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(71) Applicant *(for all designated States except US)*: **UNIVERSITE PIERRE ET MARIE CURIE (PARIS 6)** [FR/FR]; 4, Place Jussieu, F-75005 Paris (FR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **AUTRAN, Brigitte** [FR/FR]; 92 ter Bd Pereire, F-75017 Paris (FR). **HARFOUCH, Sawsan** [SY/FR]; 2 Square Claude Debussy, F-92160 Antony (FR). **VALANTIN, Marc-Antoine** [FR/FR]; 13 rue Titon, F-75011 Paris (FR). **SAMRI, Assia** [FR/FR]; 4 allée Paul Eluard, F-95680 Montlignon (FR). **GUIGUET, Marguerite** [FR/FR]; 38 rue Campo Formio, F-75013 Paris (FR). **THEODOROU, Ioannis** [GR/FR]; 39 rue Dunois, F-75013 Paris (FR).

(74) Agents: **CHAJMOWICZ, Marion** et al.; Becker & Associes, 25 rue Louis Le Grand, F-75 002 Paris (FR).

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(54) **Title**: METHODS FOR PREDICTING OUTCOME OF A HEPATITIS VIRUS INFECTION

(57) **Abstract**: The invention provides an *in vitro* method for predicting whether a patient affected with a viral hepatitis will cure spontaneously, or whether a patient affected with a viral hepatitis will be responsive to an antiviral hepatitis treatment, which method comprises determining the level of TGF-⊐β produced by T cells that are specific for the hepatitis virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-⊐β, in a biological sample from the patient.

Methods for predicting outcome of a hepatitis virus infection

The present invention relates to predictive methods to evaluate the outcome of an infection by a hepatitis virus.

Technical background of the invention:

At least 8 different hepatitis viruses are believed to exist, and include the A, B, C, D, E, F, G and cryptogenic hepatitis viruses. The hepatitis viruses are spread through a number of different virus families. Of these viruses, hepatitis C virus (HCV, a flavivirus) and hepatitis B virus (HBV, a hepadnavirus) pose the greatest public health problem in industrialized countries. Both hepatitis C and B are bloodborne diseases, although both viruses may also be transmitted perinatally and via sexual contact. Hepatitis C and B can each give rise to acute and chronic infections. A relatively low level of mortality is due to acute C and B hepatitis (primarily due to fulminant hepatitis). However, the chronic forms of each disease pose significant medical issues. HCV is currently the leading cause of liver transplants in the United States. Only few HCV infections, are symptomatic in the acute phase, which typically presents with symptoms such as jaundice, fatigue, abdominal pain and/or loss of appetite. Subclinical infections can be detected using diagnostic testing for viral antigens and/or DNA.

In the U.S., acute HCV infections are substantially less common than acute HBV infections, by a factor of approximately ten. However, due to the substantially greater risk of progression to chronic infection (about 85%), the prevalence of chronic HCV infection is two to three times greater than that for chronic HBV infection. Additionally, HCV infection carries a much greater risk of the development of chronic liver disease and liver failure.

While new antiviral drugs are currently developed, available treatments for chronic hepatitis C infection are so far limited to interferons. Interferon α2a, interferon α2b and interferon α con-1 (a recombinant, non-naturally occurring interferon 1 variant) are currently used for the treatment of chronic hepatitis virus infection, optionally in combination with ribavirin (an anti-viral drug).

However, these drugs require frequent administration and are associated with a large number of side effects, including "flu-like" symptoms (*e.g.,* fatigue, fever, myalgia), leukopenia, thrombocytopenia, nausea, vomiting, and arthralgia. One rare complication of interferon administration is hepatotoxicity, which can be fatal. Unfortunately, only about 40% of patients show any improvement with interferon treatment, and may relapse after treatment is completed.

International patent application WO2002/16949 discloses a method for detecting the extend of an inflammatory, fibrotic or cancerous disease in a patient, in particular liver fibrosis, in particular in a patient infected with hepatitis C virus, by using the serum concentration of easily detectable biological markers. However it is not concerned with predicting the response of the patient to a therapeutic treatment. Lee et al, Disease Markers 28, 2010, 273-280 discloses that a combination of plasma levels of decreased IP-10 and elevated TGF-β1 is associated with viral clearance following therapy in patients with hepatitis C virus.

