh 7.5.1 cells and cells transduced with the control lentiviral vector encoding only luciferase were used as negative and positive control groups, respectively. The transduction was performed using a classic "open" culture system (i.e., transduction in Petri dishes) but also using a closed culture system in order to improve the safety of handling. We found that there is no difference between the open and closed culture systems in terms of transduction efficiency, both of them showed high efficiency of transduction (Figure 36), indicating that the production of lentiviral vectors in the closed culture system is as efficient as in a classic open system.

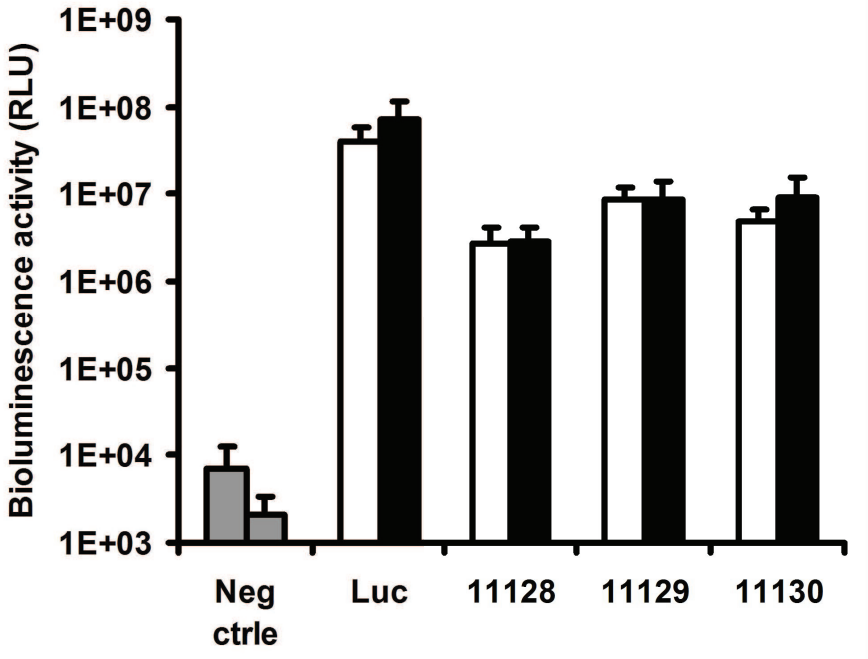


Figure 36 Comparison of transduction efficiency by lentiviral vectors produced in open and closed culture systems.

Three lentiviral vectors encoding the luciferase and two oncogenes each (11128,11129 and 11130) and a control vector encoding the luciferase only were produced in an open (white bars) and closed (black bars) culture system, then used to transduce Huh7.5.1 cells. The luciferase activity was evaluated three days later. The background of luciferase activity in non transduced control cells is shown in grey (Neg ctrle).

2.4.Lentiviral-mediated transduction of PPH in a closed cluture system

Based on the experiment above, we decided to compare the efficiency of the transduction of PPH in closed culture system with conventional open system. We used the control lentiviral vector encoding only the luciferase gene and transduced PPH either in open system (two independent experiments) or in a close culture system, using the CliniCell device. Three days later, transduced PPH were harvested and the luciferase activity was evaluated. Our result (Figure 37) showed that, as compared with the open system, the closed system provided a similar bioluminescence activity, suggesting that, using closed system, we could obtain the same transduction efficiency as seen in the conventional procedure, but with a potential advantage of safety for the manipulators.

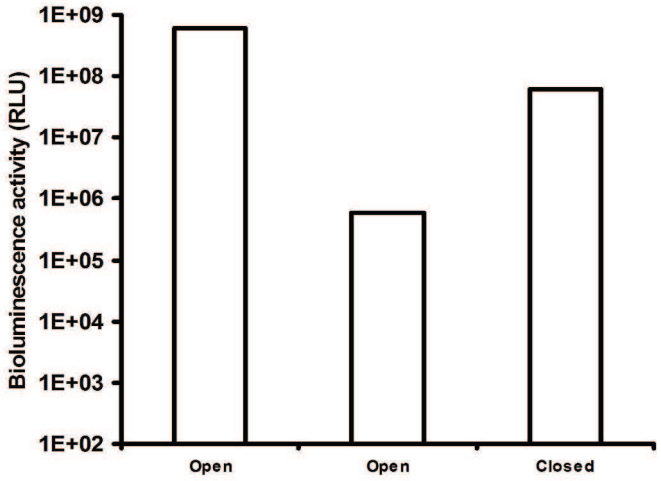


Figure 37. Comparison of the transduction efficiency of lentiviral vectors in open vs closed culture systems.

The efficiency of lentiviral-mediated transduction of PPH was evaluated by the level of luciferase activity. Each quantification was performed in triplicate.

2.5.*In vitro* evaluation of the expression of oncogenes

The expression of transgenic oncogenes was checked in two steps: first, we tested the expression of each oncogene in 293T cells at the end of the production of the lentiviral vectors, i.e., three days after 293T cell transfection with the VSV-G-Env and HIV-gag-pol plasmids plus one of three plasmids 11128, 11129 or 11130. As compared with 293T cells transfected with the 11128 or 11129 plasmids, $HRas^{G12V}$ was strongly over-expressed in 293T cells transfected with the 11130 plasmid while a slight expression of $c\text{-}myc^{T58A}$ was observed in the same cells (Figure 38). $p53^{DD}$ was over-expressed in 293T cells transfected with the 11128 plasmid, but also in cells transfected with the 11129 plasmid, as compared with cells transfected with the 11130 plasmid. Finally, $CDK4^{R24C}$ was also slightly over-expressed in 293T cells transfected with the 11129 plasmid. hTERT and Cyclin D1 were not detectable, either because of a lack of expression or a too weak expression or because of a technical problem related to the antibodies used for the detection.

In a second step, the different combinations of the three lentiviral vector groups were tested in PPH, as described above (Group A to H), in parallel with PPH transduced with control vector and non-transduced PPH. The different protein expressions were evaluated by western-blot (Figure 39). We found that $HRas^{G12V}$ (encoded by the 11130 vector) was strongly expressed in PPH transduced with the 11129+11130 and the 11128+11128+11130 combinations but not or barely in PPH transduced with the 11130 vector alone or the 11128+11129 combinations, suggesting that enforced CyclinD1 and/or CD4K expression may enhance c-myc expression. C-myc expression was barely detectable, with no clear differences among the different lanes.

The expression of p53 was also mostly evident in PPH transduced with 11128 vector or the combinations associating the 11128 and 11129 vectors, suggesting again that enforced CyclinD1 and/or CD4K expression may enhance p53 expression.

No logical and clear CDK4 expression was observed, as strong or weak signals could be observed either in PPH transduced with the CDK4-encoding 11129 vector or

in PPH non-transduced with this vector.

It may be possible that the expression of some transgenic human oncoproteins may be masked by an endogenous expression of their porcine homologous proteins, as the primary antibodies cross-react with porcine proteins. Overall, lentiviral-mediated transduction of PPH was feasible for all three vectors, as a luciferase activity was detectable *in vivo* in all groups A to H (see below Figure 36 in paragraph 2.6 "*In vivo* evaluation of transduced PPH''). Furthermore, our data suggest that transgenic oncogenes could be expressed in PPH. Especially, it seems that Ras, p53 and CDK4 were expressed at the highest level when sequential transduction was performed. However, transgenic oncoprotein expression cannot be guaranteed for all proteins and may be weak or absent for some of them.

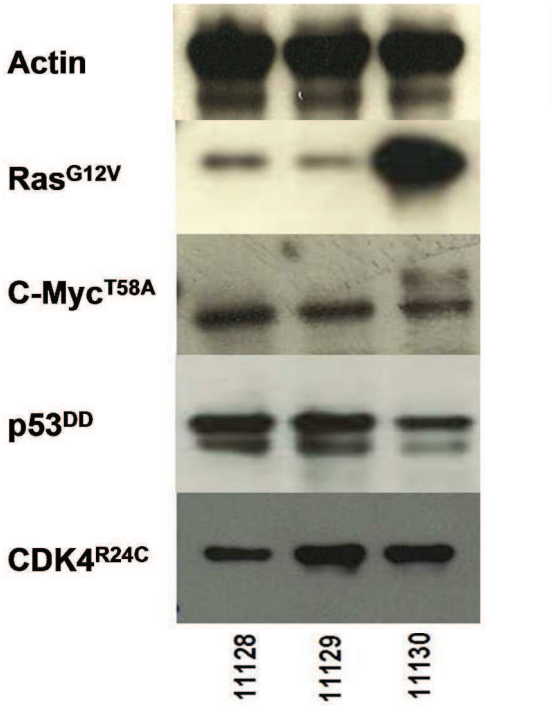


Figure 38 Western-blot of 293T cells transfected by one of the 3 plasmids 11128, 11129 or 11130.

The plasmids 11128, 11129 and 11130 encode hTERT + $p53^{DD}$, cyclinD1 + CDK^{4R24C} and c-myc^{T58A} + $hRas^{G21V}$, respectively.

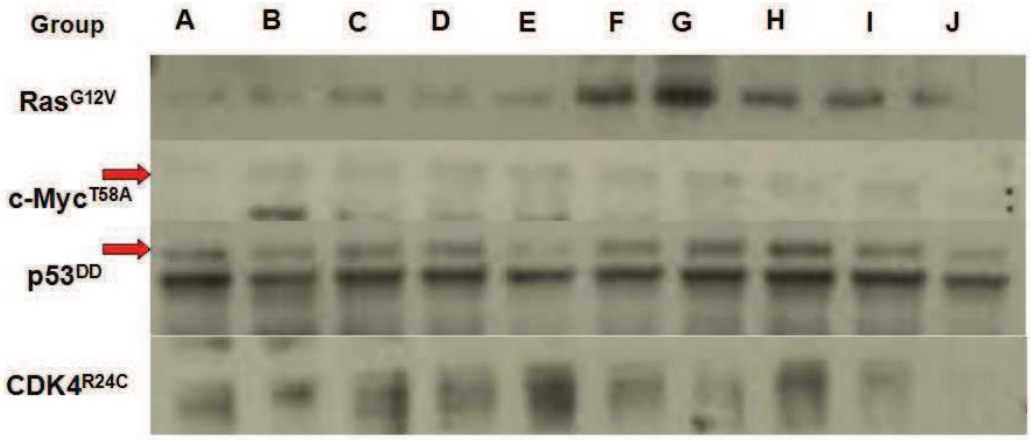


Fig 39. Western-blot tested the four protein expression in different groups PPH

PPH transfected were seeded on poly-HEMA-coated 6 well plates for 48 h. Whole cell lysates were analyzed by western blotting. GAPDH was used as a loading control. A: 11128; B: 11129; C: 11130; D, 11128+11129, E: 11128+11130; F: 11129+11130; G: 11128+11129+11130 (simultaneous transduction with all 3 vectors); H: 11128 d1+ 11129 d2+11130d3; H: VSV-g-luc; J: Non-transduced PPH. The plasmids 11128, 11129 and 11130 encode hTERT + $p53^{DD}$, cyclinD1 + CDK^{4R24C} and c-myc^{T58A} + $hRas^{G21V}$, respectively.

2.6.*In vivo* evaluation of transduced PPH

Based on the data above, we evaluated *in vivo* the growth of PPH transduced with a combination of the three oncogenes-encoding lentiviral vectors.

The eight different combinations of PPH transduction with the three lentiviral vectors were tested in NMRI-nude mice by subcutaneous injection of 1×10^6 cells in one point of flank. All the mice groups showed a positive expression of luciferase. However, we did not find differences among them because of all the increase of bioluminescence were the same baseline, suggesting that the PPH cells were not transformed to an oncogenic state (Figure 40).

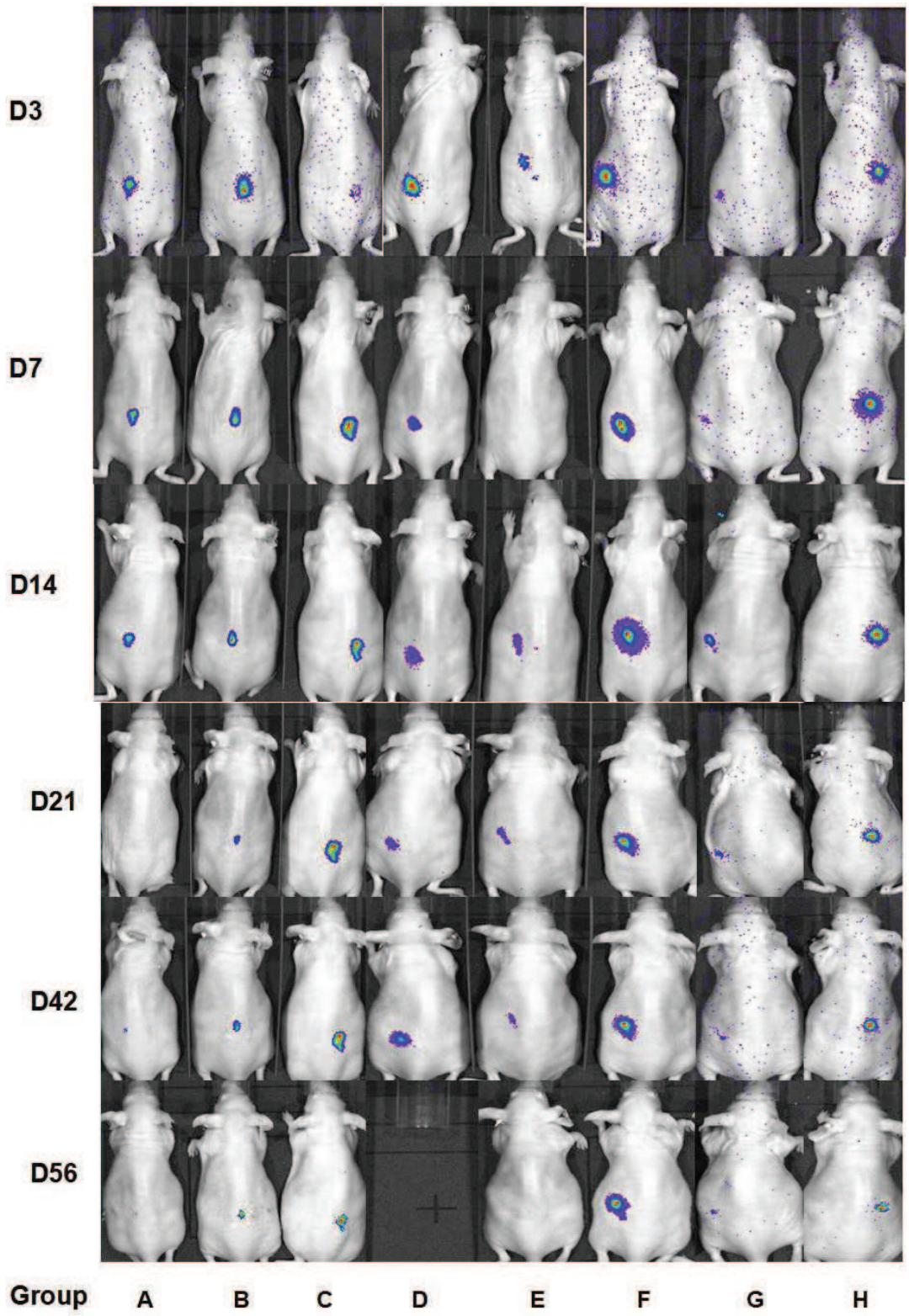


Figure 40 Evaluation of the kinetic of PPH transfected in ectopic models

Subcutaneously injection of PPH transduced by oncogenes plasmid individual or combination in NMRI-nude mice, BLI was performed at time point indicated, groups designed as: A 11128, B 11129, C 11130, D 11128+11129, E 11128+11130, F 11129+11130, G 11128+11129+11130, H 11128 d1+11129d2+11130d3. Mouse of group D at D56 was dead.

Therefore, we used a more relevant model based on immunodeficient and hepatodeficient Alb-uPA/SCID-bg mice. An orthotopic model in uPA-SCID-bg mice was established by intrasplenic injection 1×10^6 cells/mouse, using PPH transduced simultaneously with three lentiviral vectors (n=3 mice), or PPH transduced with the control vector expressing only the luciferase (n=3 mice). BLI was performed at D3 (used as the reference value) and at the following indicated time points. Unfortunately, the luciferase activity was not quantified in the cell suspensions before transplantation but the BLI was detectable in all six mice at D1. However, only two mice in the control group remained positive for BLI at later time points. During the first three weeks after cell injection, the absolute values of luciferase activity, expressed as p/s/cm²/sr, increased continuously in two mice of the control group and in the three mice of the oncogene group, indicating that both type of cells repopulated the liver, as expected (Figure 41A). However, during this period, the luciferase activity was higher in the two mice of the control group than in the three ‘’onco-mice’’ when expressed as p/s/cm²/sr (Figure 41A) but was similar in both groups when expressed as relative fold increase, i.e., as the ratio of BLI after transplantation to the BLI at D3 post-transplantation, used as a reference value (Figure 41B). This may be due to a slightly higher number of transplanted cells in the control group than in the oncogene group and/or to higher transduction efficiency in the former than in the later group. Of interest, the absolute (Figure 41A) and relative (Figure 41B) values of luciferase activity decreased in the two control mice after three weeks post-transplantation while it continued to increase in the three onco-mice, suggesting an oncogenic transformation of PPH in this group. Therefore, the mice were sacrificed and the livers were checked for *ex vivo* BLI (Figure 42), demonstrating the presence of transduced PHH in the liver. The livers were cut in two pieces in their bioluminescent area, one part was subcutaneously retransplanted in NMRI-nude mice (Figure 42) and one part was used for histological analysis (Figure 43). Similarly to what can be observed after PHH transplantation, PPH could be identified after hematoxillin-eosin staining as hypo-eosinophilic nodules. Although histological analysis showed that the regeneration nodules in the liver on onco-mice (n=2 mice) exhibited a more

"destructured" architecture than regeneration nodules observed in the livers of the two control mice, suggestive of a preneoplastic state. However, our pathologist was unable to certify for an oncogenic transformation and could not ensure that a "true" tumor was established. Furthermore, NMRI-nude mice retransplanted with liver pieces (n=2 for each group) did not develop any subcutaneous tumor, even in the onco-mice group, despite a detectable subcutaneous luciferase activity (Figure 42).

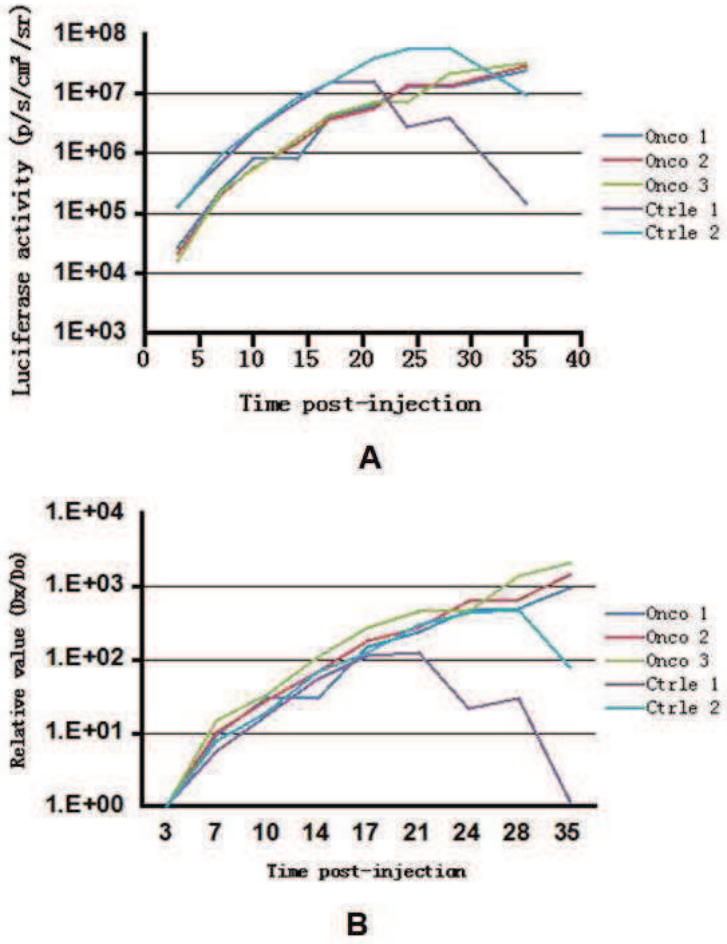


Figure 41. Monitoring of BLI in immunodeificient and hepatodeficient alb-uPA/SCID-bg mice.

(A) Data of BLI are expressed as absolute values (p/s/cm²/sr) for three mice transplanted with PPH transduced with the combination of three lentiviral vectors encoding the luciferase and two oncogenes each (Onco 1 to Onco 3) and two mice transplanted with a control lentiviral vector encoding the luciferase only (Ctrle 1 and Ctrle 2). (**B**) Data of BLI are expressed as relative value, i.e., as the ratio of absolute value at the indicated time to the absolute value at D3.

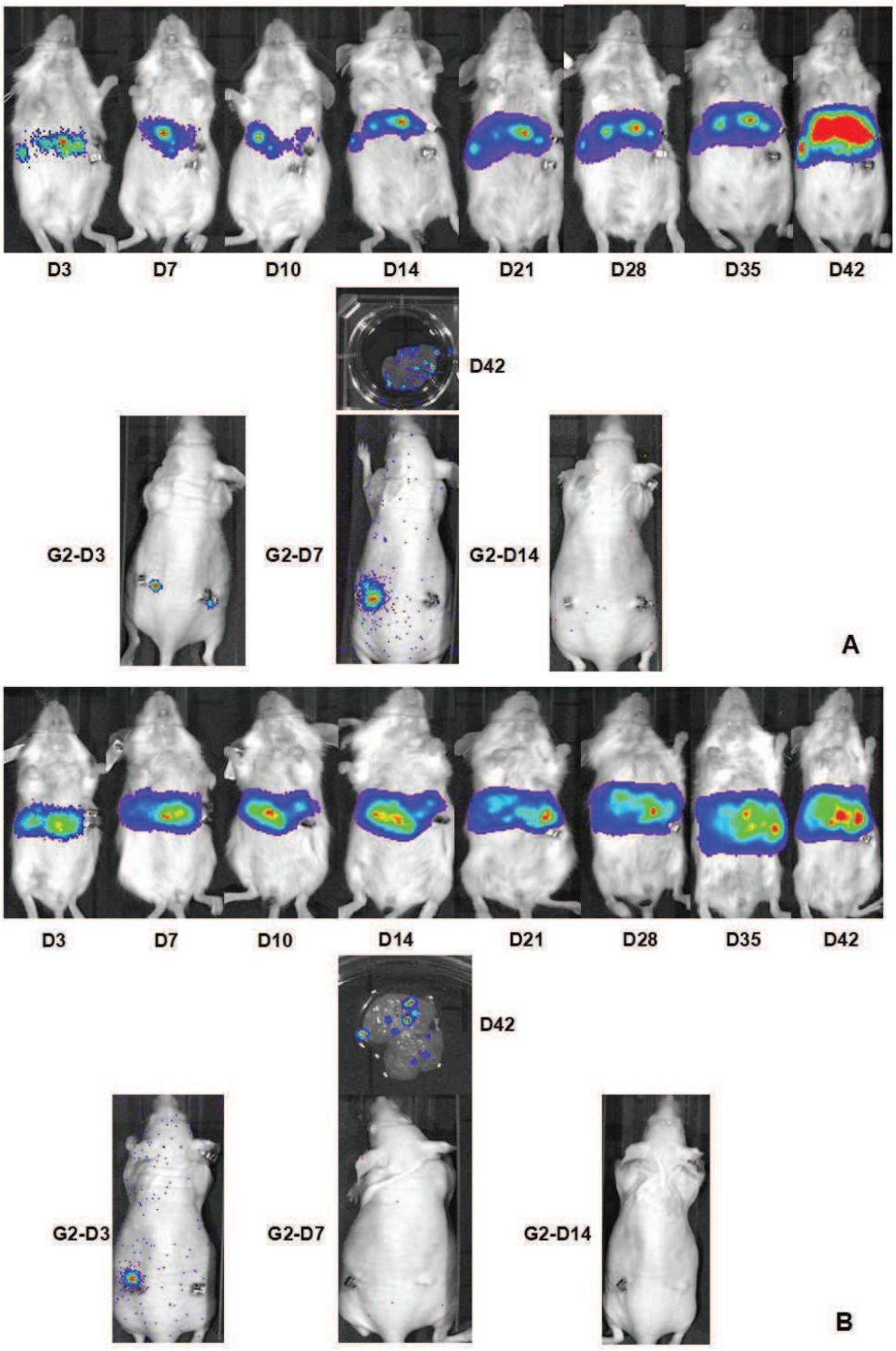


Figure 42: Evaluation of the BLI of PPH transduced with a combination of six oncogenes (A) or with a control vector (B).

For each panel A and B: Up, BLI at different time points of one Alb-uPA/SCID-bg mouse transplanted with transduced PPH representative of two mice. Middle, BLI of explanted liver species collected at d42. Bottom, BLI at different time points of one representative NMRI-nude subcutaneously retransplanted with liver tissue of the alb-uPA/SCID-bg mouse.

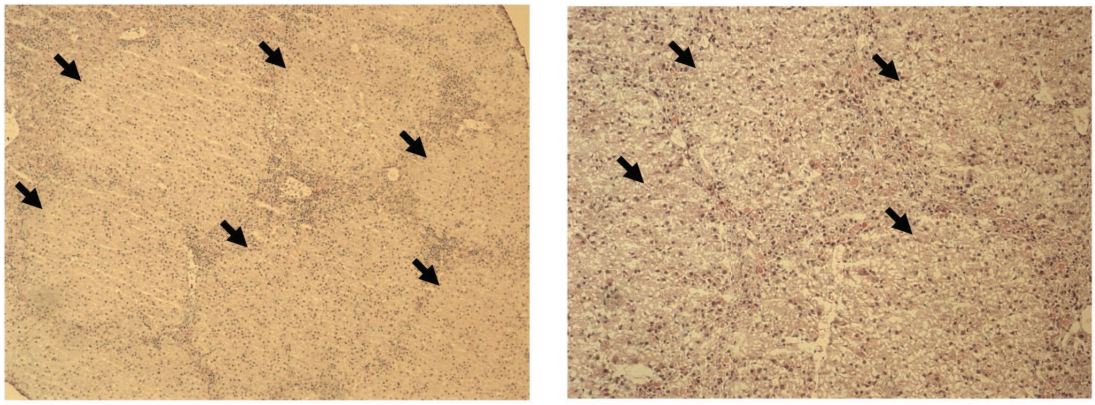


Figure 43: Hematoxillin-eosin staining of explanted livers after transplantation of PPH transduced with control vector (left) and oncogene-encoding vectors (right).

The light areas correspond to PPH-repopulated areas, due to hypo-eosinophilic regeneration nodules (arrows). The architecture is less homogeneous in the onco-mice than in control mice. Magnification x5

The study presented in this chapter is the object of a manuscript in preparation, intended for ‘’Human Gene Therapy Methods”, for which I am first author.

3. Allogenic adoptive immunotherapy for hepatocellular carcinoma using gene-modified lymphocytes expressing suicide genes.

3.1. *In vitro* characterization of gene-modified lymphocytes

Before the initiation of our study in the following paragraphs, the phenotype of GML during the production process and at the end of the production was characterized by Dr Leboeuf. These data are presented in the current paragraph in order to better understand our results.

The HSV-tk or iCasp9 suicide genes were co-expressed together with, respectively, the genes encoding the membrane markers CD34 or CD19, normally not expressed by mature T and NK cells. Therefore, the percentage of transduced cells before and after selection by immunomagnetic sorting may be determined by marking CD34 or CD19 (Figure 44).

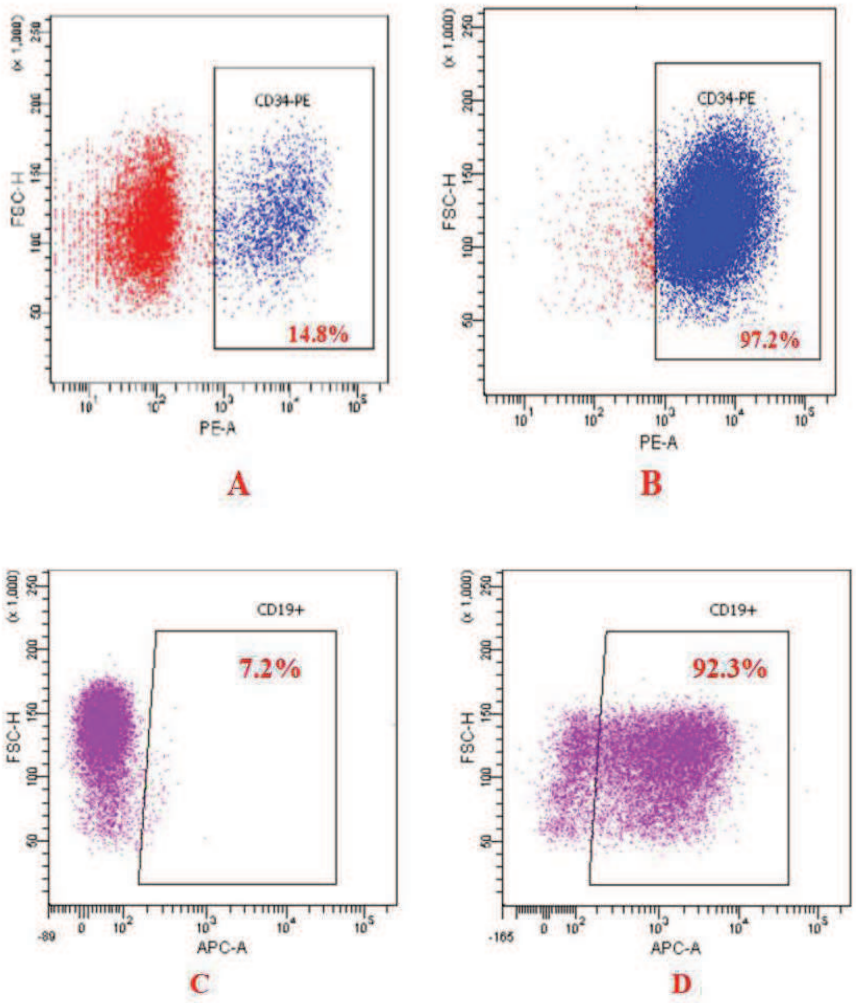


Figure 44. Two or three days after transduction, CD34 and CD19 staining

Test was performed before (A) and after (B) CD34 immunomagnetic sorting of GML transduced with the MP71-T34FT vector encoding HSV-tk and CD34; CD19 staining was performed before (C) and after (D) CD19 immunomagnetic sorting of GML transduced with the SFG.iCasp9.2A.ΔCD19 vector encoding iCasp9 and CD19.

We obtained an average transduction efficiency and a purity after immunomagnetic selection (mean ± standard error (SE)), respectively, of 12.1 ± 0.8% and 92.4%± 1.3% (n = 11) for HSV-TK/CD34 GML and 5.1± 0.8% and 95.2± 1.1% (n =6) for iCasp9/CD19 GML. To ensure that the T, NK and NKT cells are transduced equivalently, a CD34 staining was conducted on MP71-T34FT-transduced cells and percentages of T, NK and NKT cells were determined either in the total lymphocyte population before sorting (Figure 45, black bars) or in CD34 + GML after sorting (Figure 45, purple bars). It is observed that the percentages of T, NKT and NK cells are similar, indicating that all three subsets are effectively and similarly transduced.

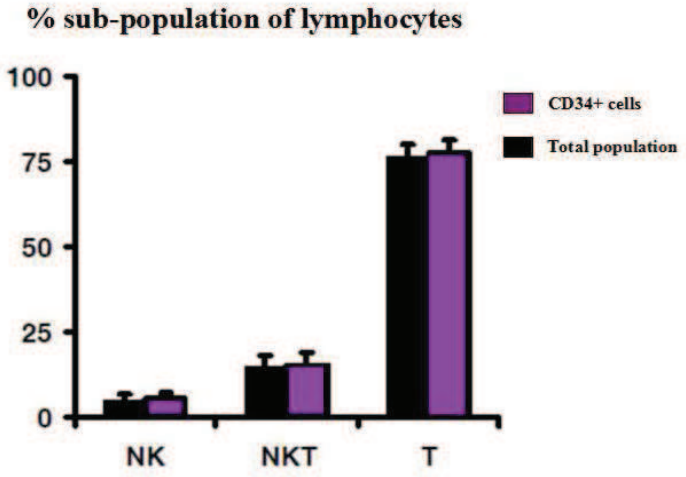


Figure 45. CD34+ purified GML (purple bars) have a similar T, NKT and NK cell sub-population repartition as total transduced lymphocytes before sorting (black bars) containing both CD34+ and CD34- cells.

The phenotype of GML was characterized at the end of the production process using the CD56 and CD3 markers that allow identification of T-cells (CD3- CD56+), NK-like T-cells (CD3+ CD56+) and NK cells (CD3- CD56+). The NK-like T-cells are defined as T-cells that also express NK cell markers such as CD56. Similarly to the conventional T lymphocytes, they express the CD3 and their TCR are provided with a α / β chains. They are not to be confused with invariant NKT (iNKT) cells, that express invariant TCR α and β chains (such as Vα24-Jα18 or Vβ11 TCRs recognizing lipids and glycolipids presented by the CD1d molecule, which is analogous to the MHC-I proteins).

The main population, was T-cells (71.8 ± 8.7%), followed by NKT cells (15.5±4.9%) and finally by NK cells (8.4±4.2). A higher proportion of NKT cells was observed among the GML as compared with the initial PBMC, which can be explained by a slightly larger expansion of NKT cells compared to the other sub-populations (Figure 46), or by the acquisition of CD56 by T-cells in culture, as has been shown previously (Kim, Lim et al. 2007). NK cells are less frequently represented among GML than among PBMC and their expansion in culture is lower than that of NKT cells (Figure 46).

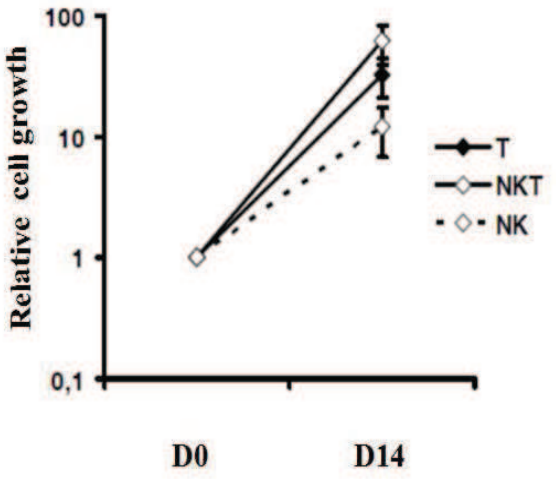


Figure 46: Relative Expansion populations of T, NK and NKT cells after 14 days of culture.

The cytotoxic activity of GML was evaluated *in vitro* toward different human cell lines, including HeLa cells, as a positive control for target cell killing and, as HCC cell lines, Huh-7, HepG2 and PLC-PRF5. Effector cells (PBMC or GML) and target cells (HeLa or HCC cell lines) were co-cultured for six days at different effector reports: target (E: C). The results were expressed either as a percentage of cytotoxicity (Figure 47A) or as lytic units 50% (UL50, Figure 47B). The GML were highly cytotoxic to Huh-7 cells (Figure 47A) as well as against the other HCC cell lines (Figure 47B), the cytotoxic activity was even higher than that observed face to HeLa cells, used as a positive control of target cell killing.

Huh-7 cells were more extensively used in our *in vitro* studies and we previously demonstrated (Leboeuf, Mailly, Wu et al., Mol Ther 2014, see Annex 1) that the cytotoxic activity of GML toward Huh-7 cells is:

- IL-2-dependent, as it is strongly impaired when the assay is performed in the absence of IL-2
- non-MHC class-I restricted, as it is not reversed by the blocking anti-MHC class I monoclonal antibody W6/32
- mostly mediated by NK-like T-cells and NK cells and, to a lesser extent, by T-cells, as demonstrated by the comparison of immunomagnetically-purified T, NKT and NK fractions of GMC

- not affected by the gene transfer process, as demonstrated by the similar cytotoxic activity observed with GML and Co cells

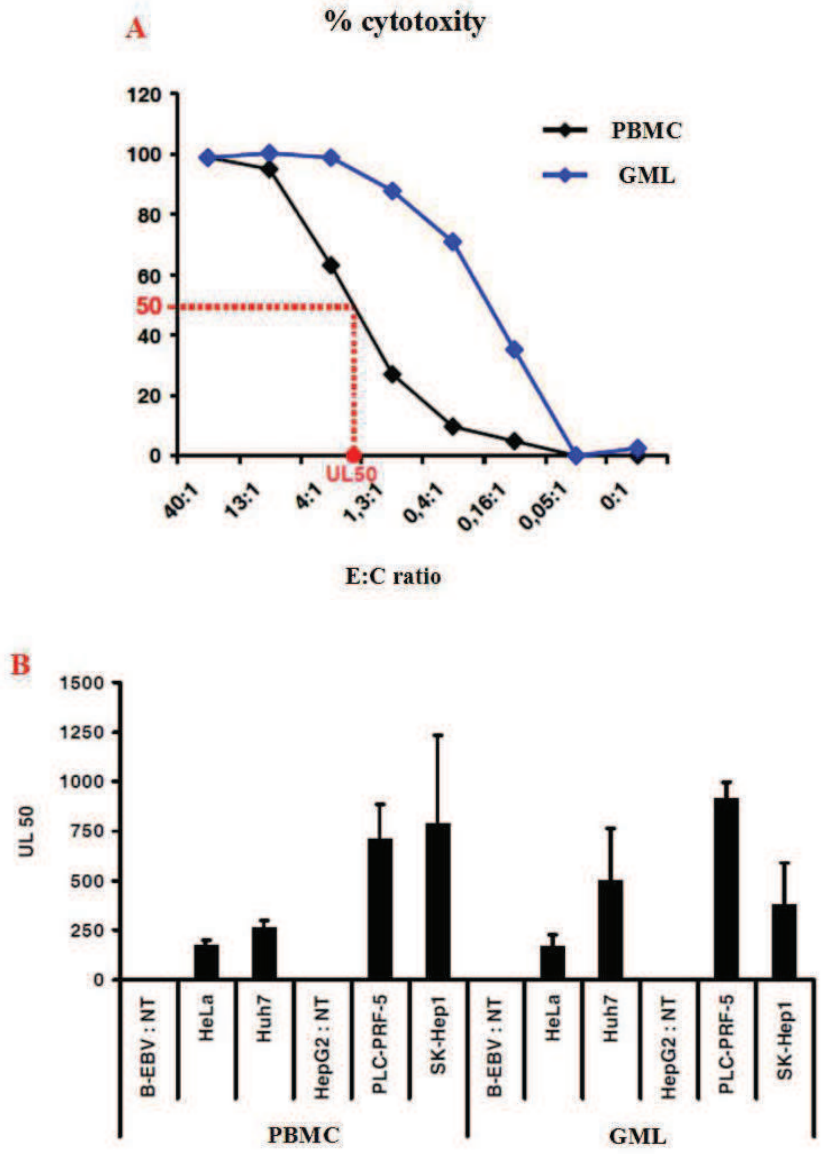


Figure 47. Cytotoxic activity of GML against different cell lines

The cell lines were co-cultured with PBMC or GML for six days at different effector reports: target (E:T). The results are expressed as percent cytotoxicity (A) or in 50% lytic units (UL50, B). The results being expressed as the number of UL50 reported 10^6 cells. NT = not tested

3.2. Prodrug sensitivity assay

The advantage of our approach relies on being able to eliminate the GML in case of allogeneic GvHD. We tested the susceptibility of HSV-tk/CD34+ GML by culturing them for one week in the presence or absence of the prodrug GCV (1 μg/ml) or CID (10 nM). Co cells (PBMC cultured and activated in parallel with GML, but non-transduced and non-selected) served as negative control. Trypan blue counts showed a reduced relative cell growth of more than 95% within one week in the presence of GCV, compared to the control culture without GCV, while GCV had no significant effect on the relative cell growth of Co cells (Figure 48A). In an independent series of experiments, a similar reduction of relative cell growth was showed in the presence of CID for CD19/iCasp9+ GML (figure 48B). As expected, the CID and GCV had no effect on the relative cell growth of HSV-tk/CD34+ and CD19/iCasp9+ GML, respectively.

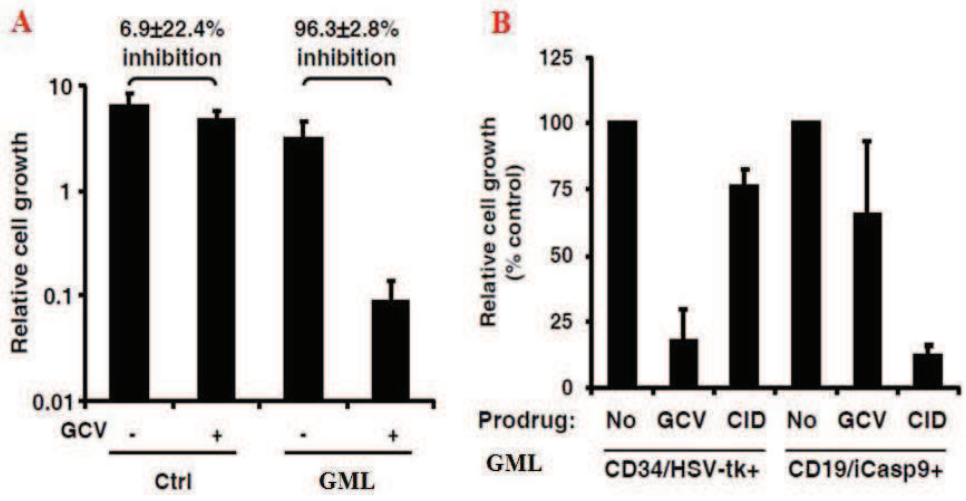


Figure 48. Prodrug sensitivity assay

In order to demonstrate the sensitivity of GML to their prodrugs, GML or Co negative control cells were cultured, at D14 of expansion, for one additional week in the presence of 500 IU/ml human Interleukin-2 with or without the prodrugs 1 µg/ml GCV or 10 nM chemical inducer of death (CID). The relative cell growth was calculated as the ratio of the number of cells at the end of the assay to the input number of cells at initiation of the assay. The percentage of inhibition of relative cell growth by the prodrugs was calculated according to the formula [1-($RCG_{PRODRUG}$/RCG)] x 100, where $RCG_{PRODRUG}$ and RCG are the relative cell growths in the presence and absence of prodrug, respectively.

3.3. *In vivo* evaluation of the antitumoral activity of gene-modified lymphocytes in an orthotopic and xenogeneic hepatocellular carcinoma model

3.3.1. GML migrate preferentially in the liver after intravenous injection

In parallel with these *in vitro* studies, *in vivo* cytotoxicity assays were performed by subcutaneous co-injections of effector (GML) and target (HeLa-Luc, Huh-7-Luc) cells (Leboeuf, Mailly, Wu et al., Mol Ther 2014, see Annex I). These studies were completed by *in vivo* studies in a more relevant model, using orthotopic xenogeneic HCC generated by intrasplenic injection of Huh7-Luc cells. My participation in this project consisted in completing experiments in this orthotopic HCC model.

Preliminary experiments were conducted to determine what mode of injection is the most effective to deliver the GML cells in the liver. Thus, GML were labeled with a fluorescent marker, the DiR, and administered intravenously (retro-orbital) or ip. The fluorescence is detected three hours after GML injection and up to 72h (Figure. 49A). GML cells located mainly in the liver and to a lesser extent in the spleen, lung and intestine in the case of intravenous injection (Figure 49B). Moreover, after the IP injection, the GML have been detected in the intestine, spleen and liver, but with a lower emission of fluorescence in the liver than after iv injection (Figure 49 B ,C and D). Twenty-four hours after their injection, GML were found surrounding the tumor (figure 49 E,F). Intravenous administration of GML is thus considered as the most suitable model for obtaining a preferential migration of the cells in the liver.

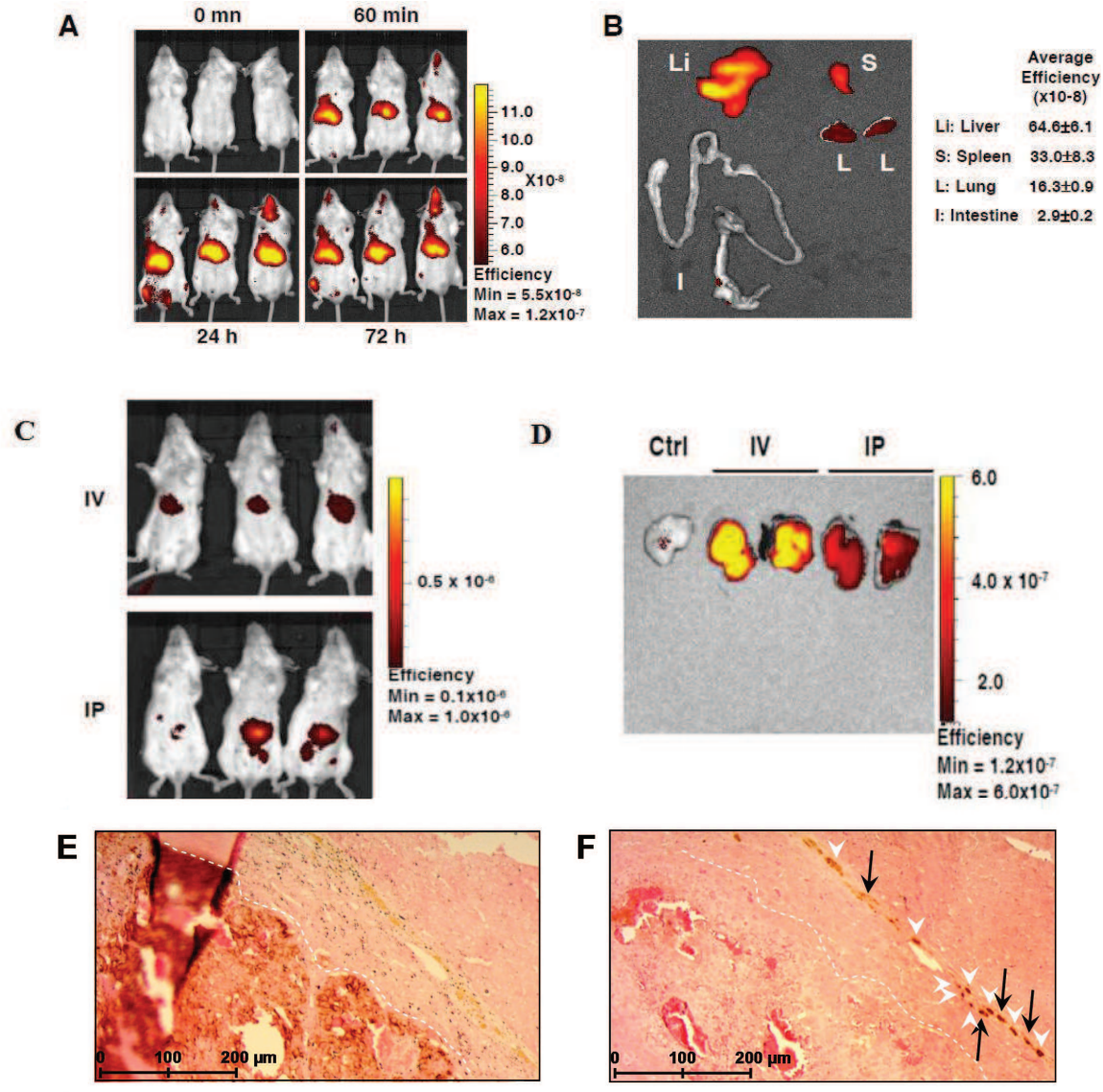


Figure 49. GML migrate preferentially in the liver.

DiR-labeled GML preferentially and rapidly migrate to the liver when intravenously injected to SCID-bg mice. (A) The *in vivo* fluorescence signal is shown for three mice representative of six. (B) Fluorescence quantification in the liver (Li), spleen (S), lungs (L), and intestine (I) 72 hours after GML injection is reported in the right part of the figure (mean ± SEM, n = 4. (C) SCID-bg mice received an i.p. or i.v. (retro-orbital) injection of 100 × 10^6 DiR-labeled GML. Fluorescence emission three hours after injection is shown for three representative mice of six per group. (D) The livers were then harvested for two mice and fluorescence was recorded. One non-injected mouse was used as a negative control of fluorescence (Ctrl). CK18 (E) and CD3 (F) immunostaining was performed on serial sections of the liver of Huh7-Luc tumor-bearing SCID-bg mice, 24h after i.v. injection of 150x10^6 GML. Allogeneic GML were found surrounding the CK18-positive tumor (E: bottom left part of the micrograph) and were mostly CD3-positive (F: white arrowheads), with some cells also CD56-positive (F: black arrows, identified on a third serial section, not shown).