There remains a need for a reliable predictive method for evaluating the outcome of an infection by a hepatitis virus in a patient, and whether a therapeutic treatment, which may have side-effects, would be effective in this particular patient.

Summary of the invention:

The invention provides in vitro methods for predicting hepatitis outcome in patients infected with a hepatitis virus, more particularly for determining a susceptibility to non-spontaneous viral clearance or to a non-response to a treatment.

A subject of the invention is an *in vitro* method for predicting whether a patient affected with a viral hepatitis will cure spontaneously, which method comprises determining the level of TGF- β produced by T cell that are specific for the hepatitis virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF- β, in a biological sample from the patient, wherein the patient is predicted to cure spontaneously when said level is not significantly higher, compared to a control group.

Another subject of the invention is an in vitro method for predicting whether a patient affected with a viral hepatitis will be responsive to an antiviral hepatitis treatment, such as a treatment with an interferon and/or an anti-viral nucleoside, e.g. ribavirin, or direct anti-viral agents (DAAs), which method comprises determining the level of TGF-β produced by T cells that are specific for the hepatitis virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-β, in a biological sample from the patient, wherein the patient is predicted to be responsive to said treatment when said level is not significantly higher compared to a control group.

In a particular embodiment, the method comprises determining the level of TGF-β, produced by T cells, upon stimulation with a hepatitis virus antigen. Especially the method may comprise stimulating Peripheral Blood Mononuclear Cells (PBMC) obtained from the biological sample of the patient with a hepatitis virus antigen, detecting TGF-β □in the culture

supernatant, and comparing with the level of TGF-β □produced in the absence of hepatitis virus antigen.

In another embodiment, the method comprises determining the level of T cells that produce TGF-β, upon stimulation with a hepatitis virus antigen. Especially the method comprises stimulating Peripheral Blood Mononuclear Cells (PBMC) obtained from the biological sample of the patient with a hepatitis virus antigen, and quantifying the subset of cells that produce TGF-β, e.g. by flow cytometry.

It is further provided a kit for evaluating the outcome of an infection by a hepatitis virus, comprising (i) means for determining the level of TGF-β produced by T cells that are specific for the hepatitis C virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-β, in a biological sample from the patient, and optionally (ii) instructions for use.

The method of the invention makes it possible to classify patients who are likely to cure, either spontaneously or after standard treatment *versus* patients who are likely to progress to liver fibrosis. New candidate drugs, or liver transplant, may be proposed to the latter.

Legends to the Figures

Figures 1A and 1B are graphs that show comparison of HCV-specific CD4+ (Figure 1A) and CD8+ (Figure 1B) TGF-β+ T cell Percentages (presented after subtraction of background), between HD (healthy donors), RE (patients with recovery) and VI patients (patients who remained viremic).

Figure 2 is a graph that shows the association trend between anti-HCV TGF-β producing T cells and progress of liver disease (12 patients).

Detailed description of the invention

The inventors have shown that patients infected with HCV who have progressed toward chronic hepatitis have a dramatically high level of CD4+ or CD8+ T cells that produce TGF-β. On that basis, they provide an *in vitro* method for predicting whether a patient affected with a viral hepatitis will cure, or is likely to cure, spontaneously, or whether a patient affected with a viral hepatitis will be, or is likely to be, responsive to an antiviral treatment, which method comprises determining the level of TGF-□β produced by T cells that are specific for the hepatitis virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-□β, in a biological sample from the patient.

Definitions

Within the context of this invention, the term **"patient"** or **"subject"** refers to any individual capable of being tested. Preferably, it is a human being, but the term includes any other mammal, such as dogs, cats, rodents, cattle, horses, monkeys, etc. The patient can be tested irrespective of the sex or age thereof. Preferably it is a human being, including males or females. It may be a child, an adolescent, or an adult of any age. The patient may be may be asymptomatic, or may show early or advanced signs of hepatitis. For example, the patient may be an individual predisposed to cure spontaneously from hepatis infection, in particular an individual carrying one or more mutations in the gene encoding IL-28B.

The term **"TGF-β"** refers to transforming growth factor beta that is a multifunctional peptide that controls proliferation, differentiation, and other functions in many cell types. Many cells synthesize ce peptide and almost all of them have specific receptors for this peptide. Any of TGF-β1, TGF-β2, and TGF-β3 is encompassed, since they all function through the same receptor signaling systems. The present invention preferably assays TGF-β1 or T cells that are specific for a hepatitis virus and that produce TGF-β1. TGF-β1 is produced as a large protein precursor (containing 390 amino acids) that is proteolytically processed to produce a mature peptide of 112 amino acids. Mature TGF-β, more particularly mature TGF-β1, is preferably assessed.

The term **"predicting"** or **"prognosing"** refers to the determination of a likelyhood for a patient infected with a hepatitis virus to cure spontaneaously or after treatment. Preferably the method is performed before initiation of therapy>.

The term **"to cure"** preferably means that viral clearance is observed. Viral clearance and alleviation of the symptoms may be observed within 1 week to 12 months, generally within 3 to 6 months after infection.

The term **"responsive"** means that the method of the invention assesses whether the patient is likely to be respond to an antiviral treatment, such as a treatment including or consisting of, an interferon and/or an anti-viral nucleoside, such as ribavirin. Interferon α2a, and even preferably pegylated Interferon α2a, combined with ribavirin, is currently the standard treatment. Alternatively, the antiviral treatment may comprise any of the below mentioned drugs. Treatments of hepatitis virus include: (1) Interferon and ribavirin; (2) Substrate-based NS3 protease inhibitors (WO 98/22496); (3) Non-substrate-based inhibitors such as 2,4,6-trihydroxy-3-nitro-benzamide derivatives (Sudo K. et al., Biochemical and Biophysical Research Communications, 238:643-647 (1997); Sudo K., et al. Antiviral Chemistry and Chemotherapy, 9:186 (1998)), including RD3-4082 and RD3-4078, the former substituted on the amide with a 14 carbon chain and the latter processing a para-

phenoxyphenyl group; (4) Thiazolidine derivatives, which show relevant inhibition in a reverse-phase HPLC assay with an NS3/4A fusion protein and NS5A/5B substrate (Sudo K. et al., Antiviral Research, 32: 9-18 (1996)), especially compound RD-1-6250, possessing a fused cinnamoyl moiety substituted with a long alkyl chain, RD4 6205 and RD4 6193; (5) Thiazolidines and benzanilides, identified in Kakiuchi N. et al. J. FEBS Letters 421, 217-220; and Takeshita N. et al. Analytical Biochemistry, 247: 242-246 (1997); (6) A phenanthrenequinone, which possesses activity against protease in a SDS-PAGE and autoradiography assay and is isolated from the fermentation culture broth of Streptomyces sp., Sch 68631 (Chu M. et al., Tetrahedron Letters, 37: 7229-7232 (1996)), and Sch 351633, isolated from the fungus Penicillium griscofuluum, which demonstrates activity in a scintillation proximity assay; (7) Selective NS3 inhibitors based on the macromolecule elgin c, isolated from leech (Qasim M. A. et al., Biochemistry, 36: 1598-1607 (1997)); (8) Helicase inhibitors (U.S. Pat. No. 5,633,358); (9) Polymerase inhibitors, such as nucleotide analogues, gliotoxin (Ferrari E. et al., Journal of Virology, 73:1649-1654 (1999)), and the natural product cerulenin (Lohmann V. et al., Virology, 249: 108-118 (1998)); (10) Antisense phosphorothioate oligodeoxynucleotides (S-ODN) complementary to sequence stretches in the 5' non-coding region (NCR) of the virus, or nucleotides 326-348 comprising the 3' end of the NCR and nucleotides 371-388 located in the core coding region of the HCV RNA; (11) Inhibitors of IRES-dependent translation; (12) Nuclease-resistant ribozymes; and (13) Miscellaneous compounds including 1-amino-alkyloyclohexanes (U.S. Pat. No. 6,034,134 to Gold et al.), alkyl lipids (U.S. Pat. No. 5,922,757 to Chojkier et al.), vitamin E and other antioxidants (U.S. Pat. No. 5,922,757 to Chojkier et al.), squalene, amantadine, bile acids (U.S. Pat. No. 5,846,964 to Ozeki et al.), N-(phosphonoacetyl)-L-aspartic acid, (U.S. Pat. No. 5,830,905 to Diana et al.), benzenedicarboxamides (U.S. Pat. No. 5,633,388 to Diana et al.), polyadenylic acid derivatives (U.S. Pat. No. 5,496,546 to Wang et al.), 2',3' dideoxyinosine (U.S. Pat. No. 5,026,687 to Yarchoan et al.), and benzimidazoles (U.S. Pat. No. 5,891,874 to Colacino et al.).