3.3.2. Evaluation of the anti-tumor activity of GML *in vivo*

Huh7 cells expressing luciferase were injected into the spleen of SCID-bg mice in order to allow their migration towards the liver through the splenic vein. Four days later, mice were injected with PBS as a negative control or with GML. *In vivo* tumor bioluminescence test was performed just before iv administration of GML and also three and seven days later after injection to assess tumor growth.

The tumor growth was slower in mice treated with GML compared to control group, all the tumor cells of several mice were even completely eliminated virtually (Figure 50A). The mean tumor growth, expressed either as absolute bioluminescent activity (Figure. 50B) or relative bioluminescent activity (Figure. 50C) and the frequency of tumor-progressing mice (Figure. 50D) were markedly reduced at D3 and D7 after intravenous (IV) GML injection, as compared to the non-treated control group. No side effects have also been observed in mice treated with GML.

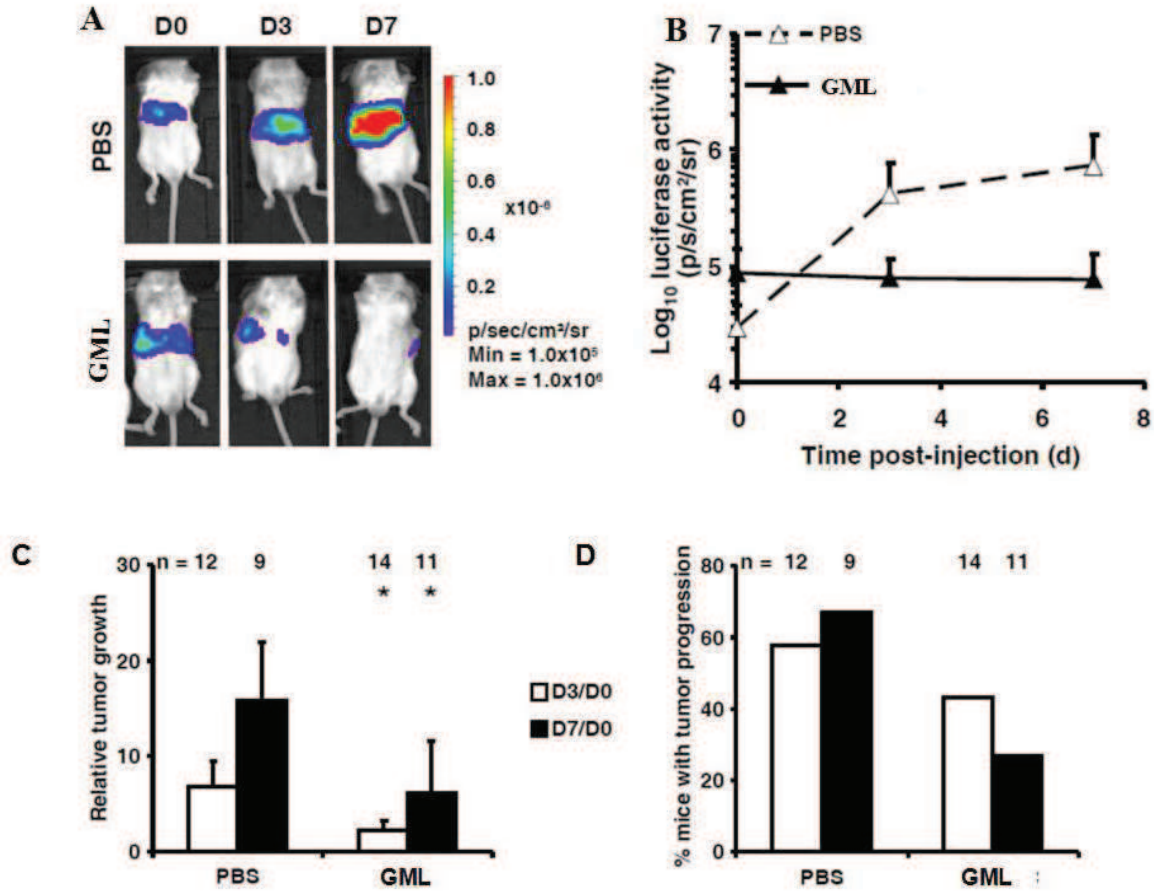


Figure 50. GML provide an antitumor effect in a humanized orthotopic model of HCC

(A) Representative experiment showing the Huh7-Luc bioluminescence in one mouse injected with phosphate-buffered saline (PBS) or GML. The injection of GML delays the tumor growth, **(B)** evaluated by the luciferase activity of Huh7-Luc cells or **(C)** expressed as relative tumor growth on D3 and D7. *P = 0.07 compared with control group with PBS injection (Mann–Whitney test). **(D)** The proportion of mice with tumor progression (i.e., with a relative tumor growth value >1) on D3 and D7 shows that GML reduce the frequency of mice with Huh7-Luc tumor progression. Data in d–f expressed as mean ± SEM are pooled results of five and three independent experiments on D3 and D7, respectively, using the indicated total number of mice (n) per group.

3.3.3. The frequency of injected NK-like T cells and NK cells are increased in the liver after injection.

In order to understand which sub-population of GML was responsible for the cytotoxic activity *in vivo*, GML were recovered from the livers at D7 post-injection and analyzed by flow cytometry for the relative repartition of NK, NK-like T and T-cells. As compared with the infused product, the frequencies of NK

and NK-like T-cells were increased while T-cells were less frequent (Figure. 51), further confirming *in vivo* that NK and NK-like T-cells are mostly responsible for the antitumor effect.

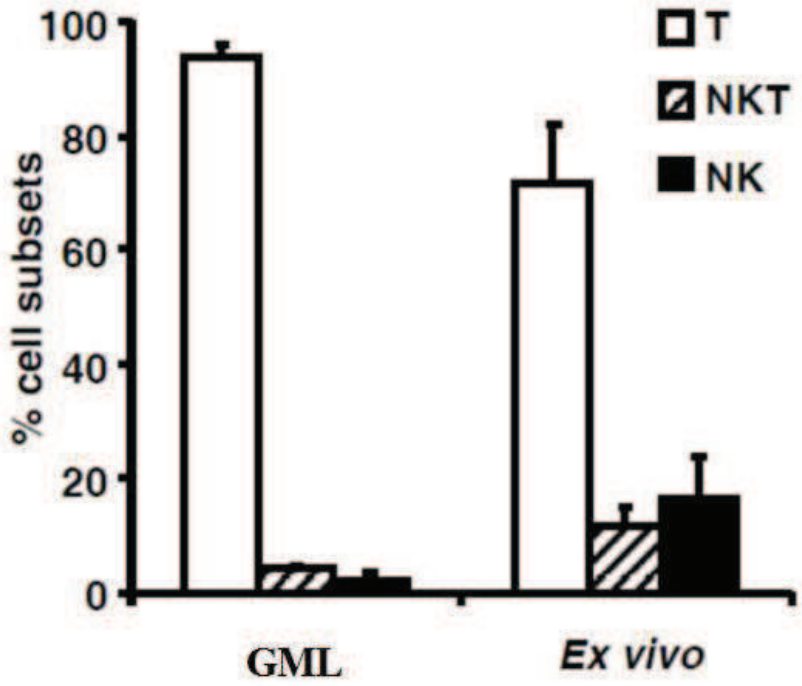


Figure 51. Sub-population of GML, *ex vivo* before injection (GML) and *in vivo* after injection (*Ex vivo*).

The relative frequencies of NK-like T-cells and NK cells harvested from the liver on D7 post-injection ("*ex vivo*"), evaluated by flow cytometry, are increased as compared with the frequencies observed on D0 in the infused GML product ("GML"). Data are expressed as mean ± SE of nine mice from three independent experiments.

3.3.4. The antitumor effect of GML expressing iCasp9/CD19

Considering that the immunogenecity of HSV-tk might contribute to the immune rejection of GML by the host, intrinsic suicide gene probably could be a better alternative to avoid the immune reaction. Therefore, we investigated *in vivo* antitumor effect of the GML expressing another suicide gene, the iCasp9. The results showed that the iCasp9 eliminated the transduced cells more rapidly than the HSV-tk suicide gene (Figure 52 A). Moreover, the antitumor effect of iCasp9+ GML was rapidly

reversed when its prodrug, the AP20187 CID, was injected to SCID-bg mice (Figure 52B). This suggests a superior efficacy of the iCasp9/CID system than the HSV-tk/GCV system in the current pre-clinical system and validates that the anti-tumor activity is in fact due to the GML.

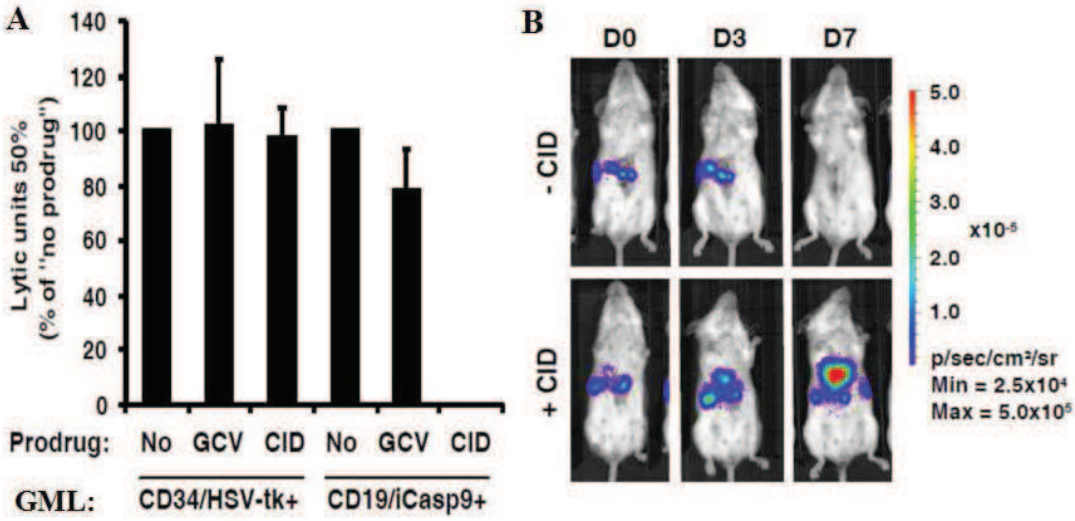


Fig 52. *In vitro* and *in vivo* efficacy of iCasp9+ GML depletion by its prodrug CID.

(A) The cytotoxic activity of GML is prevented more efficiently and more rapidly with iCasp9+ GML than with HSV-tk+ GML. The cytotoxic activity of CD34/HSV-tk+ and CD19/iCasp9+ GML was evaluated against Huh7 cells after three days of co-culture in the presence or absence of 1 μg/ml GCV or 10 nM CID. No cytotoxicity was observed with iCasp9+ GML in the presence of CID, at all tested E:T ratios, leading to no quantifiable lytic units in the presence of CID. By contrast, some cytotoxic activity was still observed with HSV-tk+ GML in the presence of GCV, leading to no or only a slight decrease of LU50 values. Data are expressed as mean±SEM LU50 of four experiments. Controls: GML without prodrug (CD34/HSV-tk+ GML: 116±59 LU50; CD19/iCasp9+ GML: 105±34 LU50). **(B)** The antitumor effect of CD19/iCasp9+ GML in the orthotopic HCC model is reversed by only one administration of chemical inducer of death (CID). Representative experiment showing the Huh7-Luc bioluminescence in one mouse injected with or without 2.5 mg/kg CID at the time of intravenous injection of 150–200 × 10^6 GML. Interleukin-2 (IL-2) was intraperitoneally injected daily.

3.3.5. Safety of GML injection

As mentioned above, the safety of the GML administration is a major event concern the pre-clinical and clinical use. Fortunately, no side effects were observed in

mice infused with GML, suggesting that antitumor activity could be safely established in absence of GvHD induction. In order to further explore the toxicity of GML, histological analyses were performed in the liver, lung, spleen, kidney and intestine of tumor-bearing mice one and seven days after GML injection.

As shown by the following, the livers presented focal zones of necrosis, occasionally associated with massive inflammatory infiltrates of neutrophils at the periphery of the necrotic hepatocytes (Figure. 53a). These zones of necrosis resulted from thrombosis (Figure. 53a, insert) that was probably induced by the intrasplenic injection of Huh-7 cells. Indeed, they were also observed in tumor-bearing control mice not injected with GML while they were not observed in non tumor-bearing control mice injected with GML alone (data not shown). Multifocal inflammatory infiltrates were also visible in the hepatic parenchyma (Figure. 53b). Lymphocytes infiltrating the lung and kidney parenchyma, the lamina propria of intestine and the white pulp of the spleen were also observed but no histopathological abnormalities were found in these organs (Figure. 54), (excepted the occasional presence of tumor cells in the spleen, due to the intrasplenic injection of Huh-7 cells), suggesting that GML were safe to murine tissues and did not induce xenogeneic GvHD.

The potential cytotoxicity of GML against normal hepatocytes was further explored in human liver-chimeric mouse model, using Alb-uPA/SCID-bg mice whose livers were repopulated between 10-20% with PHH (Figure. 53c). Two weeks after GML injection, large areas of proliferative cellular infiltration were observed in perivascular areas and, more dispersed, in the hepatic parenchyma as well as in the human clusters (Figure 53d). Despite this infiltration, the level of liver repopulation by PHH was similar in GML-injected mice compared to non-injected control mice, showing that there is no massive destruction of those cells after GML injection. This is further substantiated by the observation that human albumin serum levels remain stable in both groups (figure. 53e). As reported above in tumor-bearing SCID-bg mice, no histopathological abnormalities were observec in the spleen, lung, kidney and intestine of human liver-chimeric Alb-uPA/SCID-bg mice, injected or not with GML (data not shown).

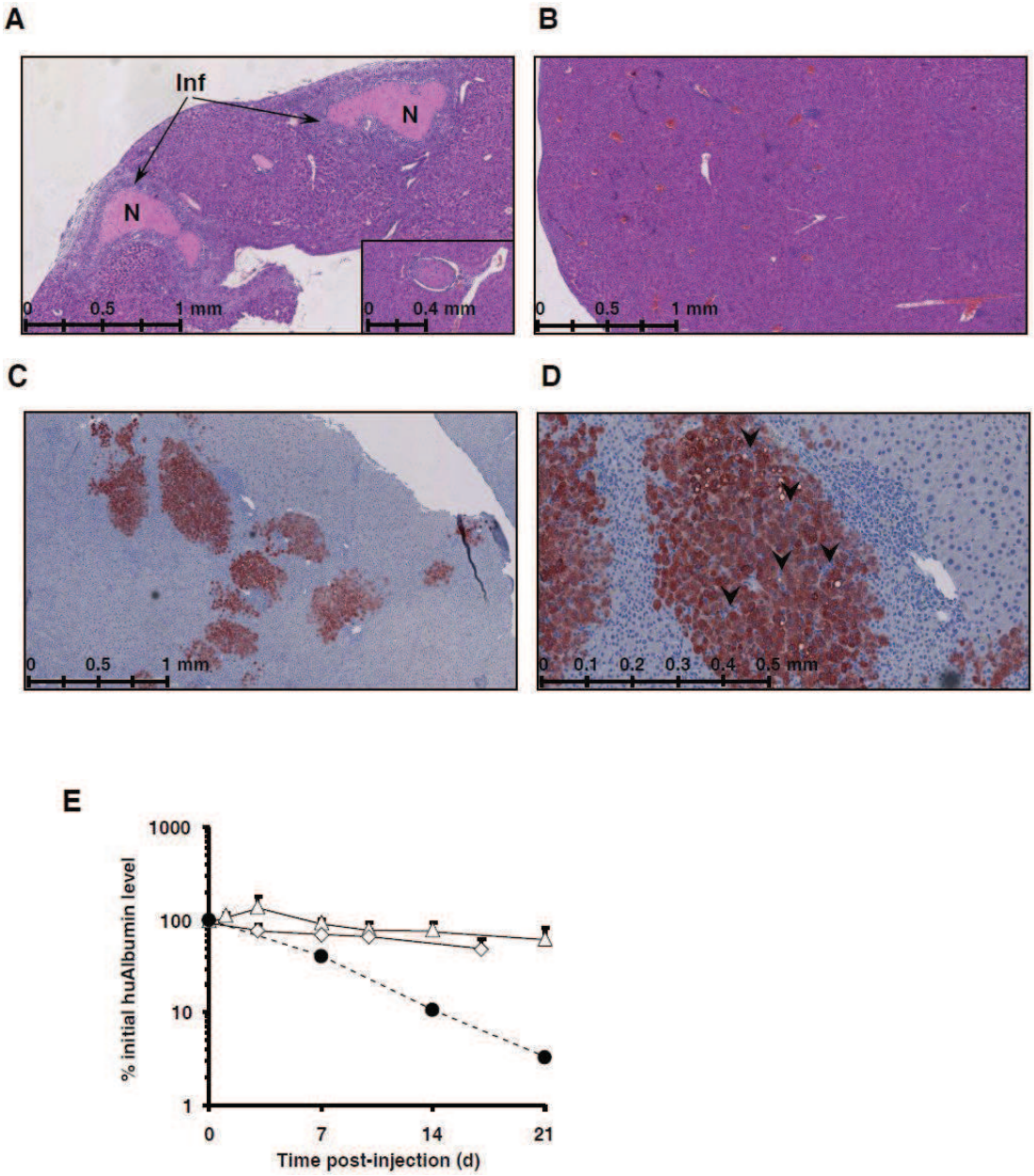


Figure 53. Histology investigation about the safety of the use of GML *in vivo*

The livers presented focal zones of necrosis, occasionally associated with massive inflammatory infiltrates of neutrophils at the periphery of the necrotic hepatocytes **(a)**, These zones of necrosis resulted from thrombosis (a insert); Multifocal inflammatory infiltrates were also visible in the hepatic parenchyma **(b)**; The liver of human liver-chimeric mouse model (Alb-uPA/SCID-bg mice) were repopulated between 10-20% with primary human hepatocytes (PHH) **(c)**; Two weeks after GML injection, large areas of proliferative cellular infiltration in perivascular areas and, more dispersed in the hepatic parenchyma as well as in the human clusters **(d)**; The human albumin serum levels remain stable in both groups **(e)**

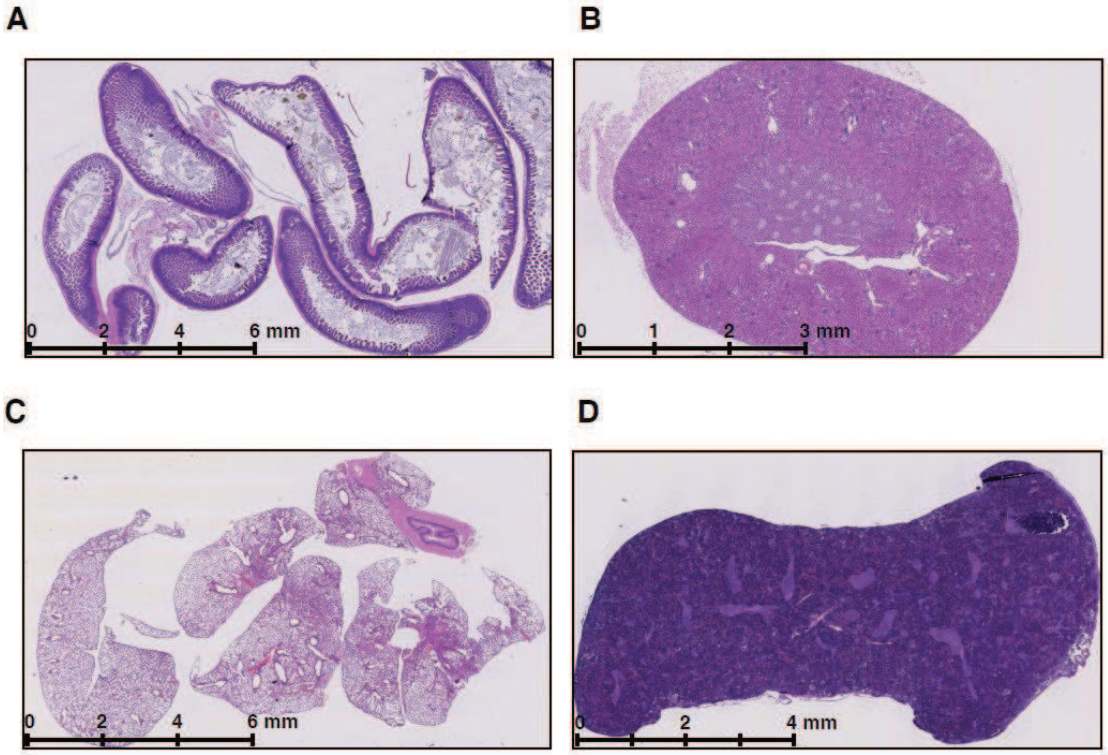


Figure 54. Administration of GML does not induce histopathological abnormalities in intestines, kidney, lung and spleen.

Seven days after i.v. injection of 150 x 10^6 GML into Huh-7-Luc tumor-bearing SCID-bg mice, hematoxillin-eosin stainings were performed on the intestine **(A)**, kidney **(B)**, lung **(C)** and spleen **(D)**. Lymphocytes infiltrated the lung and kidney parenchyma, the lamina propria of intestine and the white pulp of the spleen, but no histopathological abnormalities were found in these organs. Images are representative of four mice.

3.3.6. GML resistance to cyclosporine

Allo-immunization of the recipient's immune system against the GML might be a major limitation for our approach. This is why immunosuppressive conditioning is necessary to avoid the rejection of GML. The cytotoxic activity of GML and PBMC has been tested *in vitro* in the presence of a response dose of cyclosporine A (CsA), calcineurin inhibitor. PBMC's cytotoxicity is inhibited by CsA whereas that of GML are not affected, indicating that the GML are resistant to cyclosporine, because of their activated state (Figure 55 A B).

The resistance of the cultured cells was also proven *in vivo*: some splenocytes from FvB luciferase transgenic mice were grown similarly to human GML and used as a source of allogeneic lymphocytes. Splenocytes were injected into Balb-c recipient mice with CsA or PBS. In the absence of CsA, cultured splenocytes are rejected by the immune system of the recipient mice, and therefore do not induce GvHD. On the contrary, in presence of CsA, the immune system of the recipient mice is inhibited, leading to the non-rejection of splenocytes that can then induce GVHD. Herein, alloreactivity was used to assess the GML functionality. (Figure 55 C D).

At earlier time points post-injection, aEES from FvB/N mice could be detected by *in vivo* fluorescence in the liver, to a higher level in the presence of CsA than in its absence (Figure. 55E), indicating that CsA did not impair the preferential hepatic homing of aEES to the liver. In the presence of CsA, aEES induced a GvHD that results in weight loss (Figure. 565), prostration and ruffled fur. Although some mice occasionally died, these clinical signs were usually weak, in accordance with histological analysis demonstrating mild lesions at the intestinal level and no lesions at the hepatic level (data not shown).

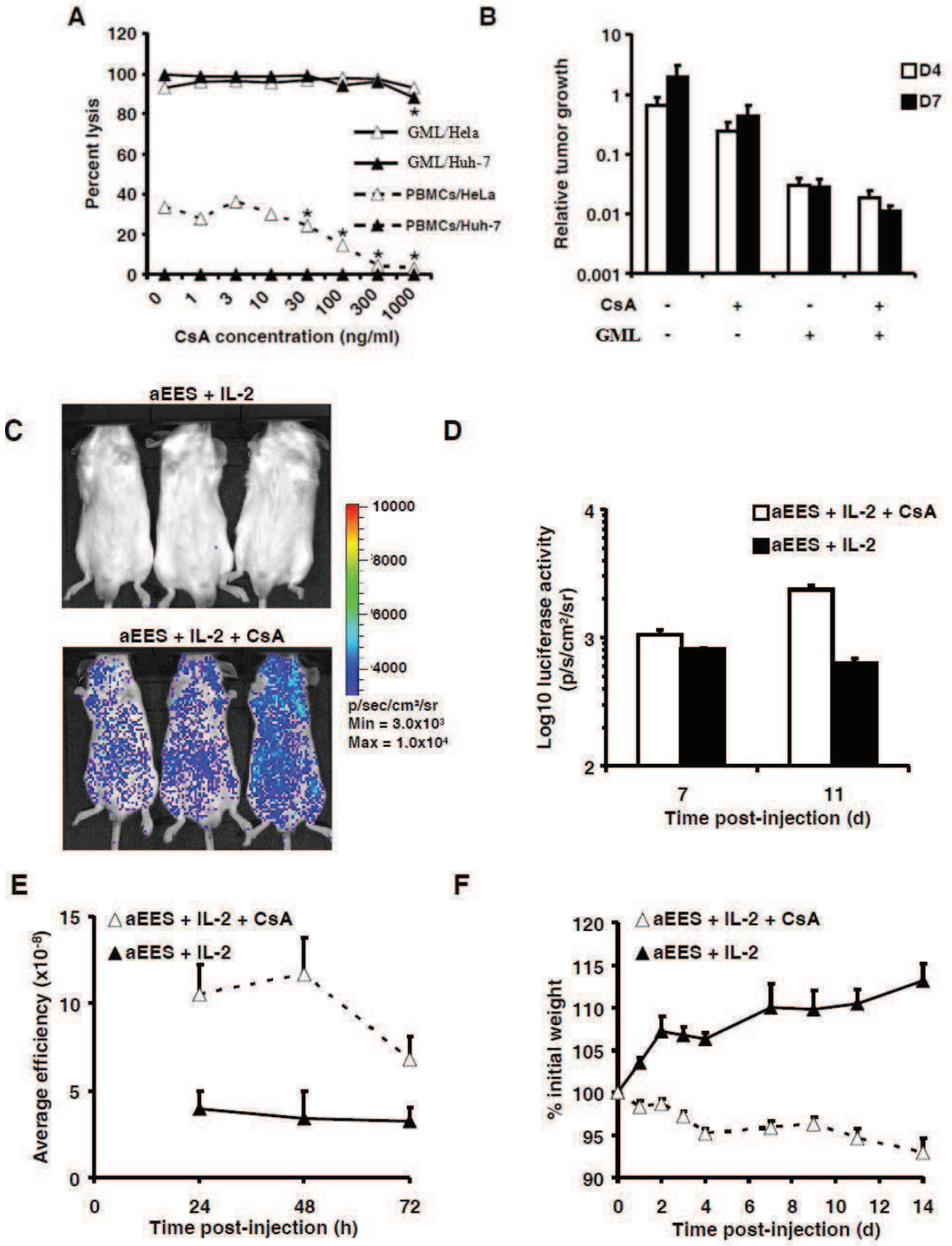


Figure 55. Resistance of expanded lymphocytes to ciclosporine A (CsA) as a mean to prevent their allorejection.

(A, B) Human allogeneic suicide gene-modified killer cells (GML) are resistant to CsA *in vitro* and *in vivo*. **(A)** The *in vitro* cytotoxicity of peripheral blood mononuclear cells (PBMCs) (positive control for sensitivity of effector cells to the inhibitory effect of CsA) and GML was assessed at an effector: target ratio of 40:1 in the presence of CsA (one experiment representative of four). *P < 0.05 compared with 0 ng/ml CsA. **(B)** *In vivo* GMLs' cytotoxic activity against Huh7-Luc cells was assessed by sc co-injection of 10^6 Huh7-Luc cells, with or without GML at an E:T ratio of 10:1 and in the absence or presence of daily intraperitoneal injections of CsA (n = 3/group). The BLI was evaluated at D4 and D7. (C-E) CsA prevents the rejection of allogeneic expanded *ex vivo* splenocytes (aEESs). **(C)**

Luciferase-expressing aEESs were injected to Balb/c recipient mice (n = 3/group) in the presence of IL-2 ± CsA and their presence was monitored by *in vivo* bioluminescence imaging on D7 (one experiment representative of four). **(D)** Quantification of the luciferase activity in mice injected with aEESs + IL-2 in the absence or presence of CsA (mean ± SE; n =3/group). **(E)** The CsA does not prevent the homing of aEESs to the liver. DiR-labeled aEESs from FvB/N mice were injected to Balb/c recipient mice in the presence of IL-2 ± CsA and were monitored by *in vivo* fluorescence gated on the liver (mean ± SE; n = 3/group). **(F)** The prevention of aEES rejection by administering CsA allows aEESs to induce a graft-versus-host disease, monitored by body weight loss (mean ± SE; n = 3 mice without CsA and n = 9 mice with CsA).

3.3.7. Allogeneic lymphocytes exhibit antitumor activity in immunocompetent mice

To investigate the antitumor activity of our approach in an immunocompetent host, we transplanted the syngeneic BNL-luc HCC murine cells into Balb/c mice. Because of a high death rate after orthotopic transplantation, we assessed the antitumor activity of aEES in a SC model. BNL-Luc were injected via SC in Balb/c mice, the tumors were treated by repeated intratumoral injections of aEES from FvB mice in the presence of IL-2+CsA. As expected, tumors showed a minimal spontaneous regression at D18 in non-treated (Figure. 56A) or IL-2-treated (data not shown) control groups. In the IL-2+CsA control group, tumors ultimately expanded at D18, after a short period of reduced growth (data not shown). In sharp contrast, a continuous tumor size decrease was observed from D7 to D18 in recipient mice treated by aEES and IL-2+CsA (Figure. 56A) and was associated with enhanced persistence of aEES in the tumor (Figure. 56B). This tumor rejection occurred without inducing any clinically detectable GvHD. These data demonstrate the proof-of-concept of our approach in an immunocompetent mouse model.

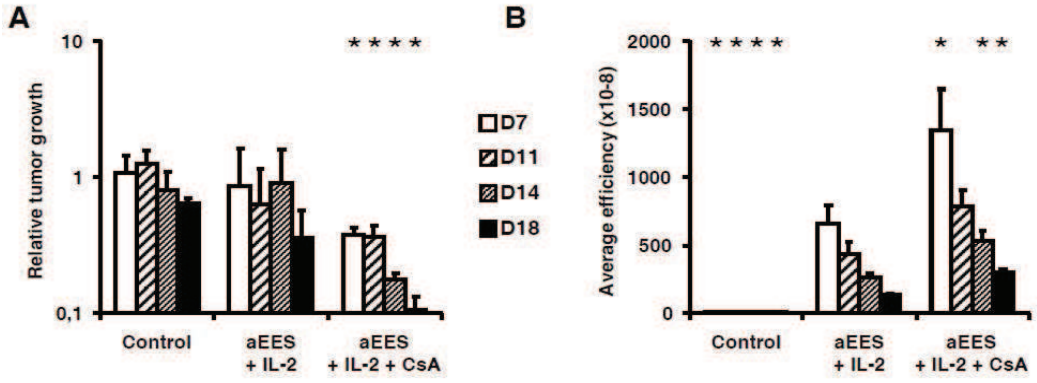


Figure 56. Antitumor activity of allogeneic *ex vivo*-expanded murine splenocytes (aEESs), used as a surrogate cell therapy product in an immunocompetent hepatocellular carcinoma (HCC) mouse model.

(A) In the presence of interleukin-2 (IL-2) and cyclosporine A (CsA), but not in the sole presence of IL-2, intratumoral injection of aEESs leads to the rejection of BNL1.MEA7.R1-Luc subcutaneous tumors. *P < 0.05 compared with the control group, injected with phosphate-buffered saline (PBS) alone (Mann–Whitney test; n = 5 mice/group). **(B)** CsA administration increases the amount of DiR-labeled aEESs present within the tumor, as monitored by *in vivo* fluorescence gated on the tumor. *P < 0.05 compared with the group injected with aEESs + IL-2 (Mann–Whitney test; n = 5 mice/group).

The study presented in this chapter is the object of a manuscript for which I am first co-author, published in ‘’Molecular Therapy” (Leboeuf C, Mailly L, Wu T, Bour G, Durand S, Brignon N, Ferrand C, Borg C, Tiberghien P, Thimme R., Pessaux P, Marescaux J, Baumert TF, Robinet E. *In vivo* proof-of-concept of adoptive immunotherapy for hepatocellular carcinoma with using allogeneic suicide gene-modified killer cells. Mol Ther. 2014 Mar; 22(3):634-44).

This manuscript is presented in annex 2.

4. Prevention of liver graft reinfection with HCV by administering CGM expressing a suicide gene

4.1. GML have antiviral activity against HCV

A phase I study conducted by Ohira et al. (Ohira, Ishiyama et al. 2009) that included seven patients with HCV infections showed that an injection of activated lymphocytes reduced the HCV viral load after LT. Before transplantation, lymphocytes from the liver graft were recovered by perfusion of the graft; then, they were *ex vivo* activated for three days, and reinjected into the patient. A decrease in viral load was observed in five patients, and two of those five exhibited undetectable HCV levels; of the latter two, one relapsed, but the other continued to exhibit persistently HCV-negative serum, which strongly suggested that the chronic infection had healed. The decrease in viral load was more pronounced and persistent when the pre-transplant viral load was low; this finding suggested that the therapeutic effect was limited by reinfection of the graft. Indeed, when activated lymphocytes were injected into the patient three days after transplantation, the graft had already been reinfected.

Consequently, we reasoned that a cell infusion given at the time of transplantation might be more efficient than an infusion performed three days after transplantation. This approach would require injecting lymphocytes that had been activated prior to the transplantation. To that end, we propose to produce a bank of "ready for use", activated lymphocytes from healthy donors that express a suicide gene. With this readily available store, at the time a patient receives a transplant, a batch of cells could be chosen from the bank and administrated immediately, without

wasting the time required for cell production and qualification. However, there is a risk that allogeneic cells might exert alloreactivity toward the patient's cells or the liver graft, which could lead to severe side effects, like GvHD or liver graft rejection, respectively. To avoid these side effects, a suicide gene could be introduced into the lymphocytes prior to injection. Thus, we wanted to determine whether our GML expressing a suicide gene have antiviral activity against HCV reinfection. We tested the antiviral activity of GML by using a replicon system that reflects only viral replication, and a HCVcc system that allows production of infectious viral particles. Huh7.5.1 cells were either electroporated with a subgenomic replicon that expresses the luciferase, or infected with HCVcc (Jc-1 clone) encoding the luciferase. Subsequently, the GML were added four hours later. After three days of co-incubation, the viral replication and production of infectious viral particles, with or without GML, were quantified by measuring the luciferase activity. The antiviral activity of GML in the replicon system was tested in co-culture and in transwell culture (no contact between Huh7.5.1 cells and GML). In parallel with each test, the survival of target cells was evaluated by crystal violet staining.

Using an HCV replicon model, we compared the antiviral activity of GML produced by CD3 activation or CD3/CD28 costimulation. A similar dose-dependent reduction of HCV replication was shown by both cell products (Figure. 57A). This antiviral effect was confirmed by using Huh7.5.1 cell infection with the infectious HCVcc Jc1 strain. In both models, we did not observe significant GML-mediated cytotoxicity toward target cells (Figure. 57B). Since GML produced after CD3/CD28 costimulation have been previously shown to be more aggressive *in vivo* than GML produced after CD3 activation, we chose the latter production protocol in further experiments. The gene transfer process did not affect the antiviral activity of *ex vivo*-expanded lymphocytes, as shown by the similar effect observed with GML compared to non-transduced control cells in the replicon model (Figure. 57C) and HCVcc model (Figure. 57D). Importantly, there was no obvious cytotoxic activity against target cells. As shown in Figures. 57C, D, the antiviral activity of GML was more potent when assessed in the presence than in the absence of exogenous IL-2.

Therefore, subsequent experiments were performed in the presence of IL-2.

In the HCVcc model, the antiviral effect of GML was more important when the co-culture was initiated the day of HCV inoculation than one to three days later (Figure. 57E), indicating that GML should be more efficient at preventing the liver graft reinfection if administered at time of liver transplantation than three days later.

However, we did not find that GML exert any significant cytotoxicity against target cells at the low E: T ratio used. It was possible that only few target cells were electroporated in the replicon model or infected in the HCVcc infection model and that specific killing of such cells was not detectable in our cytotoxicity assay. Therefore, a transwell system was performed in order to exclude a direct cell killing. GML exhibited a strong and IL-2-dependent antiviral activity (Figure. 57F), indicating that effector-target cell contacts were not required to inhibit HCV replication and that the antiviral effect was not dependent upon direct cytotoxicity.

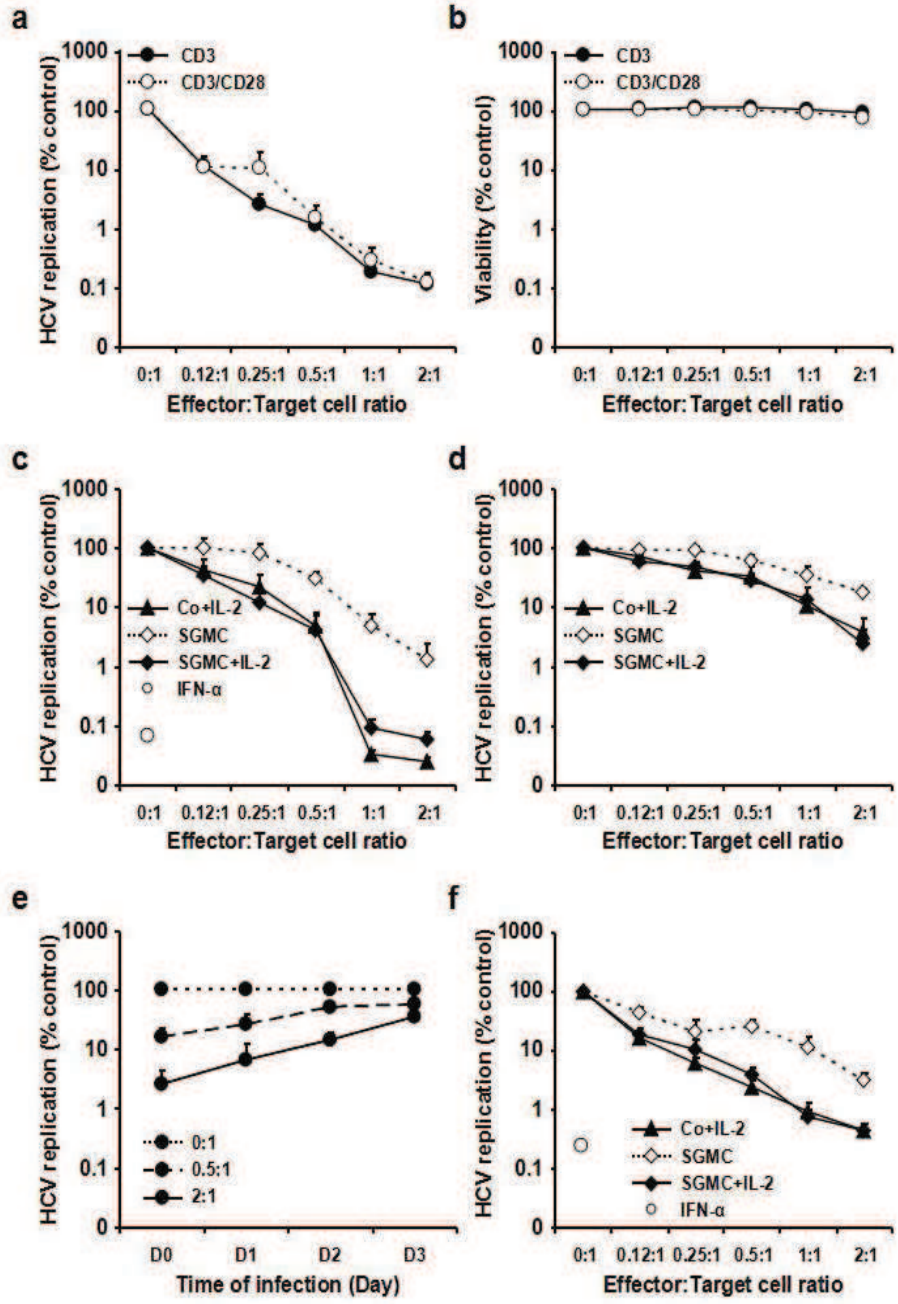


Figure 57. GML inhibit *in vitro* the HCV replication

(a) Huh7.5.1 cells electroporated with a luciferase-expressing replicon were co-cultured with graded amounts of effector cells generated after CD3 activation or CD3/CD28 costimulation. **(b)** The cytotoxicity of effector cells was evaluated by cristal violet test at the end of co-culture. **(c)** Huh7.5.1 cells electroporated were cultured in the presence or absence of IFN-γ, used as a positive control for antiviral activity, or IL-2 and with or without effector cells; **(d)** Huh7.5.1 cells were infected with HCVcc in the absence or presence of IL-2 and effector cells as indicated in (c). **(e)** The anti-viral effect is more pronounced when effector cells are incubated with target cells at time of infection. Huh7.5.1 target cells were infected with HCVcc three, two, one day before or the day of addition of GML and were cultured for three additional days in the absence or presence of effector cells at an effector:target ratio of 0.5:1 or 2:1. **(f)** Target cells were cultured as indicated in (c), but were separated from effector cells by transwells, with effector cells in the upper chamber.

4.2. Phenotypical and functional characterization of GML involved in the antiviral activity

The results of the transwell experiments suggested that the antiviral activity was mediated via soluble factors. The addition of anti-IFN-γmonoclonal antibodies (mAbs), but not anti-IFN-α or anti-IFN-β mAbs reversed the antiviral effect of GML on HCV replication, both in a transwell assay (Figure. 58a), in a co-culture assay (Figure. 58c) and in the HCVcc system (Figure. 58d). The combination of anti-IFN-α, -β and –γ mAbs did not further reverse the antiviral activity (data not shown).

Most GML are CD3+ CD56- T cells, with the remaining cells being CD3+ CD56+NK-likeT cells and CD3- CD56+ NK cells (Figure. 59b). In order to decipher which cells were involved in the antiviral activity of GML, CD56+ and CD56- cells were purified by immunomagnetic sorting. Both fractions contributed to the antiviral activity, although CD56- cells had a slightly lower antiviral activity than CD56+ cells, both in the replicon (Fig. 59a) and the HCVcc system (Fig. 59c). The cryopreservation does not affect the antiviral activity of GML, as shown by the similar antiviral activity observed between fresh and frozen cells (Fig. 59b), demonstrating that cryopreserved cells may be used adequately for clinical use.

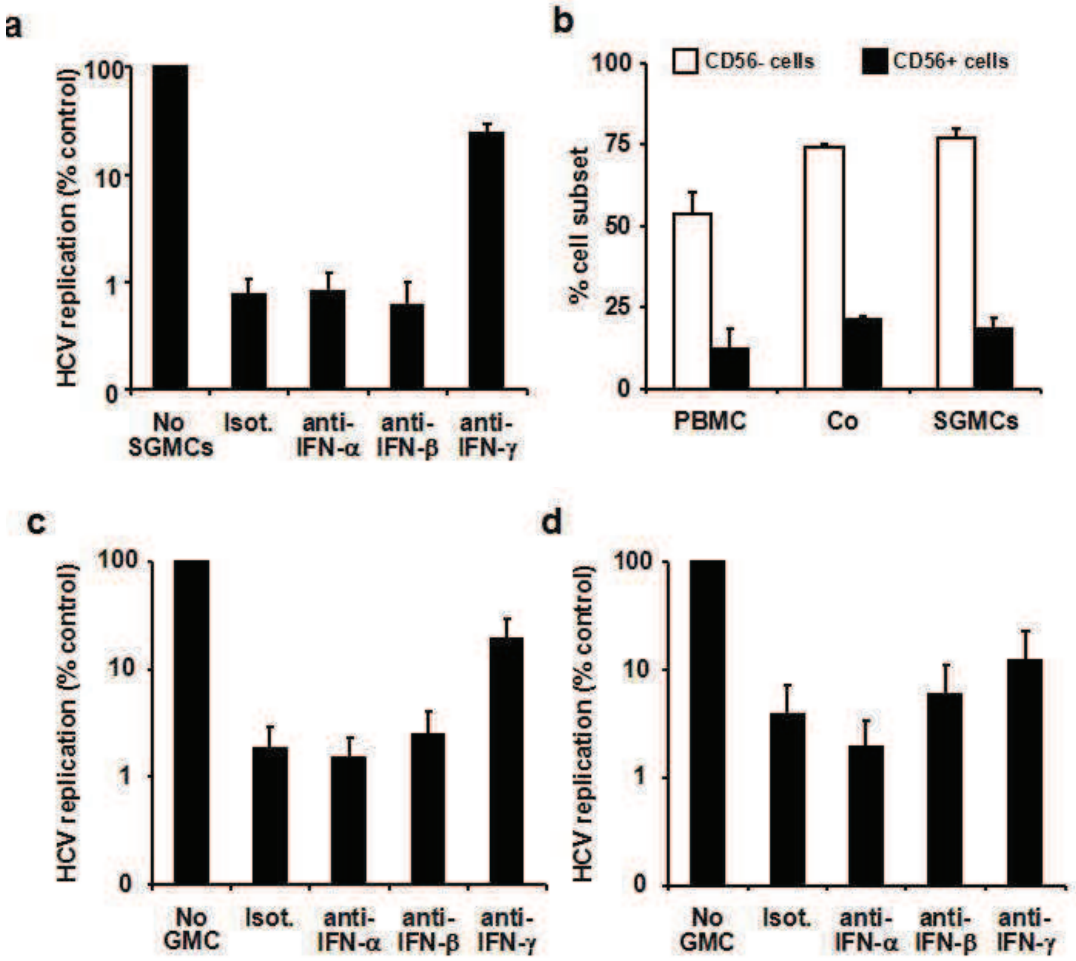


Figure 58. Phenotypical and functional characterization of GML involved in the antiviral activity

(a) GML were assessed for their antiviral activity in the replicon model at an effector:target ratio of 2:1 in the absence or presence of 5 µg/mL isotype control mAb or anti-IFN-α, IFN-β or IFN-γ mAbs. Effector cells were separated from target cells by transwells. **(b)** The frequency of CD56-negative cells and CD56-positive cells was quantified in the initial PBMC suspension, Co cells and GML by flow cytometry. GML were assessed in the replicon model **(c)** and the HCVcc infection model **(d)**at an effector: target ratio of 0.5:1 in the absence or presence of 5µg/mL isotype control mAb or anti-IFN-α, IFN-β, IFN-γ mAbs, or a combination of all three mAbs.

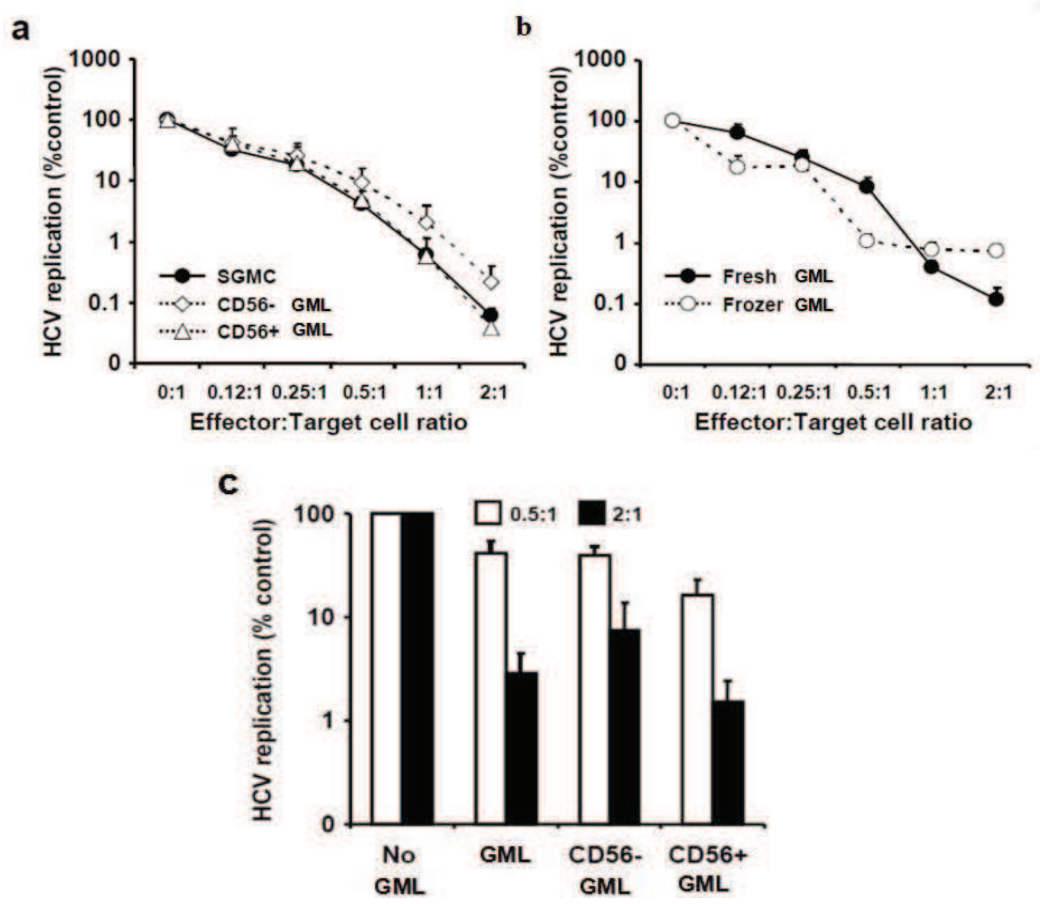


Figure 59 Phenotype of GML

(a) CD56-and CD56+ fractions from GML were compared to unsorted GML for their antiviral activity in the replicon model. **(b)** The cryopreservation does not affect the antiviral activity of GML in the replicon model, as compared to fresh GML **(c)** CD56-depleted (CD56-) and CD56-enriched (CD56+) fractions from GML were compared to unsorted GML for their anti-viral activity in the HCVcc infection model.