More recently, other anti-viral drugs have been developed, depending on polymerase and protease enzymes as targets: 1- Protease inhibitors such as telaprevir (VX-950) which is a specific peptidomimetic inhibitor of NS3/NS4a protease (Reesink HW Gastroenterology 2006, 131:997-1002) and boceprevir (SCHS03034) (Sarrazin C Gastroenterology 2007, 132:1270-1278). 2- Polymerase inhibitors of 2' and 3' substituted ribonucleoside analogues such as Valopicitabine, a prodrug of the nucleoside analogue 2-Cmethylcytidine (NM283) (Pierra C J med chem. 2006, 49:6614-6620), and non nucleoside RNA-dependent RNA polymerase inhibitors, such as benzimidazole derivatives JTK-109 and JTK-003 (Tomei L. J Virology 2004, 78(2):938-946). 3- Immune modulators, including the Toll-like receptor

agonists such as isatoribine (TLR7) (Horsmans Y, Hepatology 2005, 42:724-731), resiquimod (TLR7 and 8) (Pockros PJ, Hepatology 2007, 47:174-182), and CPG10101 (TLR9) (McHutchison JG, Hepatology 2007, 46:1341-1349).

The term **"spontaneously"** means that the patient is not treated with any antiviral drug against the hepatitis virus, such as those mentioned above. In particular the patient is not treated with an interferon and/or an anti-viral nucleoside, such as ribavirin.

The term **"biological sample"** refers to any biological sample originating from a patient. Examples of samples include biological fluids and tissue biopsies. Preferably, the sample may be blood, serum, saliva, urine or sperm. More preferably, the biological sample is a blood sample.

The term **"control"** or **"control value"** refers to a basal value corresponding to the average of the values obtained with the biological sample from healthy individuals not infected with a hepatitis virus. It may be a statistical reference value.

A level " **not significantly higher, compared to a control value"** generally means that the value is in the limits of normal values or that no statistically significant increase, for example the increase, if any, is less than two standard deviations above the mean of the concentration of TGF-β in the healthy individuals.

A level " **significantly higher, compared to a control value"** generally means that the value shows a statistically significant increase, for example the increase is at least than two standard deviations above the mean of the concentration of TGF-β in the healthy individuals.

The term **« level »** means concentration of TGF-β produced by T cells that are specific of a hepatitis virus and that produce TGF-β, or concentration or quantity of T cells that are specific of a hepatitis virus and that produce TGF-β.

The term **"T cells that are specific of a hepatitis virus and that produce TGF-β"** includes CD4+ or CD8+ T cells. The T cells may present other markers such as CD3, CD2. Preferably a sub-population of memory T cells that express CD45RA, CCR7, CD27, may be tested.

The patient to test:

The patient is infected with any hepatitis virus, preferably hepatitis C virus (HCV), or hepatitis B virus (HBV), of any genotype or strain.

Preferably the patient is infected with hepatitis C virus (HCV). In a particular aspect of the invention, the patient is co-infected with a HIV virus. In a preferred embodiment the patient is co-infected with both HIV and HCV.

In a preferred embodiment, the patient is affected with an acute viral hepatitis.

Preferably the level of TGF-β and/or of T cells that are specific for the hepatitis virus and produce TGF-β is determined in a biological sample taken from the patient within three months, preferably within two months, after an acute viral hepatitis is diagnosed.

However the method may also be useful when the patient already suffers from chronic hepatitis.

When the level of TGF-β produced by T cells that are specific for the hepatitis virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-β in a biological sample from the patient, is undetectable or not significantly higher than the normal values from a control healthy un-infected group, then the patient is predicted to cure spontaneously or to be responsive to a treatment with an interferon and/or an anti-viral nucleoside, *e.g.* ribavirin, or direct anti-viral agents (DAAs). For example an application might be that if the patient has suffered from an acute hepatitis, the physician may then propose to wait for a few months (typically 3 months), to let the possibility for the patient to recover spontaneously, without any treatment. If the patient does not show any sign of recovery after said period without treatment, then a treatment with an interferon and/or an anti-viral nucleoside, *e.g.* ribavirin, or direct anti-viral agents (DAAs) may be proposed, since the patient is likely to respond to said treatment.

When the level of TGF-β produced by T cell that are specific for the hepatitis virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-β in a biological sample from the patient, is undetectable or not significantly higher than the normal values from a control group that may be a healthy uninfected group, then the patient is predicted neither to cure spontaneaously nor with a treatment with an interferon and/or an anti-viral nucleoside, *e.g.* ribavirin. In that case, alternative therapeutic options are to be contemplated, e.g. direct anti-viral agents (DAAs) or any innovative treatment.

Methods for quantifying TGF-β or T cells that produce TGF-β :

The method of the invention comprises determining the level of TGF-β produced by T cells that are specific for the hepatitis virus, or the level of T cells that produce TGF-β upon stimulation with a hepatits virus antigen. It generally includes a step of comparing the level of T cells that produce TGF-β, or of TGF-β produced by T cells, either in absence or in presence of a hepatitis virus antigen, such as a virus protein, peptide, or a virus particle or vector. In a particular embodiment, the comparison may be performed with a hepatitis virus antigen, *vs* a non-viral or a non-hepatitis virus antigen.

It should be underlined that determining the level of TGF-β produced by T cells that are specific for the hepatitis virus does not mean determining the plasmatic level of non specific TGF-β.

Determining the level of TGF-β produced by T cells that are specific of a hepatitis virus may be performed by various methods.

Secreted TGF-β produced by T cells that are specific of a hepatitis virus may be detected in the culture supernatant by an immunoassay, after antigenic stimulation. In a particular embodiment, the method may involve stimulating Peripheral Blood Mononuclear Cells (PBMC), or purified CD4+ or CD8+ T cells, obtained from the biological sample of the patient, with a hepatitis virus antigen such as a virus protein, peptide, or a virus particle or vector. In a particular embodiment, it may involve isolating and optionally culturing the CD4+ or CD8+ T cells either before or after stimulation by a HCV antigen.

In one aspect, assays of the invention thus utilize antibodies directed to TGF-β. Such antibodies are well known in the art. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled.

The biological sample can be optionally treated in a prior step, or brought directly into contact with at least one capture antibody.

The method can be carried out according to various formats well known to those skilled in the art: in solid phase or in homogenous phase; in one step or in two steps; in a competition method, by way of non-limiting examples.

According to one preferred embodiment, the capture antibody is immobilized on a solid phase. By way of non-limiting examples of a solid phase, use may be made of microplates, in particular polystyrene microplates, such as those sold by the company Nunc, Denmark. Use may also be made of solid particles or beads, paramagnetic beads, such as those provided by Dynal or Merck-Eurolab (France) (under the trademark EstaporTM), or else polystyrene or polypropylene test tubes, etc.

An immunoassay format for detecting TGF-β by competition is also possible. Other immunoassay modes can also be envisioned and are well known to those skilled in the art.

ELISA assays, radioimmunoassays, or any other detection technique can be used for revealing the presence of the antigen-antibody complexes formed. The EliSPOT® system may be particularly useful.

Anti-TGF-β antibodies of use with the present invention may be purchased from commercial sources or may be generated using art-recognized methods. It will be appreciated that any antibody directed to TGF-β will be useful in assays of the invention. In an exemplary aspect, antibodies used with assays of the invention recognize epitopes of the extracellular portion of membrane-bound TGF-β. In another exemplary aspect, the anti- TGF-β antibodies are monoclonal antibodies.

In a particular aspect, the method of the invention involves quantifying TGF-β that is produced or released by the hepatitis virus- specific T cells, preferably within a time window of between 4 to 36h after stimulation with a hepatitis virus antigen.

In a further aspect, assays of the invention detect both membrane-bound and soluble TGF-β.