4.3. The antiviral activity of GML is not inhibited by calcineurin inhibitors

The major indication for the use of our GML is to prevent the reinfection of graft in liver transplant for the patients with chronically HCV infected. Obviously, these patients are always under immunosuppressive conditioning regimen to prevent graft rejection by the immune system. Therefore, we wanted to test the antiviral activity of our GML in the presence of immunosuppressive drugs in order to determine whether these cells are resistant to immunosuppressive drugs, as reported in

Result part 3 for their cytotoxic activity.

The calcineurin inhibitors CsA and FK506 are administered to liver-transplanted patients in order to prevent graft rejection. As we previously reported that the alloreactivity of *ex vivo*-expanded lymphocytes is resistant to CsA(Contassot, Ferrand et al. 1998), we reasoned that calcineurin inhibitors could prevent both the allo-immunization against liver graft and GML without impairing their antiviral activity. Thus, we evaluated *in vitro* the effect of CsA and FK506 on HCV replication. Electroporated Huh7.5.1 cells were incubated in the absence or presence of fixed amounts of GML in the absence or presence of graded amounts of CsA or FK506, in the replicon model (Figures. 60a, b) or HCVcc model (Figures. 60c, d). In accordance with previous reports (El-Farrash, 2007), CsA, (Figures. 60a, c) but not FK506 (Figures. 60b, d) exhibited *in vitro* an inhibitory effect at high concentration on HCV replication, which was not due to a toxic effect on the cell viability (Figure. 60). However, neither CsA nor FK506 reversed the antiviral effect of GML, indicating that GML were resistant to the immunosuppressive activity of calcineurin inhibitors. This suggests that administration of calcineurin inhibitors to liver-transplanted patients as a prophylaxis for liver graft rejection may not prevent the antiviral activity of GML.

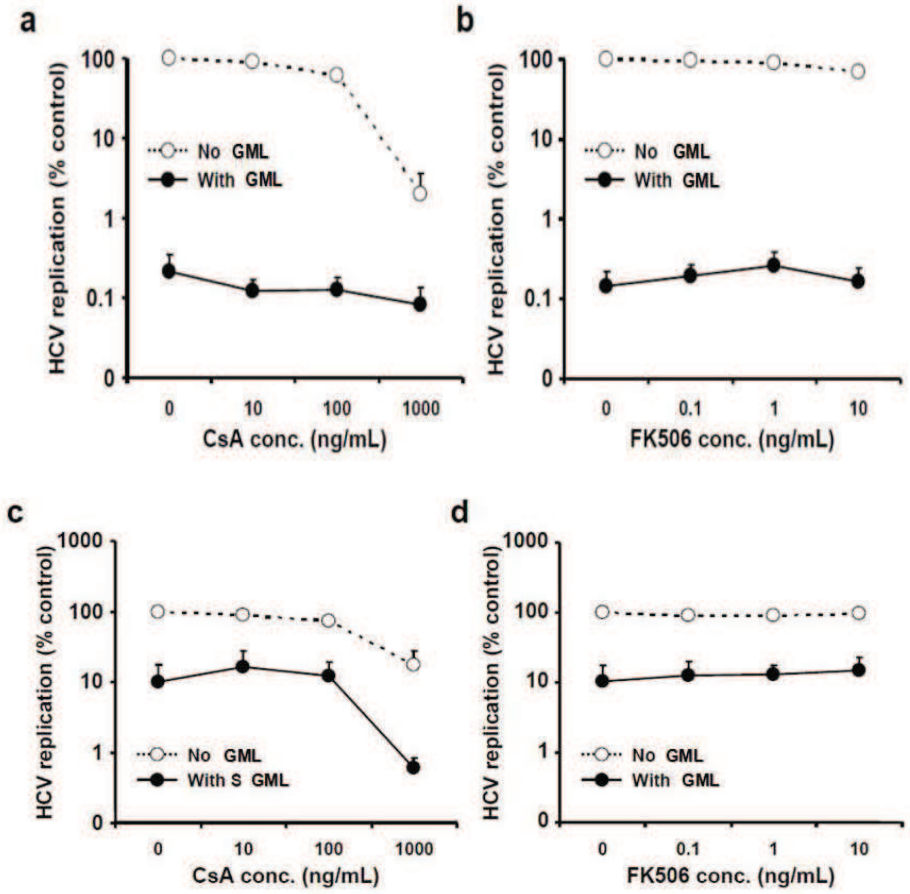


Figure 60. The antiviral activity of GML is not inhibited by calcineurin inhibitors

Huh7.5.1 target cells were incubated in the absence or presence of GML at an effector: target ratio of 0.5:1 in the replicon model **(a, b)** or at an effector: target ratio of 2:1 in the HCVcc infection model **(c, d)** and in the presence of graded amounts of CsA **(a, c)** or FK506 **(b, d)**. HCV replication was evaluated three days later.

4.4. The depletion of GML is more efficient with iCasp9 than with HSV-tk suicide gene

We then assessed the ability of two suicide genes, HSV-tk and iCasp9, to reverse the GML cytotoxicity. In order to observe both an antiviral and a cytotoxic effect, a co-culture was performed respectively at low ($\leq$ 1:1) and high (2:1 to 8:1) effector: target cell ratios, in the presence or absence of its prodrug, GCV and CID. After three days of co-culture, a slight shift of

the viability curve was observed (Figure. 61), indicating a limited inhibition of the cytotoxic activity by GCV, while, as expected, the CID did not affect the GML' cytotoxic activity. By contrast, The CID indeed completely prevented the cytotoxic activity of iCasP9+ GML at high effector: target cell ratios (Figure. 61b), up to a 40:1 ratio (data not shown), while GCV had no effect. Overall, when expressed as lytic units (Figure. 61), the GCV had no effect on HSV-tk+ GML while CID completely prevented the cytotoxicity of GML (Figure. 61c). The greatest efficacy of the iCasp9/CID system over the HSV-tk/GCV system did not result from a more pronounced GML depletion but rather from a more rapid depletion, as suggested by the observation that a similar GML depletion was finally observed when GML were cultured for one week in the presence of their respective prodrug (Figure. 61d). Moreover, the GML' antiviral activity was less affected by the prodrug treatment than their cytotoxic activity since no significant effect of GCV was observed on HSV-tk+ GML' antiviral activity (Figure. 61e) and only a partial reversal of the iCasp9+ GML' antiviral activity was induced by CID (Figure. 61f).

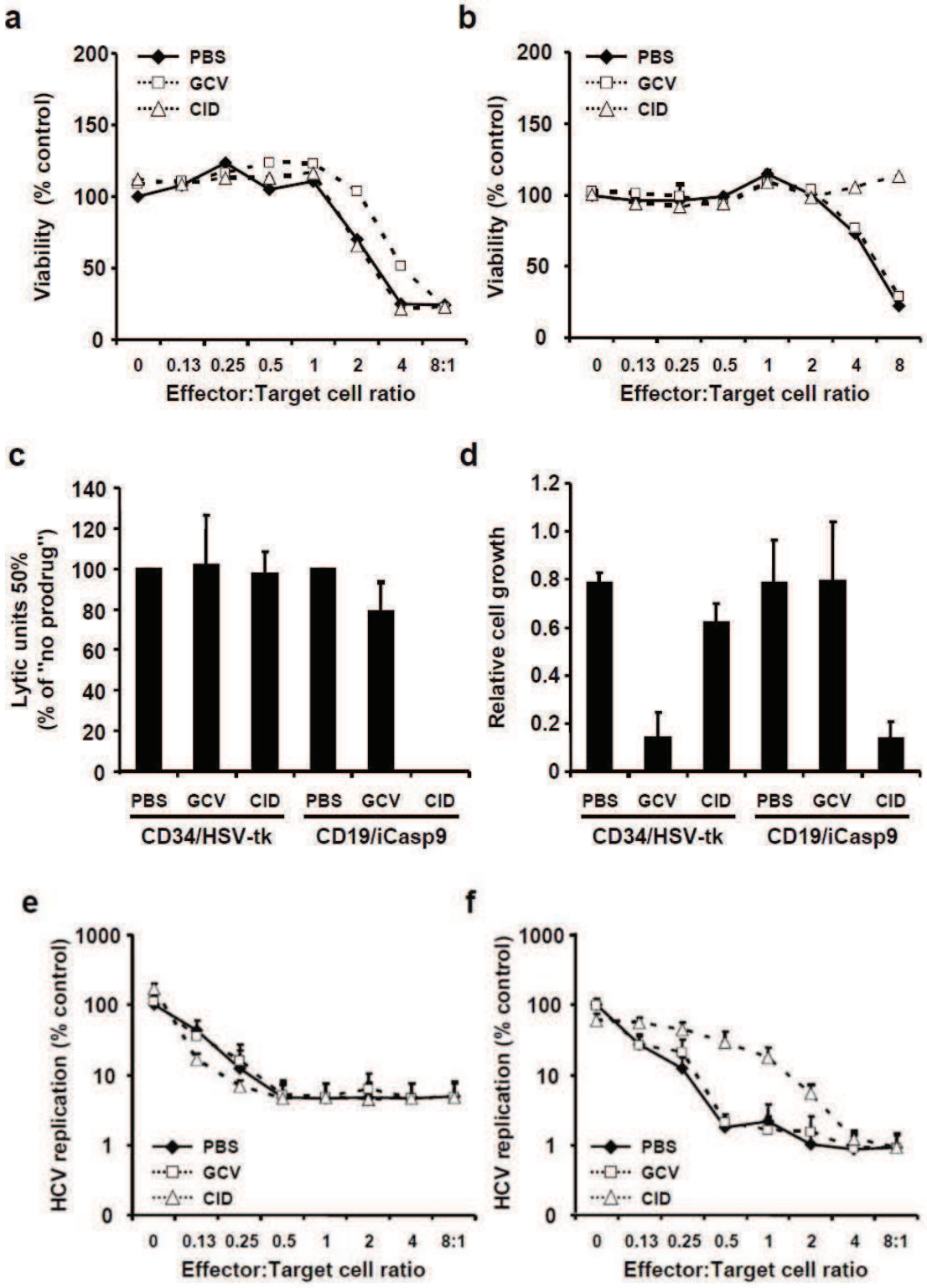


Figure 61. The depletion of GML is more efficient with iCasp9 than with HSV-tk suicide gene

GML expressing the HSV-tk **(a)** or iCasp9 **(b)** suicide gene were co-cultured with replicon-electroporated Huh7.5.1 target cells in the absence or presence of GCV, or CID at the

indicated effector: target cell ratios, from 0:1 to 8:1. The target cell viability, evaluated three days later, is expressed as % optical density of control cells **(c)** The CID completely abrogates the cytotoxic activity of iCasp9+ GML while GCV does not significantly affect the cytotoxic activity of HSV-tk+ GML after three days of co-culture with target cells. **(d)** The GCV prodrug is as efficient at depleting HSV-tk+ GML as the CID for depleting iCasp9+ GML. GML were expanded for one additional week in the presence or absence of prodrug. **(e)** The antiviral activity of HSV-tk+ GML is not efficiently reversed by GCV after three days of co-culture with target cells. **(f)** The antiviral activity of iCasp9+ GML if partly reversed by CID.

The study presented in this chapter is the object of a manuscript in revision in "Gene Therapy" (Leboeuf C, Roser-Schilder J, Lambotin M, Durand S, Wu T, Fauvelle C, Su B,, Bôle-Richard E, Deschamps M, Ferrand C, Tiberghien P, Pessaux P, Baumert TF, Robinet E. Prevention of hepatitis C virus infection by adoptive allogeneic immunotherapy using suicide gene-modified lymphocytes: an *in vitro* **proof-of-concept).**

This manuscript is presented in annex 3.

DISCUSSION

Part I

We chose a xenograft mouse model as our basic experimental model for subsequent studies because the HCC cell lines injected into mice could maintain their cellular features such as stable morphology, clonogenic capacity and molecular characteristics. In order to standardize a protocol for establishing an available orthotopic mouse model, we attempted to optimize the mode of injection of HCC cells through comparing the three most widely used injection modes: IH, IS and IPT.

Concerning the mortality, IH and IS injections showed more encouraging results compared with IPT injection. This might be explained by three reasons: first, IPT injection is technically more difficult because of the exposition and injection in the portal vein is evidently more complex than the others, as the access to the vein is uneasy, as compared with the access to the liver or to the spleen. Furthermore, the risk of hemorrhage is elevated: the hemostasis can be done only once. If the puncture in the vein has been misconducted, it is not possible to redo a second one in another location, so the manipulator has only one chance! Second, more complications happened during injection: excepted the uncontrollable peri- and post-operative hemorrhage, the cells might induce a thrombus of the portal vein or possibly an hepatic insufficiency similar to an acute hepatodeficiency leading to death within two to three hours, or similar to a chronic hepatodeficiency leading to the death within one to two days post-surgery. Third, some mice died possibly because of the longer procedure, increasing the heat loss and thereby the sensitivity to anesthesia-induced complications. The heat loss is further enhanced by viscera exposure (required to get access to the portal vein), despite attempts to reduce the heat loss by viscera washing with pre-warmed PBS. By contrast, IS and IH injections are relative simple. Each of these two approaches has its own features: tumor cells spread well in the liver after IS injection, perhaps it is more suitable for research on metastasis in the liver. However, the rate of hepatic location of tumor cells was lower in the IS group than in the IH

group: a lot of mice presented tumors either in the spleen or in the intestines after IS injection. It is possible that the tumor cells blocked the splenic vein or refluid into the intestinal veins during the process of migration towards the liver. All together, we consider that IH injection is a feasible procedure to establish mouse orthotopic models based on the lowest mortality, simpler manipulation and higher rate of mice available for experimentation.

One challenge for the orthotopic mouse HCC model is how to localize the tumor site prior to the subsequent experiments. Unlike subcutaneous models, where tumors are easy to manage and localize, precisely identifying the site of the tumor before initiating the experiment is crucial in orthotopic models, particularly in HCC models and especially in the experiments concerning the test of new strategies and agents. Indeed, the function of liver as well as the tropism of some agents (such as nanovetors or immune cells used in immunotherapy approaches) is a key element for the success of the strategy. In accordance with this, it was not possible in some mice to distinguish between intrahepatic or perihepatic tumors by BLI (the results of autopsy in these mice confirmed this hypothesis), a one-demensional test normally sufficient for the subcutaneously model. Therefore, three-dimensional devices, such as MRI, are a good alternative to distinguish the tumor sites. Our result showed that after IH injection there is a good correlation between BLI (analyzed on regions of interest of "supposed" intrahepatic tumors) and MRI (analyzed only on confirmed intrahepatic tumors) regarding the kinetic of cell in the liver, suggesting that IH injection is a valuable approach for establishing orthotopic tumors.

Exception of the precise tumor location and kinetic of tumor growth, a standardized HCC model should comprise a characterization of cell line and histology analysis. Therefore, in our future research, a reproducible experiment will be performed using aliquots of cryopreserved Huh-7-luc cells obtained from a secondary cell bank (or working cell bank) established from a primary cell bank (or master cell bank). This master cell bank has been characterized in terms of:

- absence of mycoplasma, checked twice, at time of cryopreservation of the master cell bank and one month before

- absence of 27 murine pathogens, checked by MAP PCR in order to ensure a safe injection of cells in mice

- stability of bioluminescence activity during *in vitro* culture

The identity and the clonality of Huh-7-Luc cells remain to be confirmed, respectively by genetic imprinting and by sequencing of the flanking regions of the luciferase transgene by LAM-PCR (Dr Ferrand, EFS Bourgogne/Franche-Comté) and the intensity of their bioluminescence activity must be quantified in terms of p/s/cell. Otherwise, since tumor vascularisation is a key parameter for the study of innovative HCC treatments, such as immunotherapy cell products or nanovectors, histological analysis are ongoing in order to characterize the kinetic of tumor neovascularisation, after injection of the Huh-7-Luc cells.

However, many observations are now re-evaluating the value of mouse models in the development of cancer-related research, owing to an opinion that cancer is a complex disease with intricate interactions between transformed cells, harboring oncogenic mutations, and surrounding environment, such as normal cells, stromal cells and immune cells (Hanahan and Weinberg 2000; Frese and Tuveson 2007). Several crucial differences exist when comparing tumor xenografts with transformed cell lines (such as Huh-7 or HepG2 or PLC-PRF5 cells) versus patient-derived xenografts (PDX) of primary tumor samples, such as diminished genetic heterogeneity and derangement of the normal tumor architecture in cell line-derived tumor models: importantly, the features of the tumor microenvironment could be altered or lost in cell line-derived tumor xenografts, including nearby normal tissues, stromal cells, vasculature and lymphatic circulation, and immune cells (Sikder, Huso et al. 2003). These switches in tumor cells and the microenvironment are probably relevant in the therapeutic evaluation of compounds. For example, anti-neoplastics with demonstrable activity in cell line xenografts are ineffective in the clinical setting (Johnson, Decker et al. 2001).

One possible solution to solve the problem of the differences between cancer cell lines and patients' tumors is the surgical orthotopic implantation of intact fragments of human cancer samples, directly harvested from the patient and transplanted into the

corresponding organ of immunodeficient rodents. Such PDX models have been successfully applied to breast, lung, and prostate cancer among others. In association with our previous experience, a surgical orthotopic implantation of primary HCC samples in currently under investigation, in collaboration with Pr D. Somacalle and the Dr T. Piardi (Pôle Digestif / Urologie / Néphrologie / Endocrinologie, CHU Reims). These PDX models will be used in future experiments for the evaluation of nanovectors under development, allowing for the dual imaging and treatment of HCC.

Compared with mouse models, porcine models have some predominant characteristics such as similarities with humans in body size, anatomy, and their physiological and pathophysiological responses. A lot of research has focused on using porcine models in preclinical experiments to aid development of tumor therapies in human via variety of strategies which could be divided into three categories: first, generate mimicking structures by injection of chemical products such as agarose, cellulose and glycerol (N'Djin, Melodelima et al. 2007) and liquid plastic (Hidalgo, Belani et al. 2005). Second, artificially inducing porcine tumors by administration of chemical carcinogens (Li, Zhou et al. 2006). While such procedures may be suitable for specific applications, it is labor intensive and can raise ethical concerns. Third, converting the pig's somatic cells to a tunorigenic state by transducing oncogenic cDNA. Two teams have successful established onco-pig models in pig's colorectal cells and fibroblastic cells respectively (Adam, Rund et al. 2007; Flisikowska, Merkl et al. 2012). Based on these encouraging data, we tried to establish an orthotopic porcine HCC model through introducing six oncogenes into the porcine hepatocytes in order to convert them into HCC cells. Unlike two previous reports, our project is based on porcine hepatocytes that are difficult to generate and culture *in vitro*. Thus, an accurate and effective selection of the lentiviral vector plasmid is a requirement prior to the lentiviral production. Compared with other two candidate vectors, the plasmid of pLenti CMV V5-LUC Blast showed high transduction efficiency in different cell lines or primary cells. Interestingly, when it was tested in PPH, pLenti CMV V5-LUC Blast performed also high luciferase activity, indicating that this vector has a similar or even higher transduction ability in PPH than

in PHH or even hepatoma cell lines (Huh 7.5.1).

It was proven that in human, normal somatic cells could be converted into a tumorigenic state by transducing several oncogenes in order to disrupt the tumor suppressors and activate the proto-oncoprotein. Indeed, inactivation of p53 and Rb pathways by expression of a dominant-negative p53 protein ($p53^{DD}$) and an activated cyclin-dependent kinase (CDK)/cyclin complex (cyclin D1/CDK^{4R24C}) in conjunction with activation of c-Myc, Ras and hTERT pathways via expression of oncogenic forms of c-Myc (c-Myc^{T58A}) and H-Ras (H-Ras^{G12V}) and hTERT may drive human kidney cells, mammary epithelial cells and myoblasts to a tumorigenic state (Kendall, Linardic et al. 2005). In the porcine, co-expression of hTERT, p53DD, cyclin D1, CDK4R24C, c-MycT58A and H-RasG12V also driven normal porcine fibroblast cells to a tumorigenic state (Adam, Rund et al. 2007). Based on these data, we tried to establish a porcine orthotopic HCC model by co-expression of these oncogenes. Considering PPH, VSV-G-LV is a good candidate to transfect and to deliver oncogenes. However, because of the potential risk of manipulating oncogenes-encoding lentiviral vectors, a biosafety level 3 laboratory is mandatory. However, as compared with a biosafety level 2, the biosafety level 3 enhances the protection level for the environment but not for the manipulator, whose protection is provided by handling under a class 2 biosafety cabinet. Therefore, improving the safety of the manipulator was mandatory for our project. This is why we wished to use a closed system culture for the production of lentiviral vectors and the PPH transduction. However, such a system should not impair the capacity of lentiviral production as well as transduction efficiency, as compared with classic open culture systems. Indeed, by comparing with conventional “open” system, our close culture system presents great advantages: (i) security, the whole experimental process occured in a completely closed system, decreasing the risks of contamination of cells and supernatants and (ii) it could also protect the manipulators from contacting with potential dangers of lentivirals. Interestingly, it could be used as a tool for direct cryopreservation of transduced cells in the culture cassette. Importantly, as shown in our results, the capacity of production of lentiviral vectors and transduction efficiency

are comparable with open system. Thus, this system could be used for further experiments which have risks for either the experiment or the manipulators.

Owing to the fact that difference between hepatocytes with other somatic cells, plus the whole genome of porcine is still incompletely known, we hypothesized that if the process of oncogenic transformation in PPH is not similar to other cells, meaning that the tumorigenic state is induced by different oncogenes or by different combinations of oncogenes, we designed several groups in order to investigate the effect of each of combinations of oncogenes. As shown in the results, all the experimental groups allow for a stable *in vivo* expression of luciferase for a long time (until euthanasia), suggesting that the vectors have the capacity to transducer PPH. Interestingly, in the SCID-bg mice, transplanted orthotopically, the level of luciferase expression in the PPH transduced with the combination of three vectors increased continuously, although it was lower at the beginning than in the control group. This suggests that the tumorigenic state may need several weeks to establish in orthotopic PPH models.

In vitro, we have also tested the expression of different proteins. The results showed that the most transgenic proteins were detected, particularly the expression of negative-domain of $p53^{DD}$ and proto-oncoprotein such as $c\text{-}Myc^{T58A}$ had a relative high level comparing with others.

However, in transduced PPH cells, we did not find an obvious tumorigenic state although the level of luciferase expression was substantially high in the orthotopic model. This is strengthened by the observation that when the liver tissue was subcutaneously retransplanted in secondary recipient mice, no tumor was found. Several possible reasons may be considered including: first, all the oncogenes may not be expressed at the same time, or their expression may be perturbed by endogenous PPH proteins; second, the intrinsic protein level may be lower in PPH than in other cells (reflecting that the function and proliferation capacity are different in the PPH and cell lines such as 293T cells or other somatic cells such as fibroblast cells), therefore, higher oncogene expression levels may be required. In the future experiments, in order to solve the above questions, we will focus on: (i) testing the

expression of each vector in other cell lines (such as 293T cell or Huh 7.5.1 cell) to investigate whether their expressions is influenced by intrinsic cell events (such as the proliferative status, that will be modified by starving) and (ii) comparing the expression of each plasmid in different porcine somatic cells (hepatocyte, fibroblast cells, or colorectal cells) to identify if we need to switch the oncogenes or combination of oncogenes, or the order of addition of lentiviral vectors.

Taken together, although this approach is undergoing, we expect that once it will be successfully established and validated in immunodeficient mice, we could translate the experiment in pigs. This onco-pig model might bring a lot of advantages for preclinical studies. First, the tunorigenesis switch of porcine cells is more similar to the process in humans than in mouse model. Second, the resultant tumors in pigs could reach very large sizes, allowing for a number of preclinical applications, not only surgical procedure, but also drug testing and signaling pathway investigation, etc. Third, if the PPH transformation could be realized, apart from hepoatocytes and fibroblasts, different cell types might yield tumors. We could hope then that it is possible to generate many different types of tumors potentially anywhere in the body. Finally, using our closed culture system, the entire procedure is quite safe and nearly as rapid as existing mouse xenograft models. Thus, the porcine onco-model will be a hope as a relevant model for preclinical studies of human cancers.

Part II

We propose to use GML expressing the suicide gene such as HSV-tk or iCasp9 for two major clinical applications, the treatment of HCC in combination with other therapies currently available, and the prevention of graft reinfection with HCV in liver transplantation.

Gene-modified lymphocytes as adjuvant immunotherapy of HCC

Immunotherapy based on the administration of allogeneic GML in the treatment of hematological malignancies has been shown to be beneficial for patients without major side effects. Indeed, allogeneic lymphocytes expressing the HSV-tk gene have

been widely used in several clinical studies in combination with HSCT (Bonini, Ferrari et al. 1997; Tiberghien 2001; Ciceri, Bonini et al. 2009). The latter allows reconstitution of the immune system, while GML provide a strong antileukemic activity against the residual cancer cells. Based on these anti-cancer properties against hematological malignancies, we tried to determine whether these cells could have antitumor activity against solid tumors, in particular against the HCC. The hypothesis of this new application is reinforced by the fact that another product cell therapy has been the subject of numerous clinical studies for the treatment of solid tumors, CIK cells. These cells are lymphocytes harvested from the patient's blood, subsequently activated and cultured *in vitro* and then reinjected autologously. Several clinical trials were studies in patients with colorectal cancer, renal cancer, stomach cancer or HCC (Sangiolo, Martinuzzi et al. 2008). Used in combination with standard treatments (chemotherapy, surgical resection), the CIK cells have a significant antitumor activity and significantly improve the survival of patients, especially when used in patients with HCC (Hui, Qiang et al. 2009) (Kim, Lim et al. 2007). In our study, we compared our GML with CIK cells phenotypically and functionally using different activation protocols and culture described in the literature for the production of CIK (Weng, Zhou et al. 2008; Ayello, van de Ven et al. 2009; Olioso, Giancola et al. 2009). We did not observe any significant difference in the percentage of T, NK and NK-like T in the GML and CIK cells, and the level of their potential cytotoxic against Huh7 cells *in vitro* (data not shown). Therefore, we concluded that our GML are actually genetically modified CIK, which strongly suggests that they may have an antitumor effect against HCC (Wu et al., manuscript in preparation – these results were not included in the current thesis).

The capacity of alloreactivity control of GML is a crucial component of our approach. Alloreactive cells that may be toxic to healthy tissues of the patient and may induce a GvHD or a liver transplant rejection. We opted for the transfer of HSV-tk gene or iCasp9 gene in our GML cells, which can then be eliminated conditionally by GCV or CID (Garin, Garrett et al. 2001; Di Stasi, Tey et al. 2011).

A new vector called "iCasp9-CD19" was recently developed by Team of Brenner.

The iCasp9 gene encodes one part for human caspase 9, and another for the binding protein FK506, FKBP12. In the presence of AP1903, like FK506 molecule binds with high affinity to FKBP12, iCasp9 dimerization takes place, resulting in activation of caspases 3, 6 and 7 and leading to apoptosis of the transduced cell. The transgene iCasp9 is fused to the gene encoding the membrane marker CD19, which allows the selection of cells transduced by immunomagnetic sorting. This vector was used to transduce allogeneic T lymphocytes which have been administered patients in five pediatric leukemia relapse patients following HSC transplantation. In case of GvHD (GvHD grade I in 4 patients), the patients were treated with a single dose (0.4 mg / kg) of AP1903 which permits the elimination of more than 90% of genetically modified cells in 30 minutes to end GvHD (Di Stasi, Tey et al. 2011). Given this extremely rapid elimination, it will be possible to use this vehicle for transduction of allogeneic lymphocytes in future clinical use.

The characterization of the cytotoxic activity of our GML was a central point of our work. The GML were very cytotoxic *in vitro* versus HeLa (positive control sensitivity to lysis) and cells of different lineages of HCC. The cells produce large amounts of IFN-γ and to a lesser extent of TNF-α, the two cytokines may be coordinated to mediate cytotoxicity of GML(Wang, Jaw et al. 2012). Indeed, our results show that the simultaneous addition of blocking antibodies anti-IFN-γ and TNF-α during the co-culture of target cells and GML partially inhibits the cytotoxic activity of GML, while the antibodies alone have virtually no effect.

In addition to its presence required during the expansion of GML, IL-2 is also required to ensure maximum cytotoxicity during the co-incubation with the target cells. Indeed, GML showed very little cytotoxic without IL-2. Therefore, it may be necessary to combine IL-2 to GML when administered *in vivo*, as we have done in our preclinical studies in mice. This has not been done in clinical trials of GML administration. However, in one study (Slavin, Ackerstein et al. 2010), patients treated with allogeneic activated lymphocytes (similar to our GML, but without gene transfer) received subcutaneous recombinant IL-2 (6.10^6UI/m^2 per day) for five days after administration of the cells. The IL-2, associated with indomethacin administration to

reduce fever and inflammatory reactions, was well tolerated in most patients. The duration of administration of IL-2 was limited to three days in some patients due to the occurrence of side effects (Slavin, Ackerstein et al. 2010).

In order to determine the impact of the retroviral transduction on GML functionality, whose alloreactivity has been shown to be impaired because of the gene transfer (Sauce, Tonnelier et al. 2002; Marktel, Magnani et al. 2003), the cytotoxicity of GML against Huh7 cells was compared to that of cultured non-transduced and unselected cells. No differences could be observed between GML and control cells, indicating that the retroviral-mediated transduction does not affect the cytotoxic activity of GML.

CIK cells are widely studied for their antitumor activity against HCC, and they are similar to our GML on several points:

- activation by CD3 + IL-2 (+ /-IL-1, IL-7, IL -15, IFN-γ) - but without gene transfer
- the major cytotoxic cells are NK-like cells (Shi, Zhang et al. 2004) (Wang, Liu et al. 2002), which are T lymphocytes that have acquired CD56 and NK receptors during *in vitro* culture
- non-MHC restricted cytotoxicity, involving TRAIL, IFN-γ and TNF-α

Thus, it was interesting to determine, in our GML, which subpopulation of lymphocytes was responsible for their cytotoxicity and which mechanisms of recognition and of target cell lysis were involved.

The GML mainly consist of T lymphocytes cell, NK-like cell, and finally a small percentage of NK cells. After separating by immunomagnetic sorting, NK cells were found to be most cytotoxic cells, followed by NK-like T-cells and T-cells. Considering that the most numerous cells are less cytotoxic (T-cells) and that the rarest cells are more cytotoxic (NK cells), the relative contribution of each subpopulation was examined through a test. Most cells linked to the target cells are NK-like T-cells. It strongly suggests that NK-like T-cells play a key role in the lysis of target cells (Leboeuf, Mailly, Wu et al., Mol Ther 2014, see Annex 2). Our *in vivo* analysis confirmed this result: in the liver of mice infused with GML, the frequency of NK and

NK-like T-cells was increased while the frequency of T-cells was decreased, as compared with the initial subset repartition observed in the cell suspension before infusion, indicating that the cytotoxicity of GML *in vivo* involves mainly NK and/or NK-like T-cells. These results are consistent with the phenotypic and functional properties of CIKs.

The intravenous injection of GML four days after administration of IS injection of tumor cells is a relevant model: IS injection of tumor cells allows the diffusion of cells into the portal vein and an homogeneous distribution in the liver, which was different from the IH injection model. Preliminary experiments have shown that the GML administered via intravenous injection preferentially migrate to the liver. The liver tropism may be explained by the acquisition of I-CAM-1 and V-CAM-1 molecules (Hamann, Klugewitz et al. 2000) during *in vitro* culture, and could afford to get targeted cytotoxicity in the liver while limiting the spread of effector cells throughout the body and thus limiting a possible peripheral toxicity.

However, a major obstacle to obtain an efficient cytotoxic activity of GML is the lack of accessibility to tumor cells. Indeed, tumor cells are surrounded by stroma, also themselves composed of stromal cells (fibroblasts and inflammatory cells) and a matrix containing collagen type IV, laminin and proteoglycans (Yang, Nakamura et al. 2011). The extracellular matrix is a physical barrier limiting the access and dissemination of GML in the tumor (Li, Liu et al. 2009). Therefore, a possible future development of our project could be to evaluate the possibility to develop and use another retroviral vector. This vector would combine the suicide gene and a gene encoding metalloproteinases (Mok, Boucher et al. 2007) or the relaxin (Li, Liu et al. 2009), a soluble factor inducing the synthesis of metalloproteinases. These proteins would allow the GML to degrade the extracellular matrix used as a support by the tumor cells and to infiltrate more efficiently the tumor mass.

Another important point is about alloimmunization against GML. The GML may be rejected by the recipient's immune system. This can be considered as a second safety system (in addition to the suicide gene) to avoid GML toxicity long-term. On the other hand, it would also be responsible for a reduced antitumor efficacy. We have

shown that our GML are resistant *in vitro* and *in vivo* to two calcineurin inhibitors, CsA and FK506, both of them being routinely used in transplantation. In effect, the cytotoxicity of GML is not altered during their presence. The resistance of GML to CsA and FK506 are due to their activation state due to *in vitro* cell culture, as non-manipulated PBMC were responsive to both drugs. When entering a resting cell, CsA binds to cyclophilin, a cytoplasmic protein. This complex interacts with calcineurin, which prevents the dephosphorylation of the transcription factor of NF-AT and its translocation to the nucleus for its attachment to the promoter of the gene encoding the IL-2 (Flanagan, Corthesy et al. 1991). This phenomenon inhibits resting cells to be activated, while CsA had no effect on cultured cells because they have been previously activated and NF-AT is already present in the nucleus (Contassot, Murphy et al. 1998).

Nevertheless, it seems hardly possible to use calcineurin inhibitors in immunocompetent patients with HCC, as it could suppress the patients' antitumor immunity; therefore other immunomodulatory approaches should be developed.

In the study based on the administration of (non gene-modified) activated allogeneic lymphocytes for the treatment of hematological malignancies and metastatic solid tumors (but not HCC) (Slavin, Ackerstein et al. 2010), a conditional lymphocytes depletion consisting of fludarabine (25mg/m²) or cyclophosphamide (1000 mg/m²), is carried out before infusion of allogeneic lymphocytes. This conditioning regimen allows first to create a "niche" for allogeneic lymphocytes which can then exert their antitumor activity, and second to eliminate the other recipient's regulatory T-cells that may compromise the antitumor effect of the allogeneic cells. The absence of immunosuppressive therapy following administration of allogeneic lymphocytes results in the rejection of allogeneic cells by the recipient's immune system within an estimated time up to one week, which possibly prevents the occurrence of GvHD. Indeed, the safety of this approach is based on early rejection of allogeneic cells by the immune system of recipient. The antitumor effect is assumed to be very fast and takes place before the cells are rejected, as shown by the survival without disease progression in four out of five patients with hematologic malignancies,

and 5 of 21 patients with metastatic tumors (Slavin, Ackerstein et al. 2010).

However, using the recipient's alloimmunization as a safety control system is not satisfactory. Another conditioning regimen was involving the use of FTY720, an immunomodulator inducing sequestration of lymphocytes in lymphoid organs, thus preventing their spread in the periphery (Tedesco-Silva, Szakaly et al. 2007). The molecule does not induce generalized immunosuppression because it does not affect activation and proliferation of lymphocyte. It has already been used in clinical trials for the prevention of graft rejection, particularly in the context of renal transplantation (Tedesco-Silva, Szakaly et al. 2007).

Implementing models of HCC in immunocompetent animals is a crucial objective to study the cytotoxic activity of GML while preventing their rejection by the recipient's immune system. For this purpose, tumor cells from Balb/c mice (BNL-1 cells) were similarly transduced human cells so that they express the luciferase. The BNL-1 tumor cells and splenocytes from mice of different strains (C57BL/6 or FvB) used as a source of allogeneic lymphocytes can be administered to Balb/c mice as an immunocompetent mouse HCC model allowing to assess the selectivity of tolerizing, lympho-ablating or immunosuppressive regimen toward the alloimmunization against allogeneic lymphocytes. This model has already been used to demonstrate the possibility to induce a tumor regression by allogeneic activated lymphocytes while preventing their rejection, through the use of CsA (Leboeuf, Mailly, Wu et al., Mol Ther 2014, see Annex 2).

Using GML to prevent reinfection of the graft liver by HCV

Immunotherapy based on the use of liver graft lymphocytes in cirrhotic patients who have had HCC and treated by liver transplantation was described in 2009 (Ohira, Ishiyama et al. 2009). In this phase I study, the cells present in the graft were harvested, activated and cultured for three days, then administered to transplanted patients (between 2.10^8 and 5.10^8 cells per individual) with the initial aim to prevent HCC relapse after transplantation. A very significant reduction in viral load of some patients chronically infected with HCV was demonstrated. Moreover, an antiviral

activity of CIK cells was demonstrated in patients chronically infected with HBV (Shi, Fu et al. 2009). Based on these data, we focused on another potential clinical indication of our GML, namely their antiviral activity against HCV, for the prevention of graft reinfection after liver transplantation.

Although all three lymphocyte subsets (T, NK and NK-like T cells) are involved in the antiviral activity of GML via the production of IFN-γ, it seems that the observed effect is mediated by NK cells and NK-like T-cells rather than by T-cells. These results correlated with those published by Ohira et al. (Ohira, Ishiyama et al. 2009). The antiviral activity of GML is not altered by retroviral transduction, and does not seem to be improved by replacing the monoclonal antibody CD3 by co-immobilized CD3/CD28 antibodies. Furthermore, the antiviral activity of GML is not affected by the calcineurin inhibitors FK506 and CsA, which makes this approach feasible in patients with transplantation, receiving in each case an immunosuppression to prevent the rejection of the liver graft.

During the study of Ohira et al., five patients out of seven patients who were chronically infected with HCV and who had been transplanted and administered with *ex vivo*-activated liver graft lymphocytes showed their viral load decreasing (Ohira, Ishiyama et al. 2009). Viral RNA was not detected in two of them, a patient having eradicated even the infection. The activated hepatic lymphocytes had a more potent antiviral effect when the initial viral load was low. Conversely, with a high viral load, the antiviral effect was less potent. Hence, we reasoned that, at three days after liver transplantation, the liver graft may have already been reinfected; however, at the time of transplantation, the liver graft would be less likely to be reinfected. Therefore, the activated lymphocytes should be delivered at the time of transplantation. In such a case, this approach requires cell preparation prior to the acquisition of the liver graft; thus, the GML must be produced from other sources. Therefore, we considered the possibility of using third-party allogeneic GML produced from healthy blood donors. Furthermore, the prior *ex vivo* retroviral-mediated transfer of a suicide gene into the GML would allow their specific *in vivo* killing by the administration of a prodrug in case of side effects.

In our study, we showed that GML provided a potent antiviral effect at low effector-target cell ratios that are non-toxic for target cells. We tested GML on two *in vitro* HCV replication models, including the widely used HCVcc infection model. We observed that the SGML-mediated antiviral effect was more efficient when GML were added at time of HCV inoculation, and that the later the time of GML addition, the lower the efficacy. This finding confirmed our hypothesis that activated lymphocytes, such as GML, should be infused at the time of transplantation. The observation that an antiviral effect can be observed at low, non-toxic effector-target cell ratios suggest that the number of cells that need to be activated in order to produce the IFN-γ involved in the antiviral effect is lower than the number of cells required to induce a cytotoxic effect. This interpretation is substantiated by the observation that the CID-induced depletion of GML abrogated completely their cytotoxic activity, but only partially their antiviral effect: at high effector-target cell ratios, few GML may escape the CID-induced death and may be sufficient to generate an antiviral effect, but not a cytotoxic one.

Similarly to liver lymphocytes (Ohira, Ishiyama et al. 2009), peripheral blood mononuclear cells (PBMCs) subjected to CD3+IL-2 activation present an IFN-γ-mediated antiviral activity toward HCV and an antitumor activity toward HCC, both provided mostly by CD56+ cells, including CD3- CD56+ NKs and CD3+ CD56+ NK-like T cells (Doskali, Tanaka et al. 2011). These studies are consistent with our present results and with our recent report demonstrating that high concentrations of GML provided NK and NK-like T-cell-mediated antitumor effects in an HCC model (Leboeuf, Mailly et al. 2014). However, since they were generated after short-term (three days) activation with IL-2, this raises the question of the similitude of such activated lymphocytes, as well as of our GML, with "Lymphokine-Activated Killer" (LAK) cells. Indeed, the clinical use of LAK cells was limited owing to a massive release of cytokines and subsequent pulmonary oedema requiring intensive care. Our GML seem to be closer to ''Cytokine-Induced Killer'' (CIK) cells than to LAK cells in terms of protocol of activation (CD3+IL-2 for GML vs CD3+IL-2±IFN-γ ±IL-1 for CIK cells vs IL-2 for LAK cells) and culture

duration (two to four weeks for GML and CIK cells vs three to five days for LAK cells). One may hypothesize that this may lead to differences between LAK vs SGML (or CIK cells), as an example in terms of expression of homing molecules (thus, of homing properties) and/or exhaustion (thus, of potential for cytokine release, proliferation and cytotoxicity). Indeed, GML, evaluated in phase I to phase III clinical trials in patients transplanted with hematopoietic stem cells, induced limited toxicities (Bonini, Ferrari et al. 1997; Ciceri, Bonini et al. 2009; Zhou, Di Stasi et al. 2014) and, to the best of our knowledge, no pulmonary oedema.

Using a humanized HCC mouse model, we previously demonstrated that, when infused intravenously, GML preferentially homed to the liver and weakly migrate to the lungs (Leboeuf, Mailly, Wu et al., Mol Ther 2014, see Annex 2). This property is of advantage to improve the efficacy while limiting the peripheral spreading of GML. Indeed, infusing GML could allow reaching high local concentrations of IFN-γ, close to the target cells while leading to low peripheral IFN-γ concentrations. In this respect, this should be of great advantage, in terms of tolerability, over infusing exogenous recombinant IFNs, such as IFN-γ or IFN-α: the IFN-α toxicity is a strong limitation of the current pegylated-IFN-α-based regimen, due to the doses required to reach efficient concentrations in the liver, that also lead to high concentrations in peripheral, non-target organs and induce severe side effects. However, the use of allogeneic GML is associated with complex immunological interactions involving three immunological partners: the patient's immune system, the liver graft lymphocytes, and the allogeneic GML, generated from a third-party donor. Nevertheless, GML also have the advantage of being resistant to CsA inhibition. Thus, an immunosuppressive regimen with CsA can control the first two partners: (1) the recipient immune system alloreactivity toward the liver graft (prevention of liver graft rejection) and toward GML, and (2) the alloreactivity of grafted liver lymphocytes toward the recipient tissues (hepatic GvHD). Alternatively, the third partner, the GML, can be controlled with the suicide gene approach without affecting the first two partners.

One important issue is to determine whether there is any advantage in using

third-party GML, which are genetically different from the liver graft, compared to using autologous liver graft-derived lymphocytes. This hypothesis is technically challenging to investigate, because HCV replication is typically evaluated *in vitro*, with established hepatoma cell lines. Indeed, primary human hepatocytes are mostly refractory to HCV infection *in vitro*. Thus, comparing the anti-viral activity of GML toward autologous vs. allogeneic primary human hepatocytes remains unfeasible. Indirect observations have suggested that alloreactivity may provide additional benefits. When activated lymphocytes were restimulated with CD3 mAbs 24 h before evaluation of their antiviral activity, their ability to decrease HCV replication improved (Doskali, Tanaka et al. 2011). Also, allogeneic HSC transplantation (Henrich, Hu et al. 2013), but not autologous HSC transplantation (Cillo, Krishnan et al. 2013), was reported to lead to loss of detectable HIV, which was temporally correlated with full donor chimerism, but also to the development of GVHD, a complication of HSC transplantation associated with alloreactivity. Although the mechanisms leading to a HIV cure after HSC transplantation remain to be elucidated, and although they may be different from those involved in the prevention of HCV replication, those results suggested that alloreactivity may add benefit to an antiviral effect. Taken together, our results have provided *in vitro* proof-of-concept that an adoptive immunotherapy can be safely applied to the efficient prevention of liver graft reinfection by HCV.

In the further project, a lot of work needs to be done before putting the GML into clinical use: first, apart from the evaluation of the antiviral activity of GML through replicon systems and HCVcc, the next step is to provide the *in vivo* proof-of- concept. We plan to use our HCV infection model in human liver-chimeric Alb-uPA/SCID-bg mice to test our GML. These immuno- and hepato-deficient mice reconstituted with human hepatocytes are a model of HCV infection for which we already have a strong experience as a result of the study of the prevention of HCV infection. Based on this mouse model, several crucial questions need to be solved such as the dose of GML administration leading to efficient *in vivo* antiviral effect without cytotoxicity toward PHH and murine hepatocytes. Second, because of the

variety of HCV phenotypes seen in clinic, we will try to test the efficiency of our GML against different subsets of HCV such as HCV gt1a, 1b, 4 etc. Third, compare and combine our GML strategy with other classic or innovative antiviral therapy strategies, to develop the best effect, curable and lowest side-effect therapeutic outcome. So it will be interesting to study the effect of GML in both prevention and treatment of infection.

Creating a bank of ready-to-use allogeneic GML

Whatever the clinical application, our ultimate purpose is to create a bank of allogeneic GML as "ready-to-use" effector cells. Compared with allogeneic autologous approaches (CIK, liver cells), the advantages of our approach are numerous. First, it will be possible to establish a production of GML at very large scale. Lymphocytes from healthy donors are much easier to grow and expand than those from patients who are often subject to heavy treatments. We felt that from a sample of 10.9 CMN, it may be possible to get 50 x 10.9 GML, meaning that it could allow to perform 100 injections on the basis of the average dose used in the study of Ohira et al. (5 x 10.6 cells / kg). Second, cells are ready-to-use, which means that they can be administered to the patient immediately, at the time of liver transplantation for example. So there is no loss of time associated with the production and qualification of the cells. Third, if a clinical batch for a given patient cannot be administered for medical reasons or other reasons (such as non-compliance), the cells can still be used for another patient, thereby preventing the loss of clinical lots produced in vain.

Thus, the creation of a bank of ready-to-use cells would be extremely beneficial from a medical, logistic and financial point of view and relevant in terms of industrial development in comparison with conventional approaches autologous immunotherapies which are to produce, for one specific batch of cells for each patient.

DISCUSSION (version française)

Partie I

Nous avons choisi un modèle de xénogreffe de cellules hmaines de CHC (lignée Huh-7 exprimant le gène rapporteur de la luciferase : Huh-7-Luc) chez la souris comme modèle expérimental de base pour des études ultérieures, car les lignées cellulaires de CHC injectées à des souris sont susceptibles de maintenir leurs caractéristiques cellulaires telles que la stabilité de la morphologie, la capacité clonogénique et les caractéristiques moléculaires. Afin de normaliser un protocole pour l'établissement d'un modèle orthotopique de CHC chez la souris, nous avons essayé d'optimiser le mode d'injection des cellules de CHC en comparant les trois modes d'injection les plus utilisés: intrahépatique (IH), intrasplénique (IS) et intraportal (IPT).

En ce qui concerne la mortalité, les injections IH et IS ont montré des résultats plus encourageants par rapport à l'injection IPT. Cela peut s'expliquer par trois raisons: d'abord, l'injection IPT est techniquement plus difficile en raison de l'exposition et l'injection dans la veine porte est évidemment plus complexe que les autres car l'accès à la veine est mal aisé par rapport à l'accès au foie ou à la rate. En outre, le risque d'hémorragie est élevé: l'hémostase ne peut être faite qu'une seule fois. Si la ponction dans la veine a été mal conduite, il n'est pas possible de refaire une deuxième ponction dans un autre endroit, de sorte que le manipulateur n'a qu'une seule chance! Deuxièmement, plus de complications se sont produites lors de l'injection: exceptée l'hémorragie péri- et post-opératoire, incontrôlable, les cellules peuvent induire un thrombus de la veine porte ou éventuellement une insuffisance hépatique semblable à un hepatodeficience aiguë conduisant à la mort dans les 2-3 heures, ou semblable à une hepatodeficience chronique conduisant à la mort dans les 1-2 jours après la chirurgie. En troisième lieu, des souris sont mortes probablement en raison de la procédure plus longue, ce qui augmente la perte de chaleur et ainsi la sensibilité à des complications induites par l'anesthésie. La perte de chaleur est encore renforcée par l'exposition des viscères (requis pour accéder à la veine porte), malgré les tentatives

de réduire la perte de chaleur par les viscères par lavage avec du PBS préchauffé. En revanche, les injections IS et IH sont relativement simples. Chacune de ces deux approches a ses propres caractéristiques: les cellules cancéreuses se propagent bien dans le foie après injection IS, qui est peut-être plus appropriée pour des recherches portant sur les métastases dans le foie. Toutefois, le taux de localisation hépatique des cellules tumorales était plus faible dans le groupe de IS dans le groupe IH: un lot de souris a présenté des tumeurs soit dans la rate et/ou dans les intestins après injection IS. Il est possible que les cellules tumorales ont bloqué la veine splénique ou ont reflué dans les veines intestinales pendant le processus de migration vers le foie. Globalement, nous considérons que l'injection IH est une procédure préférable pour établir des modèles orthotopiques de CHC chez la souris sur la base de la plus faible mortalité, la manipulation simple et le taux de souris disponibles pour l'expérimentation plus élevé.

L'une des difficultés pour le modèle orthotopique de CHC chez la souris est la manière de localiser le site de la tumeur avant d'utiliser les animaux en expérimentation. Contrairement aux modèles sous-cutanés, où les tumeurs sont faciles à gérer et à localiser, identifier précisément le site de la tumeur avant de lancer l'expérience est crucial dans les modèles orthotopiques, en particulier dans les expériences relatives à l'essai de nouvelles stratégies et agents thérapeutiques. En effet, la fonction de foie ainsi que le tropisme de certains agents (tels que nanovecteurs ou les cellules immunitaires utilisées dans les approches d'immunothérapie) est un élément clé pour le succès de la stratégie. Il n'était pas possible dans certaines souris d'établir une distinction entre des tumeurs intrahépatiques ou périhépatiques par BLI (les résultats de l'autopsie chez ces souris ont confirmé cette hypothèse), cette modalité d'imagerie en deux dimensions étant normalement suffisante pour les modèles sous-cutanés. Par conséquent, les dispositifs d'imagerie en trois dimensions, tels que l'IRM, sont une bonne alternative pour distinguer les sites tumoraux. Nos résultats ont montré que; après l'injection IH il ya une bonne corrélation entre BLI (analyse sur les régions d'intérêt de ''soi-disant'' tumeurs intra-hépatiques) et IRM (analysé uniquement sur les tumeurs intra-hépatiques confirmées) en ce qui concerne

la cinétique de croissance des cellules Huh-7 dans le foie, ce qui suggère que l'injection IH est une approche intéressante pour l'établissement de tumeurs orthotopiques.

En plus de la caractérisation de la localisation précise de la tumeur et de la cinétique de croissance de la tumeur, un modèle standardisé de CHC devrait comprendre une caractérisation de la lignée cellulaire et l'analyse histologique. Par conséquent, dans nos recherches futures, la reproductibilité des expériences sera assurée en utilisant des aliquotes de cellules Huh-7-luc cryoconservés obtenus à partir d'une banque secondaire de cellules établie elle-même à partir d'une banque primaire de cellules. Cette banque primaire de cellules a été caractérisée en termes de:

-absence de mycoplasmes, contrôlée deux fois, au moment de la cryoconservation de la banque primaire de cellules et un mois auparavant

-absence de 27 agents pathogènes murins, vérifiés par PCR afin d'assurer une sécurité des injections de cellules chez les souris

-stabilité de l'activité de bioluminescence au cours de la culture in vitro

L'identité et la clonalité des cellules Huh-7-Luc restent à confirmer, respectivement par empreinte génétique et par séquençage des régions flanquantes du transgène luciférase par LAM-PCR (Dr Ferrand, EFS Bourgogne / Franche-Comté). L'intensité de leur activité de bioluminescence doit également être quantifiée en termes de photons/seconde/cellule. Puisque vascularisation de la tumeur est un paramètre clé pour l'étude de traitements CHC innovants, tels que les produits cellulaires d'immunothérapie ou les nanovecteurs, une analyse histologique est en cours afin de caractériser la cinétique de la néovascularisation tumorale après l'injection des cellules Huh-7-Luc.

Cependant, de nombreuses observations sont maintenant en train de réévaluer la valeur des modèles de souris dans le développement de recherches liés au cancer, du fait que le cancer est une maladie complexe avec des interactions imbriquées entre les cellules transformées, des mutations oncogéniques, et l'environnement des cellules tumorales, tel que les cellules normales, les cellules stromales et les cellules immunitaires (Hanahan et Weinberg, 2000; Frese et Tuveson 2007). Par analogie avec

d’autres types de tumeur, on peut supposer que plusieurs différences fondamentales existeraient si l'on comparait des xénogreffes de tumeurs de lignées transformées de cellules (telles que des cellules Huh-7 ou HepG2 ou PLC-PRF5) par rapport à des xénogreffes d'échantillons de tumeurs primaires provenant de patients (modèles PDX), telles qu’une hétérogénéité génétique plus importante et une architecture tumorale plus conservée dans des modèles PDX que dans les tumeurs dérivées de cellules de lignées. Surtout, les caractéristiques du microenvironnement de la tumeur, telles que les tissus voisins normaux, les cellules stromales, vasculaires, la circulation lymphatique et les cellules immunitaires, pourraient être modifiés ou perdues dans les xénogreffes de tumeurs dérivées de la lignée cellulaire (Sikder et al. 2003). Ces changements dans les cellules tumorales et le micro-environnement sont probablement utiles dans l'évaluation thérapeutique de composés. Par exemple, des anti-néoplasiques ayant une activité démontrée dans des xénogreffes de lignées cellulaires ne sont pas efficaces dans la pratique clinique (Johnson et al. 2001).

Une solution possible pour résoudre le problème des différences entre les lignées cellulaires de cancer et les tumeurs des patients est l'implantation chirurgicale orthotopique de fragments intactes d’échantillons de cancer humain, directement récoltées à partir du patient et transplantés dans l'organe correspondant de rongeurs immunodéprimés. De tels modèles PDX ont été appliquées avec succès pour les cancers du sein, du poumon, de la prostate et le cancer du colon, entre autres. En association avec notre expérience précédente, une implantation chirurgicale orthotopique des échantillons CHC primaires actuellement à l'étude, en collaboration avec le Pr D. Somacalle et le Dr T. Piardi (Pôle Digestif / Urologie / Néphrologie / Endocrinologie, CHU Reims). Ces modèles PDX seront utilisés dans des expériences futures pour l'évaluation des nanovecteurs en cours de développement, permettant de réaliser simultanément l’imagerie et le traitement du CHC.

Par rapport aux modèles de souris, les modèles porcins ont des caractéristiques prédominantes comme les similitudes avec l'homme en termes de taille du corps, d'anatomie, et de réponses physiologiques et physiopathologiques. Un grand nombre de chercheurs a mis l'accent sur l'utilisation de modèles de porc dans des études

précliniques pour aider au développement de thérapies antitumorales chez l'homme. Diverses stratégies ont été envisagées pour établir un modèle de CHC chez le cochon, qui peuvent être divisées en trois catégories: d'abord, générer des tumeurs ‘’fantômes’’ imitant les structures de la tumeur par injection de gels tels que l'agarose, la cellulose ou le glycérol (N'Djin et al., 2007) et le plastique liquide (Hidalgo et al., 2005). Deuxièmement, induire artificiellement des tumeurs porcines par l'administration de substances chimiques cancérogènes (Li et al., 2006). Bien que ces procédures puissent être utilisées pour des applications spécifiques, il nécessite beaucoup de main-d'œuvre et peut soulever des préoccupations éthiques. Troisièmement, la conversion de cellules somatiques du porc en un état tumorigène par transfert d’oncogènes. Deux équipes ont établis avec succès des modèles de tumeur à partir de cellules colorectales et des cellules fibroblastiques respectivement (Adam et al 2007;. Flisikowska et al 2012.).

En effet, il a été prouvé que chez l'homme, les cellules somatiques normales pourraient être converties dans un état tumorigène par transduction de plusieurs oncogènes afin de perturber les suppresseurs de tumeurs et activer des proto-oncoprotéines. En effet, l'inactivation des voies de la p53 et de Rb par l'expression d'une protéine p53 dominant-négative (p53DD), l’activation de la cycline D1 et d’une kinase cycline-dépendante (complexe cycline D1/CDK4^{R24C}) en conjonction avec l'activation des voies de c-Myc, Ras et de hTERT, par l'expression de formes oncogéniques de c-Myc (c-Myc^{T58A}) et H-Ras (H-Ras^{G12V}) peut conduire des cellules rénales humaines, des cellules épithéliales mammaires et les myoblastes dans un état tumorigène (Kendall et al. 2,005). Chez le porc, la co-expression de hTERT, p53DD, la cycline D1, CDK4^{R24C}, c-Myc^{T58A} et H-Ras^{G12V} a également transformé des cellules de fibroblastes de porc normale en un état tumorigène (Adam et al., 2007). Sur la base de ces données, nous avons essayé d'établir un modèle de CHC orthotopique porcine par la co-expression de ces oncogènes.

Sur la base de ces données encourageantes, nous avons essayé d'établir un modèle porcin de CHC orthotopique par l'introduction par voie lentivirale de ces six oncogènes dans les hépatocytes porcins afin de les convertir en cellules de CHC.

Contrairement aux deux études précédentes, notre projet est basé sur des hépatocytes porcins, qui sont difficiles à générer et cultiver in vitro. Ainsi, une sélection précise et efficace du plasmide accepteur dans lequel seront clonés les oncogènes est une condition préalable à la production de vecteurs lentiviraux. Par rapport aux deux autres vecteurs candidats, le plasmide de CMV pLenti V5-LUC Blast a montré une haute efficacité de transduction dans des lignées cellulaires ou dufférentes cellules hépatocytaires primaires. Fait intéressant, quand il a été testé dans des hépatocytes porcins primaires (HPP), le plasmide pLenti CMV V5-LUC Blast a permis également d'obtenir une activité luciférase élevée, ce qui indique que ce vecteur a une capacité de transduction similaire ou même plus élevée dans les HPP que dans les hépatocytes primaires humaines (HPH) ou même que dans la lignée cellulaire d'hépatome Huh 7.5.1.

Cependant, en raison du risque potentiel de manipuler des vecteurs lentiviraux codant des oncogènes, un laboratoire de biosécurité de niveau 3 est obligatoire. Cependant, par rapport à un niveau de biosécurité 2, la prévention des risques biotechnologiques lavel 3 améliore le niveau de protection pour l'environnement, mais pas pour le manipulateur, dont la protection est assurée par la manipulation sous un poste de sécurité microbiologique de classe II (que ce soit dans un laboratoire avec confinement de niveau 2 ou de niveau 3). Par conséquent, l'amélioration de la sécurité du manipulateur était obligatoire pour notre projet. C'est pourquoi nous avons souhaité utiliser une culture en système fermé pour la production de vecteurs lentiviraux et la transduction de HPP. Toutefois, un tel système ne devrait pas nuire à la capacité de production de lentivirus ainsi qu'à l'efficacité de transduction, par rapport aux systèmes classiques de culture ouverts. En effet, en comparant avec le système classique «ouvert», notre système de culture clos a présenté de grands avantages: (i) la sécurité, l'ensemble du processus expérimental est réalisé dans un système complètement fermé, diminuant les risques de contamination des cellules et des surnageants et (ii), il peut également protéger les manipulateurs d'entrer en contact avec les vecteurs lentiviraux potentiellement dangereux. De façon intéressante, il peut être utilisé comme outil pour la cryoconservation directe des cellules transduites dans

la cassette de culture. Il est important de noter, comme le montrent nos résultats, que la capacité de production de vecteurs lentiviraux et l'efficacité de transduction sont comparables à ce qui est obtenu en système ouvert. Ainsi, ce système clos pourrait être utilisé pour d'autres expériences de transfert de gène qui présentent des risques pour le manipulateur.

En raison de la différence entre les hépatocytes avec d'autres cellules somatiques et du fait que l'ensemble du génome porcin est encore incomplètement connu, nous avons supposé qu'il est possible que le processus de transformation oncogénique dans les HPP ne soit pas similaire à celui d'autres types de cellules, ce qui signifie que l'état tumorigène pourrait être induit par différents oncogènes ou par différentes combinaisons d'oncogènes, Nous avons produit trois vecteurs lentiviraux différents codant tous la luciferase et soit hTERT/p53^{DD}, soit cyclin D1/CDK4^{R24C}, soit c-Myc^{T58A}/HRas^{G12V}.

Nous avons transduit des HPP avec chaque vecteur seul ou en combinaison afin d'étudier l'effet de chacune des combinaisons d'oncogènes. Comme le montrent les résultats, tous les groupes expérimentaux permettent une expression stable de la luciférase in vivo pendant une longue période (jusqu'à l'euthanasie), ce qui suggère que les vecteurs sont capables de transduire les HPP. Fait intéressant, dans les souris SCID-bg, transplanté en situation orthotopique avec des HPP transduits avec les 3 vecteurs, le niveau d'expression de la luciférase augmente de façon continue, bien qu'il soit plus faible au début de l'expérience que dans le groupe témoin. Ceci suggère que l'état tumorigène peut avoir besoin de plusieurs semaines avant d'établir des modèles orthotopiques de CHC.

In vitro, nous avons également testé l'expression de protéines différentes. Les résultats ont montré que les protéines transgéniques ont été détectées. Notamment l'expression du dominant négatif de p53^{DD} et les proto-oncoprotéines telles que c-Myc^{T58A} ont été détectées à un niveau relativement élevé comparé aux autres protéines transgéniques.

Cependant, dans les foies de souris transplantées en situation orthotopique avec des HPP transduits avec les trois vecteurs, nous n'avons pas trouvé un état

tumorigène évident même si le niveau d'expression de la luciférase était sensiblement élevée. Ceci est renforcé par l'observation que, lorsque le tissu hépatique a été retransplanté par voie sous-cutanée dans des souris receveuses secondaires, aucune tumeur n'a été trouvée. Plusieurs raisons possibles peuvent être envisagées, y compris: d'abord, tous les oncogènes peuvent ne pas être exprimés en même temps, ou leur expression peut être perturbée par des protéines endogènes des HPP; d'autre part, le niveau de la protéine transgénique peut être inférieure dans les HPP à celle d'autres cellules (ce qui reflète le fait que la fonction et la capacité de prolifération sont différents dans les HPP, des lignées cellulaires telles que les cellules 293T ou d'autres cellules somatiques telles que les cellules fibroblastiques). Par conséquent, des niveaux plus élevés d'expression de l'oncogène peuvent être nécessaires dans les HPP. Dans les expériences futures, afin de résoudre les questions ci-dessus, nous allons nous concentrer sur: (i) le test de l'expression de chaque vecteur dans d'autres lignées cellulaires (telles que les cellules 293T ou Huh 7.5.1) afin de déterminer si leur expression est influencée par des événements cellulaires intrinsèques (par exemple, l'état de prolifération, qui sera modifiée par déprivation de sérum) et (ii) la comparaison de l'expression de chaque plasmide dans différentes cellules somatiques porcine (hépatocytes, cellules fibroblastes, cellules ou colorectal) afin d'identifier si nous devons changer les oncogènes ou les combinaison d'oncogènes uilisées, ou l'ordre d'addition de vecteurs lentiviraux.

Dans l'ensemble, bien que cette approche soit en cours, nous nous attendons à ce que, une fois qu'il sera établi et validé chez la souris immunodéficientes avec succès, nous pourrions transférer cette expérience chez le porc. Ce modèle porcin pourrait apporter beaucoup d'avantages pour les études précliniques. En premier lieu, la commutation de la tunorigénèse de cellules porcines est probablement plus proche du processus chez l'homme que les modèles de souris. Deuxièmement, les tumeurs qui en résultent dans les porcs pourraient atteindre de très grandes dimensions, ce qui permettrait un certain nombre d'applications précliniques, non seulement pour la procédure chirurgicale, mais également la validation de nouvelles molécules antitumorales ou l'investigation de la voie de signalisation. En troisième lieu, si la

transformation des cellules, outre des hepatocytes et des fibroblastes, peut être réalisée, différents types de cellules peuvent produire des tumeurs. Nous pourrions alors espérons qu'il est possible de générer de nombreux types de tumeurs potentiellement n'importe où dans le corps. Enfin, en utilisant notre système de culture fermée, toute la procédure est tout à fait sure et presque aussi rapide que les modèles de xénogreffe de souris existants. Ainsi, l'onco-modèle porcin sera un espoir comme un modèle pertinent pour des études précliniques de cancers humains.

Partie II

Nous proposons d'utiliser des lymphocytes génétiquement modifiés (LGM) exprimant un gène suicide comme HSV-tk ou iCasp9 pour deux applications cliniques majeures, le traitement du CHC en combinaison avec d'autres thérapies actuellement disponibles, et la prévention de la réinfection du greffon par le VHC chez les patients recevant une transplantation hépatique.

Les lymphocytes génétiquement modifiés comme immunothérapie adjuvante du CHC

L'immunothérapie basée sur l'administration de LGM allogéniques dans le traitement des hémopathies malignes a été montré comme étant bénéfique pour les patients et ceci sans effets secondaires majeurs. En effet, les lymphocytes allogéniques exprimant le gène HSV-tk ont été largement utilisés dans plusieurs études cliniques en combinaison avec une greffe de cellules souches hématopoïétique (CSH) (Bonini et al 1997; Tiberghien et al. 2001; Ciceri et al. 2009.). Cette dernière permet la reconstitution du système immunitaire, tandis que les LGM fournissent une forte activité antileucémique contre les cellules cancéreuses résiduelles. Sur la base de ces propriétés anti-tumorales contre des tumeurs malignes hématologiques, nous avons essayé de déterminer si ces cellules pourraient avoir une activité anti-tumorale contre les tumeurs solides, en particulier contre le CHC. L'hypothèse de cette nouvelle application clinique est renforcée par le fait qu'un autre produit de thérapie cellulaire a fait l'objet de nombreuses études cliniques pour le traitement de tumeurs solides, des

cellules CIK. Ces cellules sont récoltées à partir de lymphocytes du sang du patient et ensuite activées en culture in vitro, puis réinjectées en situation autologue. Plusieurs essais cliniques ont été réalisées chez les patients atteints de cancer colorectal, cancer du rein, cancer de l'estomac ou CHC (Sangiolo et al., 2008). Utilisé en combinaison avec les traitements standards (de la chimiothérapie, la résection chirurgicale), les cellules CIK ont une activité anti-tumorale significative et aident de manière significative à améliorer la survie des patients, en particulier lorsqu'elles sont utilisées chez les patients atteints de CHC (Hui et al., 2009) (Kim et al., 2007). Dans notre étude, nous avons comparé notre LGM avec des cellules CIK phénotypiquement et fonctionnellement au moyen de protocoles d'activation différentes et culture décrits dans la littérature pour la production de CIK (Weng et al 2008;. Ayello et al, 2009;. Olioso et al., 2009). Nous n'avons pas observé de différence significative entre les cellules CIK et les LGM en terme de pourcentage de T, NK et NK-T et au niveau de leur potentiel cytotoxique contre les cellules Huh7 in vitro (données non présentées). Par conséquent, nous avons conclu que nos LGM sont des CIK génétiquement modifiés, ce qui suggère fortement qu'ils pourraient avoir un effet antitumoral contre le CHC (Wu et al, manuscrit en préparation -. Ces résultats n'ont pas été inclus dans la thèse en cours).

La capacité de contrôle de l’alloréactivité des LGM est une composante essentielle de notre approche. Les cellules alloréactives peuvent être toxiques pour les tissus sains du patient et peuvent induire une (réaction du greffon contre l’hôte) GvHD ou un rejet de greffe de foie. Nous avons opté pour le transfert des gènes suicide HSV-tk ou iCasp9 dans nos cellules LGM, qui peuvent ensuite être éliminées conditionnellement par leurs prodrogues respectives, le ganciclovir (GCV) et l’inducteur chimique de mort (CID, également appelée AP1903) (Garin et al, 2001; Di Stasi et al 2011).

Un nouveau vecteur appelé "iCasp9-CD19" a été récemment mis au point par l'équipe de Brenner. Le gène codant pour iCasp9 comprend une partie de la caspase 9 humaine (en l’occurrence, le dommaine effecteur de la caspase), et le domaine de liaison du FK506 de la protéine de liaison de FK506, FKBP12. En présence d'AP1903,

un analogue non immunosuppresseur du FK506 qui se lie avec une haute affinité à FKBP12, une dimérisation de iCasp9 a lieu, ce qui entraîne l'activation des caspases 3, 6 et 7 et conduisant à l'apoptose de la cellule transduite. Le transgène du iCasp9 est fusionné au gène codant pour le marqueur membranaire CD19, ce qui permet la sélection de cellules transduites par tri immunomagnétique. Ce vecteur a été utilisé pour la transduction des lymphocytes T allogéniques qui ont été administrés à cinq patients pédiatriques atteints de leucémie en rechute après greffe de CSH. En cas de GvHD (grade de GvHD I sur 4 patients), les patients ont été traités avec une dose unique (0,4 mg / kg) de AP1903 qui permet l'élimination de plus de 90% des cellules génétiquement modifiées en 30 minutes pour mettre fin à la GvHD (Di Stasi et al., 2011). Compte tenu de cette élimination très rapide, il sera possible d'utiliser ce vecteur pour la transduction des lymphocytes allogéniques en situation clinique.

La caractérisation de l'activité cytotoxique de nos LGM a été un point central de notre travail. Le LGM sont très cytotoxiques in vitro contre les cellules HeLa (contrôle positif de sensibilité à la lyse) et les cellules de différentes lignées de CHC. Les cellules produisent de grandes quantités d'IFN-γ et, dans une moindre mesure, de TNF-α, les deux cytokines peuvent coopérer pour médier la cytotoxicité de LGM (Wang, et al. 2012). En effet, nos résultats montrent que l'addition simultanée d'anticorps bloquants anti-IFN-γ et de TNF-α au cours de la co-culture de cellules cibles et de LGM inhibe partiellement l'activité cytotoxique de LGM, tandis que les anticorps seuls n’ont pratiquement aucun effet.

En plus de la présence nécessaire d’IL-2 au cours de l'expansion de LGM, l'IL-2 est également nécessaire pour assurer la cytotoxicité maximale au cours de la co-incubation des LGM avec les cellules cibles. En effet, les LGM se sont montrés très peu cytotoxiques sans IL-2. Par conséquent, il peut être nécessaire de combiner l'IL-2 aux LGM lorsqu'ils sont administrés in vivo, comme on l'a fait dans les études précliniques chez la souris. Cela n'a pas été fait dans les essais cliniques d'administration de LGM chez les patients ayant reçu une allogreffe de CSH. Cependant, dans une étude (Slavin et al., 2010) des patients traités par lymphocytes activés allogéniques (semblables à nos LGM, mais sans transfert de gènes) ont reçu

également de l'IL-2 recombinante sous-cutanée (6.106UI / m2 par jour) pendant 5 jours après l'administration des cellules. L'IL-2, associée à l'administration d'indométhacine pour réduire la fièvre et les réactions inflammatoires, a été bien tolérée chez la plupart des patients. La durée de l'administration d'IL-2 a été limitée à trois jours chez certains patients en raison de l'apparition d'effets secondaires (Slavin et al. 2010).

Afin de déterminer l'impact de la transduction rétrovirale sur la fonctionnalité des LGM, dont l'alloréactivité a été montré pour être compromise en raison du transfert de gène (Sauce et al, 2002; Marktel et al 2003), la cytotoxicité des LGM contre les cellules Huh7 a été comparée à celle des cellules non transduites cultivées et non sélectionnés. Aucune différence n'a pu être observée entre LGM et des cellules témoins, ce qui indique que la transduction par voie rétrovirale n'affecte pas l'activité cytotoxique des LGM.

Les cellules CIK sont largement étudiées pour leur activité anti-tumorale contre le CHC, et elles sont similaires à nos LGM sur plusieurs points:

-activation par CD3 + IL-2 (+ / -IL-1, IL-7, IL -15 et/ou IFN-γ) - mais sans transfert de gène.

-les cellules cytotoxiques sont principalement des lymphocytes de type NK (Shi et al., 2004) (Wang et al., 2002), qui sont les lymphocytes T qui ont acquis l'expression de CD56 et de récepteurs NK pendant la culture in vitro

- cytotoxicité non restreinte par le CMH, médiée par TRAIL, IFN-γ et TNF-α

Ainsi, il était intéressant de déterminer, dans nos LGM, quelle sous-population de lymphocytes était responsable de leur cytotoxicité et quels mécanismes de reconnaissance et de lyse des cellules cibles ont été impliqués.

Le LGM se composent essentiellement de cellules lymphocytes T, cellules NK-like, et enfin un faible pourcentage de cellules NK. Après séparation par tri immunomagnétique, les cellules NK ont été jugés les plus cytotoxiques des cellules, suivie des cellules T de type NK puis des cellules T. Considérant que la plupart des cellules sont moins cytotoxiques (cellules T) et que les cellules les plus rares sont plus cytotoxiques (cellules NK), la contribution relative de chaque sous-population a été

examinée par un test de liaison des cellules effectrices aux cellules cibles. La plupart des cellules liées à des cellules cibles sont des cellules T de type NK. Ceci suggère fortement que les cellules T de type NK jouent un rôle clé dans la lyse des cellules cibles (Leboeuf, Mailly, Wu et al., Mol Ther 2014, voir l'annexe 2). L'analyse in vivo confirme ce résultat: dans le foie de souris perfusées avec le LGM, la fréquence des cellules NK et les cellules T NK-like a été augmenté, tandis que la fréquence des cellules T a été réduite, par rapport à la répartition de la sous-ensemble initial observé dans la suspension de cellules avant la perfusion, ce qui indique que la cytotoxicité in vivo des LGM consiste principalement en cellules NK et / ou cellules T de type NK. Ces résultats sont en accord avec les caractéristiques phénotypiques et fonctionnelles des CIKs.

L'injection intraveineuse de LGM, quatre jours après l'administration de l'injection de cellules tumorales est un modèle pertinent: l'injection IS des cellules tumorales permet la diffusion de cellules dans la veine porte et une distribution homogène dans le foie, ce qui est différent du modèle d'injection IH . Des expériences préliminaires ont montré que le LGM administré par injection intraveineuse migrent préférentiellement vers le foie. Le tropisme hépatique peut être expliqué par l'acquisition de I-CAM-1 et V-CAM-1 molécules (Hamann et al., 2000) pendant la culture in vitro, et pourrait permettre d'obtenir la cytotoxicité ciblée dans le foie tout en limitant la propagation des cellules effectrices à travers le corps et une toxicité limitant ainsi possible périphérique.

Cependant, un obstacle majeur pour obtenir une activité cytotoxique efficace de LGM est le manque d'accessibilité à des cellules tumorales. En effet, les cellules tumorales sont entourées par un stroma, aussi lui-même composé de cellules stromales, de fibroblastes et de cellules inflammatoires) et d'une matrice contenant du collagène de type IV, la laminine et des protéoglycanes (Yang et al. 2011). La matrice extracellulaire est une barrière physique limitant l'accès et la diffusion de LGM dans la tumeur (Li et al. 2009). Par conséquent, un développement possible de notre projet, pour l'avenir, pourrait être d'évaluer la possibilité de développer et d'utiliser un autre vecteur rétroviral. Ce vecteur combiner aitle gène suicide et un gène codant pour une

métalloprotéinase (Mok et al., 2007) ou la relaxine (Li et al., 2009), un facteur soluble induisant la synthèse des métalloprotéinases. Ces protéines pourraient permettre au LGM de dégrader la matrice extracellulaire servant de support aux cellules tumorales et à infiltrer de manière plus efficace de la masse tumorale.

Un autre point important est l'allo-immunisation contre les LGM. Les LGM peuvent être rejetés par le système immunitaire du receveur. Cela peut être considéré comme un deuxième dispositif de sécurité (en plus du gène suicide) pour éviter la toxicité des LGM pendant une longue durée. D'un autre côté, il serait aussi responsable d'une efficacité anti-tumorale réduite. Nous avons montré que notre LGM sont résistants in vitro et in vivo à deux inhibiteurs de la calcineurine, CsA et FK506, les deux étant utilisés habituellement en transplantation. En effet, la cytotoxicité des LGM n'est pas modifiée en leur présence. La résistance de LGM à la CsA et au FK506 sont en raison de leur état d'activation due à la culture in vitro de cellules puisque des cellules mononucléées non manipulées sont sensibles à ces deux médicaments. Lors de son entrée dans une cellule au repos, la CsA se lie à la cyclophiline, une protéine cytoplasmique. Ce complexe interagit avec la calcineurine, ce qui empêche la déphosphorylation du facteur de transcription de NF-AT et sa translocation vers le noyau pour sa fixation sur le promoteur du gène codant pour l'IL-2 (Flanagan, Corthesy et al. 1991). Ce phénomène empêche les cellules au repos d'être activées, tandis que la CsA n'a aucun effet sur des cellules en culture, car elles ont été préalablement activées et NF-AT est déjà présent dans le noyau (Contassot et al. 1998).

Néanmoins, il ne semble guère possible d'utiliser des inhibiteurs de la calcineurine chez les patients immunocompétents atteints de CHC, car ils pourraient supprimer l'immunité anti-tumorale des patients; donc d'autres approches immunomodulatrices devraient être développés.

Dans l'étude d'administration de lymphocytes allogéniques activés (non génétiquement modifiées) pour le traitement d'hémopathies malignes et de tumeurs solides métastatiques (mais pas de CHC) (Slavin, Ackerstein et al., 2010), une dépletion conditionnelle des lymphocytes constituée de fludarabine (25mg / m^2) ou de

cyclophosphamide (1000 mg / m²), est effectuée avant la perfusion de lymphocytes allogéniques. Ce conditionnement permet en premier lieu de créer une «niche» pour les lymphocytes allogéniques qui peuvent alors exercer leur activité anti-tumorale, et en second lieu d'éliminer les cellules T régulatrices de l'hôte qui pourraient compromettre l'effet anti-tumoral des cellules allogéniques. L'absence de traitement immunosuppresseur après l'administration de lymphocytes allogéniques entraîne le rejet de cellules allogéniques par le système immunitaire du receveur dans un délai jusqu'à une semaine, ce qui empêche peut-être l'apparition de GvHD. En effet, la sécurité de cette approche est basée sur le rejet précoce des cellules allogéniques par le système immunitaire du receveur. L'effet anti-tumoral est supposé être très rapide et avoir lieu avant que les cellules n'aient été rejetées, comme l'a montré la survie sans progression de la maladie chez les patients 4 sur 5 d'une hémopathie maligne, et 5 des 21 patients atteints de tumeurs métastatiques (Slavin et al., 2010).

Cependant, l'utilisation de l'allo-immunisation du destinataire en tant que système de contrôle de sécurité n'est pas satisfaisante. Un autre conditionnement possible pourrait impliquer l'utilisation de FTY720, un immunomodulateur induisant la séquestration des lymphocytes dans les organes lymphoïdes, empêchant ainsi leur propagation dans la périphérie (Tedesco-Silva et al., 2007). La molécule n'induit pas une immunosuppression généralisée parce qu'elle n'affecte pas l'activation et la prolifération des lymphocytes. Elle a déjà été utilisée dans des essais cliniques pour la prévention du rejet de greffe, en particulier dans le contexte de la transplantation rénale (Tedesco-Silva et al. 2007).

La mise en œuvre des modèles de CHC chez des individus immunocompétents est un objectif crucial pour étudier l'activité cytotoxique de LGM, tout en empêchant leur rejet par le système immunitaire du receveur. A cette fin, des cellules tumorales de souris Balb/c (cellules BNL-1) ont été transduites de façon similaire aux cellules humaines pour qu'elles expriment la luciférase. Les cellules tumorales BNL-1 et les splénocytes de souris de souches différentes (C57BL / 6 ou FvB) utilisés en tant que source de lymphocytes allogéniques peuvent être administrés à des souris Balb/c comme un modèle de carcinome hépatocellulaire de souris immunocompétentes. Ceci

permet d'évaluer la sélectivité et l'efficacité de traitements immunosuppresseurs, inducteurs de tolérance, ou lympho-ablatifs sur la prévention de l'allo-immunisation contre les lymphocytes allogéniques. Ce modèle a déjà été utilisé pour démontrer la possibilité d'induire une régression de la tumeur par des lymphocytes allogéniques activés, tout en empêchant leur rejet, par l'utilisation de la CsA (Leboeuf, Mailly, Wu et al., Mol Ther 2014, voir l'annexe 2).

Utilisation de LGM pour la prévention de la réinfection du greffon hépatique par le VHC

L'immunothérapie basée sur l'utilisation de lymphocytes issus du greffon hépatique chez les patients cirrhotiques ayant un CHC et traités par une greffe de foie a été décrite en 2009 (Ohira, Ishiyama et al. 2009). Dans cette étude de phase I, les cellules présentes dans le greffon ont été récoltées, activées et mises en culture pendant trois jours, puis administrées à des patients transplantés (entre 2x10.8 et 5x10.8 cellules par individu) dans le but d'empêcher la rechute du CHC initial après la transplantation. Une réduction très significative de la charge virale de certains patients chroniquement infectés par le VHC a été démontrée. Par ailleurs, une activité anti-virale des cellules CIK a été démontrée chez les patients infectés de manière chronique par le VHB (Shi et al. 2009). Sur la base de ces données, nous nous sommes concentrés sur une autre indication clinique potentielle de nos LGM, à savoir leur activité anti-virale contre le VHC, pour la prévention de la réinfection du greffon après transplantation hépatique.

Bien que les trois sous-ensembles de lymphocytes (T, NK et les cellules T de type NK) sont impliqués dans l'activité anti-virale des LGM par la production d'IFN-γ, il semble que l'effet observé soit médiée par les cellules NK et T de type NK plutôt que par les cellules T. Ces résultats corrélent avec ceux publiés par Ohira et al. (Ohira et al., 2009). L'activité anti-virale de LGM n'est pas modifiée par la transduction rétrovirale et ne semble pas être améliorée en remplaçant l'anticorps monoclonal CD3 par des anticorps CD3/CD28 co-immobilisés. Par ailleurs, l'activité anti-virale des LGM n'est pas affectée par les inhibiteurs de la calcineurine CsA et FK506, ce qui rend cette approche réalisable chez les patients présentant une greffe, recevant à

chaque fois une immunosuppression pour prévenir le rejet de la greffe du foie.

Au cours de l'étude de Ohira et al., cinq patients sur sept patients qui ont été infectés de façon chronique par le VHC et qui avait été transplantés et administrés avec des lymphocytes du greffon du foie activés ex vivo ont vu leur charge virale diminuer (Ohira et al., 2009) . L'ARN viral a été indétectable dans deux d'entre eux, un patient ayant même éradiqué l'infection. Les lymphocytes hépatiques activés ont eu un effet antiviral plus puissant lorsque la charge virale initiale était faible. A l'inverse, avec une charge virale élevée avant transplantation, l'effet antiviral post-transplantation était moins puissant. Nous avons pensé que, lors de l'injection des cellules activées, trois jours après la transplantation du foie, le greffon de foie a peut-être déjà été réinfecté. Par conséquent, si l'efficacité de l'immunothérapie dépend du niveau d'infection du foie lors de l'administration des cellules, les lymphocytes activés doivent être délivrés au moment de la transplantation, le greffon du foie étant alors moins susceptible d'être réinfecté à ce moment-là. Dans un tel cas, cette approche nécessite une préparation de cellules avant l'acquisition de la greffe du foie; ainsi, les LGM doivent être produits à partir d'autres sources. Par conséquent, nous avons examiné la possibilité d'utiliser des LGM allogénique tierce partie, produits à partir de donneurs de sang sains. En outre, en cas d'effets secondaires, le transfert préalable d'un gène suicide par transduction rétrovirale dans les LGM permettrait leur élimination spécifique in vivo par l'administration d'une prodrogue.

Dans notre étude, nous avons montré que les LGM fournissent un effet antiviral puissant à de faibles rapports de cellules effectrices:cibles qui ne sont pas toxiques pour les cellules cibles. Nous avons testé les LGM sur deux modèles in vitro de la réplication du VHC, y compris le modèle largement utilisé d'infection par du virus produit en culture cellulaire (HCVcc). Nous avons observé que l'effet antiviral des LGM était plus efficace lorsque les LGM ont été ajoutés au moment de l'inoculation du VHC, et que l'efficacité diminue si l'on retarde le moment de l'addition des LGM. Ce résultat confirme notre hypothèse selon laquelle les lymphocytes activés, tels que les LGM, doivent être perfusés au moment de la transplantation plutôt que trois jours après. L'observation selon laquelle un effet antiviral peut être observée à de faibles

rapports de cellules effectrices : cibles, non toxiques, suggèrent que le nombre de cellules qui doivent être activés pour produire l'IFN-γ impliqué dans l'effet antiviral est inférieur au nombre de cellules nécessaires pour induire un effet cytotoxique. Cette interprétation est étayée par l'observation que la dépletion des LGM par le CID abroge complètement leur activité cytotoxique, mais seulement partiellement leur effet antiviral: à taux de cellules effectrices cibles élevées, quelques LGM peuvent échapper à la mort induite par CID, ce qui peut être suffisant pour générer un effet antiviral, mais pas cytotoxique.

De même que pour les lymphocytes du foie (Ohira et al. 2009), des cellules mononucléées du sang périphérique (PBMC) soumis à une activation CD3 + IL-2 présentent une activité antivirale contre le VHC, médiée par l'IFN-γ, et une activité anti-tumorale contre le CHC, les deux fournis principalement par les cellules CD56+, y compris les cellules NK CD3- CD56+ et les cellules T de type NK CD3+ CD56+ (Doskali et al., 2011). Ces études sont en accord avec nos résultats actuels et notre récent article démontrant que de fortes concentrations de LGM fournissent des effets anti-tumoraux à médiation cellulaire de type NK et T de type NK dans un modèle de CHC (Leboeuf, Mailly, Wu et al. 2014). Cependant, les cellules utilisées dans les études de Ohira et al. et Doskali et al. ont été générées après activation à court terme (3 jours) par l'IL-2, ce qui soulève la question de la similitude de ces lymphocytes activés, ainsi que de notre LGM, avec les cellules LAK (cellules "tueuses activées par les lymphokines"). En effet, l'utilisation clinique des cellules LAK a été limitée en raison d'une libération massive de cytokines, pouvant conduire occasionnellement à un œdème pulmonaire ultérieur nécessitant des soins intensifs. Nos LGM semblent être plus proches des cellules CIK que des cellules LAK en termes de protocole d'activation (CD3+IL-2 pour les LGM vs CD3+IL-2+IFN-γ+/-IL-1 pour les cellules CIK vs IL-2 pour les cellules LAK) et la durée de culture (2-4 semaines pour les LGM et cellules CIK vs 3-5 jours pour les cellules LAK). On peut émettre l'hypothèse que cela peut conduire à des différences entre LAK vs LGM (ou cellules CIK), par exemple en termes d'expression de molécules d’adhérence (donc, des propriétés de migration) et / ou d'épuisement (donc, du potentiel pour la libération de cytokines, la

prolifération et la cytotoxicité). En effet, les LGM, évalués dans des essais cliniques en phase I à III chez les patients transplantés avec des CSH, induisent des toxicités limitées (Bonini et al 1997;. Ciceri et al 2009; Zhou et al 2014) et, au mieux de notre connaissance, pas d'œdème pulmonaire.

En utilisant un modèle de CHC sur souris humanisée, nous avons démontré que, lorsqu'ils sont perfusés par voie intraveineuse, les LGM migrent de préférence vers le foie et faiblement vers les poumons (Leboeuf, Mailly, Wu et al., Mol Ther 2014, voir l'annexe 2). Cette propriété est un avantage pour améliorer l'efficacité des LGM tout en limitant leur dissémination périphérique. En effet, la localisation hépatique préférentielle des LGM pourrait permettre d'atteindre des concentrations locales élevées de l'IFN-γ, à proximité des cellules cibles tout en conduisant à de faibles concentrations d'IFN-γ périphériques. À cet égard, cela devrait être d'un grand avantage, en termes de tolérance, par rapport à l'administration d'IFNs recombinants exogènes, telles que l'IFN-γ ou l'IFN-α: la toxicité des IFNs est en effet une forte limitation du traitement à based'IFN-α pégylé : en raison des doses nécessaires pour atteindre des concentrations efficaces dans le foie, l'administration d'IFN-α pégylé conduit également à de fortes concentrations dans les organes périphériques (qui ne sont pas les organes cibles du traitement), induisant ainsi des effets secondaires graves. Cependant, l'utilisation de LGM allogénique est associée à des interactions immunologiques complexes impliquant trois partenaires immunologiques: le système immunitaire du patient, les lymphocytes du greffon de foie, et les LGM allogéniques, générés à partir d'un donneur tiers. Néanmoins, les LGM ont également l'avantage d'être résistants à l'inhibition de la CsA. Ainsi, un traitement immunosuppresseur avec CsA peut contrôler les deux premiers partenaires: (1) l'alloréactivité du système immunitaire du receveur envers la greffe de foie (prévention du rejet de greffe du foie) et envers les LGM, et (2) l'alloréactivité des lymphocytes du foie greffé envers les tissus du receveur (GvHD hépatique). Alternativement, l'alloréactivité du troisième partenaire, les LGM, peut être contrôlée grâce au gène suicide sans affecter les deux premiers partenaires.

Une question importante est de déterminer s'il existe un avantage à utiliser des

LGM d’un donneur tierce partie, qui sont génétiquement différents du greffon de foie, par rapport à l'utilisation de lymphocytes ‘’autologues’’ dérivés du foie greffé. Cette hypothèse est techniquement difficile à étudier, parce que la réplication du VHC est généralement évaluée in vitro, avec des lignées cellulaires d'hépatome établies. En effet, les hépatocytes humains primaires sont la plupart du temps réfractaires à l'infection par le VHC in vitro. Ainsi, comparer l'activité anti-virale de LGM envers des hépatocytes primaires humains autologues demeure impossible. Des observations indirectes ont suggéré que alloréactivité peut offrir des avantages supplémentaires à une approche ‘’autologue’’. Lorsque des lymphocytes activés ont été restimulés avec un anticorps CD3 24 h avant l'évaluation de leur activité antivirale, leur capacité à diminuer la réplication du VHC est améliorée (Doskali et al. 2011). En outre, la transplantation allogénique de CSH (Henrich et al. 2013), mais pas la transplantation autologue de CSH (Cillo et al. 2013), a été associée à une diminution de la charge virale détectable du VIH, qui a été temporellement corrélée avec un chimérisme donneur complet, mais également au développement d’une GvHD, une complication de la greffe associée à l’alloréactivité. Bien que les mécanismes conduisant à la guérison du VIH après greffe de CSH restent à élucider, et même si ils peuvent être différents de ceux qui sont impliqués dans la prévention de la réplication du VHC, ces résultats suggèrent que l’alloréactivité peut apporter un avantage pour augmenter un effet anti-viral. Pris dans leur ensemble, nos résultats ont fourni la preuve de concept in vitro qu’une immunothérapie adoptive peut être appliquée en toute sécurité à la prévention efficace de la réinfection de la greffe de foie par le VHC.

Dans ce nouveau projet, beaucoup de travaux doivent être fait avant de permettre l'utilisation clinique de LGM: d'abord, faisant suite à l'évaluation de l'activité anti-virale des LGM à travers des systèmes de réplicons et HCVcc, l'étape suivante consiste à fournir à la preuve de concept in vivo. Nous prévoyons d'utiliser notre modèle d'infection par le VHC dans des souris Alb-uPA/SCID-bg chimériques pour un foie humanisé pour tester nos LGM. Ces souris immuno-déficientes et hépato-reconstituées avec des hépatocytes humains sont un modèle d'infection par le VHC pour lesquels nous avons déjà une solide expérience. Sur la base de ce modèle

de souris, plusieurs questions cruciales doivent être résolues telles que la dose d'administration de LGM menant à un effet antiviral efficace in vivo sans cytotoxicité envers les hépatocytes humains. Deuxièmement, en raison de la variété des génotypes du VHC vus en clinique, nous allons essayer de tester l'efficacité de nos LGM contre divers génotypes tels que les génotypes 1a, 1b, 4 etc… Troisièmement, comparer et combiner notre stratégie LGM avec d'autres stratégies de thérapie anti-virales classiques ou innovantes, afin d'obtenir le meilleur effet curatif avec le moins d'effets secondaires possibles. Il sera donc intéressant d'étudier l'effet des LGM dans la prévention et le traitement de l'infection.

Création d'une banque de LGM allogéniques prêts à l'emploi

Quelle que soit l'application clinique, notre but ultime est de créer une banque de LGM allogéniques comme cellules effectrices "prêts à l'emploi". Par rapport aux approches autologues (CIK, cellules du foie), les avantages de notre approche sont nombreux. Tout d'abord, il sera possible d'établir une production de LGM à très grande échelle. Les lymphocytes de donneurs sains sont beaucoup plus faciles à cultiver et expandre que ceux des patients qui sont souvent soumis à des traitements lourds. Nous avons pensé que d'un échantillon de 10.9 CMN, il peut être possible d'obtenir 50x10.9 LGM, ce qui signifie qu'il pourrait permettre de réaliser 100 injections sur la base de la dose moyenne utilisée dans l'étude de Ohira et al. (5x10.6 cellules / kg). Deuxièmement, les cellules sont prêtes à l'emploi, ce qui signifie qu'elles peuvent être administrées au patient immédiatement, au moment de la transplantation du foie par exemple. Il n'y a donc pas de perte de temps associée à la production et à la qualification des cellules. Troisièmement, si un lot clinique pour un patient donné ne peut être administré pour des raisons médicales ou d'autres raisons (comme la non-conformité ou la non compliance du patient), les cellules peuvent encore être utilisés pour un autre patient, évitant ainsi la perte de lots cliniques produits en vain.

Ainsi, la création d'une banque de cellules prêtes à l'emploi serait extrêmement benefique d'un point de vue médical et pertinent en termes de développement

industriel, logistique et financier en comparaison avec les approches classiques d'immunothérapies autologues qui consistent à produire un lot particulier de cellules pour chaque malade.

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Annex 1

Detailed results of IS, IPT and IH injections

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Injection	Mouse strain	Death			Tumor engraftment				Tumor location (N mice with tumor at the indicated location)			% Tumor location / grafted mice (reference: column A)			% Tumor location/evaluated mice (reference: column D)		
		N grafted mice	N dead mice	% Death	N evaluated mice	% evaluated mice	N mice with tumor	% mice with tumor	Intrahepatic	Extrahepatic	Intrahepatic & extrahepatic	Intrahepatic	Extrahepatic	Intrahepatic & extrahepatic	Intrahepatic	Extrahepatic	Intrahepatic & extrahepatic
IS	NMRI-nude	17	0	0,0	17	100,0	15	88,2	7	6	2	41,2	35,3	11,8	41,2	35,3	11,8
	SCID-bg	90	9	10,0	81	90,0	55	67,9	27	23	5	30,0	25,6	5,6	33,3	28,4	6,2
	NMRI-nude + SCID-bg	107	9	8,4	98	91,6	70	71,4	34	29	7	31,8	27,1	6,5	34,7	29,6	7,1
IPT	NMRI-nude	41	12	29,3	29	70,7	15	51,7	10	3	2	24,4	7,3	4,9	34,5	10,3	6,9
	SCID-bg	113	54	47,8	59	52,2	46	78,0	20	20	6	17,7	17,7	5,3	33,9	33,9	10,2
	NMRI-nude + SCID-bg	154	66	42,9	88	57,1	61	69,3	30	23	8	19,5	14,9	5,2	34,1	26,1	9,1
IH	NMRI-nude	161	9	5,6	152	94,4	145	95,4	93	28	24	57,8	17,4	14,9	61,2	18,4	15,8
	SCID-bg	50	2	4,0	48	96,0	27	56,3	17	8	2	34,0	16,0	4,0	35,4	16,7	4,2
	NMRI-nude + SCID-bg	211	11	5,2	200	94,8	172	86,0	110	36	26	52,1	17,1	12,3	55,0	18,0	13,0

Column C = (B/A)x100; Column E = (D/A)x100; Column G = (F/D)x100; Columns K to M = ([H to J]/A)x100; Columns N to P = ([H to J]/D)x100

Annex 2

In vivo proof-of-concept of adoptive immunotherapy for hepatocellular carcinoma with using allogeneic suicide gene-modified killer cells.