Another known assay for the quantification of active TGF-βis described in Abe et al., 1994, Anal Biochem, 216:276-284. The assay relies on the ability of TGF-β to stimulate PAI-1 and utilises a TGF-β responsive cell containing an expression vector having a TGF-β response element (the PAI-1 promoter) and a structural region encoding an indicator molecule, for example luciferase. TGF-β present in a test sample induces activation of the PAI-1 promoter. This results in transcription and the expression of the indicator molecule. Measurement of the amount of indicator molecule determines the amount of TGF-β responsible for the induced activation.

The method of the invention may comprise determining the level of other markers of treatment outcome, but preferably not IP-10. In a preferred aspect of the invention, assaying the level of TGF-β produced by T cells upon stimulation by a hepatitis virus antigen only is sufficient, and is alone useful to evaluate the outcome of the infection in the patient. In a particular embodiment, the method further comprises detecting polymorphisms, especially single-nucleotide polymorphisms (SNPs) in IL-28b gene (see Thomson et al, Gastroenterology. 2010, 139(1):120-9, and international patent application WO2011/013019).

In another embodiment, the method of the invention comprises determining the level of T cells that are specific for the hepatitis virus and that produce TGF-β.

The first steps may be identical as those described above, by stimulating Peripheral Blood Mononuclear Cells (PBMC) obtained from the biological sample of the patient, with a hepatitis virus antigen, *e.g.* a virus protein, peptide, or a virus particle or vector. The subset of cells that produce TGF-β is then quantified, *e.g.* by flow cytometry, *e.g.* by following a standard protocol of intracytoplasmic cytokine staining (see *e.g.* Prussin and Metcalfe, Journal of Immunological Methods, 1995, 188:117-128).

In one aspect, assays of the invention analyze antibody staining of cells using flow cytometry. Such flow cytometry methods are well known and well- characterized in the art.

In one aspect, assays of the invention include the steps of applying an anti- TGF-β antibody to a population of cells and detecting whether the antibody is able to stain the cells. Cells stained by the antibody express TGF-β on their surface. Detection of such stained cells can be accomplished using methods known in the art. In one exemplary embodiment, cells stained for surface TGF-β are detected using flow cytometry. It will be appreciated that stains for both membrane-bound and intracellular TGF-β can be utilized in accordance with the present invention.

Kits may be useful to perform such methods. In particular, it is described a kit for evaluating the outcome of an infection by a hepatitis virus, *i.e.* for predicting whether a patient affected with a viral hepatitis will cure spontaneously, or whether a patient affected with a viral hepatitis will be responsive to an antiviral hepatitis treatment, comprising (i) means for determining the level of TGF-β produced by T cells that are specific for the hepatitis C virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-β, in a biological sample from the patient, and optionally (ii) instructions for use.

The means for determining the level of TGF-β produced by T cells that are specific for the hepatitis C virus and/or the level of T cells may include reagents, such as anti-TGF-β antibodies, or staining agents, optionally in combination with a hepatitis virus antigen, such as a hepatitis virus antigenic peptide.

The examples and figures illustrate the invention without limiting its scope.

Examples:

Example 1: Lack of TGF-β production by Hepatitis C virus-specific T cells during HCV acute phase is associated with HCV clearance.

Methods:

Patients

Acute HCV infection was prospectively diagnosed on liver enzymes increase during routine monitoring of HIV infection and confirmed by two consecutive positive HCV-RNA detections in patients previously HCVRNA or HCV-Ab-negative within the past 8 months. Patients were prospectively studied for 15 months from diagnosis. Patients who did not spontaneously clear HCV 3 months after diagnosis were proposed a treatment with pegylated-IFN-α-2a and ribavirin at standard dosages for 6 months. Rapid virologic response was defined when HCV

RNA became undetectable after 4 weeks of treatment (RVR). Sustained virologic response (SVR) defined by a negative PCR 6 months (M) after treatment discontinuation.

Immunological assays

Blood samples

Blood samples were collected quarterly. Peripheral blood mononuclear cells (PBMCs) were isolated after Ficoll gradient centrifugation and either used directly or cryopreserved in liquid nitrogen and then used after thawing ensuring more than 85% viability.

Peptides

Four hundred and twenty-nine 18-mer HCV synthetic peptides (strain H77, genotype 1a) overlapping by 11 amino acids were pooled in 18 pools of 17–30 peptides each. Control peptides were HIV-p24 peptides (five pools of 11, 15-mer peptides overlapping on 11 amino acids).