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In Vivo Proof of Concept of Adoptive Immunotherapy for Hepatocellular Carcinoma Using Allogeneic Suicide Gene-modified Killer Cells

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Cell therapy based on alloreactivity has completed clinical proof of concept against hematological malignancies. However, the efficacy of alloreactivity as a therapeutic approach to treat solid tumors is unknown. Using cell culture and animal models, we aimed to investigate the efficacy and safety of allogeneic suicide gene-modified killer cells as a cell-based therapy for hepatocellular carcinoma (HCC), for which treatment options are limited. Allogeneic killer cells from healthy donors were isolated, expanded, and phenotypically characterized. Antitumor cytotoxic activity and safety were studied using a panel of human or murine HCC cell lines engrafted in immunodeficient or immunocompetent mouse models. Human allogeneic suicide gene-modified killer cells (aSGMKCs) exhibit a high, rapid, interleukin-2–dependent, and non–major histocompatibility complex class I-restricted *in vitro* cytotoxicity toward human hepatoma cells, mainly mediated by natural killer (NK) and NK-like T cells. *In vivo* evaluation of this cell therapy product demonstrates a marked, rapid, and sustained regression of HCC. Preferential liver homing of effector cells contributed to its marked efficacy. Calcineurin inhibitors allowed preventing rejection of allogeneic lymphocytes by the host immune system without impairing their antitumor activity. Our results demonstrate proof of concept for aSGMKCs as immunotherapy for HCC and open perspectives for the clinical development of this approach.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. Although early-stage tumors can be curatively treated using surgical approaches, treatment options for advanced HCCs are limited.[1] Hepatic resection is the treatment of choice for HCC in non-cirrhotic patients, but this group accounts for a minority of patients.[2] Liver transplantation is the best therapeutic option for HCC as it simultaneously removes the tumor and the underlying cirrhosis, thereby minimizing the HCC recurrence risk. However, this option is limited by organ shortage and its restriction to patients with limited disease. Minimally invasive treatments such as percutaneous treatments or radiofrequency ablation are alternatives for early-stage HCC patients who are not eligible for surgical resection or transplantation. Transarterial chemoembolization may offer palliative benefits for patients with intermediate-stage HCC.[3] Although the multikinase inhibitor, sorafenib, was the first agent to demonstrate a survival benefit for patients with locally advanced or metastatic HCC, treatment options for patients with advanced HCC remain limited.[1] Therefore, development of novel therapies for patients with advanced HCC or early-stage HCC not eligible for standard of care remains a major unmet medical need.

Cancer immunotherapy is emerging in preclinical and clinical evaluation.[4] Allogeneic hematopoietic stem cell (HSC) transplantation has demonstrated that alloreactivity provides one of the most powerful antitumor effects in immunotherapy of hematological malignancies. Indeed, an HSC graft contains pluripotent HSCs (that will reconstitute complete long-term hematopoiesis) and immune cells, such as T cells. Up to 10% of these T cells are alloreactive and recognize differentially expressed human leukocyte antigen (HLA) determinants. Thus, they are responsible for a severe complication of HSC transplantation, graft-versus-host disease (GvHD), but also for graft rejection prevention and graft-versus-leukemia effect. In addition to T cells, natural killer (NK) cells also provide alloreactivity through the recognition of NK receptor ligands mismatches.[5] Furthermore, alloreactivity may be efficient for the treatment of solid tumors.[6] However, if allogeneic adoptive immunotherapy is highly efficient, it can elicit toxicities that need to be controlled. *Ex vivo* retroviral-mediated transfer

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of a "suicide" gene, such as the herpes simplex virus-thymidine kinase (HSV-tk) or the inducible caspase 9 (iCasp9) in donor lymphocytes allows their specific killing by their respective prodrug, ganciclovir (GCV)[7] or chemical inducer of death (CID).[8] Clinical trials demonstrated that allogeneic suicide gene-modified killer cell (aSGMKC)–induced GvHD can be controlled by their prodrugs[8,9] and that aSGMKCs are able to exert a strong graft-versus-leukemia effect.[9,10] However, the effect of this cell therapy product (CTP) against solid tumors is unknown. Using HCC as a model for solid tumors, here we show that aSGMKCs have a potent non–major histocompatibility complex (MHC) class I-restricted antitumor activity *in vitro* and *in vivo* and open a previously unknown perspective for the clinical treatment of HCC.

RESULTS

Production and phenotypic analysis of aSGMKCs for HCC immunotherapy

Allogeneic SGMKCs from healthy blood donors were produced as shown in **Supplementary Figure S1a**. Prior to its evaluation in preclinical HCC models, we analyzed the phenotype of the CTP. As shown in **Table 1**, most aSGMKCs are $CD3^+CD56^-$ T cells, followed by $CD3^+CD56^+$ NK-like T cells. In contrast, $CD3^-CD56^+$ NK cells make up the smallest proportion within aSGMKCs. This phenotype is similar to that of control cells and shows increased NK-like T cells frequencies, as compared with peripheral blood mononuclear cells (PBMCs) (**Table 1**).[11] While control cells were not GCV sensitive, growth of aSGMKCs was GCV inhibited (**Supplementary Figure S2**). Alloreactivity of aSGMKCs is impaired, as compared with PBMCs, in terms of proliferative response after allostimulation by PBMCs,[12,13] B-EBV cell lines[11,14,15] or HCC cell lines (**Supplementary Figures S3 and S4**) and in terms of allo- or xenogeneic GvHD induction in animal models.[14,16–18] The impairment of aSGMKCs' alloreactivity can be reversed by replacing CD3 activation with CD3/CD28 costimulation and/or by replacing interleukin (IL)-2 with IL-7.[14] Thus, we evaluated the cytotoxicity against Huh7 cells or HeLa cells (as a positive control for target cell lysis) of aSGMKCs generated from PBMCs after CD3 versus CD3/CD28 activation and expansion with IL-2 versus IL-7 (**Figure 1a**). CD3+IL-2-activated cells exhibited the highest cytotoxic activity, expressed as lytic units 50% (**Supplementary Figure S1b**). Replacing CD3 activation by CD3/CD28 costimulation and/or IL-2 by IL-7 was associated with a lower cytotoxicity (**Figure 1a**). Therefore, only $CD3^+$IL-2 activation was used to produce aSGMKCs in subsequent experiments.

Table 1 Relative distribution of T, NK-like T, and NK cells in aSGMKCs, as compared with PBMCs or control cells.

	PBMCs (n = 18)	Control cells (n = 7)	aSGMKCs (n = 22)
T cells ($CD3^+CD56^-$)	64.0 ± 2.1[a]	81.0 ± 3.6[b]	77.5 ± 2.8[b]
NK-like T cells ($CD3^+CD56^+$)	6.3 ± 1.0	11.4 ± 3.7	10.8 ± 1.5[b]
NK cells ($CD3^-CD56^+$)	10.4 ± 1.0	2.8 ± 0.8[b]	5.0 ± 1.7[b]

aSGMKCs, allogeneic suicide gene-modified killer cells; NK, natural killer; PBMCs, peripheral blood mononuclear cells.
[a]Data are indicated as the percentage (mean ± SEM) of T, NK-like T, and NK cells gated within the total PBMCs, control cells, or aSGMKCs. [b]$P < 0.05$ versus PBMCs (Student's paired t-test).

aSGMKCs demonstrate broad and sustained activity against hepatoma cells

Next, we studied aSGMKCs' cytotoxic activity against hepatoma cells. Allogeneic SGMKCs were tested against Huh7 and PLC-PRF5 hepatoma cells. SK-Hep1, an endothelial cell line, was also assessed in parallel. All target cells were sensitive to aSGMKCs killing (**Figure 1b**). Because Huh7 cells exhibited an intermediate sensitivity to killing, we chose this cell line to further characterize the aSGMKCs' cytotoxicity. PBMCs exhibited significant cytotoxic activity after 3 or 6 days of coculture with target cells, whereas aSGMKCs cytotoxic activity was maximal after only 1 day of coculture (**Figure 1c**), indicating that aSGMKCs are ready to exert their cytolytic potential. The gene transfer process did not affect aSGMKCs' cytotoxic activity, as shown by the similar level of cytotoxicity observed with control cells generated in parallel (**Figure 1c**).

Mechanism of action

Allogeneic SGMKCs' cytotoxic activity is IL-2 dependent, while IL-7 cannot replace IL-2 during the cytotoxicity assay (**Figure 1d**). Moreover, neither the W6/32 monoclonal antibody, a pan anti-HLA class I antibody inhibiting $CD8^+$ T cells proliferative response in mixed lymphocyte reaction (data not shown), nor an isotype control monoclonal antibody significantly affected aSGMKCs' cytotoxicity (**Figure 1e**). These data demonstrated that the cytotoxic activity is non–MHC class I restricted. To go further, we analyzed the lytic potential of T, NK-like T, and NK cells sorted from aSGMKCs compared with that of unsorted aSGMKCs. Purified T cells had a significantly lower cytotoxicity than unsorted aSGMKCs, whereas purified NK-like T or NK cells displayed an increased cytotoxicity (**Figure 1f**). These data suggest that NK-like T and NK cells are mostly responsible for aSGMKCs' cytotoxic activity against hepatoma cells.

In vivo cytotoxicity of aSGMKCs in xenograft HCC models

In vivo cytotoxicity assays were performed using HeLa-Luc cells as a positive control for lysis sensitivity and Huh7-Luc cells as hepatoma target cells in xenograft HCC mouse models. When increasing aSGMKCs amounts were subcutaneously coinjected with fixed target cell numbers into immunodeficient mice, a dose-dependent reduction of the relative tumor growth was observed for HeLa-Luc (**Figure 2a,c**) and Huh7-Luc cells (**Figure 2b,d**), even at low effector:target ratios (3:1). However, tumor escape in some mice led to an increase of the mean relative Huh7 tumor growth on D7 or D14. Therefore, the frequency of mice with tumor progression was also evaluated and was shown to be dose-dependently reduced (**Figure 2e,f**). High effector:target ratios allowed for complete tumor eradication in most animals (**Figure 2a,b**), as demonstrated by the low percentage of animals with tumor progression (**Figure 2e,f**).

Antitumor activity of aSGMKCs against orthotopic HCC

In a next step, we investigated whether aSGMKCs exhibit antitumor activity in a more relevant model, using an orthotopic HCC model. In contrast to the classical xenograft models, Huh7 cells are injected intrasplenically, resulting in tumor growth in the liver.

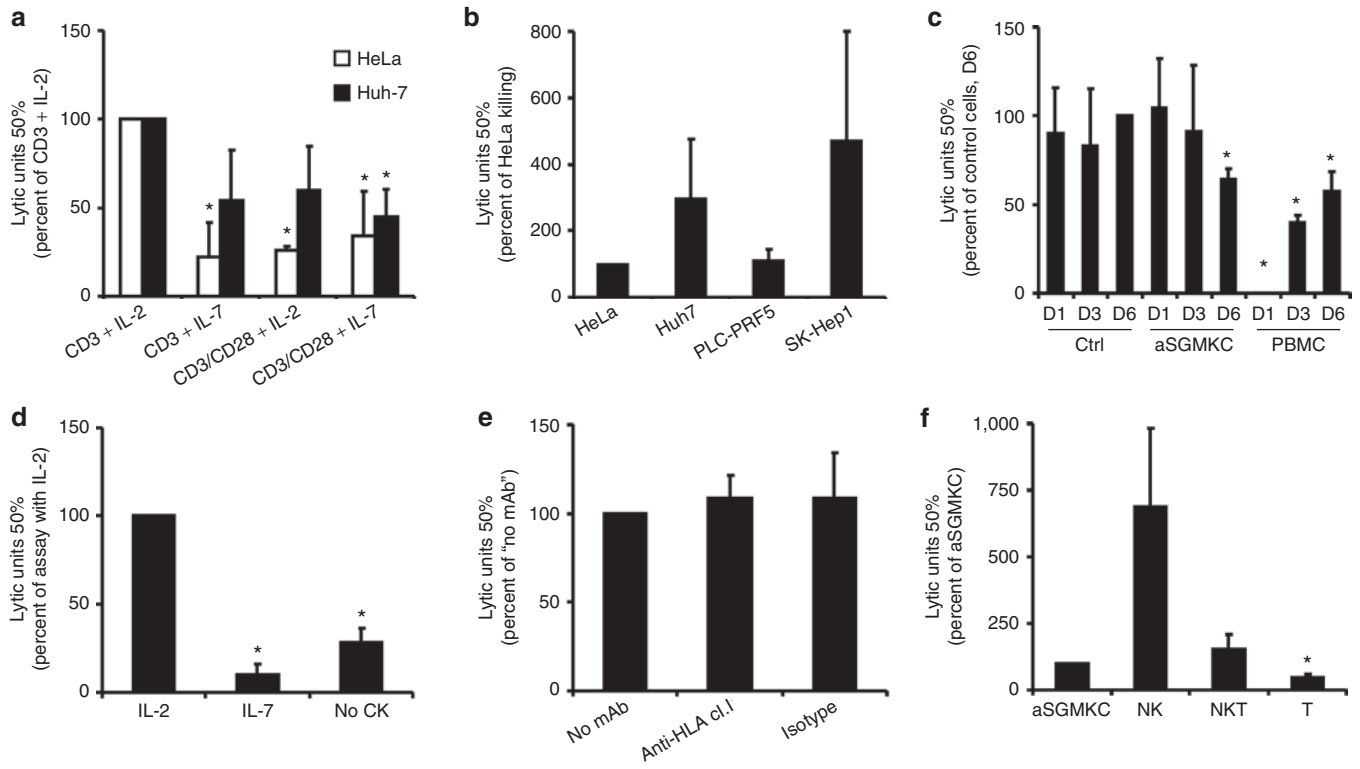

Figure 1 ***In vitro*** **cytotoxicity of allogeneic suicide gene-modified killer cells (aSGMKCs) against hepatoma cells.** (**a**) Peripheral blood mononuclear cells (PBMCs) were expanded for 2 weeks after CD3 or CD3/CD28 monoclonal antibodies (mAbs) ± IL-2 or IL-7 activation before evaluation of their cytotoxic activity. Control groups: CD3 + IL-2: 653±145 lytic units 50% ((LU50) (HeLa) and 224±109 LU50 (Huh7); $n = 4$. (**b**) The cell lines Huh7, PLC-PRF5, and SK-Hep1 are sensitive to aSGMKCs-mediated killing. Control group: HeLa cells killing: 172±22 LU50; $n = 6$. (**c**) The cytotoxic activity of effector cells (PBMCs, control cells or aSGMKCs from the same donors) was evaluated on D1, D3, or D6 of coculture with Huh7 cells. Control group: control (ctrl) cells on D6, 218±64 LU50; $n = 4$. (**d**) aSGMKCs' cytotoxic activity was assessed without any cytokine (no CK) and in the presence of IL-2 or IL-7 during coculture with target cells. Control group: IL-2, 164.4±46.5 LU50; $n = 5$. (**e**) aSGMKCs' cytotoxic activity is not inhibited by the addition of an anti–human leukocyte antigen (HLA) class I mAb during the coculture. Control group: culture with "no mAb", 210±98 LU50; $n = 4$. (**f**) Cytotoxic activity of immunomagnetically purified (see fraction purities in **Supplementary Table S1**) T, natural killer (NK)-like T, and NK cells was tested in parallel with unsorted aSGMKCs. Control group: unsorted aSGMKCs, 154±33 LU50; $n = 4$. Data are expressed as mean ± SEM of the control group. In all panels, $^*P < 0.05$ compared with the control group (Mann–Whitney test). IL, interleukin.

Intravenous (i.v.) aSGMKCs injection led to a rapid (**Figure 3a** and **Supplementary Figure S5a**) and preferential (**Figure 3b**) liver homing, which is more pronounced than after intraperitoneal (i.p.) injection (**Supplementary Figure S5b,c**). Twenty-four hours after their injection, aSGMKCs were found surrounding the tumor (**Supplementary Figure S5d–f**). These cells were $CD3^+$, of which some were also $CD56^+$. Huh7-Luc orthotopic tumor growth quantification was performed by *in vivo* bioluminescence imaging (BLI) before and after aSGMKCs i.v. injection (**Figure 3c**). The mean tumor growth, expressed either as absolute bioluminescent activity (**Figure 3d**) or relative bioluminescent activity (**Figure 3e**), and the frequency of tumor-progressing mice (**Figure 3f**) were markedly reduced on D3 and D7 after i.v. aSGMKCs injection, as compared with the nontreated control group. Allogeneic SGMKCs were recovered from the livers on D7 postinjection and analyzed by flow cytometry for the relative repartition of NK, NK-like T, and T cells. As compared with the infused product, the frequencies of NK and NK-like T cells were increased, whereas T cells were less frequent (**Figure 3g**), suggesting that NK and NK-like T cells may be more likely to be involved in the antitumor effect. In order to further demonstrate that the antitumor effect was performed by aSGMKCs, repeated administrations of GCV for 4 days were initiated at the time of aSGMKCs infusion. However, no effect on aSGMKCs-induced tumor regression was observed (data not shown), possibly because aSGMKCs-mediated killing of tumor cells could be more rapid than GCV-induced killing of aSGMKCs. Therefore, the antitumor effect of aSGMKCs modified to express iCasp9, a suicide gene known to eliminate the transduced cells more rapidly than the HSV-tk suicide gene (**Supplementary Figure S6a,b**)[19] was evaluated in the absence or presence of its prodrug, the AP20187 CID. The antitumor effect was reversed by only one injection of CID at the time of $iCasp9^+$ aSGMKCs infusion (**Figure 3h** and **Supplementary Figure S6c**). This suggests a superior efficacy of the iCasp9/CID system than the HSV-tk/GCV system in the current preclinical system and validates that the antitumor activity is in fact due to the aSGMKCs.

Safety of aSGMKCs injection

No clinical side effects were observed in mice infused with aSGMKCs, suggesting that antitumor activity could be safely established in absence of GvHD induction. To further explore the toxicity of aSGMKCs, histological analyses were performed in the liver, intestine, kidney, lung, and spleen of tumor-bearing mice on D1 and D7 after aSGMKCs injection.

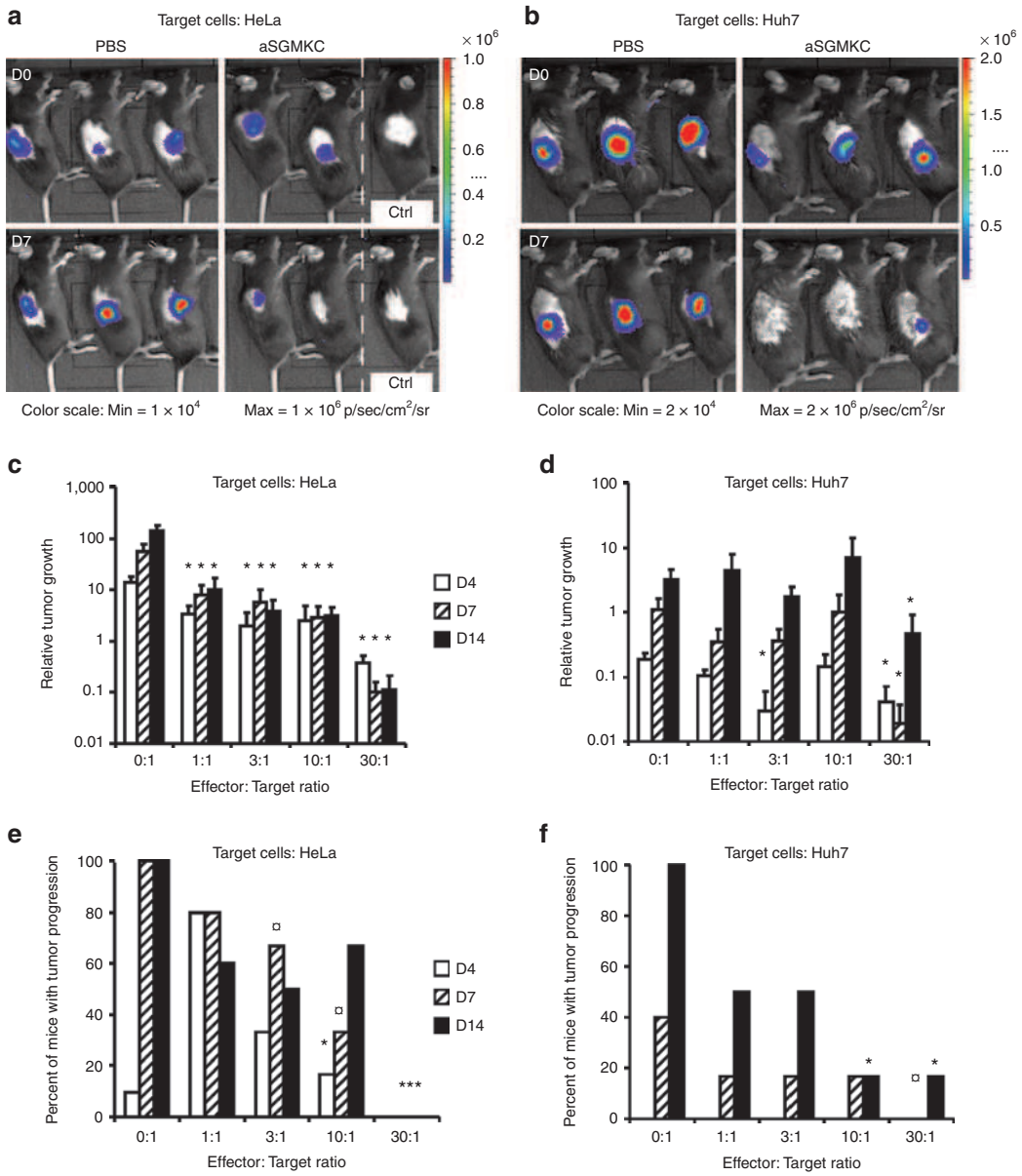

Figure 2 ***In vivo*** **cytotoxicity of allogeneic suicide gene-modified killer cells (aSGMKCs) against HeLa-Luc and Huh7-Luc cells.** Mice were subcutaneously (s.c.) coinjected either with HeLa-Luc (**a**,**c**,**e**) or Huh7-Luc (**b**,**d**,**f**) cells and aSGMKCs. (**a**,**b**) The bioluminescence activity of target cells is shown at the time of injection (D0) and on D7 for representative mice s.c. injected with phosphate-buffered saline (PBS) or aSGMKCs at an effector:target (E:T) ratio of 30:1. Control (ctrl): negative control of bioluminescence (no target cell injection). (**c**,**d**) aSGMKCs dose-dependently delay or reduce (values <1) the relative tumor growth (mean ± SEM). (**e**,**f**) aSGMKCs dose-dependently reduce the proportion of mice with tumor progression (i.e., with a relative tumor growth value >1) of (**e**) HeLa-Luc and (**f**) Huh7-Luc cells. Data in **c**–**f** are pooled results of six mice from two independent experiments using three mice per group. (**a**–**f**) Daily intraperitoneal injections of interleukin-2 (IL-2) were performed in all mice. **P* < 0.05 compared with control group without aSGMKCs injection (i.e., E:T ratio = 0:1), at the respective time points (**c**,**d**: Mann–Whitney test; **e**,**f**: Fisher exact test).

The livers presented focal zones of necrosis associated with massive inflammatory infiltrates of neutrophils at the periphery of the necrotic hepatocytes (**Figure 4a**). These zones of necrosis resulted from thrombosis (**Figure 4a**, insert) that were probably induced by the intrasplenic injection of Huh7 cells. Indeed, they were also observed in tumor-bearing control mice not injected with aSGMKCs, whereas they were not observed in non tumor-bearing control mice injected with aSGMKCs alone (data not shown). Multifocal inflammatory infiltrates of small size, found in the livers of all mice, were mostly CD3 positive (**Figure 4b**). No histopathological abnormalities were found in the intestines, kidney, and lung (**Supplementary Figure S7a–c**). As expected, the spleen displayed marked white pulp hypoplasia, but no histopathological abnormalities were observed (**Supplementary Figure S7d**), excepted the presence of neoplasia in some spleens, due to the intrasplenic injection of Huh7 cells. All together, these results suggest that aSGMKCs were safe to murine tissues and did not induce xenogeneic GvHD.

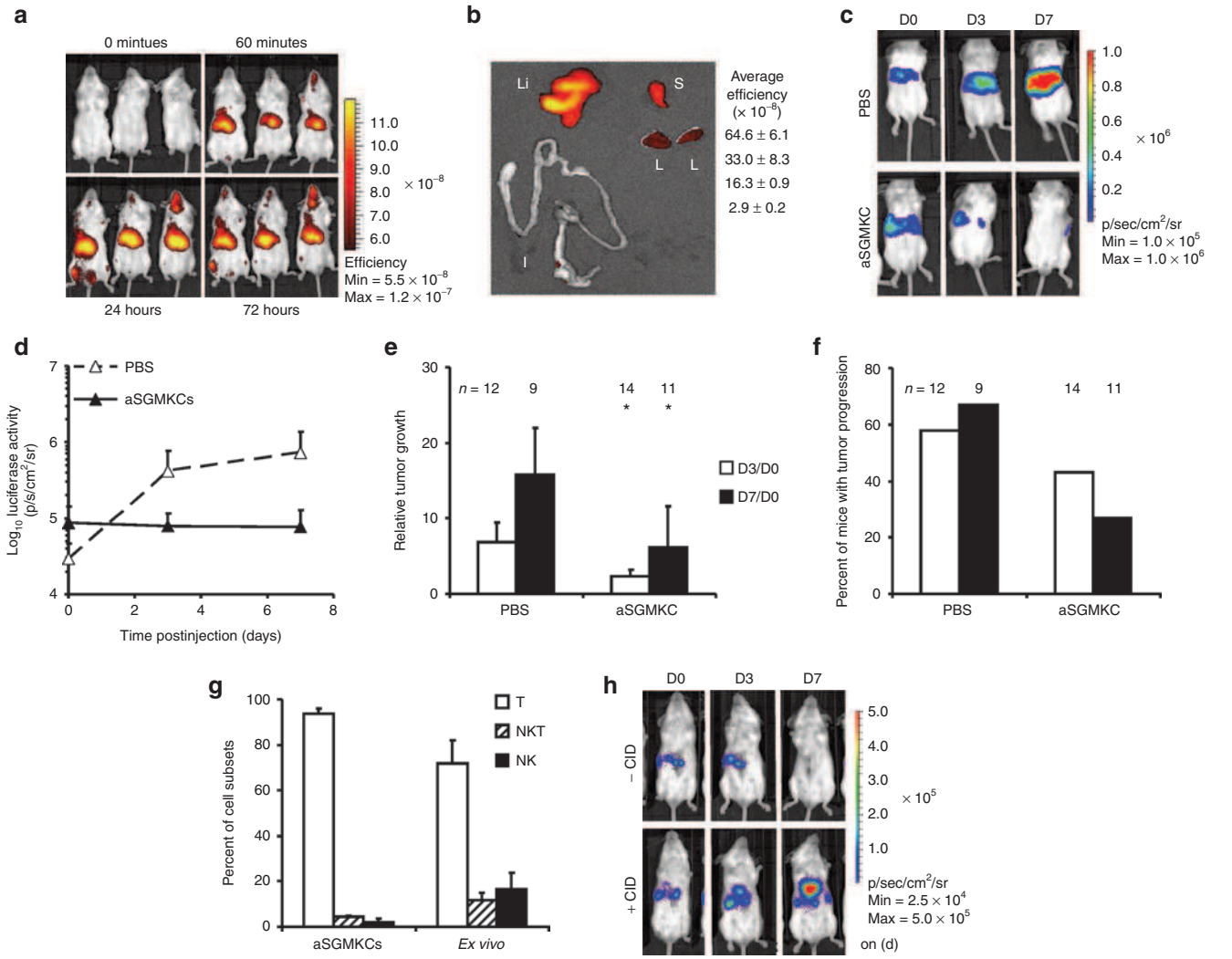

Figure 3 Antitumor effect of allogeneic suicide gene-modified killer cells (aSGMKCs) in a humanized orthotopic model of hepatocellular carcinoma (HCC). (**a**,**b**) DiR-labeled aSGMKCs preferentially and rapidly migrate to the liver when intravenously injected to severe combined immunodeficiency–beige mice. (**a**) The *in vivo* fluorescence signal is shown for three mice representative of six. (**b**) Fluorescence quantification in the liver (Li), spleen (S), lungs (L), and intestine (I) 72 hours after aSGMKCs injection is reported in the right part of the figure (mean ± SEM, $n = 4$ mice). (**c–f**) aSGMKCs provide an antitumor effect in a humanized orthotopic model of HCC. (**c**) Representative experiment showing the Huh7-Luc bioluminescence in one mouse injected with phosphate-buffered saline (PBS) or aSGMKCs. The injection of aSGMKCs delays the tumor growth, (**d**) evaluated by the luciferase activity of Huh7-Luc cells or (**e**) expressed as relative tumor growth on D3 and D7. *$P = 0.07$ compared with control group with PBS injection (Mann–Whitney test). **(f)** The proportion of mice with tumor progression (i.e., with a relative tumor growth value >1) on D3 and D7 shows that aSGMKCs reduce the frequency of mice with Huh7-Luc tumor progression. Data in **d–f** expressed as mean ± SEM are pooled results of five and three independent experiments on D3 and D7, respectively, using the indicated total number of mice (*n*) per group. (**g**) The relative frequencies of T, natural killer (NK), and NK-like T cells harvested from the liver on D7 postinjection ("*ex vivo*") are increased, as compared with the frequencies observed on D0 in the infused aSGMKCs product ("aSGMKCs"). Data are expressed as mean ± SEM of nine mice from three independent experiments. (**h**) The antitumor effect of CD19/iCasp9$^+$ aSGMKCs in the orthotopic HCC model is reversed by only one administration of chemical inducer of death (CID). Representative experiment showing the Huh7-Luc bioluminescence in one mouse injected with or without 2.5 mg/kg CID at the time of intravenous injection of 150–200 × 10^6 aSGMKCs. Interleukin-2 (IL-2) was intraperitoneally injected daily. (**a–h**) Daily intraperitoneal injections of IL-2 were performed in all mice.

The potential cytotoxicity of aSGMKCs against normal hepatocytes was further explored in human liver-chimeric mouse model, using albumin promoter-driven urokinase-like plasminogen activator transgenic severe combined immunodeficiency–beige (SCID-bg) mice whose livers were repopulated between 10 and 20% with primary human hepatocytes (**Figure 4c**). Two weeks after aSGMKCs injection, large areas of proliferative cellular infiltration were observed in perivascular areas and, more dispersed, in the hepatic parenchyma as well as in the human clusters (**Figure 4d**). Despite this infiltration, the level of liver repopulation by primary human hepatocytes was similar in aSGMKCs-injected mice compared with noninjected control mice, showing that there is no massive destruction of those cells after aSGMKCs injection. This is further substantiated by the observation that human albumin serum levels remain stable in both groups (**Figure 4e**). As reported above in tumor-bearing SCID-bg mice, no histopathological abnormalities were observed in the spleen, lung, kidney, and intestine of

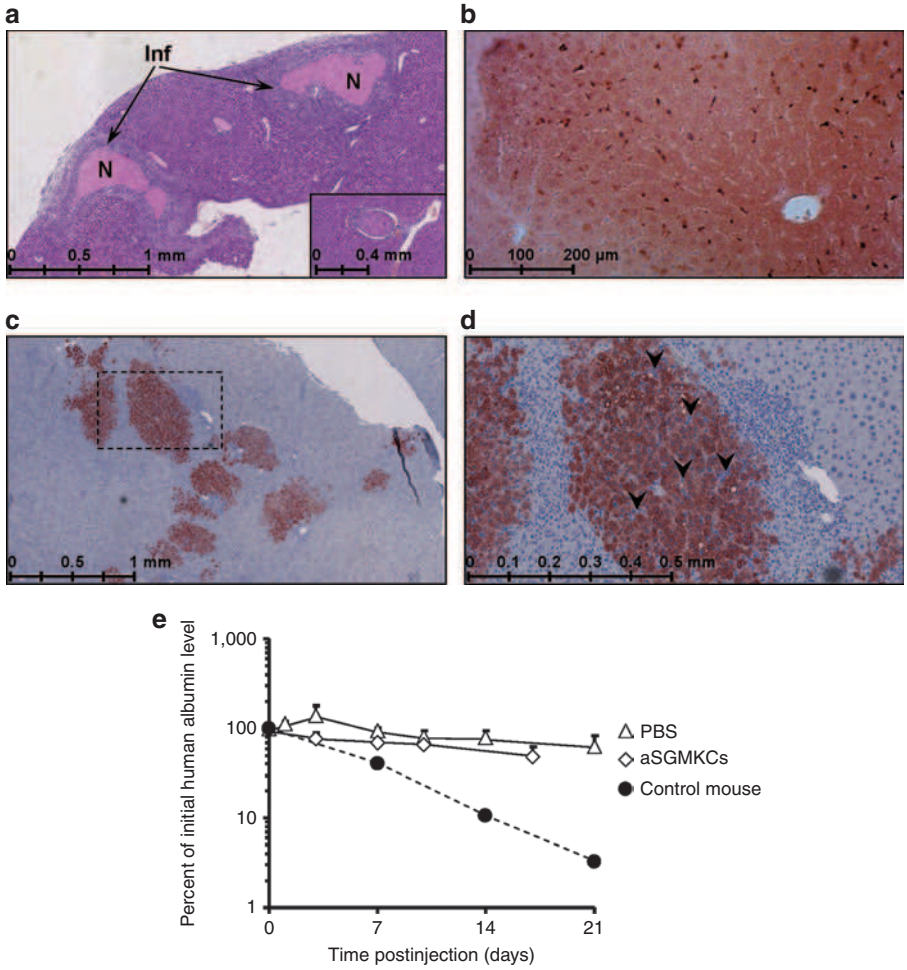

Figure 4 Absence of significant cytotoxicity of allogeneic suicide gene-modified killer cells (aSGMKCs) toward primary murine and human hepatocytes. (**a**) Hematoxilin–eosin staining shows infiltrates of neutrophils (Inf) at the periphery of focal zones of necrosis (N) resulting from Huh7 cell–induced thrombus (insert). (**b**) Seven days after intravenous injection of 180×10.6 aSGMKCs, lymphocytes infiltrating the hepatic parenchyma do not induce histopathological abnormalities (CD3 immunostaining and eosin counterstaining). (**c**) Primary human hepatocytes revealed by human cytokeratin-18 immunohistochemistry in the liver of chimeric Alb-uPA/severe combined immunodeficiency–beige (SCID-bg) mice 2 weeks after aSGMKCs injection. (**d**) Magnification of the dashed area in **c**, showing infiltrates of proliferating lymphocytes (arrowhead) in the human areas. (**e**) Similar serum levels of human albumin in chimeric Alb-uPA/SCID-bg mice infused (white diamonds, $n = 5$) or not (white triangles, $n = 12$) with aSGMKCs (mean ± SEM: $72 \pm 22 \times 10^6$ cells/mouse). Data are expressed as percent (mean ± SEM) of initial human albumin concentration that were 377±157 µg/ml in the control group and 220±99 µg/ml in the aSGMKCs-infused group. Human albumin levels of one historical control chimeric mouse (black circles, dashed line) that spontaneously lost its graft are shown as example of decreased serum human albumin. PBS, phosphate-buffered saline.

human liver-chimeric Alb-uPA/SCID-bg mice, injected or not with aSGMKCs (data not shown).

CsA can control the potential rejection of allogeneic lymphocytes

Allogeneic SGMKCs rejection by the recipient's immune system could be a clinical limitation to our approach. Allogeneic SGMKCs' cytotoxic activity is resistant to the immunosuppressive effect of the calcineurin inhibitor, cyclosporin A (CsA), both *in vitro* (**Figure 5a**) and *in vivo* (**Figure 5b**). Thus, we reasoned that CsA could prevent the rejection of aSGMKCs, without impairing their antitumor activity (**Supplementary Figure S8**). To address this question, we used a murine immunocompetent HCC mouse model. As a surrogate model of human aSGMKCs, we used allogeneic *ex vivo*-expanded splenocytes (aEESs), generated from spleen cells and known to be also resistant to CsA.[18] This mouse model allowed us to assess the safety and efficacy of this approach in an immunocompetent host.

To investigate and confirm that CsA was able to prevent the rejection of the CTP by the recipient's immune system, aEESs from luciferase-transgenic FvB/N mice (H-2^q) were infused into immunocompetent recipient Balb/c mice (H-2^d) in the presence of IL-2, with and without CsA. In the absence of CsA, aEESs were not detectable by BLI, indicating that they have been rejected by the host's immune system. By contrast, when CsA was administered, luciferase-transgenic aEESs became detectable on D11 (**Figure 5c**,**d**), suggesting that CsA prevents allogeneic CTP rejection in an immunocompetent host. At earlier time points postinjection, aEESs from FvB/N mice could be detected by *in vivo* fluorescence in the liver, to a higher level in the presence of

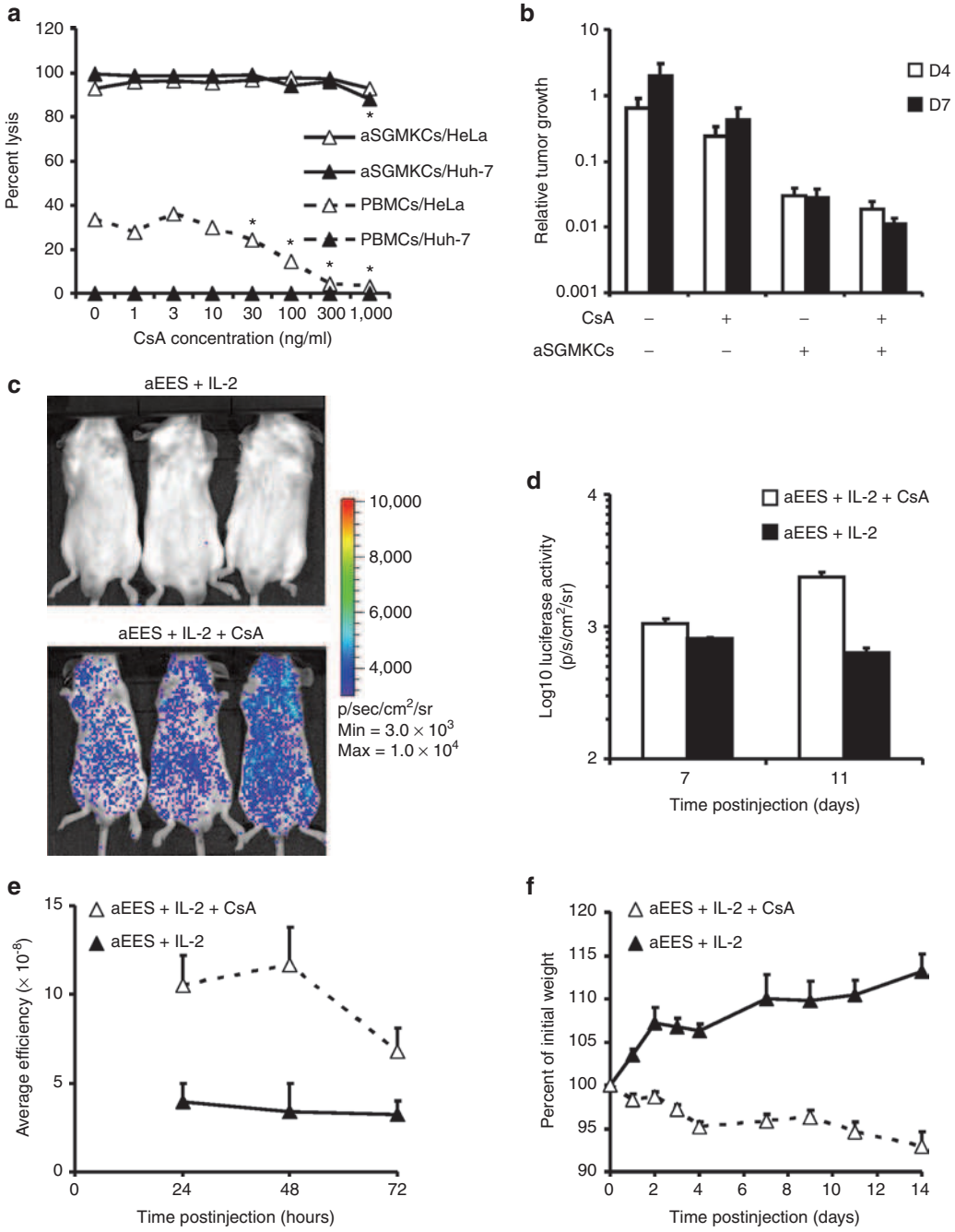

Figure 5 Resistance of expanded lymphocytes to ciclosporine A (CsA) as a mean to prevent their allorejection. (**a**,b) Human allogeneic suicide gene-modified killer cells (aSGMKCs) are resistant to CsA *in vitro* and *in vivo*. (**a**) The *in vitro* cytotoxicity of peripheral blood mononuclear cells (PBMCs) (positive control for sensitivity of effector cells to the inhibitory effect of CsA) and aSGMKCs was assessed at an effector:target ratio of 40:1 in the presence of CsA (one experiment representative of four). **P* < 0.05 compared with 0 ng/ml CsA. (**b**) *In vivo* aSGMKCs' cytotoxic activity against Huh7-Luc cells was assessed, as reported in **Figure 2b**,**d**, in the absence or presence of daily intraperitoneal injections of CsA (*n* = 3/group). (**c**–**e**) CsA prevents the rejection of allogeneic expanded *ex vivo* splenocytes (aEESs). (**c**) Luciferase-expressing aEESs were injected to Balb/c recipient mice (*n* = 3/group) in the presence of IL-2 ± CsA and their presence was monitored by *in vivo* bioluminescence imaging on D7 (one experiment representative of four). (**d**) Quantification of the luciferase activity in mice injected with aEESs + IL-2 in the absence or presence of CsA (mean ± SEM; *n* = 3/group). (**e**) The CsA does not prevent the homing of aEESs to the liver. DiR-labeled aEESs from FvB/N mice were injected to Balb/c recipient mice in the presence of IL-2 ± CsA and were monitored by *in vivo* fluorescence gated on the liver (mean ± SEM; *n* = 3/group). (**f**) The prevention of aEES rejection by administering CsA allows aEESs to induce a graft-versus-host disease, monitored by body weight loss (mean ± SEM; *n* = 3 mice without CsA and *n* = 9 mice with CsA). IL, interleukin.

CsA than in its absence (**Figure 5e**), indicating that CsA did not impair the preferential hepatic homing of aEESs to the liver. In the presence of CsA, aEESs induced a GvHD that results in weight loss (**Figure 5f**), prostration, and ruffled fur. Although some mice occasionally died, these clinical signs were usually weak, in accordance with histological analysis demonstrating mild lesions at the

intestinal level and no lesions at the hepatic level (data not shown). This is in agreement with the observation that aEESs exhibited a decreased alloreactivity compared with fresh spleen cells,[16] as evidenced in immunodeficient recipient Rag2$^{-/-}$ IL-2Rγ$^{-/-}$ Balb/c mice (**Supplementary Figure S9a–d**).

Allogeneic lymphocytes exhibit antitumor activity in immunocompetent mice

To investigate the antitumor activity of our approach in an immunocompetent host, recipient Balb/c mice were transplanted with syngeneic BNL1MEA.7R.1-Luc HCC murine cells. Assessment of the antitumor activity of aEESs in an orthotopic BNL1MEA.7R.1-Luc model was not possible because of a high death rate after BNL1MEA.7R.1-Luc transplantation. Therefore, in order to mimic HCC treatment in an immunocompetent host, established subcutaneous BNL1MEA.7R.1-Luc tumors were treated by repeated intratumoral injections of aEESs from FvB mice in the presence of IL-2 + CsA. As expected, tumors showed a minimal spontaneous regression on D18 in nontreated (**Figure 6a**) and IL-2–treated (data not shown) control groups. In the IL-2 + CsA control group, tumors ultimately expanded on D18, after a short period of reduced growth (data not shown). In sharp contrast, a continuous tumor size decrease was observed from D7 to 18 in recipient mice treated by aEESs and IL-2 + CsA (**Figure 6a**) and was associated with enhanced persistence of fluorescent aEESs in the tumor (**Figure 6b**). This tumor rejection occurred without inducing any clinically detectable GvHD. These data demonstrate proof of concept of this approach in an immunocompetent mouse model.

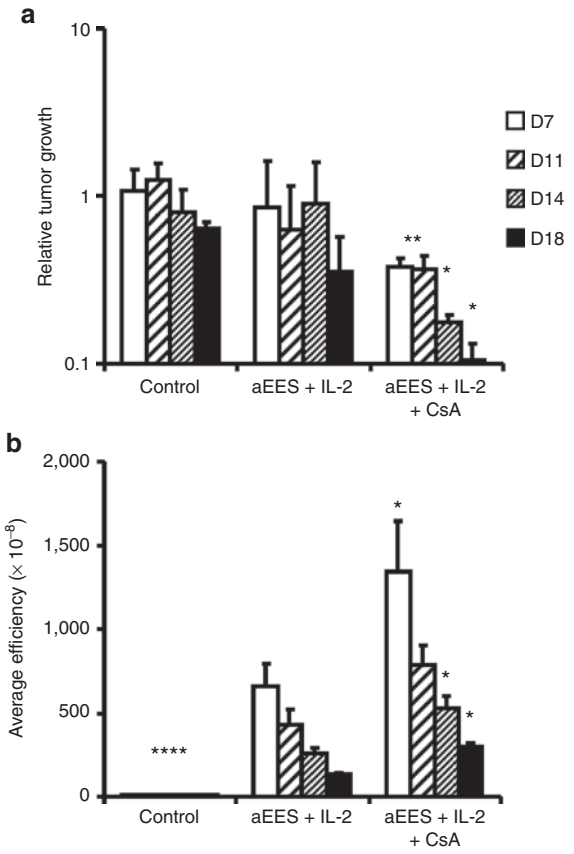

Figure 6 Antitumor activity of allogeneic *ex vivo*-expanded murine splenocytes (aEESs), used as a surrogate cell therapy product in an immunocompetent hepatocellular carcinoma (HCC) mouse model. (**a**) In the presence of interleukin-2 (IL-2) and cyclosporine A (CsA), but not in the sole presence of IL-2, intratumoral injection of aEESs leads to the rejection of BNL1.MEA7.R1-Luc subcutaneous tumors. *P < 0.05 compared with the control group, injected with phosphate-buffered saline (PBS) alone (Mann–Whitney test; n = 5 mice/group). (**b**) CsA administration increases the amount of DiR-labeled aEESs present within the tumor, as monitored by *in vivo* fluorescence gated on the tumor. *P < 0.05 compared with the group injected with aEESs + IL-2 (Mann–Whitney test; n = 5 mice/group).

DISCUSSION

Addressing an unmet medical need for the treatment of advanced HCC or early-stage HCC not eligible to standard of care, we have demonstrated *in vivo* proof of concept of aSGMKCs for HCC immunotherapy. We have uncovered for the first time that aSGMKCs exhibit a marked antitumor efficacy in state-of-the-art *in vitro* and *in vivo* models for HCC including an immunocompetent mouse model for HCC.

Addressing a future clinical development, we demonstrate that the aSGMKCs administration is safe in preclinical animal models. Because aSGMKCs are not tumor antigen specific, they could spread anywhere in the body and be harmful against any cell type. Suicide gene transfer is therefore necessary to control any unwanted side effects of aSGMKCs infusion. The *ex vivo* culture required for retroviral-mediated gene transfer is associated with a modification of lymphocytes' migratory properties, leading to a preferential hepatic tropism and thus limiting aSGMKCs spreading throughout the body. Furthermore, this hepatic tropism is also of advantage to target HCC and may be relevant for targeting intrahepatic metastasis of nonhepatic tumors such as colorectal cancer. With the aim to further increase the local concentration of aSGMKCs, we will consider the possibility to perform image-guided intratumor injections of aSGMKCs to improve their antitumor efficacy and limit their peripheral secondary effects. A second consequence of *ex vivo* culture is a decreased alloreactivity in terms of proliferation[12,13] and GvHD induction[14,16–18]: this is of advantage to limit hepatic toxicity, because the liver is a preferential target organ of GvHD. It should be noted that no clinical signs of xenogeneic GvHD were observed in SCID-bg mice receiving aSGMKCs under conditions where an antitumor effect was observed. This suggests that the antitumor effect may be mediated by a different mechanism than GvHD and may relate to a differential involvement of NK and/or NK-like T cells versus T cells in the cytotoxic activity versus GvHD induction.[20] We hypothesize that NK and/or NK-like T cells present in aSGMKCs are already activated and ready to kill target cells, whereas alloreactive T cells may need more time to expand and induce GvHD. Therefore, a therapeutic window may exist, allowing NK and NK-like T cells to provide an antitumor effect before the induction of GvHD. Despite the reduced ability of aSGMKCs to induce GvHD, the suicide gene transfer as a safety control system remains mandatory, as severe GvHD may still occur in small fraction of patients.[9,10] Indeed, numerous *in vitro* and *in vivo* preclinical studies have proven the efficacy and safety of the HSV-tk gene as a suitable transgene allowing to control alloreactivity (for a review,

see ref. [7]) and GvHD after HSC transplantation.[9,10] Indeed, aSGMKCs-induced GvHD has been controlled by GCV in all clinical trials reported to date. Our results suggesting a superior efficacy of the iCasp9/CID system than the HSV-tk/GCV system will prompt us to further explore iCasp9$^+$ aSGMKCs, especially using orthotopic models of HCC based on the use of primary tumor samples, which are more relevant than cell line-based models.[21] The iCasp9/CID system has the interest of not requiring the proliferation of target cells to induce apoptosis, which is an advantage because aSGMKCs have a reduced proliferative capacity.

We also demonstrate that potential rejection of aSGMKCs can be controlled by CsA. Since patients are immunocompetent, immune responses against aSGMKCs will occur, leading to their rejection. This will limit the risk of long-term aSGMKCs toxicity, but also their short-term antitumor efficacy. Thus, effector cells must be cytotoxic enough to quickly eliminate the tumor before being rejected by patient's immune cells. In phase I/II clinical trials of administration of partially HLA-matched EBV-specific allogeneic T cells for treatment of EBV-induced lymphoma, complete responses were observed in 14 of 33 patients despite alloimmunization against this "ready-for-use" allogeneic CTP. This suggests that HLA mismatch may not represent a strong limitation to allogeneic adoptive immunotherapy if short-term antitumor effects are desired.[22,23] Similarly, in a phase I trial of infusion of "intentionally HLA-mismatched IL-2 activated killer" cells produced from normal donors, rejection of infused intentionally HLA-mismatched IL-2 activated killer cells was expected to occur within 1 week and was considered as a safety mechanism to avoid GvHD.[24] Infusion of intentionally HLA-mismatched IL-2 activated killer cells was safe and, among 35 patients with metastatic solid tumors, only one grade I GvHD was observed.[24] Of importance, our data in immunocompetent recipient mice demonstrate the feasibility of obtaining antitumor effects while preventing the rejection of effector cells through CsA administration. This result further strengthens the proof of concept that human aSGMKCs could lead to a solid tumor regression, provided that their rejection is prevented.

The production of a bank of ready-for-use allogeneic CTP has been successfully used to treat hematological immunotherapies in patients.[22,23,25] Compared with autologous immunotherapies, this approach allows an easier large-scale development. Indeed, CTP production is easier when using lymphocytes from normal donors, which usually expand more efficiently than those from heavily treated patients. Second, a ready-for-use allogeneic CTP can be rapidly infused to patients, without the waste of time required for the production and qualification of a patient-specific CTP (i.e., an autologous or patient-directed CTP). Finally, there is no risk of losing a clinical-grade CTP batch: indeed, if a frozen cell batch planned to be infused to a given patient cannot be finally administered, it can still be used later for another patient.

Cytokine-induced killer cells, namely *ex vivo*-activated autologous PBMCs expanded in similar conditions as our aSGMKCs but without gene transfer,[26,27] have been shown to exhibit potent non–MHC-restricted NK-like T cell–mediated cytotoxic activity especially against HCC cell lines[27,28] and low potential for GvHD induction,[29] similarly to our observations with aSGMKCs. Randomized clinical trials[30,31] evaluating cytokine-induced killer therapy associated with transarterial chemoembolization and/or radiofrequency have demonstrated their ability to reduce HCC recurrence and to increase median time to relapse, relapse-free survival, and overall survival in HCC patients. Compared with previously described autologous cytokine-induced killer immunotherapies, our innovative approach based on aSGMKCs alloreactivity presents significant advantages and progress by providing rapid availability, the possibility to control side effects through the introduction of a suicide gene and an additional mechanism for tumor recognition, leading to a more potent antitumor effect. This concept is supported by clinical studies of allogeneic HSC transplantation demonstrating that (i) leukemic patients were at lower risk of relapse when transplanted with allogeneic compared with syngeneic or autologous graft[32] and (ii) delayed infusions of aSGMKCs[33] or allogeneic cytokine-induced killer cells[34] have anti-leukemic effects.

Collectively, our results provide a significant conceptual advancement in the immunotherapy of solid tumors by providing for the first time *in vivo* proof of concept for using aSGMKCs as immunotherapy for HCC. Furthermore, our results open the perspective for a clinical evaluation of aSGMKCs as an immunotherapy in patients with advanced HCC or early-stage HCC not eligible to standard of care.

MATERIALS AND METHODS

Detailed Materials and Methods are available in **Supplementary Materials**.

Production of aSGMKCs. Allogeneic SGMKCs were produced as previously described[14] and is detailed in **Supplementary Figure S1a**. PBMCs from 23 blood donors were activated with CD3 monoclonal antibody and human IL-2 and transduced on day (D) 3 with the MP71-T34FT retroviral vector encoding the human CD34 truncated form fused to the HSV-tk[35] or the SFG.iCasp9.2A.ΔCD19 retroviral vector (provided by M.K. Brenner, Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX) encoding the human CD19 and iCasp9,[8] immunomagnetically selected on D5 and expanded until D14. Transduced and CD34- or CD19-selected cells are referred as aSGMKCs, whereas nontransduced cells expanded in parallel for 14 days are referred as control cells. Effector cells (aSGMKCs or, when indicated, control cells or PBMCs) were then qualified for phenotype and GCV sensitivity and functionally characterized for *in vitro* and *in vivo* cytotoxicity, as outlined in **Supplementary Figure S1a**.

Cell lines. HeLa, Huh7, PLC-PRF5, and SK-Hep1 human cell lines were provided by the American Type Cell Culture (LGC Standards, Molsheim, France) and the Balb/c-derived HCC murine cell line BNL1MEA.7R.1[36] was kindly provided by Jean-François Dufour (University of Bern, Switzerland).

Animals. Eight- to 12-week-old C57BL/6 Rag2$^{-/-}\gamma_c^{-/-}$ mice were provided by Naoko Uchiyama (Central Institute for Experimental Animals, Kawasaki, Japan), C.B-17 severe combined immunodeficiency–beige (SCID-bg) mice were obtained from Taconic (Ejby, Denmark), Balb/c and FvB/N mice were provided by Janvier (Le Genest Saint Isle, France), luciferase-transgenic FvB/N (FvB/N-Luc) mice were provided by Robert S. Negrin (Stanford University, CA), and uPA/SCID-bg mice were kindly provided by Maura Dandri (Hamburg University, Germany). Animal experimentations received the approval of the local ethical committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg, approval n°AL/34/41/02/13).

In vitro *cytotoxicity assay.* Target cells (HeLa, Huh7, PLC-PRF5, and SK-Hep1) and graded amounts of effector cells were cocultured for 1–6

days in the presence of IL-2 and, when indicated, of anti-HLA class I monoclonal antibody (5 µg/ml; clone W6/32; Tebu, Le-Perray-en-Yvelines, France). Subsequently, remaining viable, adherent target cells were stained with crystal violet. Data are expressed as lytic units 50% of the experimental group normalized to the lytic units 50% values of the control group (**Supplementary Figure S1b**).

In vivo *cytotoxicity assay.* *In vivo* cytotoxicity assays were performed as previously described.[37] Luciferase-expressing target cells (HeLa-Luc and Huh7-Luc) were subcutaneously injected into C57BL/6 Rag2$^{-/-}\gamma_c^{-/-}$ immunodeficient recipient mice in the absence or presence of graded amounts of aSGMKCs, with daily i.p. IL-2 injection. Relative tumor growth was obtained by calculating the ratio of *in vivo* BLI on the indicated day to BLI on D0.

Orthotopic xenogeneic tumor model. SCID-bg mice with hepatic localization of intrasplenically injected Huh7-Luc cells were i.v. injected with aSGMKCs or vehicle and were daily i.p. injected with IL-2. Relative tumor growth was monitored by BLI. Homing of aSGMKCs was analyzed by *in vivo* fluorescence of DilC18(7) tricarbocyanine (DiR; Invitrogen, Cergy-Pontoise, France)-labeled aSGMKCs. CD19/iCasp9$^+$ aSGMKCs were injected with or without 2.5 mg/kg CID (AP20187 or BB Homodimerizer; Ozyme, Montigny le Bretonneux, France).

Murine immunocompetent tumor model. Murine splenocytes were expanded as previously described.[16] To evaluate the prevention of aEES rejection, Balb/c mice were i.v. injected with aEESs from FvB/N-Luc mice, daily i.p. injected with IL-2 ± CsA and monitored for the presence of aEESs by BLI. To evaluate the antitumor activity of aEESs, Balb/c mice were subcutaneously injected with BNL1.MEA7.R1-Luc cells. One week later, they received one intratumoral injection per day for 4 days of aEESs from FvB/N mice. Mice were daily i.p. injected with IL-2 + CsA and monitored by BLI for relative tumor growth. The GvHD was monitored clinically as described by Cooke *et al*.[38] and was quantified by body weight loss.

Statistical analysis. Data are expressed as mean ± SEM and were compared with the Mann–Whitney or Fisher exact nonparametric tests, excepted for **Table 1**, where normal data distribution allowed to perform a Student's paired *t*-test.

SUPPLEMENTARY MATERIAL

Figure S1. Outline of experimental protocols for aSGMKCs production and determination of Lytic Units 50% (LU50).
Figure S2. Allogeneic SGMKCs, but not control cells, are sensitive to the suicide gene prodrug.
Figure S3. Hepatoma cells have a low allostimulating potential.
Figure S4. Allogeneic SGMKCs have a decreased proliferative response to hepatoma cells, as compared to PBMCs.
Figure S5. Intravenous injection is more efficient than i.p. injection for favoring hepatic homing of aSGMKCs.
Figure S6. Efficacy of CD19/iCasp9$^+$ aSGMKCs depletion by the prodrug CID.
Figure S7. Administration of aSGMKCs does not induce histopathological abnormalities in intestines, kidney, lung and spleen.
Figure S8. Experimental design for adoptive allogeneic immunotherapy in immunocompetent recipients.
Figure S9. Allogeneic *ex vivo*-expanded splenocytes are less alloreactive than fresh SC.
Table S1. Relative distribution of T, NK-like T, and NK cells before and after immunomagnetic sorting of T, NK-like T and NK cells from aSGMKCs.

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Supplementary Materials

Materials and Methods

Cell lines

HeLa, Huh7, PLC-PRF5, HepG2 and SK-Hep1 human cell lines (American Type Cell Culture, LGC Standards, Molsheim, France) and the Balb/c-derived HCC murine cell line BNL1MEA.7R.1*[1]* (kindly provided by Dr F. Dufour, University of Bern, Switzerland) were cultured in Dulbecco's Modified Eagle Medium (DMEM, PAA Laboratories) supplemented with non-essential amino acids (GIBCO®, Invitrogen, Cergy Pontoise, France), 10µg/ml gentamycin (GIBCO®) and 10% (human cell lines) or 20% (BNL1MEA.7R.1 cell line) fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany). HeLa, Huh7 and PLC-PRF5 cells stably expressing luciferase (HeLa-Luc, Huh7-Luc, PLC-PRF5-Luc) were produced by transduction using a retroviral vector generated from a luciferase-encoding pCLNCX vector (Dr. Lorang, NIH, Bethesda, MD, USA). BNL1MEA.7R.1-Luc cells were transduced with luciferase-encoding VSV-G pseudoparticles.

Phenotyping

Effector cells were stained with phycoerythrin (PE)-cyanin (Cy)7-conjugated mAb specific for CD34 (Becton-Dickinson (BD), San Diego, CA, USA), Pacific Blue-conjugated CD3 mAb (BD) or PE-Cy5-conjugated CD56 mAb (Miltenyi Biotec) and analyzed with a LSRII flow cytometer (BD) and the Diva software (BD).

Prodrug sensitivity assay

In order to demonstrate the sensitivity of aSGMKCs to their prodrugs (Fig. S2 & S6a), aSGMKCs or, as a negative control, control cells, were cultured at D14 of expansion for one additional week in the presence of 500 IU/ml human Interleukin-2 (IL-2, Proleukin™, Novartis Pharma, Dorval, Québec) with or without the prodrugs 1 µg/ml GCV (Cymevan; Syntex, Puteaux, France) or 10 nM chemical inducer of death (CID, AP20187, Clontech Laboratories, Saint Germain en Laye, France). The relative cell growth was calculated as the ratio of the number of cells at the end of the assay (D14+7, see **Fig. S1a**) to the input number of cells at initiation of the assay (D14). The percentage of inhibition of relative cell growth by the prodrugs was calculated according to the formula [1-($RCG_{PRODRUG}$/RCG)] x 100, where $RCG_{PRODRUG}$ and RCG are the relative cell growths in the presence and absence of prodrug, respectively.

In vitro *cytotoxicity*

Target cells (HeLa, Huh7: 1500 cells/well; SK-Hep1: 2500 cells/well; PLC-PRF5: 20000 cells/well) were cultured in the absence or presence of effector cells (aSGMKCs, control cells or PBMCs) at E:T ratios 40:1 to 0.05:1 (serial 1/3 dilutions) in the presence of 500 IU/ml IL-2 or, when indicated, 20 ng/ml human IL-7 (Cytheris, Issy-les-Moulineaux, France) before evaluation of target cell killing. The effect of prodrugs on aSGMKCs'cytotoxicity was evaluated by performing the assay in the presence or absence of 1 µg/mL GCV or 10 nM CID (AP20187 or BB Homodimerizer; Ozyme, Montigny le bretonneux, France). For the evaluation of cytotoxicity in the presence of Cyclosporin A (CsA, Sandimmun, Novartis Pharma, Rueil-Malmaison, France), effector and target cells were cocultured at the E:T ratio of 40:1 in the absence or presence of the indicated CsA concentrations. After one to six days of co-culture, non-adherent effector cells and dead target cells were removed by washing with

PBS, and remaining viable, adherent, target cells were stained for 15min at room temperature (RT) with crystal violet (Sigma, Lyon, France) before being washed three times with tap water and lysed in PBS 1% w/v SDS for 10 min at RT. Absorbance was read at 560 nm on a Mithras LB940 microplate reader (Berthold, Thoiry, France). Data are expressed as percent killing (determined from serial dilutions of target cells) at the indicated effector:target ratios, then as lytic units 50% (LU50), calculated as the reverse of the number of cells per 10^6 effector cells required to kill 50% of target cells (**Fig. S1b**). LU50 of the experimental groups were normalized to the LU50 values of their control group, in order to normalize inter-experimental variations (**Fig. S1b**), and expressed as mean ± SE. HepG2 target cells killing could not be evaluated in this assay because target cells did not adhere strongly enough to allow for efficient staining by crystal violet.

Mixed lymphocyte reaction (MLR)

Responder cells (PBMC - used as a positive control of alloresponse – or aSGMKCs from the same donors) were stained as previously described[2] with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, the Netherlands). Triplicates of $2x10^5$ responder cells were cultured in the absence or presence of 60 Gy-irradiated allogeneic stimulating cells (EBV-transformed B-cell line - used as a positive control of allostimulation - or HCC hepatoma cell lines Huh7, HepG2, PLC-PRF5 and SK-Hep1) at a 4:1 responder:stimulator cell ratio. Cells were resuspended in RPMI 1640 medium supplemented with 10% normal human serum (EFS Bourgogne/Franche-Comté, Besançon, France) and cultured in round-bottomed 96-well plates in a final volume of 200 µl. After 6 days, the proliferative response in the MLR was evaluated by flow cytometry analysis of the dilution of CFSE staining, as previously described[2]: cells with reduced CFSE content ($CFSE^{low}$) were identified as alloreactive cells, while cells that did not lose CFSE staining ($CFSE^{high}$) were

identified as non-alloreactive cells. Data are expressed as the percentage of $CFSE^{low}$ cells (i.e. frequency of alloreactive cells) and as proliferative index. The proliferation index is calculated as the ratio of the median fluorescence intensity (MFI) of CFSE for $CFSE^{high}$ cells to the MFI of CFSE for $CFSE^{low}$ cells. This ratio indicates the fold decrease of MFI of proliferating cells, normalized to the MFI of non-proliferating cells[2]. The greater the number of cell divisions undergone by the alloreactive cells, the higher the proliferation index.

In vivo *cytotoxicity*

1×10^6 luciferase-expressing target cells were SC co-injected into C57BL/6 $Rag2^{-/-}$ $\gamma_c^{-/-}$ immunodeficient recipient mice in the absence or presence of graded amounts of aSGMKCs at the indicated E:T cell ratio in PBS. Daily IP injections of IL-2 (10^6 IU/kg) were performed during all experiments. Luciferase activity, measured by BLI using an IVIS 50 camera (Caliper Lifesciences, Roissy, France) after IP injection of 100µL luciferin (20 mg/ml; Caliper Lifesciences), was determined at the time of target cell injection and four (D4), seven (D7), and 14 (D14) days later. Tumor cell bioluminescence, analyzed with Living Image 3.1 software (Caliper Lifesciences), was expressed as photons/second/cm^2/steradian (p/s/cm^2/sr). Relative tumor growth was obtained by calculating the ratio of bioluminescence at the indicated day to the bioluminescence at D0. A relative tumor growth value <1 indicates tumor cell elimination.

Animal experimentations were performed after approval by the local ethical committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg, approval n°AL/34/41/02/13).. For ethical reasons, mice were not monitored until tumor-related death but were all sacrificed at the end of experiments.

Orthotopic xenogeneic tumor model

SCID-bg mice were intrasplenically injected with 1×10^6 Huh7-Luc, allowing them to migrate to the liver through the splenic vein. Four days later, luciferase activity was measured by BLI. Animals with hepatic localization of Huh7-Luc cells were selected for the experiment and $200x10^6$ aSGMKCs in 100µL PBS or vehicle were IV-injected under isoflurane anesthesia (D0) in the retro-orbital sinus. Daily IP injections of IL-2 (10^6IU/kg) were performed and luciferase activity was measured by BLI three and seven days after aSGMKCs injection, as indicated above. For ethical reasons, mice were not monitored until tumor-related death but were all sacrificed at the end of experiment.

In vivo *evaluation of expanded murine allogeneic splenocytes in immunocompetent recipients*

Splenocytes from FvB/N or FvB/N-Luc mice were resuspended at $0,2x10^6$ cells/ml in complete medium consisting in RPMI 1640 supplemented with penicillin-streptomycin (100U/ml and 100µg/ml respectively, GIBCO®), 10% v/v fetal bovine serum, 50µM β-mercaptoethanol and IL-2 (500 UI/ml) and activated by 5 µg/ml concanavalin A (Sigma) as previously described [3]. Activated splenocytes were then expanded in complete medium until day 14 at a cell density of 0.2 to $0.5x10^6$ cells/ml. For the evaluation of *in vivo* aEES survival, Balb/c mice were IV-injected at D0 with $10x10^6$ aEES from FvB/N-Luc mice and were daily IP injected (i) with either PBS or 50mg/kg/d CsA starting from D-2 before cell injection and (ii) with 10^6 IU/kg IL-2 starting from the day of cell injection. *In vivo* cell survival and expansion was monitored by BLI as described above. To evaluate the anti-tumor activity of aEES, Balb/c mice were SC-injected with $10x10^6$ BNL1.MEA7.R1-Luc cells. One week later, aEES from FvB/N mice were daily injected into the palpable tumor for four days ($50x10^6$ cells/day, from D0 to D3) with daily IP injections of 10^6 IU/kg IL-2 and 50 mg/kg

CsA from D0 to D14. Control groups were daily injected with PBS into the tumor from D0 to D3 and were daily IP-injected with PBS, IL-2 or IL-2+CsA from D0 to D14. Mice were monitored for relative tumor growth by BLI as described above.

Analysis of aSGMKCs or aEES homing by in vivo *fluorescence*

Allogeneic SGMKCs or *ex vivo* expanded murine splenocyteswere labeled by I ncubation in culture medium with 1.5 µg/ml DilC18(7) tricarbocyanine (DiR, D12731, Invitrogen) for 1h at 37°C, washed once with PBS, and resuspended in PBS at 7.5×10^8 cells/ml. 100-150 × 10^6 DiR-labeled aSGMKCs were IV or IP injected to SCID-bg mice or $50x10^6$ aEES were IV injected to Balb/c mice and the *in vivo* fluorescence signal was detected at the indicated time after epi-illumination with an IVIS 50 camera using a 675nm excitation filter and ICG (810-875nm) emission filter. The fluorescence emission was expressed as average of efficiency (% emission radiance - p/s/cm²/sr - to illumination irradiance - µw/cm²).

Human liver-chimeric mice

Human liver-chimeric mice were produced by transplanting cryopreserved primary human hepatocytes into the spleen of 3 weeks old Alb-uPA/SCID-bg mice as previously described[4]. Human albumin levels were quantified in mouse sera by ELISA according to manufacturer's instructions (Bethyl Laboratories, Euromodex, Souffelweyersheim, France).

Histological analysis

For hematoxilin-eosin staining and immunostaining, the organs (liver, spleen lung, intestine, kidneys) were fixed in 4% paraformaldehyde, processed, embedded in paraffin prior to

sectioning (10 microns) and stained as previously described[4]. For staining of Huh7-Luc cells or human hepatocytes, tissue sections were incubated with 1/25-diluted anti-hCK18 mAb (M7010, clone DC10; Dako France, Les Ulis, France) overnight at 4°C. For staining of aSGMKCs, tissues sections were incubated with 1/100-diluted human CD3 mAb (ref, clone , Dako) or CD56 mAb (ref, clone, Dako). Following washing, immunostained cells were detected using the peroxidase Vectastain Elite ABC kits (Vector Laboratories, Clinisciences, Nanterre, France).

Fig. S1. Outline of experimental protocols for aSGMKCs production and determination of Lytic Units 50% (LU50). (a) aSGMKCs production. D0: Activation of PBMCs (10^6 cells/ml) from healthy volunteer blood donors (Etablissement Français du Sang Alsace – EFS-, Strasbourg, France) with 10 ng/ml CD3 mAb (OKT3, Jansen-Cilag, Levallois-Perret, France) and expansion in RPMI 1640 medium supplemented with 10% normal human serum (EFS Bourgogne/Franche-Comté) and 500 IU/ml human IL-2, referred as complete culture medium. When indicated, CD3 mAb was replaced with 10^6 CD3/CD28 mAb-coated beads/ml (Invitrogen, Cergy Pontoise, France) and/or IL-2 was replaced by 20 ng/ml human IL-7. D3: Transduction of activated cells by a MP71-T34FT retroviral vector (EUFETS GmbH, Idar-Oberstein, Germany) encoding the naturally truncated form of human CD34 (tCD34), used as a selection marker and fused to the splice-corrected form of the HSV-tk (scHSV-tk), used as a suicide gene*[5]*. Cells were spin-transduced (1000 g for 3h at 32°C) at 10^6 cells/ml in the retroviral supernatant supplemented with 1000 IU/ml IL-2 and 5 µg/ml protamin sulfate (Sigma) as previously described[6]. D5: Immunomagnetic selection of CD34-expressing (i.e. transduced) cells with a CD34 Microbeads kit (Miltenyi Biotec SAS, Paris, France). Mean ± SE of percent CD34-positive cells before (transduction efficiency) and after (aSGMKCs purity) are indicated for 23 MACS® sorts, together with one representative CD34 expression profile in flow cytometry before and after selection. D5-D14: Expansion in complete culture medium (0,5-1.10^6 cells/ml). D14: Qualification of effector cells (aSGMKCs or, when indicated, control cells or PBMCs) for phenotype (CD34, CD3 and CD56 markers) and GCV sensitivity (one-week culture with GCV). Functional characterization for *in vitro* (effector:target cells co-cultures) and *in vivo* (SC co-injection of effector and target cells) cytotoxicity assays and for anti-tumor assays (orthotopic tumors and delayed IV-injection of effector cells). **(b) Determination of Lytic Units 50% (LU50).** The percent of target cell killing (in the example illustrated in the figure: Huh7 cells, 1500 cells/well) by aSGMKCs

(black triangles, full line) and PBMCs (white triangles, dashed line) was plotted against the E:T ratio. LU50 were calculated as the reverse of the number of cells per 10^6 effector cells required to kill 50% of target cells. In this representative experiment, the cytotoxic activity was calculated to be 233.1 LU50 for PBMCs, used as the control group, and 1709.4 LU50 for aSGMKCs, used as the experimental group. Therefore, LU50 of the experimental group represented 733% of LU50 of the control group.

Fig. S2. Allogeneic SGMKCs, but not control cells, are sensitive to the suicide gene prodrug. Relative cell growth of control cells (Ctrl) or aSGMKCs in the presence or absence of GCV was calculated as the ratio of the number of cells at the end of the GCV assay to the input number of cells at initiation of the GCV assay (mean ± SE, n=4 donors). For aSGMKCs, but not control cells, GCV-induced inhibition of relative cell growth (mean ± SE) is above the threshold of 80% inhibition, defined as the minimal value allowing for aSGMKCs clinical use (see [7]).

Fig. S3. Hepatoma cells have a low allostimulating potential. Responder cells (aSGMKCs -black bars- or PBMCs -white bars-, used as a positive control of alloreactive cells) were stained with CFSE and co-cultured with stimulating cells. Percent of proliferating ($CFSE^{low}$) cells was determined by flow cytometry at D6 of co-culture (mean ± SE, n=6 donors). The hepatoma cell lines are less efficient at inducing a proliferative response of aSGMKCs or PBMCs than B-EBV cells, used as a positive control for allostimulation. *: $P < 0.05$ compared to B-EBV stimulation (Mann-Whitney test).

Fig. S4. Allogeneic SGMKCs have a decreased proliferative response to hepatoma cells, as compared to PBMCs. Flow cytometry profiles of CFSE staining (from one representative experiment of 6 donors) 6 days after allostimulation of PBMCs (left) or aSGMKCs (right) by the indicated stimulating cells. The proliferation indexes, indicated in each quadrant, show that, whatever the stimulating cells, alloreactive aSGMKCs exhibit less cell divisions than alloreactive PBMCs and that aSGMKCs are therefore functionally impaired in terms of alloreactivity, in agreement with our previous data reported after B-EBV stimulation[2].

Fig. S5. Intravenous injection is more efficient than IP injection for favoring hepatic homing of aSGMKCs. (a) 150 × 10^6 DiR-labeled aSGMKCs were IV injected to SCID-bg mice and fluorescence emission gated on the liver was recorded at the indicated time. (Mean±SE, n=6 mice). **(b)** SCID-bg mice received an IP or IV (retro-orbital) injection of 100 × 10^6 DiR-labeled aSGMKCs. Fluorescence emission three hours after injection is shown for three representative mice of six per group. **(c)** The livers were then harvested for two mice and fluorescence was recorded. One non-injected mouse was used as a negative control of fluorescence (Ctrl). Similar results were observed at 24 h after injection (data not shown). **(d-f)** Human CK18 (d), CD3 (e) and CD56 (f) immunostaining was performed on serial sections of the liver of Huh7-Luc tumor-bearing SCID-bg mice, 24h after IV-injection of 150x10.6 aSGMKCs. Allogeneic SGMKCs were found surrounding the tumor and were mostly CD3-positive, with some cells being also CD56-positive (arrows).

Fig. S6. Efficacy of CD19/iCasp9+ aSGMKCs depletion by their prodrug CID (a) The relative cell growth of HSV-tk+ and iCasp9+ aSGMKCs was evaluated after one week culture in the absence or presence of GCV or CID. Relative cell growth of control cells : HSV-tk+

aSGMKCs: 0.87±0.10; iCasp9+ aSGMKCs: 2.38±1.32 (n=4). **(b)** The cytotoxic activity of aSGMKCs is prevented more efficiently and more rapidly with iCasp9+ aSGMKCs than with HSV-tk+ aSGMKCs. The cytotoxic activity of CD34/HSV-tk+ and CD19/icasp9+ aSGMKCs was evaluated against Huh7 cells after 3 days of co-culture in the presence or absence of 1 µg/mL GCV or 10 nM CID. No cytotoxicity was observed with iCasp9+ aSGMKCs in the presence of CID, at all tested E:C ratios, leading to no quantifiable lytic units in the presence of CID. By contrast, some cytotoxic activity was still observed with HSV-tk+ aSGMKCs in the presence of GCV, leading to no or only a slight decrease of LU50 values. Data are expressed as mean ± SE LU50 of four experiments. Controls: aSGMKCs without prodrug (CD34/HSV-tk+ aSGMKCs: 116±59 LU50; CD19/iCasp9+ aSGMKCs: 105±34 LU50). **(c)** The anti-tumor effect of CD19/iCasp9+ aSGMKCs in the orthotopic HCC model is reversed by only one administration of CID. Representative experiment showing the Huh7-luc bioluminescence in one mouse injected with or without 2.5 mg/kg CID at time of IV injection of 150-200x10^6 aSGMKCs. IL-2 was IP injected daily.

Fig. S7. Lymphoid infiltration by aSGMKCs in intestines, kidney, lung and spleen do not induce histopathological abnormalities. Seven days after IV injection of 150 x 10^6 aSGMKCs into Huh-7-Luc tumor-bearing SCID-bg mice, hematoxillin-eosin were performed on the intestines **(a)**, kidney **(b)**, lung **(c)** and spleen **(d)**. Images are representative of 4 mice.

Fig. S8. Experimental design for adoptive allogeneic immunotherapy in immunocompetent recipients. Allogeneic *ex vivo*-expanded splenocytes (aEES, depicted in light blue) from FvB/N donor mice (H-2^q) were considered as being functionally equivalent to human aSGMKCs and used as a surrogate cell therapy product. These cells were injected to

Balb/c recipients (H-2^d) harboring a syngeneic HCC tumor (BNL1.MEA7.R1-Luc cell line, depicted in pink) will be rejected by the recipient's immune system. The administration of the immunosuppressive drug CsA inhibits the alloimmunization of the recipient's immune system against aEES and thus prevents their rejection. Since aEES are resistant to the immunosuppressive activity of CsA, they can thus exert their anti-tumor activity and induce a tumor regression. The infusion of IL-2 together with aEES further improves their anti-tumor activity.

Fig. S9. Allogeneic *ex vivo*-expanded splenocytes are less alloreactive than fresh SC. $Rag2^{-/-}$ IL-2R$\gamma^{-/-}$ Balb/c mice (n=3/group) were IV-injected with 20 x 10^6 aEES or fresh SC from FvB/N-Luc mice. (**a**) The luciferase activity, evaluated at the indicated time, show that fresh SC expanded over time while aEES did not. One mouse injected with aEES died at D8 without overt clinical signs of GvHD. (**b**) Quantification of luciferase activity (p/s/cm²/sr, mean ± SE) in mice injected with fresh SC or aEES. Background of luciferase activity was evaluated on control mice that were not injected with cells from FvB-Luc mice. (**c**) The weight of recipient mice decreased after injection of fresh SC but not aEES, indicating that only fresh SC induce GvHD. (**d**) At the end of the experiment, 2 mg luciferine were injected and the spleen (S), lungs (L), intestines (I) and liver (Li) of recipient mice were harvested 10mn later and evaluated for luciferase activity. The luciferase activity is higher in organs of fresh SC-injected mice (n=3) than aEES-injected ones (n=2), consistent with a decreased potential of aEES to induce GvHD.

Table S1. Relative distribution of T, NK-like T and NK cells before and after immunomagnetic sorting of T, NK-like T and NK cells from aSGMKCs.

	Before sorting	Sorted fractions		
		T cells	NK-like T cells	NK cells
T cells ($CD3^+CD56^-$)	71.8±8.7	89.7±5.8	2.1±0.9	0.7±0.4
NK-like T cells ($CD3^+CD56^+$)	15.5±4.9	7.1±4.8	87.1±4.7	1.9±0.4
NK cells ($CD3^-CD56^+$)	8.4±04.2	1.0±0.8	10.0±5.2	96.5±1.3

Data are indicated as the percentage of T, NK-like T and NK cells in the indicated fractions (mean ± SE, n=4).

References

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4. Mercer, DF*, et al.* (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nature medicine* **7**: 927-933.
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7. Tiberghien, P*, et al.* (2001). Administration of herpes simplex-thymidine kinase-expressing donor T cells with a T-cell-depleted allogeneic marrow graft. *Blood* **97**: 63-72.

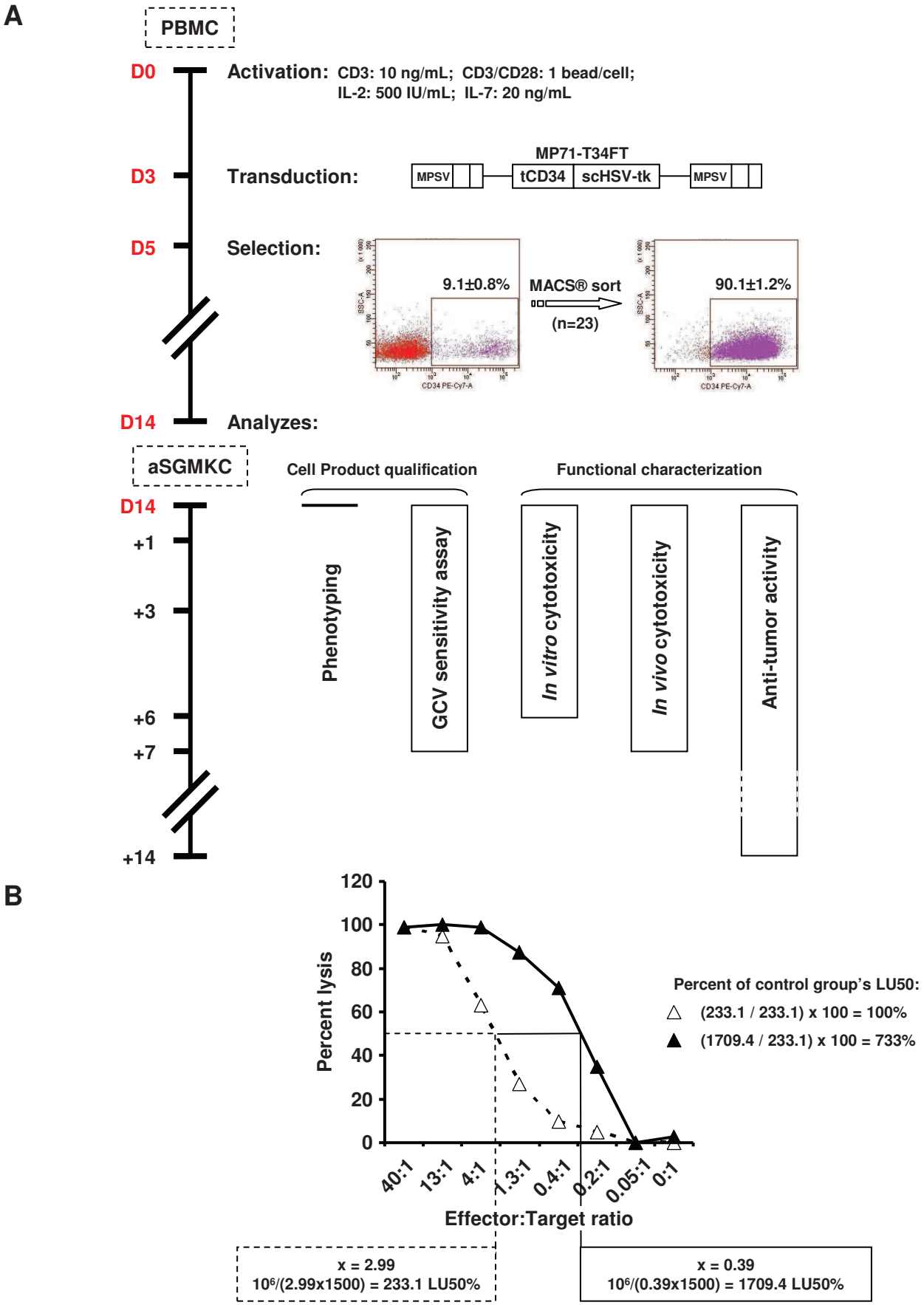

A
PBMC
D0
Activation: CD3: 10 ng/mL; CD3/CD28: 1 bead/cell;
IL-2: 500 IU/mL; IL-7: 20 ng/mL
D3
Transduction:
MP71-T34FT
MPSV
tCD34
scHSV-tk
MPSV
D5
Selection:
9.1±0.8%
MACS® sort
(n=23)
90.1±1.2%
SSC-A
CD34 PE-Cy7-A
D14
Analyzes:
aSGMKC
Cell Product qualification
Functional characterization
D14
+1
+3
+6
+7
+14
Phenotyping
GCV sensitivity assay
In vitro cytotoxicity
In vivo cytotoxicity
Anti-tumor activity
B
Percent lysis
120
100
80
60
40
20
0
40:1
13:1
4:1
1.3:1
0.4:1
0.2:1
0.05:1
0:1
Effector:Target ratio
Percent of control group's LU50:
(233.1 / 233.1) x 100 = 100%
(1709.4 / 233.1) x 100 = 733%
x = 2.99
10⁶/(2.99x1500) = 233.1 LU50%
x = 0.39
10⁶/(0.39x1500) = 1709.4 LU50%

Fig. S1

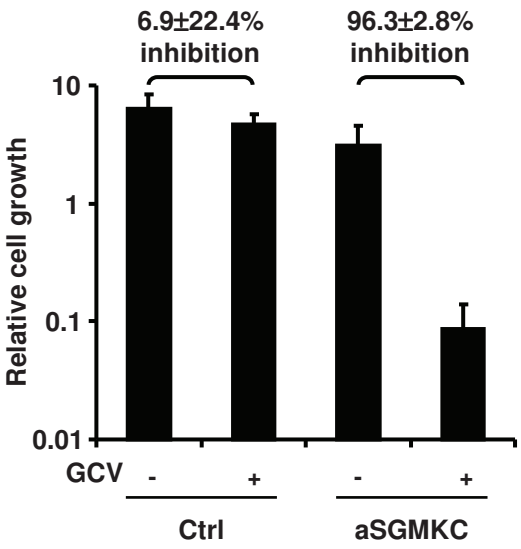

6.9±22.4%
inhibition
96.3±2.8%
inhibition
10
1
0.1
0.01
Relative cell growth
GCV
-
+
-
+
Ctrl
aSGMKC

Fig. S2

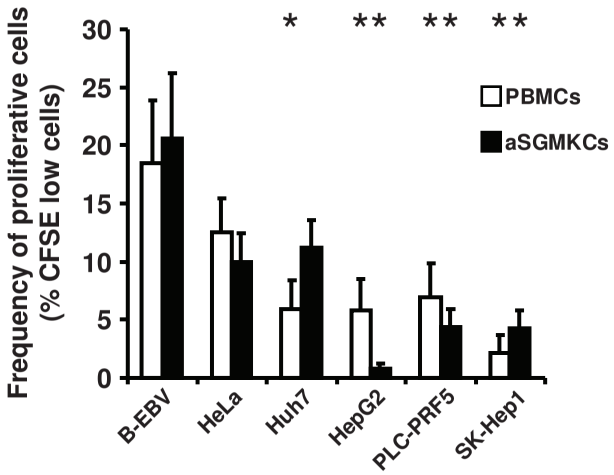

Frequency of proliferative cells
(% CFSE low cells)
30
25
20
15
10
5
0
*
**
**
**
PBMCs
aSGMKCs
B-EBV
HeLa
Huh7
HepG2
PLC-PRF5
SK-Hep1

Fig. S3

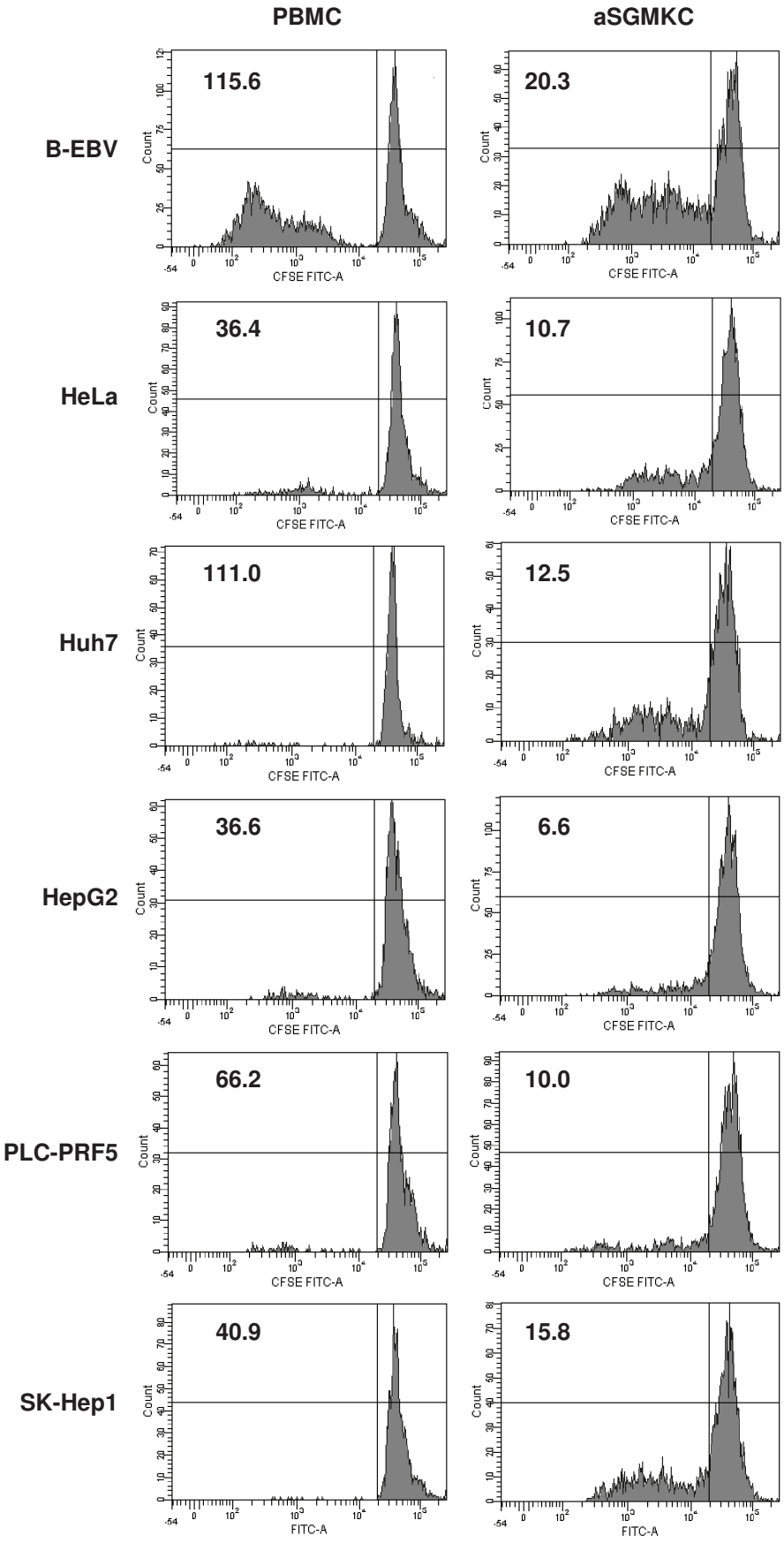

PBMC
aSGMKC
B-EBV
HeLa
Huh7
HepG2
PLC-PRF5
SK-Hep1
115.6
20.3
36.4
10.7
111.0
12.5
36.6
6.6
66.2
10.0
40.9
15.8
Count
CFSE FITC-A
FITC-A

Fig. S4

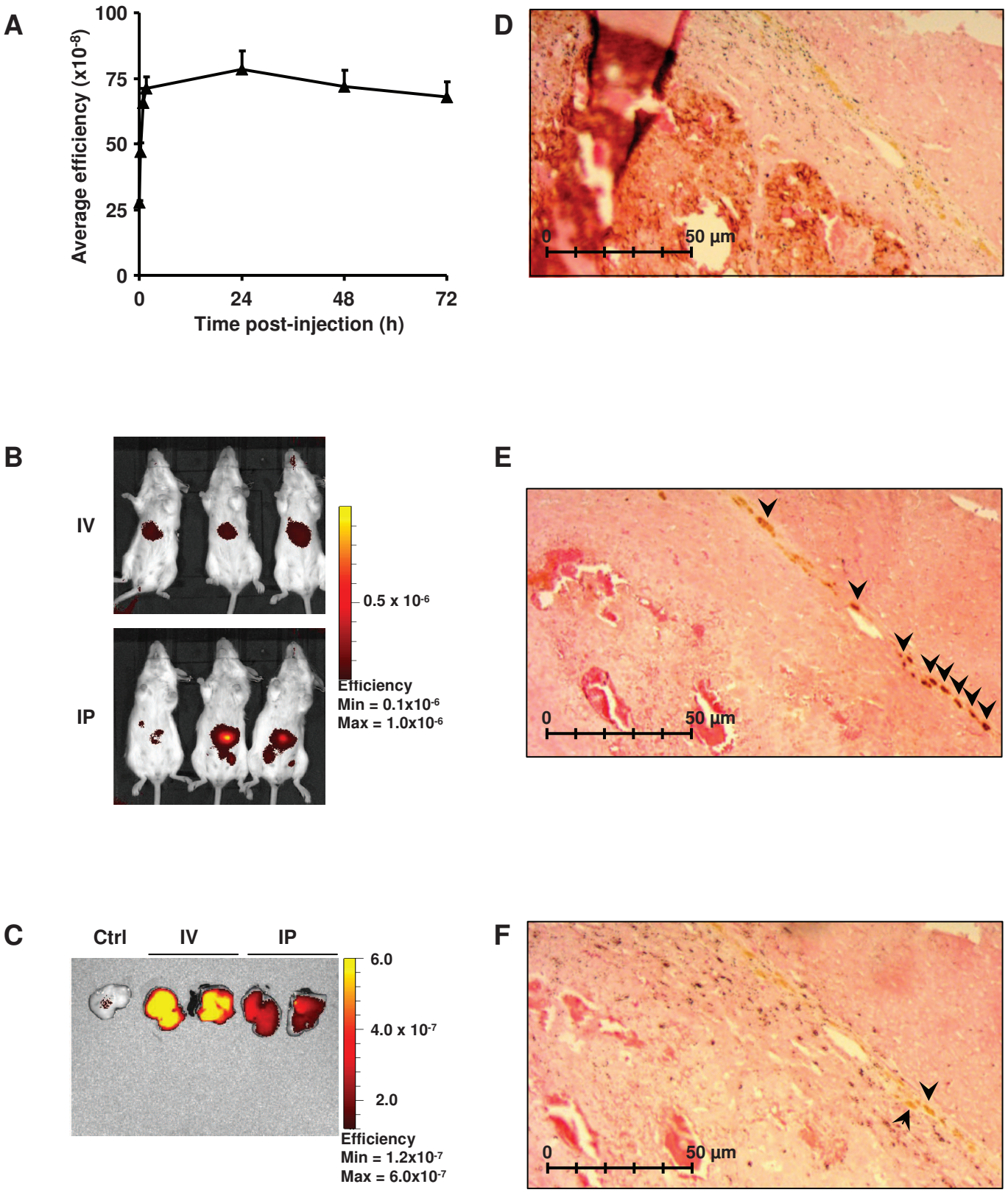
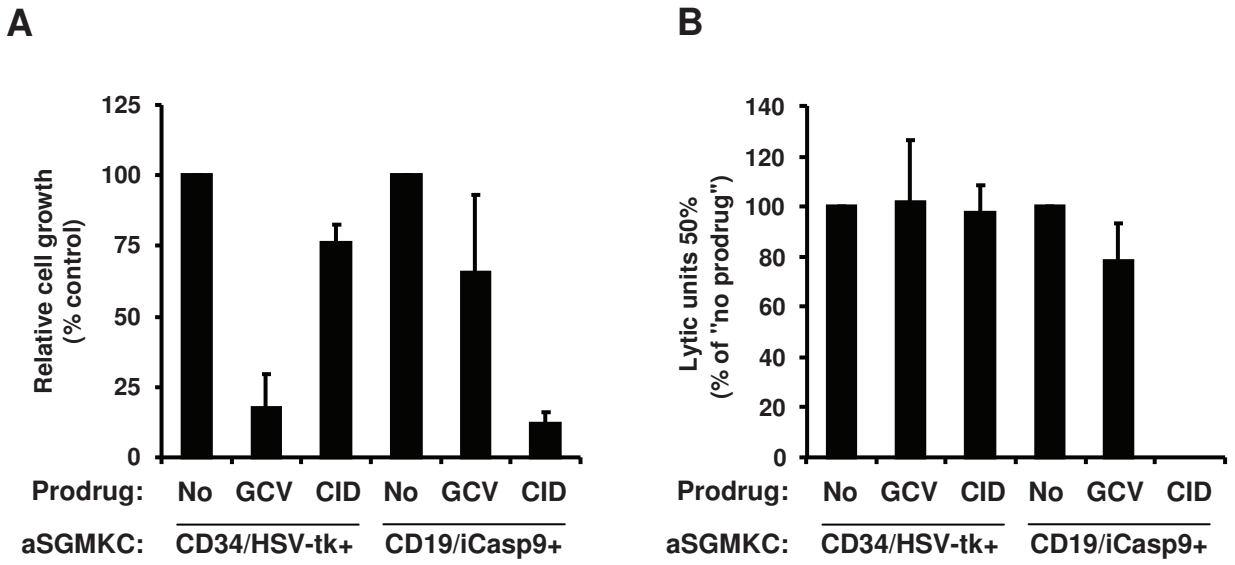

A
100
75
50
25
0
Average efficiency (x10-8)
0
24
48
72
Time post-injection (h)
D
0
50 µm
B
IV
IP
0.5 x 10-6
Efficiency
Min = 0.1x10-6
Max = 1.0x10-6
E
0
50 µm
C
Ctrl
IV
IP
6.0
4.0 x 10-7
2.0
Efficiency
Min = 1.2x10-7
Max = 6.0x10-7
F
0
50 µm

Fig. S5


A
Relative cell growth (% control)
125
100
75
50
25
0
Prodrug: No GCV CID No GCV CID
aSGMKC: CD34/HSV-tk+ CD19/iCasp9+
B
Lytic units 50% (% of "no prodrug")
140
120
100
80
60
40
20
0
Prodrug: No GCV CID No GCV CID
aSGMKC: CD34/HSV-tk+ CD19/iCasp9+