IFN-γ ELISpot assays

Enzyme-linked immunospot (ELISPOT) assays were performed as described in Schnuriger, et al, 2009, AIDS, 23:2079-2089. Cryopreserved PBMCs (2*105 per well) were incubated in duplicates in ELISpot plates coated with anti-IFN-g antibody, with 10µg/ml of HCV peptide pool (Core, Env1, NS2, and NS4) in medium with 10% fetal calf serum for 20 h, in the presence or absence of blocking monoclonal antibodies (MAbs) or their isotype controls, anti-IL-10 and anti-TGF-β1, -2, and -3 (clone DII) or immunoglobulin G1 (IgG1) and IgG2b isotype controls (R&D Systems, Minneapolis, MN) simultaneously added to the wells at the optimized concentration (10 µg/ml). Phytohemagglutinin (Sigma-Aldrich, St.Louis, MO; 1 µg/ml) and medium alone served as positive and negative controls respectively.

Antigen-specific spot-forming cell (SFC) frequencies were measured on an automated microscope (Zeiss, Munich, Germany) and expressed after subtraction of patient background. Results were considered positive if a minimum of 50 SFCs/106 cells were detected above background. This threshold was chosen as more than 2 SDs above any response observed in the peripheral blood of 10 healthy controls.

The effect of adding mAbs was considered positive when the number of SFC in the presence of mAbs is 3X > the number of SFC in the presence of isotypes controls which was similar to the cells stimulated without any addition.

Intracellular cytokine staining ICS assays:

Cryopreserved PBMCs were incubated overnight with 5µg/ml of HCV peptide pool (Core, Env1, NS2, and NS4), with brefeldin-A added 1 h later. Positive and negative controls were

LPS, SEB, or PMA/Ionomycine and medium alone. Cells stimulated with core and NS2 and those stimulated with Env1 and NS4 were pooled together just before staining with anti-CD3/PB, anti-CD8/APC-CY7, anti-CD161/PE-CY5, anti-CD27/FITC (Becton-Dickinson/BD), anti-CD4/PE-CY7, and anti-CD45RA/ECD (Beckman Coulter/BC), anti-CCR7/APC or Alexa647 (R&D or BD), then fixed, permeabilized and stained with anti-IFN-g/ Alexa 700 (BD) and anti-TGF- β /PE (IQ Products).

Data acquisition was performed on a FACSLSRII flow cytometer (Becton-Dickinson). A minimum of 500 000 cells were recorded and cytokine-producing cells were analyzed (Flow jo, Becton-Dickinson) within the CD8 or CD4 T cells population. Results were expressed as percentages of cytokine-positive cells after background (unstimulated cells) subtraction. Cell frequencies were considered positive, if greater than 0.1% (mean value obtained for unstimulated cells) for IFN-γ+ cells, and greater than 0.16% and 0.2% for CD4+ and CD8+TGF-β+ cells, respectively. This threshold was chosen as more than 2 standard deviation above the response observed in healthy donors (HD).

Results:

Patients' characteristics and clinical outcome:

We have tested twenty three co-infected patients with available samples prior to therapeutic decision out of a series of 38 acutely infected patients. Patients included: four spontaneous recoveries SR, six patients refusing therapy and evolved to chronic infection CH, and thirteen patients accepting the therapeutic option, out of these 9 responded to treatment (one being re-infected and 8 with SVR), and four non responders to treatment (with one relapse after treatment discontinuation). Altogether, we considered two groups of patients: group A= patients with recovery RE, and B= patients who remained viremic VI. At inclusion, the median blood CD4+ T lymphocytes count was 540/mm^3 (IQR: 374-1032), and 18/23 (78%) patients had received antiretroviral therapy (ARV). These HCV strains were mainly of genotype 4 (74%), (table-1).

Frequency of HCV-specific regulatory cytokines in the presence of HCV/HIV co-infection:

We had previously shown in these 38 acutely infected patients weak circulating anti-HCV T cells producing IFN-γ by ELISpot assays (Schnuriger, et al, 2009, AIDS, 23:2079-2089) that contrasted with robust memory T cells in RE patients. We then decided to investigate the mechanisms underlying these weak anti-HCV responses. We first examined whether addition of neutralizing mAbs against IL-10 and TGF-β-1,-2, and -3, or their isotypes controls, could restore IFN-γ production by ELISpot assays against HCV antigens core, E1, NS2 and

NS4 (against which no response was observed in most patients), and against HIV-P24 as a control.