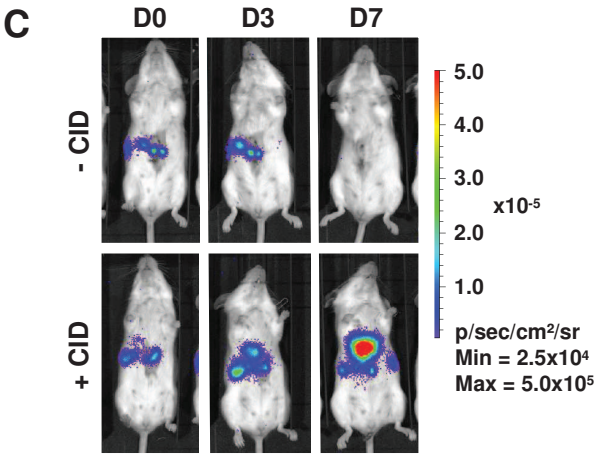

C
D0
D3
D7
- CID
+ CID
5.0
4.0
3.0
x10-5
2.0
1.0
p/sec/cm²/sr
Min = 2.5x10⁴
Max = 5.0x10⁵

Fig. S6

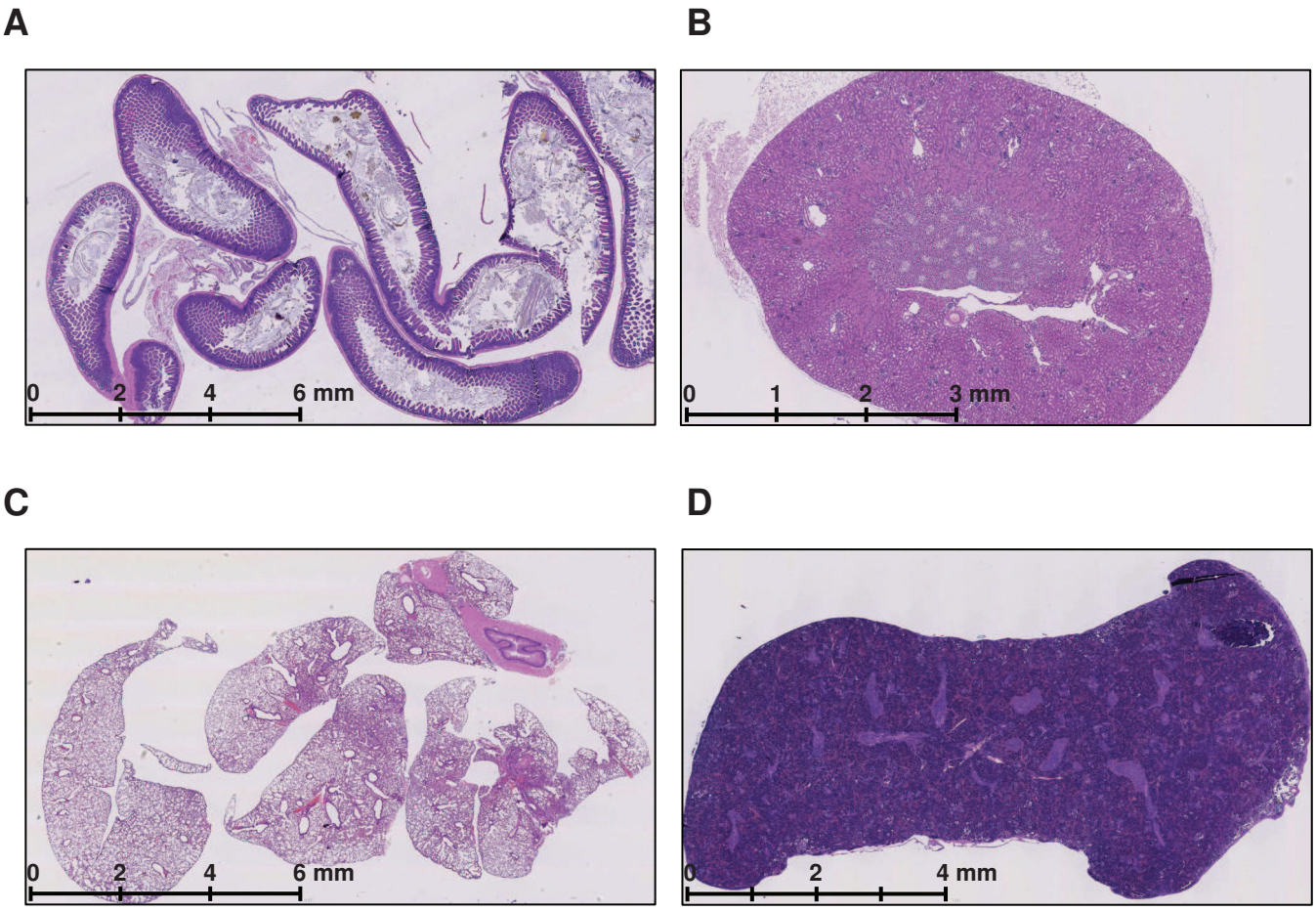


Fig. S7

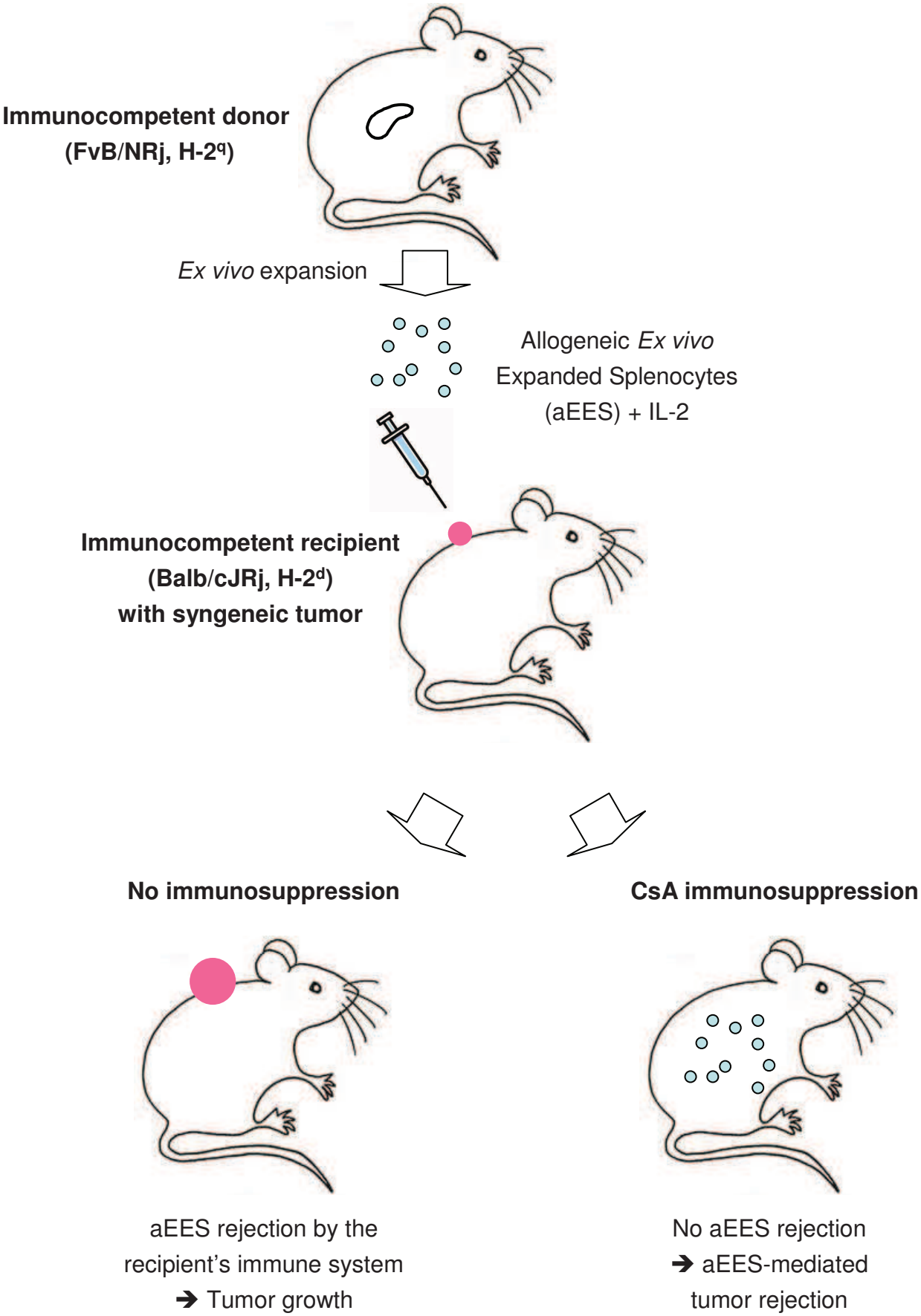

Immunocompetent donor
(FvB/NRj, H-2q)
Ex vivo expansion
Allogeneic Ex vivo
Expanded Splenocytes
(aEES) + IL-2
Immunocompetent recipient
(Balb/cJRj, H-2d)
with syngeneic tumor
No immunosuppression
CsA immunosuppression
aEES rejection by the
recipient's immune system
➔ Tumor growth
No aEES rejection
➔ aEES-mediated
tumor rejection

Fig. S8

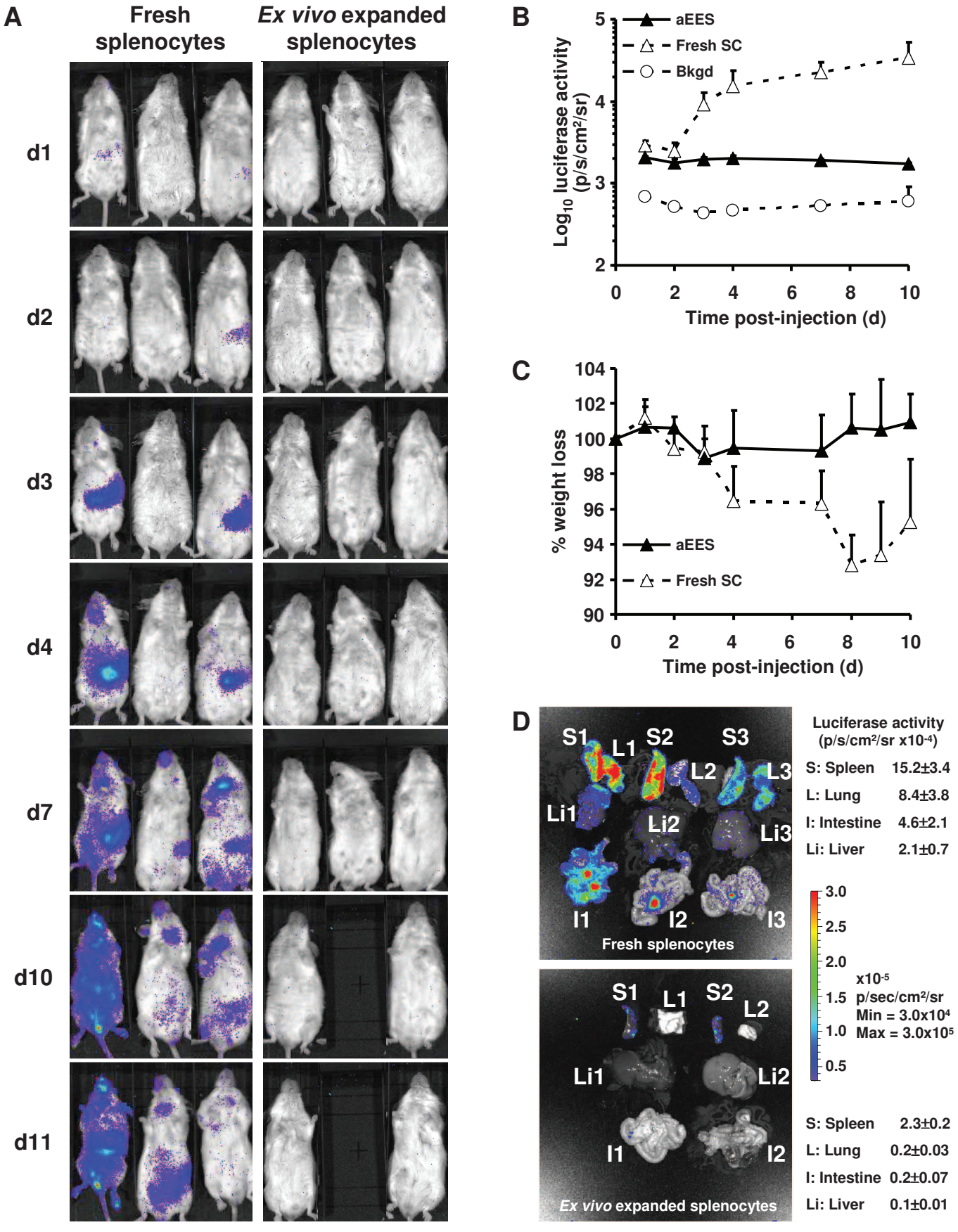

A
Fresh splenocytes
Ex vivo expanded splenocytes
d1
d2
d3
d4
d7
d10
d11
B
aEES
Fresh SC
Bkgd
Log10 luciferase activity (p/s/cm²/sr)
Time post-injection (d)
C
% weight loss
aEES
Fresh SC
Time post-injection (d)
D
S1
L1
S2
S3
L2
L3
Li1
Li2
Li3
I1
I2
I3
Fresh splenocytes
Luciferase activity (p/s/cm²/sr x10⁻⁴)
S: Spleen 15.2±3.4
L: Lung 8.4±3.8
I: Intestine 4.6±2.1
Li: Liver 2.1±0.7
x10⁻⁵ p/sec/cm²/sr
Min = 3.0x10⁴
Max = 3.0x10⁵
Ex vivo expanded splenocytes
S: Spleen 2.3±0.2
L: Lung 0.2±0.03
I: Intestine 0.2±0.07
Li: Liver 0.1±0.01

Fig. S9

Annex 3

Prevention of hepatitis C virus infection by adoptive allogeneic immunotherapy using suicide gene-modified lymphocytes: an *in vitro* proof-of-concept

Leboeuf C, Roser-Schilder J, Lambotin M, Durand S, Wu T, Fauvelle C, Su B,, Bôle-Richard E, Deschamps M, Ferrand C, Tiberghien P, Pessaux P, Baumert TF, Robinet E.

Gene Therapy, in revision

Prevention of hepatitis C virus infection by adoptive allogeneic immunotherapy using suicide gene-modified lymphocytes: an *in vitro* proof-of-concept

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Running title: Prevention of hepatitis C virus infection by allogeneic suicide gene-modified lymphocytes

Abstract

Hepatitis C virus-induced, end-stage liver disease is a major indication for liver transplantation, but systematic graft reinfection accelerates liver disease recurrence. Transplantation recipients may be ineligible for direct-acting antivirals, due to toxicity, resistance, or advanced liver disease. Adoptive immunotherapy with liver graft-derived, *ex-vivo* activated lymphocytes was previously shown to prevent hepatitis C virus-induced graft reinfections. Alternatively, the applicability and therapeutic efficacy of adoptive immunotherapy may be enhanced by "ready for use" suicide gene-modifed lymphocytes from healthy blood donors; moreover, conditional, prodrug-induced cell suicide may prevent potential side effects. Here, we demonstrate that allogeneic suicide gene-modified lymphocytes could potently, dose-dependently, and time-dependently, inhibit viral replication. The effect occurs at effector:target cell ratios that exhibits no concomitant cytotoxicity toward virus-infected target cells. The effect, mediated mostly by CD56+ lymphocytes, is IL-2-dependent, IFN-γ-mediated, and importantly, resistant to calcineurin inhibitors. Thus, post-transplant immunosuppression may not interfere with this adoptive cell immunotherapy approach. Furthermore, these cells are indeed amenable to conditional cell suicide; in particular, the inducible caspase 9 suicide gene is superior to the herpes simplex virus-thymidine kinase suicide gene. Our data provide *in vitro* proof-of-concept that allogeneic, third-party, suicide gene-modified lymphocytes may prevent hepatitis C virus-induced liver graft reinfection.

Introduction

Chronic hepatitis C virus (HCV) infections are a major public health problem, due to the high prevalence (3% of global population; i.e., 170 million people worldwide [1]) and the severity of HCV-related complications, including liver cirrhosis and hepatocellular carcinoma (HCC). HCV-induced end-stage liver disease is a major indication for liver transplantation (LT).[2] However, LT is associated with accelerated recurrence of liver disease, due to systematic graft reinfections.[3] There is currently no preventive vaccine for HCV infection. The available treatments, which combine ribavirin and pegylated Interferon-α (PEG-IFN-α), are limited by major secondary effects, low effectiveness (50% treatment failure), and high costs.[4] New treatments for HCV infections are currently under development. Many comprise different combinations of direct-acting antiviral drugs, like a protease and a polymerase inhibitor, which target several viral proteins essential for HCV replication and maturation. Those therapies were tested in treatment-naïve and treatment-experienced patients, with preliminary results suggesting good efficacy with limited treatment durations. Patients with HCV infection have been reported to be efficiently treated with interferon-free sofosbuvir-based regimens both before[5] and after[6] liver transplantation, with high rates of sustained virological responses, little resistance development, absence of drug-drug interactions, and virtually no toxicity. Despite these promising results, novel directly antiviral drugs will not prevent recurrent hepatitis C in all patients with LT and a fraction of patients that await LT may be still ineligible for these new compounds, due to toxicity, resistance, or advanced liver disease. Moreover, drug interactions with calcineurin inhibitors still limit their use in patients with LTs[7], for whom no authorization for temporary use has been delivered. Thus, new approaches remain mandatory to provide a therapeutic option for preventing HCV reinfection of the liver graft, at least for specific subgroups of patients.

A phase I study conducted by Ohira et al.[8] that included seven patients with HCV infections showed that an injection of activated lymphocytes reduced the HCV viral load after LT. Before transplantation, lymphocytes from the liver graft were recovered by perfusion of the graft; then, they were *ex vivo* activated for three days, and reinjected into the patient. A decrease in viral load was observed in five patients, and two of those five exhibited undetectable HCV levels; of the latter two, one relapsed, but the other continued to exhibit persistently HCV-negative serum, which strongly suggested that the chronic infection had healed. The decrease in viral load was more pronounced and persistent when the pre-transplant viral load was low; this finding suggested that the therapeutic effect was limited by reinfection of the graft. Indeed, when activated lymphocytes were injected into the patient three days after transplantation, the graft had already been reinfected.

Consequently, we reasoned that a cell infusion given at the time of transplantation might be more efficient than an infusion performed three days after transplantation. This approach would require injecting lymphocytes that had been activated prior to the transplantation. To that end, we propose to produce a bank of "ready for use", activated lymphocytes from healthy donors that express a suicide gene. With this readily available store, at the time a patient receives a transplant, a batch of suicide gene-modified lymphocytes (SGMLs) could be chosen from the bank and administrated immediately, without wasting the time required for SGML production and qualification. However, there is a risk that allogeneic SGMLs might exert alloreactivity toward the patient’s cells or the liver graft, which could lead to severe side effects, like graft-versus-host disease (GvHD) or liver graft rejection, respectively. To avoid these side effects, a suicide gene could be introduced into the lymphocytes prior to injection. Then, when side effects were detected, the SGMLs could be specifically eliminated by administering a prodrug that targets the suicide gene. These SGMLs are currently being tested as a gene therapy product in a phase III-clinical study of patients that received haplo-identical

allogeneic hematopoietic stem cell (HSC) transplantation. The SGMLs were successfully and safely infused into dozens of patients without major side effects.[9-12] Currently, those patients have undergone up to 15 years follow-up[12-14] (for review, see [15]).

In this study, we investigated whether allogeneic SGMLs generated from healthy donors could provide a potent antiviral effect toward HCV-infected cells, as an *in vitro* proof-of-concept that such immunotherapy could be considered in patients with HCV infections that received a LT. Our findings provide perspective on the clinical use of SGMLs for preventing HCV reinfection of a liver graft.

Results

Suicide gene-modified cells inhibit *in vitro* HCV replication

With an HCV replicon-infected cell model, we first evaluated the antiviral activity of SGMLs. The SGMLs were generated after CD3 activation or CD3/CD28 costimulation to induce low and high alloreactivity, respectively.[16, 17] Both SGML preparations produced a similar dose-dependent reduction in HCV replication; they achieved an approximately 3-log reduction in HCV replication at an effector:target cell ratio of 2:1 (Fig. 1a). We confirmed this antiviral effect in a more relevant cell culture model system, where Huh7.5.1 cells were infected with the infectious HCVcc Jc1 strain (Fig. S1a). In both cell models, the antiviral effect was observed without significant SGML-mediated cytotoxicity toward the target cells (Figs. 1b, S1b). Previous studies that evaluated various GvHD models[17] found that SGMLs produced after CD3/CD28 costimulation were more aggressive *in vivo* than SGMLs produced after CD3 activation. Therefore, we used only the latter product for subsequent experiments.

The gene transfer process did not affect the antiviral activity of *ex vivo*-expanded lymphocytes; a similar result was obtained when SGMLs were compared to non-transduced expanded control cells (referred to as Co cells) in both the HCV replicon model (Fig. 1c) and the HCVcc model (Fig. 1d). Again, the antiviral effect was not associated with cytotoxic activity against target cells (Figs. S1c, d). In accordance with their known interleukin-2 (IL-2) dependence, the antiviral activity of SGMLs was more potent in the presence than in the absence of exogenous IL-2 (Figs. 1c, d). Therefore, subsequent *in vitro* experiments were performed in the presence of 500 IU/mL IL-2.

The magnitude of inhibition of HCV RNA replication was lower in the HCVcc model than in the replicon model, raising the question of whether this was due to different efficiencies in transfection vs infection or because the HCVcc model constitutes the complete replication

cycle of the virus rather than just sugenomic RNA replication. The percentage of transfected vs infected cells in Huh7.5.1. cells, quantified by flow cytometry after staining of the HCV core protein, was similar in both models at D1 of culture but remained constant at D2 and D3 in the replicon model, while it continuously increased in the HCVcc model and was finally higher than in the replicon model (Fig. S2). These results suggest that such a higher percentage of infected cells may account, at least in part, for a less efficient inhibition of RNA replication by SGMLs.

In the HCVcc model, the antiviral effect of SGMLs was more pronounced when the coculture was initiated on the day of HCV inoculation compared to cocultures initiated one to three days later (Fig. 1e). This result supported our hypothesis that SGMLs would be more efficient at preventing liver graft reinfection in an administration at the time of LT compared to an administration three days later. Cryopreservation did not affect the antiviral activity of SGMLs, as shown by the similar antiviral activity observed between fresh and frozen cells (Fig. 1f). This result demonstrated that cryopreserved cells may be adequate for clinical use.

Phenotypical and functional characterization of SGMLs involved in antiviral activity

Although the low effector:target cell ratio facilitated a lack of significant SGML-mediated cytotoxicity against target cells, we could not exclude the possibility that only a few target cells were electroporated in the HCV replicon model or infected in the HCVcc infection model; the specific killing of only a few cells would not be detected in our cytotoxicity assay. Therefore, to exclude direct cell killing, antiviral assays in the HCV replicon system were performed in a transwell configuration, where effector and target cells were cultured in separate chambers, but soluble factors could diffuse between the chambers. Again, SGMLs exhibited strong, IL-2-dependent antiviral activity (Fig. 2a). This result indicated that effector-target cell contact was not required for inhibiting HCV replication, and the antiviral

effect was not dependent upon receptor-mediated cytotoxicity. These results suggested that the antiviral activity was mediated, at least in part, by soluble factors.

The addition of anti-IFN-γ monoclonal antibodies (mAbs), but not anti-IFN-α or anti-IFN-β mAbs, blocked the antiviral effect of SGMLs on HCV replication. The results were similar in a transwell assay (Fig. 2b), in a coculture assay (Fig. S3a), and in the HCVcc system (Fig. S3b). The combination of anti-IFN-α, -β, and –γ mAbs did not further block the antiviral activity (data not shown).

Most SGMLs are CD3+ CD56- T cells; the remaining cells are CD3+ CD56+ ''NK-like'' T cells and CD3- CD56+ NK cells (Fig. 2c and Sauce et al.[16]). To decipher which of these cells were involved in the antiviral activity of SGMLs, CD56+ and CD56- cells were purified by immunomagnetic sorting. We found that both fractions contributed to the antiviral activity, although CD56- cells had a slightly lower antiviral activity than CD56+ cells. The results were similar in the HCV replicon (Fig. 2d) and the HCVcc systems (Fig. S3c), and again, no cytotoxic activity was detected against target cells (Figs. S3d, e).

The antiviral activity of SGMLs is not inhibited by calcineurin inhibitors

One potential limitation of our approach was the potential for allo-immunization. Indeed, the patient could develop an immune response against SGMLs, and that would result in SGML elimination. This effect would be undesirable for two reasons: (1) SGML rejection would reduce their therapeutic effect, and (2) a cross-reaction could contribute to hepatic graft rejection. The calcineurin inhibitors, Cyclosporin A (CsA) and Tacrolimus (FK506), are typically administered to patients with LTs to prevent graft rejection. We previously reported that the alloreactivity of *ex vivo*-expanded lymphocytes was resistant to CsA.[18] Therefore, we reasoned that calcineurin inhibitors might prevent allo-immunization against both the liver graft and the SGMLs, but would not impair SGML antiviral activity. Thus, we evaluated *in*

vitro the effects of CsA and FK506 on HCV replication. Electroporated Huh7.5.1 cells were incubated in the absence or presence of fixed amounts of SGMLs with increasing concentrations of CsA or FK506, in the HCV replicon model (Figs. 3a, b) or in the HCVcc model (Figs. 3c, d). In accordance with previous reports,[19] a high concentration of CsA (Figs. 3a, c), but not FK506 (Fig. 3b, d), exhibited an inhibitory *in vitro* effect on HCV replication, and this effect was not due to a toxic effect on cell viability (Fig. S4). However, neither CsA nor FK506 blocked the antiviral effect of SGMLs (Fig. 3), indicating that SGMLs were resistant to the immunosuppressive activity of calcineurin inhibitors. Similar results were observed when exogenous IL-2 was omitted in the culture (Fig. S5), indicating that a possible reversal of the SGML antiviral activity by calcineurin inhibitors is not overcome by the addition of exogenous IL-2. These results suggested that administration of calcineurin inhibitors to recipients of LTs as a prophylaxis for liver graft rejection may not prevent the antiviral activity of SGMLs.

SGML depletion is more efficient with the iCasp9 than with the HSV-tk suicide gene

We assessed the ability of the HSV-tk suicide gene to reverse SGML-induced cytotoxicity. We prepared cocultures at low (≤1:1) and high (2:1 to 8:1) effector:target cell ratios, in the presence or absence of the prodrug, ganciclovir (GCV), added at the initiation of culture. After three days of coculture, a slight shift in the viability curve was observed (Fig. 4a), indicating that GCV caused a small inhibition in the cytotoxic activity of HSV-tk+ SGMLs against the target cells. This weak GCV-induced depletion of HSV-tk+ SGMLs prompted us to examine SGMLs that expressed the iCasp9 suicide gene. The new-generation, iCasp9 suicide gene was reported to confer more rapid, efficient killing than the HSV-tk suicide gene, when cells were exposed to its prodrug, a chemical inducer of death (CID)[20]. We found that the addition of the CID at initiation of the culture indeed completely prevented the cytotoxic

activity of iCasp9+ SGMLs at high effector:target cell ratios (Fig. 4b), up to a 40:1 ratio (data not shown); as expected, GCV had no effect on these iCasp9+ SGMLs. Conversely, also as expected, the CID did not affect the cytotoxic activity of HSV-tk+ SGMLs (Fig. 4a). When evaluated in terms of lytic units (calculated as shown in Fig. S6), GCV had no effect on HSV-tk+ SGMLs, but CID completely prevented the cytotoxicity of iCasp9+ SGMLs (Fig. 4c). The greater efficacy of the iCasp9/CID system over the HSV-tk/GCV system may not result from a more pronounced SGML depletion, but rather, from a more rapid depletion. This was suggested by the observation that, after one week of SGML cultures in the presence of their respective prodrugs, both prodrugs caused a similar level of SGML depletion (Fig. 4d). To further evaluate the effect of SGML depletion on antiviral activity, iCasp9+ SGMLs were cocultured with target cells in the absence or presence of CID. The antiviral activity, evaluated at non cytotoxic effector:taget cell ratios (Fig. 4e), was associated with an IFN-γ production (Fig. 4f) and the SGML depletion by CID was associated with the abrogation of the antiviral effect (Fig. 4e) and of the IFN-γ production (Fig. 4f).

Discussion

LT is the treatment of choice for end-stage liver diseases, but the systematic reinfection of the graft, associated with an accelerated recurrence of liver disease after transplantation limits its use in patients with HCV infections. A phase I clinical trial performed by Ohira et al.[8] demonstrated that liver graft-derived, *ex vivo*-activated lymphocytes infused three days after transplantation could provide an antiviral effect that could prevent liver graft HCV reinfection. In that study, the activated hepatic lymphocytes had a more potent antiviral effect when the initial viral load was low. Conversely, with a high viral load, the antiviral effect was less potent. Hence, we reasoned that, at three days after liver transplantation, the liver graft may have already been reinfected; however, at the time of transplantation, the liver graft would be less likely to be reinfected. Therefore, the activated lymphocytes should be delivered at the time of transplantation. However, this approach requires cell preparation prior to the acquisition of the liver graft; thus, the SGMLs must be produced from other sources. Therefore, we considered the possibility of using third-party allogeneic SGMLs acquired from healthy blood donors. Indeed, this cell therapy product contains *ex vivo*-activated lymphocytes. We previously demonstrated that SGMLs could be safely infused into patients that had received HCTs. In that study, we showed that SGMLs could specifically control occasional side effects, such as the induction of GvHD.[12] Furthermore, the prior *ex vivo* retroviral-mediated transfer of a suicide gene into the SGMLs allowed their specific *in vivo* killing with the administration of a prodrug, delivered at the time side effects were detected.

In the present study, we showed that SGMLs provided a potent antiviral effect at low effector:target cell ratios that are non-toxic for target cells. We tested SGMLs on two *in vitro* HCV replication models, including the widely used HCVcc infection model. We observed that the SGML-mediated antiviral effect was more efficient when SGMLs were added at time

of HCV inoculation, and that the later the time of SGMLs addition, the lower the efficacy. This finding confirmed our hypothesis that activated lymphocytes, such as SGMLs, should be infused at the time of transplantation. The observation that an antiviral effect can be observed at low, non-toxic effector:target cell ratios suggest that the number of cells that need to be activated in order to produce the IFN-γ involved in the antiviral effect is lower than the number of cells required to induce a cytotoxic effect. This interpretation is substantiated by the observation that the CID-induced depletion of SGMLs abrogated completely their cytotoxic activity, but only partially their antiviral effect: at high effector-target cell ratios, few SGMLs may escape the CID-induced death and may be sufficient to generate an antiviral effect, but not a cytotoxic one.

Similarly to liver lymphocytes[8], peripheral blood mononuclear cells (PBMCs) subjected to CD3+IL-2 activation present an IFN-γ-mediated antiviral activity toward HCV and an anti-tumor activity toward HCC, both provided mostly by CD56+ cells, including CD3- CD56+ NKs and CD3+ CD56+ NK-like T cells.[21] These studies are consistent with our present results and with our recent report demonstrating that high concentrations of SGMLs provided NK and NK-like T cell-mediated anti-tumor effects in an HCC model[22] . However, since they were generated after short-term (3 days) activation with IL-2, this raises the question of the similitude of such activated lymphocytes, as well as of our SGMLs, with “Lymphokine-Activated Killer” (LAK) cells. Indeed, the clinical use of LAK cells was limited owing to a massive release of cytokines and subsequent pulmonary oedema requiring intensive care. Our SGMLs seem to be closer to ‘’Cytokine-Induced Killer’’ (CIK) cells than to LAK cells in terms of protocol of activation (CD3+IL-2 for SGMLs vs CD3+IL-2±IFN-γ ±IL-1 for CIK cells vs IL-2 for LAK cells) and culture duration (2-4 weeks for SGMLs and CIK cells vs 3-5 days for LAK cells). One may hypothesize that this may lead to differences between LAK vs SGML (or CIK cells), as an example in terms of expression of homing molecules (thus, of

homing properties) and/or exhaustion (thus, of potential for cytokine release, proliferation and cytotoxicity). Indeed, SGMLs, evaluated in phase I to III clinical trials in patients transplanted with hematopoietic stem cells, induced limited toxicities[9-12] and, to the best of our knowledge, no pulmonary oedema.

Using a humanized HCC mouse model, we previously demonstrated that, when infused intravenously, SGMLs preferentially homed to the liver and weakly migrate to the lungs.[22] This property is of advantage to improve the efficacy while limiting the peripheral spreading of SGMLs. Indeed, infusing SGMLs could allow reaching high local concentrations of IFN-γ, close to the target cells while leading to low peripheral IFN-γ concentrations. In this respect, this should be of great advantage, in terms of tolerability, over infusing exogenous recombinant IFNs, such as IFN-γ or IFN-α: the IFN-α toxicity is a strong limitation of the current pegylated-IFN- α -based regimen, due to the doses required to reach efficient concentrations in the liver, that also lead to high concentrations in peripheral, non-target organs and induce severe side effects. However, the use of allogeneic SGMLs is associated with complex immunological interactions involving three immunological partners: the patient's immune system, the liver graft lymphocytes, and the allogeneic SGMLs, generated from a third-party donor. Nevertheless, SGMLs also have the advantage of being resistant to CsA inhibition. Thus, an immunosuppressive regimen with CsA can control the first two partners: (1) the recipient immune system alloreactivity toward the liver graft (prevention of liver graft rejection) and toward SGMLs, and (2) the alloreactivity of grafted liver lymphocytes toward the recipient tissues (hepatic GvHD). Alternatively, the third partner, the SGMLs, can be controlled with the suicide gene approach without affecting the first two partners.

One important issue is to determine whether there is any advantage in using third-party SGMLs, which are genetically different from the liver graft, compared to using autologous

liver graft-derived lymphocytes. This hypothesis is technically challenging to investigate, because HCV replication is typically evaluated *in vitro*, with established hepatoma cell lines. Indeed, primary human hepatocytes are mostly refractory to HCV infection *in vitro*. Thus, comparing the anti-viral activity of SGMLs toward autologous vs. allogeneic primary human hepatocytes remains unfeasible. Indirect observations have suggested that alloreactivity may provide additional benefits. When activated lymphocytes were restimulated with CD3 mAbs 24 h before evaluation of their antiviral activity, their ability to decrease HCV replication improved.[21] Also, allogeneic HSC transplantation[23], but not autologous HSC transplantation[24], was reported to lead to loss of detectable HIV, which was temporally correlated with full donor chimerism, but also to the development of GVHD, a complication of HSC transplantation associated with alloreactivity. Although the mechanisms leading to a HIV cure after HSC transplantation remain to be elucidated, and although they may be different from those involved in the prevention of HCV replication, those results suggested that alloreactivity may add benefit to an anti-viral effect.

To date, clinical experiences with SGMLs in an allogeneic context have shown that the risk of potential GvHD induction is low, particularly in the liver, and that, even when side effects occur, they can be efficiently controlled with the suicide gene approach.[9, 12, 25] Therefore, we propose to produce a bank of "ready for use" allogeneic SGMLs. Then, when a patient receives a transplantation, a batch of SGMLs could be selected from the bank and infused into the patient during transplantation, without wasting the time required for SGML production and qualification. Taken together, our results have provided *in vitro* proof-of-concept that an adoptive immunotherapy can be safely applied to the efficient prevention of liver graft reinfection by HCV. Additional *in vivo* experiments with human liver-chimeric mouse models of HCV infections[26] will be required to demonstrate further the feasibility of our approach.

Materials and Methods

Production of SGMLs. Allogeneic SGMLs were produced as previously described.[22]. After informed consent from healthy blood donors, PBMCs were isolated by ficoll centrifugation of donated blood. Harvested PBMCs were activated with a CD3 mAb (10 ng/mL OKT3, Jansen-Cilag, Levallois-Perret, France) and human IL-2 (500 IU/mL Proleukin™, Novartis Pharma, Dorval, Québec). Activated PBMCs were cultured for 3 days, and on day 3, they were transduced with one of two retroviral vectors. One was the MP71-T34FT vector, which encoded a truncated form of the human CD34 sequence fused to the HSV-tk sequence.[17, 27] The other retroviral vector (SFG.iCasp9.2A.ΔCD19; provided by Pr M.K. Brenner, Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX) encoded the human CD19 and iCasp9 sequences.[25] Transduced cells were immunomagnetically selected on day 5 and expanded until day 14. These transduced and CD34-selected or CD19-selected cells are referred to as SGMLs. In parallel, non-transduced cells were expanded for 14 days to serve as control cells and are referred to as Co cells. Effector cells (SGMLs or, when indicated, Co cells or PBMCs) were qualified for phenotype and prodrug sensitivity. Also, they were functionally characterized *in vitro* for antiviral activity and cytotoxicity.

The percentage of transduced selected cells out of the total numbers of CD34+ or CD19+ cells before immunomagnetic selection (i.e., the transduction efficiency) was determined by immunostaining with Phycoerythrin (PE)-conjugated CD34 mAb or Allophycocyanin (APC)-conjugated CD19 mAb (Miltenyi Biotec) and flow cytometry analyses (LSRII, Becton-Dickinson (BD), San Diego, CA, USA). The transduction efficiency was 13.1±3.1% (n=10) for CD34/HSV-tk+ SGMLs and 26.1±8.1% (n=6) for CD19/iCasp9+ SGMLs. The purity of the positive fraction after immunomagnetic sorting was 93.6±0.7% (n=10) for CD34/HSV-tk+ SGMLs and 97.2±0.9% (n=6) for CD19/iCasp9+ SGMLs. The sensitivity of SGMLs to the

corresponding prodrug was confirmed by culturing HSVtk+ SGMLs in the absence or presence of 1 μg/mL ganciclovir sodium (GCV; Cymevan; Syntex, Puteaux, France), or by culturing iCasp9+ SGMLs in the presence or absence of 10 nM of CID (BB homodimerizer; Ozyme, St Quentin en Yvelines, France). The relative cell growth was defined as the absolute number of cells obtained at the indicated time of culture to the number of input cells at the initiation of the culture.

Evaluation of SGML antiviral activity: *The HCV replicon model.* Huh7.5.1 cells were cultured in Dulbecco's Modified Eagle Medium (PAA Laboratories) supplemented with 10% fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany), non-essential amino acids (GIBCO®, Invitrogen, Cergy Pontoise, France) and 10 μg/mL gentamycin (GIBCO®). Huh7.5.1 cells were electroporated with an HCV replicon that comprised RNA corresponding to the HCV genome, genotype 2a, isolate JFH1, which was defective for virus entry, due to deletions of E1 and E2 envelope proteins and the luciferase gene (JFH1ΔE1E2-luc). In the present study, the Huh7.5.1 cells served as ‘’target cells’’.

Different amounts of SGML cells (effectors) were co-incubated with fixed amounts of target cells to produce effector:target cell ratios that ranged from 0.125:1 to 2:1 (co-cultures that allowed contact between effector and target cells). The same cell numbers were also cultured in separate compartments (transwell cultures, which did not allow contact between effector and target cells) using transwells with a pore size of 0.4 μm (Sigma). Unless otherwise indicated, all cultures were performed in the presence of 500 IU/ml IL-2. Target cells alone were cultured in the presence of 100 ng/mL IFN-γ (Clinisciences, Nanterre, France) to serve as a positive control of viral replication inhibition. After 3 days of culture, SGMLs (non-adherent cells) and dead target cells were removed by washing, and the remaining viable

target cells (adherent cells) were lysed with 0.1% w/v SDS in PBS. Luciferin was then added to measure luciferase activity, which reported viral replication within target cells.

We also investigated which soluble factor(s) were involved in SGML antiviral activity. During the co-incubation of SGMLs and target cells, we added antibodies (final concentration of 5 μg/ml; eBioscience SAS, Paris, France) that specifically blocked IFN-α, IFN-β, and IFN-γ (and an isotype control). The quantification of IFN-γ in culture supernatants was performed using a Legend Max™ human IFN-γ ELISA kit (BioLegend, San Diego, CA) according to the manufacturer's instructions.

Where indicated, after 14 days of expansion, CD56- (T lymphocytes) and CD56+ (NK and NKT cells) populations were isolated from SGMLs with PE-conjugated CD56 antibody with the anti-PE Multisort kit (Miltenyi Biotec). The separation was performed with the autoMACS Separator (Miltenyi Biotec). The purity of each cell fraction was determined by immunostaining with Pacific Blue-conjugated CD3 mAb (Becton Dickinson) and PE-conjugated CD56 mAb (Miltenyi Biotec), and performing flow cytometry (LSRII, Becton Dickinson). The antiviral activities of CD56- and CD56+ fractions were tested at different effector:target cell ratios, as previously described.

We intended to use these SGMLs in an immunosuppressive context; therefore, we tested SGML resistance to calcineurin inhibitors. To that end, Huh7.5.1 and SGMLs were cocultured at an effector:target cell ratio of 0.5:1 in the presence of different concentrations of either CsA or FK506 (Tacrolimus).

The cell culture produced-HCV model. The cell culture produced-HCV (HCVcc) model allowed the production of infectious viral particles. Briefly, Huh7.5.1 cells were infected with the recombinant virus, Jc1 HCVcc (genotype 2a/2a chimera), which also expressed luciferase. This laboratory prototype strain, based on JFH1,[28] has been used extensively to study virus-

host interactions and to evaluate antivirals *in vitro* and *in vivo*.[28-32] At different days post-infection, SGMLs were added to HCVcc-infected Huh7.5.1 cells at effector:target cell ratios of 0.5:1 or 2:1. Three days after adding SGMLs, Huh7.5.1 cells were lysed and tested for luciferase activity. Huh7.5.1 cell viability was evaluated with crystal violet staining, as previously described. The antiviral activities of CD56- and CD56+ subsets of SGMLs were also tested in this system at an effector:target cell ratio of 0.5:1.

Evaluation of SGML cytotoxic activity. In each assay, effector and target cells were cocultured in parallel with the antiviral assays to monitor target cell viability. After three days of coculture, non-adherent effector cells and dead target cells were removed by washing with PBS; the remaining viable, adherent, target cells were stained for 15 min at room temperature with crystal violet (Sigma, Lyon, France). Cells were then washed three times with tap water and lysed with 1% w/v SDS in PBS for 10 min at room temperature. Viable cell density was determined by the absorbance at 560 nm, measured on a Mithras LB 940 microplate reader (Berthold, Thoiry, France). Cell viability was expressed as the percent optical density of treated cells compared to control cells. We also calculated the inverse of the number of cells per 10^6 effector cells required to detect a 50% decrease in optical density (lytic units 50%; LU50; Fig. S6). The LU50 values of the experimental groups were normalized to the LU50 values of the corresponding control groups to control for inter-experimental variations. The results are expressed as the mean LU50±SEM.

Supplementary information is available at ISMEJ's website

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Figure legends

Figure 1. Dose and time-dependent inhibition of HCV replication by suicide gene-modified lymphocytes (SGMLs) in the absence of cytotoxicity against target cells (a) Inhibition of HCV replication by SGMLs. Fixed amounts of Huh7.5.1 cells were electroporated with a luciferase-expressing hepatitis C virus (HCV) replicon and cocultured with increasing numbers of effector cells (black circles, full line: SGMLs generated after CD3 activation; white circles, dashed line: SGMLs generated after CD3/CD28 costimulation). Data are expressed as the mean ± SE %HCV replication compared to that in control (target cells cultured alone: $6.19\times10^6\pm0.51\times10^6$ relative light units -RLU; n=6). **(b)** Target cell viability at different effector:target cell ratios. Target cells were stained with crystal violet at the end of the coculture experiments. Data correspond to experiments shown in (a), and cell viability is expressed as the mean±SE % optical density of cells cultured with SGMLs compared to that of control (target cells: 0.51 ± 0.13; n=6). **(c)** Antiviral activity of SGMLs. Huh7.5.1 cells infected with the HCV replicon were cultured in the presence or absence of either varying numbers of effector cells, with or without 500 IU/mL IL-2 (a cofactor required for effector cell survival and proliferation) or in the presence or absence of 100 ng/mL IFN-γ (a positive control for antiviral activity, in the absence of effector cells; white circle). SGMLs: white diamonds, dashed line, n=4; non-transduced expanded control lymphocytes (Co) + IL-2: black triangles, full line, n=4; SGMLs + IL-2: black diamonds, full line, n=5. HCV replication was evaluated by quantifying luciferase activity three days after HCV infection. Data are expressed as the mean±SE %HCV replication compared to that in control (target cells alone, cultured without IL-2 or IFN- γ: $5.03\times10^6 \pm 3.53\times10^6$ RLU; n=4). **(d)** Effect of IL-2 on antiviral activity in the HCVcc model. Huh7.5.1 cells infected with HCVcc were cultured as described in (c). HCV replication was evaluated by quantifying luciferase activity three days

after infection. Antiviral activity is expressed as the mean±SE %HCV replication compared to that in control (target cells cultured without IL-2: $1.43\times10^6\pm0.62\times10^6$RLU; n=3). **(e)** Effect of time on antiviral activity of SGMLs. At different times after infection, effector cells were incubated with Huh7.5.1 target cells infected with HCVcc. The cocultures were maintained for three days at effector:target cell ratios of 0:1 (dotted line), 0.5:1 (dashed line), or 2:1 (full line). Data are expressed as the mean ± SE %HCV replication compared to that in control (target cells alone: $0.44\times10^6\pm0.11\times10^6$ RLU on day 0; $0.98\times10^6\pm0.29\times10^6$ RLU after 1 day; $1.42\times10^6\pm0.44\times10^6$ RLU after 2 days; $1.57\times10^6\pm1.11\times10^6$ RLU after 3 days; n=4). **(f)** Effect of freezing on antiviral activity of SGMLs. Cryopreservation did not affect the antiviral activity of SGMLs (white circles, dashed line) in the HCV replicon model, compared to fresh SGMLs (black circles, full line). Data are expressed as the mean ± SE %HCV replication compared to that in control (target cells alone: $1.60\times10^5\pm0.80\times10^5$ RLU; n=3).

Figure 2. SGML antiviral activity is mediated by IFN-γ, mostly from CD56+ cells. (a) Target cells were cultured as indicated in (Fig. 1c), but the target cells were separated from the effector cells in transwells, where the effector cells were placed in the upper chamber, and the target cells were placed in the lower chamber, with a permeable membrane (pore size: 0.4 μm) between the chambers. HCV replication was evaluated by quantifying luciferase activity three days later, expressed as the mean ± SE %HCV replication compared to that in control (target cells alone, cultured without IL-2: $3.96\times10^6\pm2.55\times10^6$ RLU; n=4). **(b)** SGMLs were assessed for antiviral activity in the HCV replicon model at an effector:target cell ratio of 2:1. Effector cells were cultured separate from target cells in transwells in the absence or presence of 5 μg/mL anti-IFN-α, anti-IFN-β, anti-IFN-γ, or isotype (isot) control mAbs. Data are expressed as the mean ± SE %HCV replication compared to that in control (target cells cultured without SGMLs: $4.71\times10^6 \pm 3.14\times10^6$ RLU; n=3). **(c)** The frequency of CD56-

negative cells (white bars) and CD56-positive cells (black bars) was quantified by flow cytometry; PBMC: the initial suspension, Co cells: non-transduced, expanded lymphocytes . Data are expressed as the mean±SE % cell subsets compare to the total number of cells (n=3). **(d)** CD56-depleted (CD56-; white diamonds, dashed line) and CD56-enriched (CD56+; white triangles, dashed line) fractions of SGMLs were compared to unsorted SGMLs (black circles, full line) for antiviral activity in the HCV replicon model. Data are expressed as the mean±SE %HCV replication compared to that in control (target cells alone: $7.62\times10^6\pm5.96\times10^6$ RLU; n=4).

Figure 3. SGML antiviral activity is resistant to calcineurin inhibitors. Huh7.5.1 target cells were incubated in the absence (white circles, dashed lines) or presence (black circles, full lines) of SGMLs at an effector:target cell ratio of 0.5:1 in the HCV replicon model **(a, b)** or at an effector:target cell ratio of 2:1 in the HCVcc infection model **(c, d)**. All cultures were performed in the presence of 500 IU/mL IL-2 and were exposed to increasing amounts of CsA **(a, c)** or FK506 **(b, d)**. HCV replication was evaluated three days later. Data are expressed as the mean ± SE %HCV replication compared to that in control (target cells cultured without SGMLs nor calcineurin inhibitor: (a) $7.56\times10^6\pm6.25\times10^6$, n=4; (b) $6.90\times10^6\pm5.85\times10^6$, n=4; (c) $2.64\times10^5\pm0.93\times10^5$, n=4; (d) $4.86\times10^5\pm1.53\times10^5$, n=4).

Figure 4. SGML depletion was more efficient with iCasp9/CID than with HSV-tk/GCV. SGMLs that expressed either the HSV-tk **(a)** or the iCasp9 **(b)** suicide gene were cocultured with Huh7.5.1 target cells electroporated with the HCV replicon. Cocultures were not exposed (PBS: black diamonds, full line) or exposed to either GCV (white squares, dashed line) or CID (white triangles, dashed line) at the indicated effector:target cell ratios (from 0:1 to 8:1). The target cell viability, evaluated three days later, is expressed as the mean ± SE %optical

density compared to that of control (target cells alone: optical density = 0.97 ± 0.03 for one donor representative of three). **(c)** The effect of prodrugs on SGML cytotoxicity. HSV-tk+ SGML (left) and iCasp9+ SGML (right) cytotoxicities were measured after three days of coculture with target cells in the presence or absence of prodrugs, CID or GCV. Data are expressed as the mean ± SE %LU50 compared to that of control effector:target cocultures without prodrug (controls=100%). Control measurements were: CD34/HSV-tk SGMLs: 116 ± 59 LU50 (n=3); CD19/iCasp9 SGMLs: 105 ± 34 LU50 (n=4). **(d)** The efficiencies of GCV and CID in SGML depletion. SGMLs were expanded for one week in the absence (PBS) or presence of a prodrug. Data are expressed as the mean±SE relative cell growth during this week (n=4). **(e, f)** The effects of the CID prodrug on the antiviral activity and IFN- production by SGMLs. Huh7.5.1 target cells electroporated with the HCV replicon were cocultured with iCasp9+ SGMLs at the indicated effector:target cell ratios, in the absence (white bars) or presence (black bars) of CID and were evaluated three days later for HCV replication **(e)** and IFN-γ concentration in the culture supernatant **(f)**. Date in (e) are expressed as the mean±SE %HCV replication compared to that in control (target cells cultured without SGMLs and without CID: $1.09 \times 10^6 \pm 0.02 \times 10^6$ RLU; n=3). Data in (f) are expressed as ng/ml IFN-γ (mean±SE, n=3).

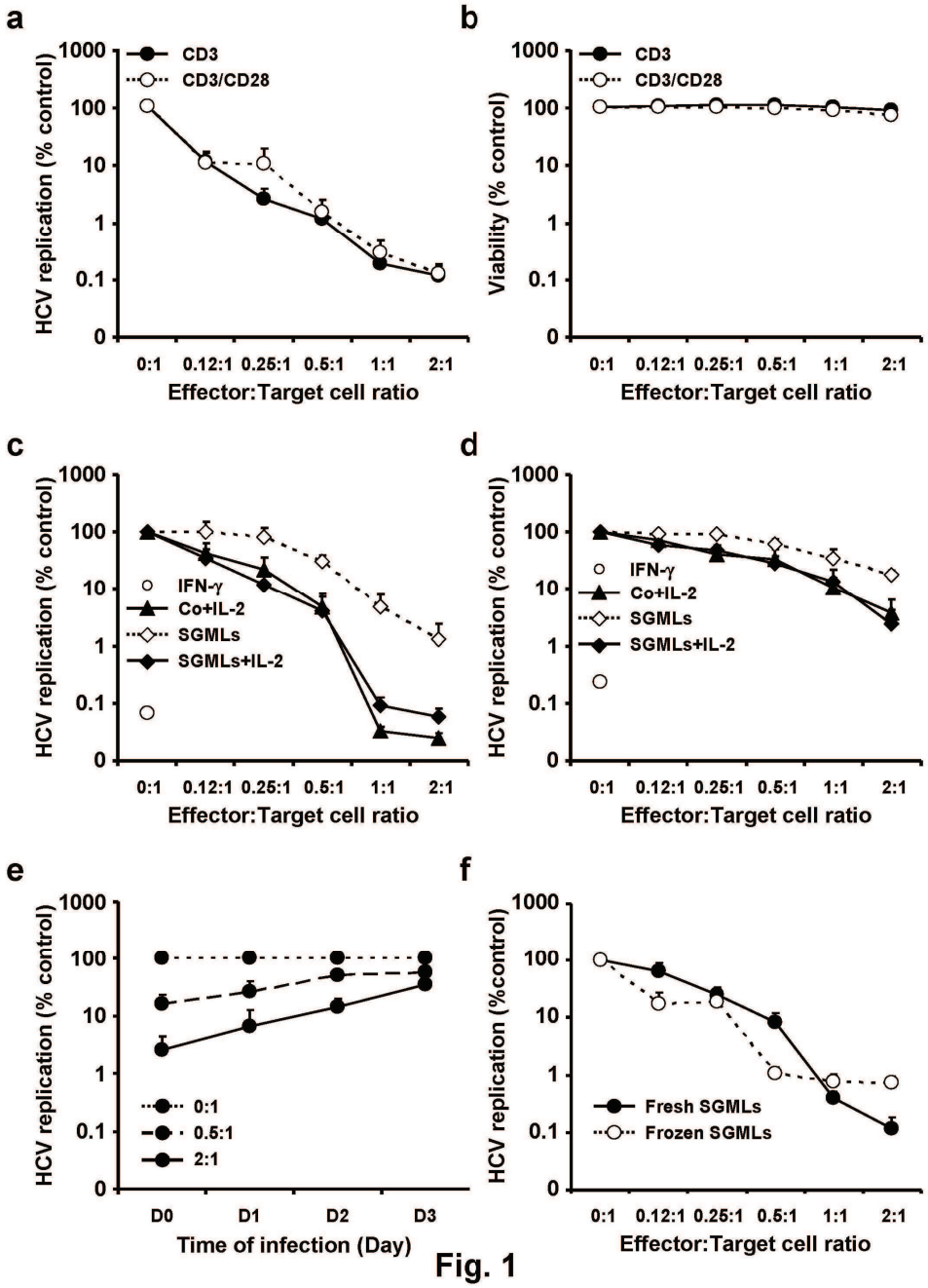
a
HCV replication (% control)
1000
100
10
1
0.1
0
CD3
CD3/CD28
0:1
0.12:1
0.25:1
0.5:1
1:1
2:1
Effector:Target cell ratio
b
Viability (% control)
CD3
CD3/CD28
Effector:Target cell ratio
c
HCV replication (% control)
IFN-γ
Co+IL-2
SGMLs
SGMLs+IL-2
Effector:Target cell ratio
d
HCV replication (% control)
IFN-γ
Co+IL-2
SGMLs
SGMLs+IL-2
Effector:Target cell ratio
e
HCV replication (% control)
0:1
0.5:1
2:1
D0
D1
D2
D3
Time of infection (Day)
f
HCV replication (%control)
Fresh SGMLs
Frozen SGMLs
Effector:Target cell ratio

Fig. 1

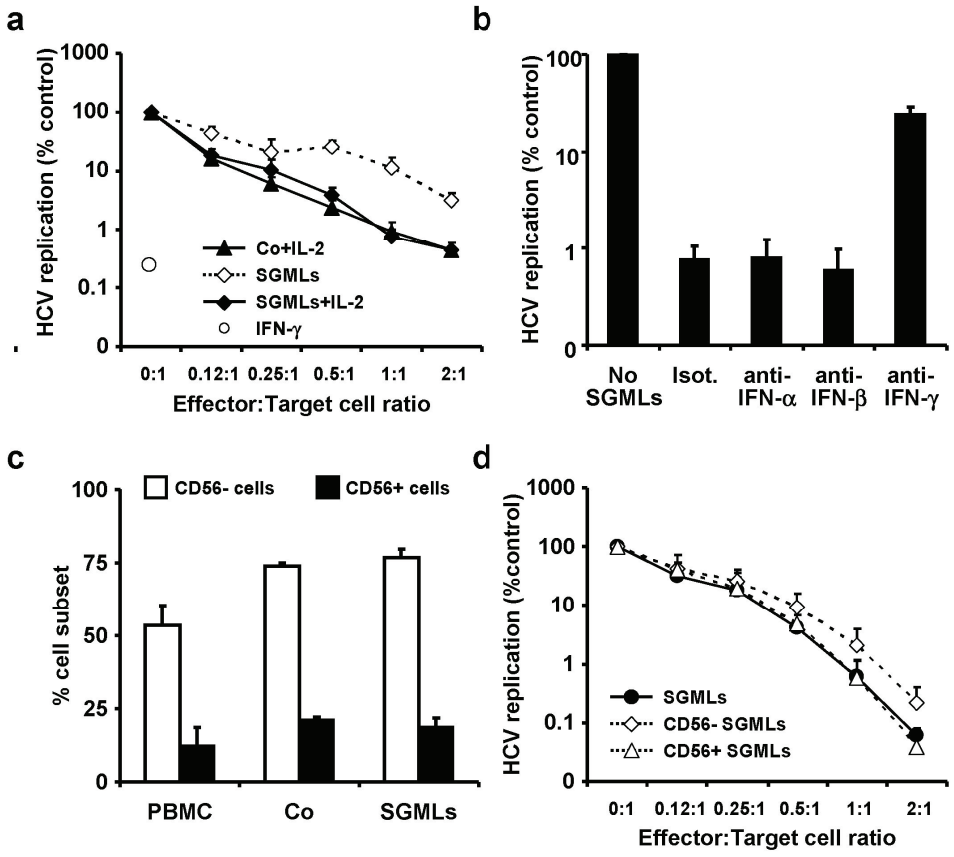
a
HCV replication (% control)
1000
100
10
1
0.1
0
Co+IL-2
SGMLs
SGMLs+IL-2
IFN-γ
0:1
0.12:1
0.25:1
0.5:1
1:1
2:1
Effector:Target cell ratio
b
HCV replication (% control)
100
10
1
0
No SGMLs
Isot.
anti-IFN-α
anti-IFN-β
anti-IFN-γ
c
% cell subset
100
75
50
25
0
CD56- cells
CD56+ cells
PBMC
Co
SGMLs
d
HCV replication (%control)
1000
100
10
1
0.1
0
SGMLs
CD56- SGMLs
CD56+ SGMLs
0:1
0.12:1
0.25:1
0.5:1
1:1
2:1
Effector:Target cell ratio

Fig. 2

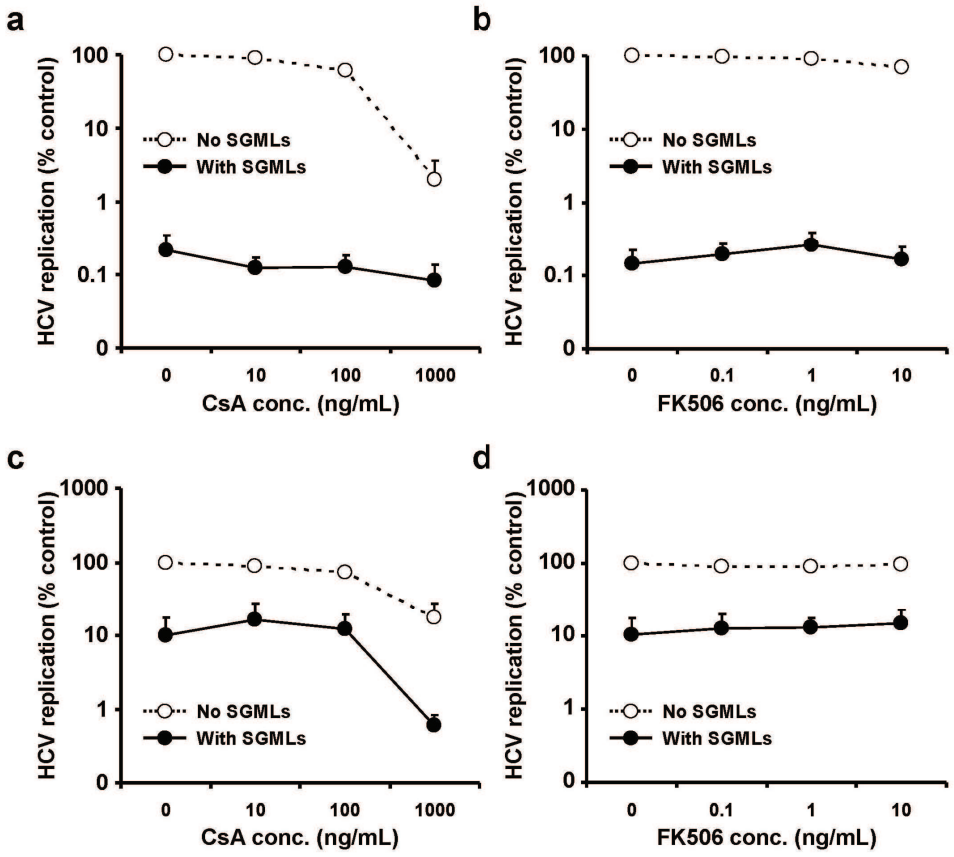
a
b
c
d
HCV replication (% control)
100
10
1
0.1
0
No SGMLs
With SGMLs
0
10
100
1000
CsA conc. (ng/mL)
0
0.1
1
10
FK506 conc. (ng/mL)

Fig. 3

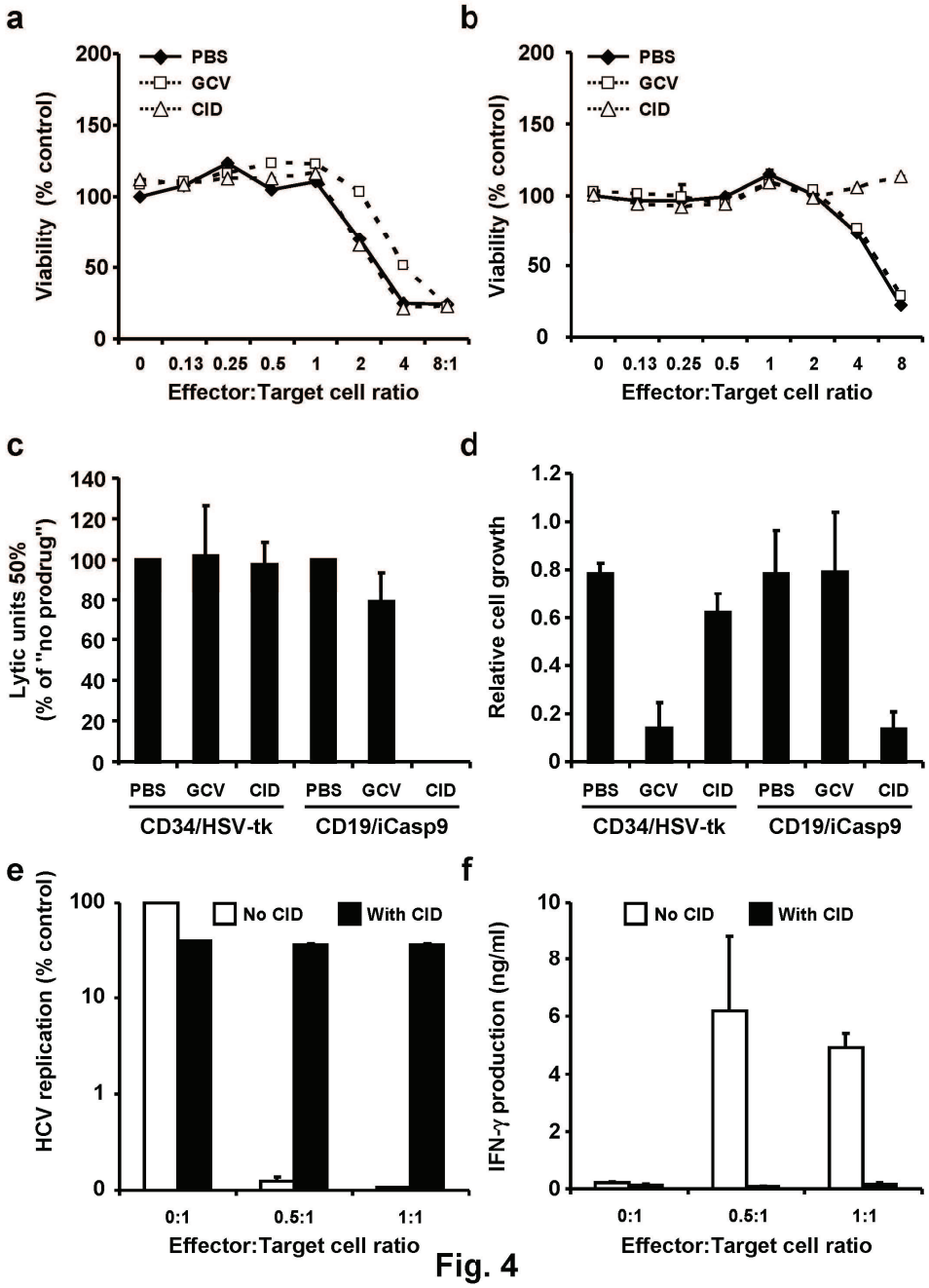
a
b
PBS
GCV
CID
Viability (% control)
200
150
100
50
0
0 0.13 0.25 0.5 1 2 4 8:1
0 0.13 0.25 0.5 1 2 4 8
Effector:Target cell ratio
c
d
Lytic units 50%
(% of "no prodrug")
140
120
100
80
60
40
20
0
Relative cell growth
1.2
1.0
0.8
0.6
0.4
0.2
PBS GCV CID PBS GCV CID
CD34/HSV-tk
CD19/iCasp9
e
f
HCV replication (% control)
100
10
1
No CID
With CID
0:1
0.5:1
1:1
IFN-γ production (ng/ml)
10
8
6
4
2

Fig. 4

Supplementary Figures

Figure S1. Inhibition of HCV replication and cytotoxicity assay in HCVcc and replicon culture models (a) SGMLs generated after CD3 or CD3/CD28 costimulation were assessed for antiviral activity against HCVcc-infected Huh7.5.1 target cells at an effector:target ratio of 0.5:1 (white bars) and 2:1 (black bars). Data are expressed as the mean±SE %HCV replication compared to that in control target cells cultured alone (controls=100%). The control measurement was $4.76\times10^5\pm0.98\times10^5$ RLU n=6). **(b)** SGML cytotoxicity against HCVcc-infected Huh7.5.1 target cells generated after CD3 or CD3/CD28 costimulation was assessed as in Fig. 1b. Data correspond to experiments shown in Fig. S1a, and they are expressed as the mean±SE % optical density compared to that of control HCVcc-infected target cells cultured alone (controls=100%). The control measurement was 3.28±0.08 (n=3). **(c)** Cytotoxic activity of effector cells against electroporated Huh7.5.1 target cells. Effector cells were SGMLs (white diamonds, dashed line: n=4); Co cells + IL-2 (black triangles, full line:, n=4); and SGMLs + IL-2 (black diamonds, full line:, n=5). Data correspond to experiments shown in Fig. 1c, and they are expressed as the mean±SE % optical density compared to that of control target cells cultured alone without IL-2 (controls=100%). The control measurement was 1.00±0.31 (n=5). **(d)** Effector cells were evaluated for their cytotoxic activity against HCVcc-infected Huh7.5.1 target cells. Data correspond to experiments shown in Fig. 1d, and they are expressed as the mean±SE % optical density compared to that of target cells cultured alone without IL-2 (controls=100%). The control measurement was 2.09±0.47 (n=3).

Figure S2. Comparison of the frequency of HCV-positive target cells in the replicon vs HCVcc model. Huh7.5.1. cells electroporated with JFH1ΔE1E2-luc RNA (white bars) or infected with HCVcc Jc1-luc (black bars) were stained at the indicated time points with an

anti-HCV core monoclonal antibody (mAb) (clone C7-50; Thermo Scientific) and a secondary phycoerythrin-labelled goat anti-mouse antibody (Beckman Coulter, Villepintes, France), then analysed for the frequency of core protein-positive cells using a LSR II flow cytometer (Becton Dickinson, Le Pont de Claix, France).

Figure S3. Mechanism of action and cell subsets involved in the antiviral activity of SGMLs. (a) SGMLs were assessed for antiviral activity in the HCV replicon model at an effector:target ratio of 0.5:1, cultured in the absence or presence of 5 µg/mL anti-IFN-α, anti-IFN-β, anti-IFN-γ, or isotype control (isot) mAbs, or a combination of all three anti-IFN mAbs. Data are expressed as the mean±SE %HCV replication compared to that in control target cells cultured with no SGMLs (controls=100%). The control measurement was $6.11\times10^{6}\pm4.81\times10^{6}$ RLU (n=3). **(b)** SGMLs were assessed for antiviral activity in the HCVcc infection model at an effector:target ratio of 0.5:1, cultured in the absence or presence of 5 µg/mL anti-IFN-α, anti-IFN-β, anti-IFN-γ, isotype control (isot) mAbs, or a combination of all three anti-IFN mAbs. Data are expressed as the mean±SE %HCV replication compared to that in control target cells cultured with no SGMLs (controls=100%). The control measurement was $3.22\times10^{5}\pm0.87\times10^{5}$ RLU (n=3). **(c)** CD56-depleted (CD56- SGMLs) and CD56-enriched (CD56+ SGMLs) fractions from SGMLs were compared to unsorted SGMLs for antiviral activity in the HCVcc infection model at an effector:target ratio of 0.5:1 (white bars, n=6) or 2:1 (black bars, n=3). Data are expressed as the mean±SE %HCV replication compared to that in control target cells (controls=100%). The control measurement was $3.99\times10^{5}\pm0.40\times10^{5}$ RLU (n=6). **(d)** Effector cells were evaluated for cytotoxic activity against electroporated Huh7.5.1 target cells. Data correspond to experiments shown in Fig. 2c, and they are expressed as the mean±SE % optical density compared to that of control target cells cultured alone (controls=100%). The control measurement was 0.56±0.10 (n=4). **(e)**

Effector cells were evaluated for cytotoxic activity against HCVcc-infected Huh7.5.1 target cells. Data correspond to experiments shown in Fig. S2c, and they are expressed as the mean±SE % optical density compared to that of control target cells cultured alone (controls=100%). The control measurement was 3.19±0.16 (n=6).

Figure S4. Target cell viability in the presence of calcineurin inhibitors. Target cells were cultured in the absence (white circles, dashed lines) or presence (black circles, full lines) of SGMLs at an effector:target ratio of 0.5:1 in the HCV replicon model **(a, b)** or at an effector:target ratio of 2:1 in the HCVcc infection model **(c, d)**. Cocultures were exposed to varying amounts of CsA **(a, c)** or FK506 **(b, d)**. Data correspond to experiments shown in Fig. 3, and they are expressed as the mean±SE % optical density compared to that of control target cells cultured without SGMLs or calcineurin inhibitor (controls=100%). The control measurements were: (a) 1.29±0.74 (n=4); (b) 1.23±0.76 RLU (n=4); (c) 2.54±0.42 (n=4); (d) 2.90±0.30 (n=4).

Figure S5. SGML antiviral activity is resistant to calcineurin inhibitors, even in the absence of IL-2.

Huh7.5.1 target cells incubated in the absence (white circles, dashed lines) or presence (black circles, full lines) of SGMLs at an effector:target ratio of 0.5:1 in the HCV replicon model **(a, b)** or at an effector:target ratio of 2:1 in the HCVcc infection model **(c, d)**. All cultures were performed in the absence of IL-2 and were exposed to increasing amounts of CsA **(a, c)** or FK506 **(b, d)**. HCV replication was evaluated three days later. Data are expressed as the %HCV replication compared to that in control target cells cultured without SGMLs nor calcineurin inhibitor (control=100%). Control RLUs were: (a) $1.87\times10^5\pm0.60\times10^5$, n=3 (b) $1.67\times10^5\pm0.37\times10^5$, n=3 (c) $3.98\times10^5\pm0.49\times10^5$, n=3 (d) $8,07\times10^5\pm2.89\times10^5$, n=3).

Figure S6. Determination of Lytic Units 50% (LU50). The viability of target cells was expressed as the % optical density compared to that of target cells cultured alone (control=100%). Target cells were cocultured with CD34/HSV-tk+ SGMLs in the absence (black diamonds, full line) or presence (white squares, dashed line) of GCV. Target cell viability was plotted against the E:T ratio. The LU50 was calculated as the inverse of the number of cells per 10^6 effector cells required to kill 50% of target cells. In this representative experiment, the cytotoxic activity was calculated to be 250.5 LU50 in the absence of GCV, which served as the control group, and 147.0 LU50 in the presence of GCV, which was the experimental group. Therefore, the LU50 of the experimental group represents (147.0/250.5) ×100 = 58.7% of the LU50 of the control group.

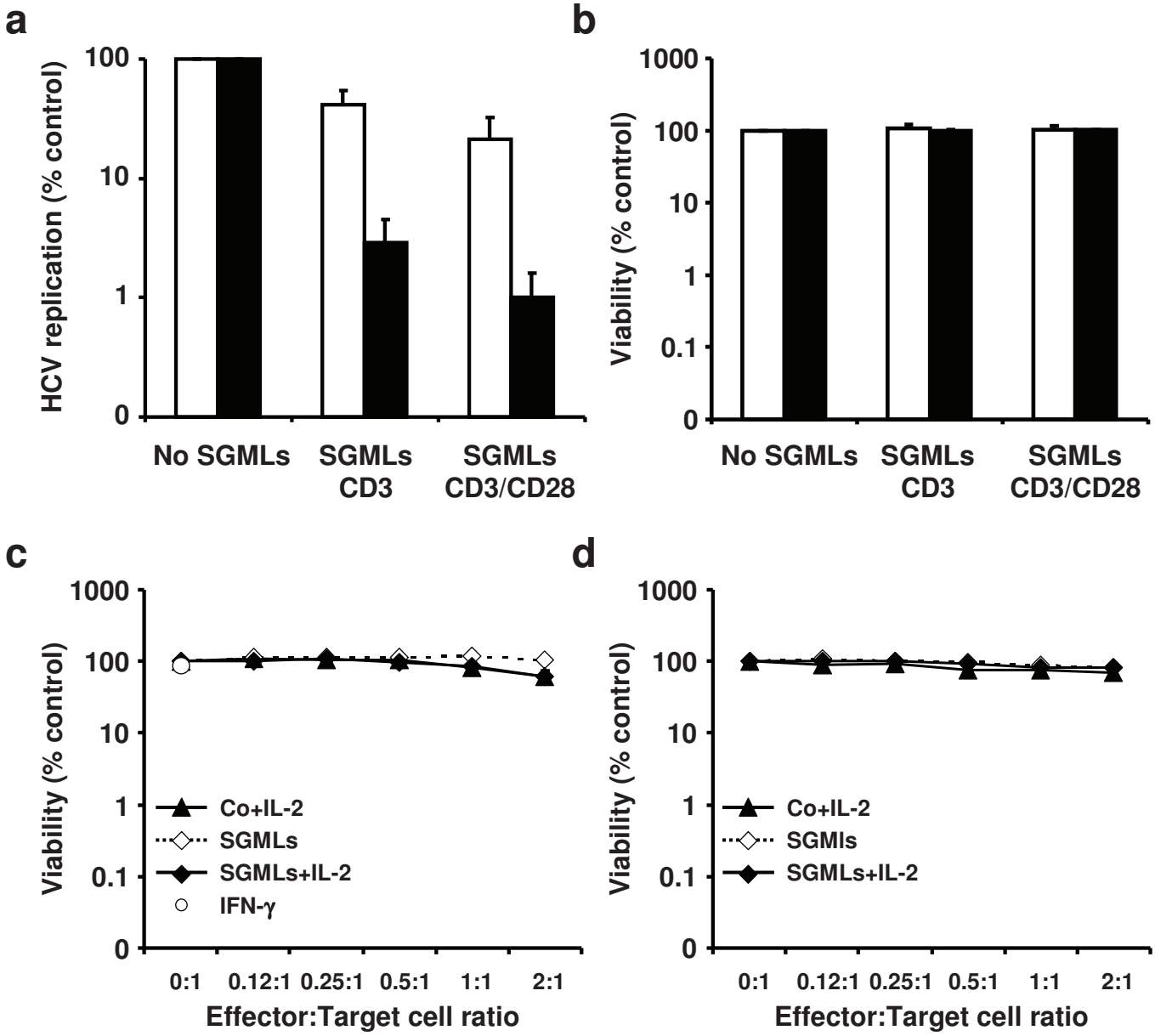
a
HCV replication (% control)
100
10
1
0
No SGMLs
SGMLs CD3
SGMLs CD3/CD28
b
Viability (% control)
1000
100
10
1
0.1
0
No SGMLs
SGMLs CD3
SGMLs CD3/CD28
c
Viability (% control)
1000
100
10
1
0.1
0
Co+IL-2
SGMLs
SGMLs+IL-2
IFN-γ
0:1
0.12:1
0.25:1
0.5:1
1:1
2:1
Effector:Target cell ratio
d
Viability (% control)
1000
100
10
1
0.1
0
Co+IL-2
SGMls
SGMLs+IL-2
0:1
0.12:1
0.25:1
0.5:1
1:1
2:1
Effector:Target cell ratio

Fig. S1

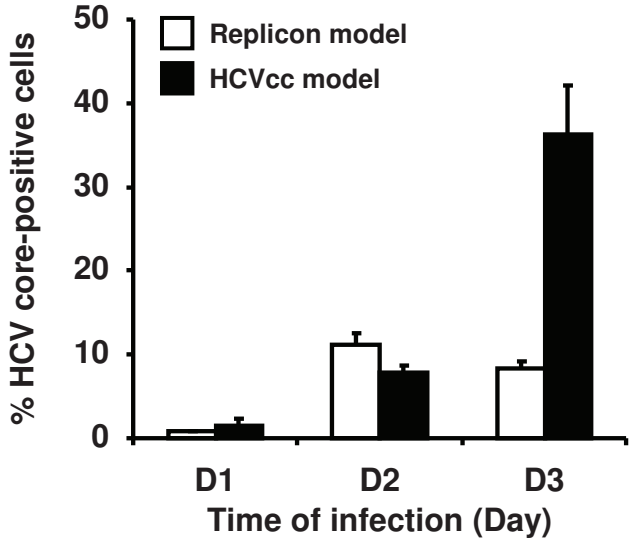
50
40
30
20
10
0
% HCV core-positive cells
Replicon model
HCVcc model
D1
D2
D3
Time of infection (Day)

Fig. S2

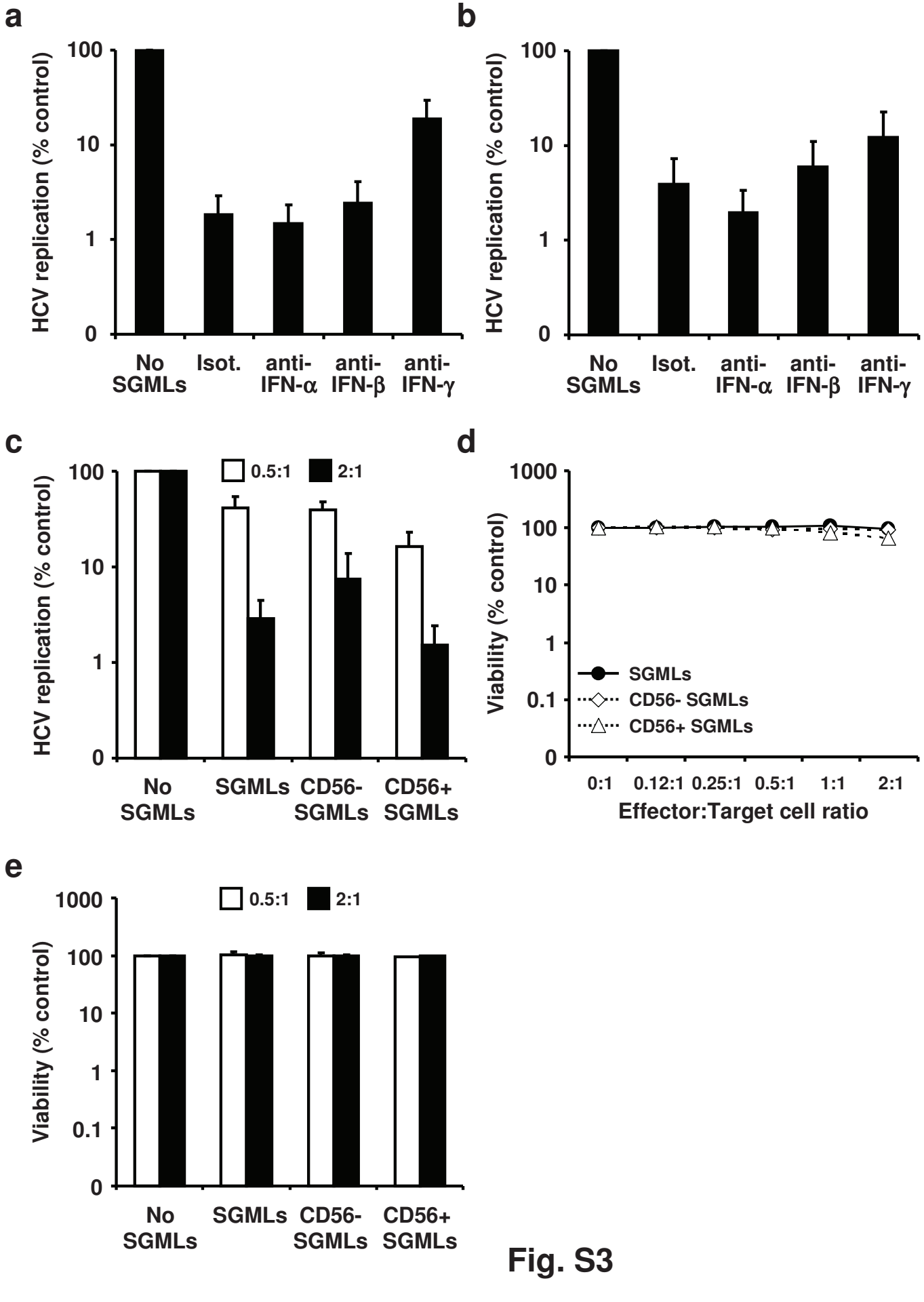
a
HCV replication (% control)
100
10
1
0
No SGMLs
Isot.
anti-IFN-α
anti-IFN-β
anti-IFN-γ
b
HCV replication (% control)
100
10
1
0
No SGMLs
Isot.
anti-IFN-α
anti-IFN-β
anti-IFN-γ
c
HCV replication (% control)
0.5:1
2:1
100
10
1
0
No SGMLs
SGMLs
CD56- SGMLs
CD56+ SGMLs
d
Viability (% control)
1000
100
10
1
0.1
0
SGMLs
CD56- SGMLs
CD56+ SGMLs
0:1
0.12:1
0.25:1
0.5:1
1:1
2:1
Effector:Target cell ratio
e
Viability (% control)
0.5:1
2:1
1000
100
10
1
0.1
0
No SGMLs
SGMLs
CD56- SGMLs
CD56+ SGMLs

Fig. S3

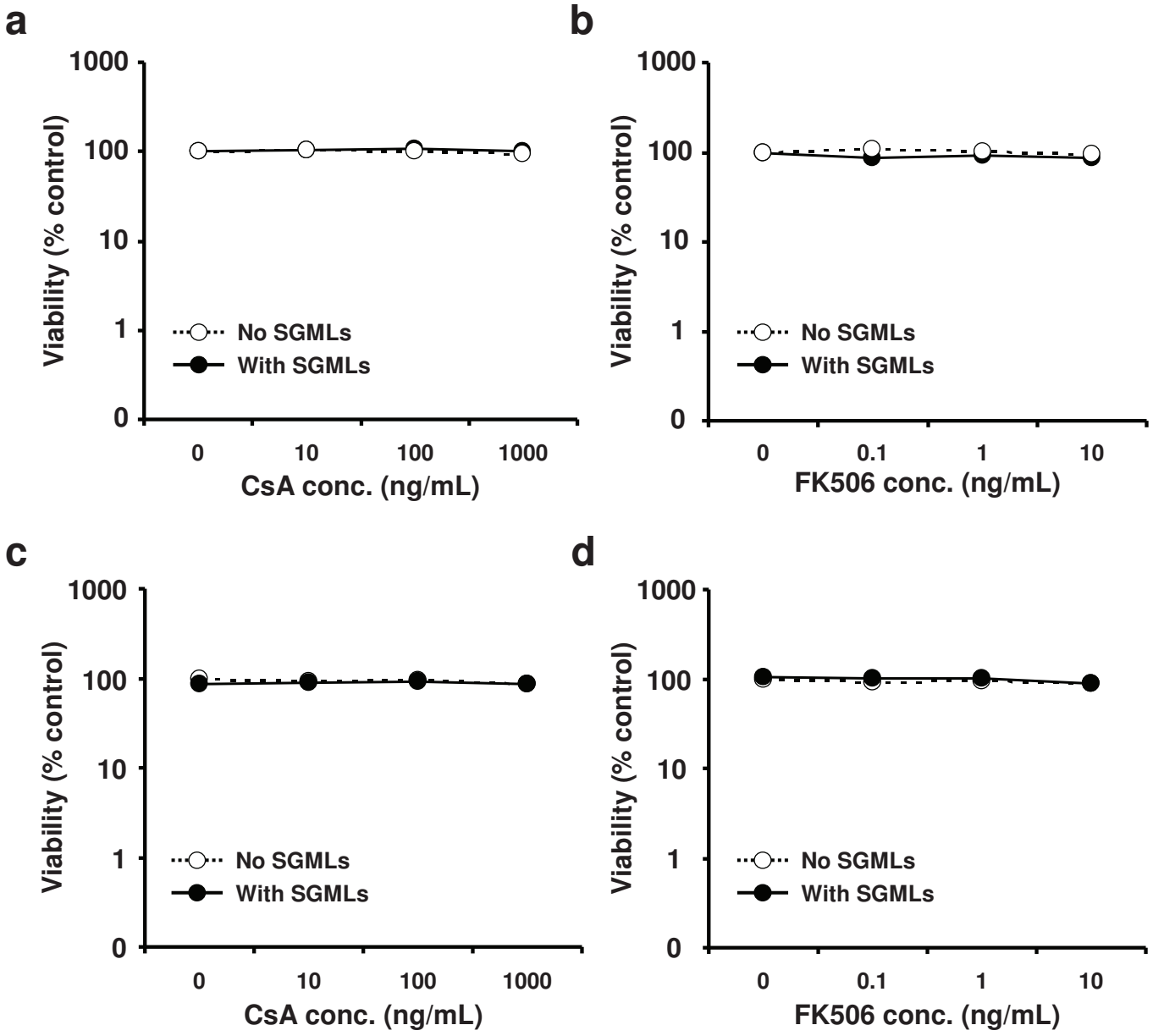
a
1000
100
10
1
0
Viability (% control)
No SGMLs
With SGMLs
0
10
100
1000
CsA conc. (ng/mL)
b
1000
100
10
1
0
Viability (% control)
No SGMLs
With SGMLs
0
0.1
1
10
FK506 conc. (ng/mL)
c
1000
100
10
1
0
Viability (% control)
No SGMLs
With SGMLs
0
10
100
1000
CsA conc. (ng/mL)
d
1000
100
10
1
0
Viability (% control)
No SGMLs
With SGMLs
0
0.1
1
10
FK506 conc. (ng/mL)

Fig. S4

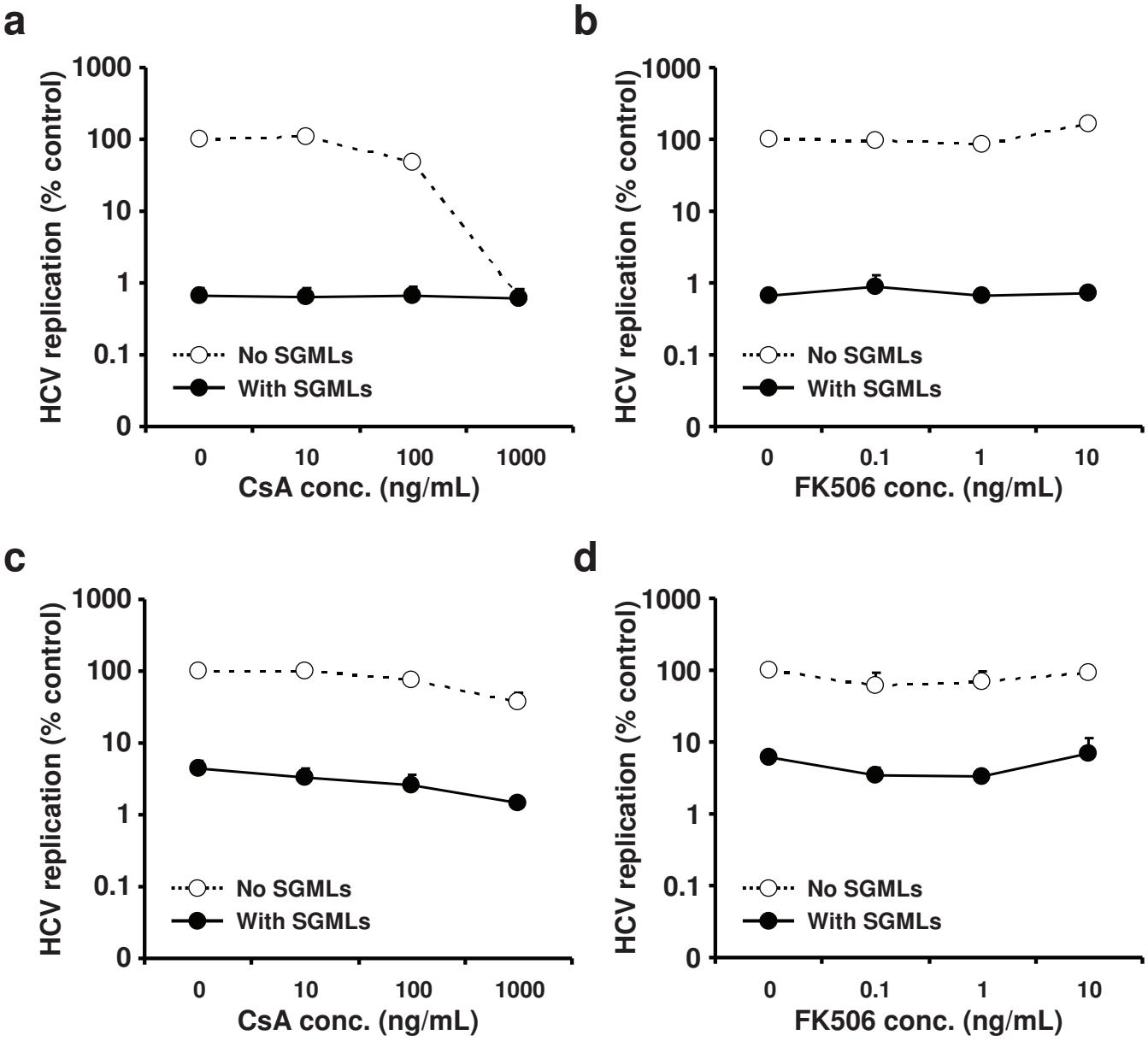
a
b
c
d
HCV replication (% control)
1000
100
10
1
0.1
0
No SGMLs
With SGMLs
0
10
100
1000
CsA conc. (ng/mL)
0
0.1
1
10
FK506 conc. (ng/mL)

Fig. S5

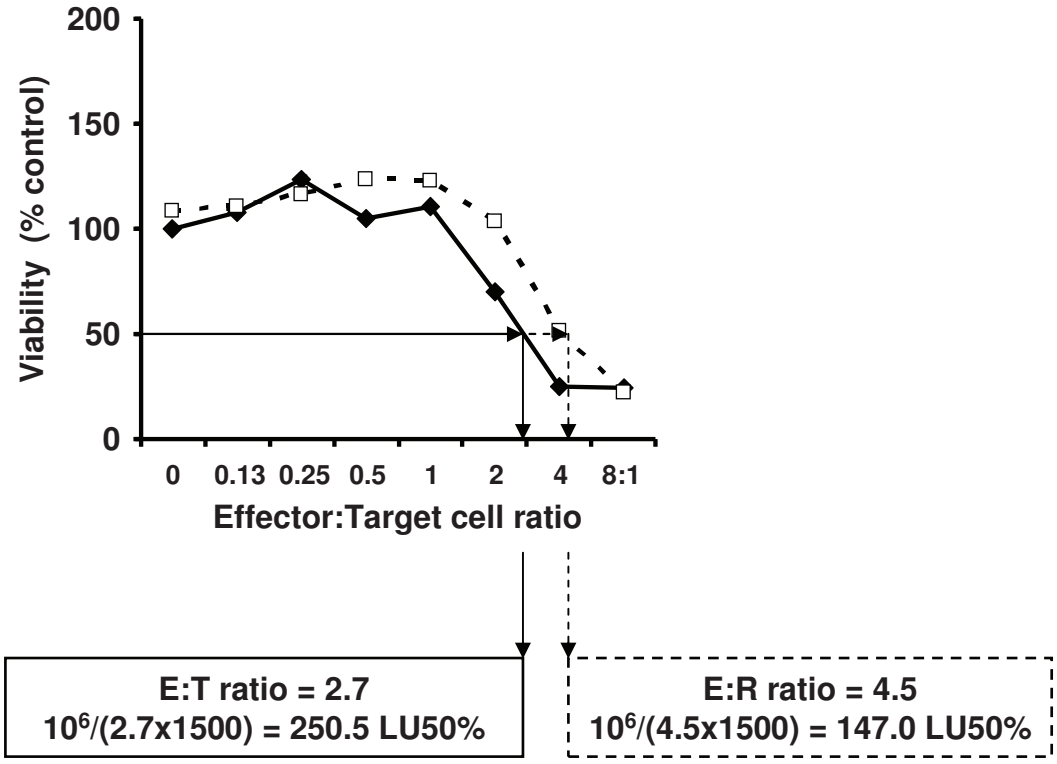
200
150
100
50
0
Viability (% control)
0
0.13
0.25
0.5
1
2
4
8:1
Effector:Target cell ratio
E:T ratio = 2.7
10^6/(2.7x1500) = 250.5 LU50%
E:R ratio = 4.5
10^6/(4.5x1500) = 147.0 LU50%

Fig. S6

Tao Wu

Evaluation préclinique de thérapies innovantes pour le carcinome hépatocellulaire et l'infection chronique par le virus de l'hépatite C

RESUME.

L'infection par le virus de l'hépatite C (VHC) représente un problème majeur de santé publique du fait de sa prévalence élevée et de la sévérité de ses complications, cirrhose et carcinome hépatocellulaire (CHC). Les objectifs du projet de thèse sont (i) de mettre en place et caractériser des modèles orthotopiques de CHC chez le petit animal (xénogreffe orthotopique de la lignée de CHC humaine Huh-7 exprimant le gène rapporteur de la luciférase chez la souris immunodéficiente) et chez le gros animal (transplantation autologue d'hépatocytes de cochon préalablement transformés ex vivo par transfert par voie lentivirale d'une combinaison de six oncogènes) et (ii) d'apporter la preuve de concept d'une approche innovante d'immunothérapie adoptive allogénique du CHC et de l'infection chronique par le VHC, par administration de lymphocytes génétiquement modifiés (LGM) allogéniques exprimant un gène de toxicité conditionnelle, ou gène suicide. Un tel gène suicide permet le contrôle des LGM, conduisant à leur élimination conditionnelle en cas d'effets secondaires indésirables. Ainsi, nous avons montré que, à dose élevée, ces LGM exercent in vitro une activité cytotoxique vis-à-vis de lignées de CHC humaines et in vivo une activité anti-tumorale vis-à-vis de tumeurs orthotopiques Huh-7. A faible dose, les LGM présentent une activité anti-virale vis-à-vis du VHC sans induire de toxicité. Ces résultats ouvrent la perspective à une approche originale d'immunothérapie du CHC, associée aux traitements actuels et de prévention de la réinfection du greffon hépatique par le VHC lors de la transplantation.

Mots clés : Immunothérapie adoptive, Lymphocytes génétiquement modifiés exprimant un gène suicide, Modèles animaux, Carcinome hépatocellulaire, Hépatite C, Oncogènes.

ABSTRACT.

The hepatitis C virus (HCV) infection is a major problem of public health, due to its high prevalence and to the severity of its complications, cirrhosis and hepatocellular carcinoma (HCC). The aims of the thesis project are (i) to set up and characterize orthotopic HCC models in the small animal (orthotopic transplantation in immunodeficient mice of the human HCC cell line Huh-7, expressing the luciferase reporter gene) and in the large animal (autologous transplantation of porcine hepatocytes previously ex vivo-transformed by lentiviral-meidated transfer of a combination of six oncogenes) and (ii) to provide the proof-of-concept of an innovative adoptive allogeneic immunotherapy approach for the treatment of HCC and prevention of liver graft reinfection by HCV, through the administration of allogeneic suicide gene-modified lymphocytes (GML). Such a suicide gene allows for the control of GML, leading to their conditional elimination in case of undesirable side effects. Thus, we have demonstrated that, at high dose, these GML present an in vitro cytotoxic activity toward HCC cell lines and an in vivo antitumoral effect against orthotopic Huh-7 tumors. At low level, the GML have an antiviral activity against HCV, without toxicity against target cells. These results open the perspective for an original approach of immunotherapy for the treatment of HCC in association with current treatments and for the prevention of liver graft reinfection by HCV at time of liver transplantation.

Key words: Adoptive immunotherapy, Allogeneic suicide gene-modified killer cell, Animal models, Hepatocellular carcinoma, Hepatitis C virus, Oncogenes.