Overall, blockade of these regulatory cytokines amplified IFN-γ responses in 4 out of 11 tested patients, against at least one HCV-peptide pool. Addition of mAbs lead to 9 IFN-γ responses with 3/10 responses against core, 2/10 against E1, 2/9 against NS2 and 2/10 against NS4, while isotypes controls did only weakly (as shown in table-2). The amplifying effect appeared to be more pronounced in 3 VI patients. Furthermore, no significant increase in the T-cell response to HIV-P24 was observed, suggesting that this effect could be specific to HCV.

TGF-β secreting HCV-specific T-cells are induced during acute HCV-HIV coinfection:

The restoration of IFN-γ production in the presence of blocking IL-10 and TGF-β Abs, though at low level, raised the question whether this phenomenon was related to IL-10 or TGF-β production by HCV-specific CD4 or CD8 T cells.

We thus evaluated whether we could detect T cells producing one of those cytokines (IL-10 or TGF-β) upon HCV stimulation. As IL-10 production was not detectable even after the control positive stimulation, we concentrated our effort on TGF-β production. In contrast, we detected TGF-β production in T cells after stimulation with a pool of HCV core, NS2, NS4, and Env1 peptides together, but not against HIV P24 used as control.

We detected anti-HCV TGF-β producing T-cells in 9 out of the 23 tested patients (39 %). The source of TGF-β production was both CD4+ and CD8+ T-cells, with median values 0.58 % (IQR: 0.2-0.81) of CD4+ TGF-β+ and 0.64 % (IQR: 0.27-0.78) of CD8+TGF-β+ (table-3). In contrast, HCV-stimulated IFN-γ production could not be detected in PBMC from any patient. In healthy donors no TGF-β+ cells could be detected after stimulation with HCV peptides. Interestingly, HCV-specific TGF-β+ cells were detectable in 4 of the 4 patient's samples in whom anti-TGF-β + anti-IL-10 mAbs could restore IFN- γ responses, and in 3 of the 6 patient's samples in whom no restoration was observed.

<u>Lack of early TGF-β production by HCV-specific T cells predicts HCV clearance during acute HCV hepatitis in HIV co-infected patients</u>

We then tested whether this HCV-specific TGF-β production observed prior to therapeutic decision was associated to HCV outcome. SR and SVR were observed in 12 out of the 14 patients without TGF-β+ detectable cells, while only in 1 out of the 9 patients with TGF-β+ cells (p=0.0007). Comparing RE and VI patients revealed that percentages of HCV-specific TGF-β producing T cells, both CD4 and CD8, were significantly lower in RE than in VI patients (p=0.003 and 0.004 for CD4+TGF-β+ and CD8+TGF-β+ T-cells, respectively). Same differences were observed when comparing HCV-specific TGF-β producing T cells against

only Core and NS2 stimulation (p=0.01 and 0.01, for CD4+TGF-β+ and CD8+TGF-β+ T-cells, respectively). Interestingly, the proportion of HCV-specific TGF-β+ cells from RE patients did not differ from those of HD. In contrast, HD TGF-β+ cells were significantly lower from VI patients (p=0.004 and 0.02, for CD4+TGF-β+ and CD8+TGF-β+ T-cells, respectively), (**Figures 1A and 1B**).

Differentiation status of TGF-β producing HCV-specific T cells:

In order to determine the phenotype of the TGF-β+ cells, we analysed the expression of T cell differentiation markers, and of CD161. We found that CD4+TGF-β+ cells displayed, half a central memory CM (CD45RA-CCR7+) 40% (IQR: 12-66) and half an effector memory EM (CD45RA-CCR7-) 46% (IQR: 26-82) phenotype. In contrast, CD8+TGF-β+ cells were mainly EM with median expression 67% (IQR: 63-72). CD4+ and CD8+TGF-β+ cells were both CD161-, with median proportion of 76% (IQR: 67-87), and 76% (IQR: 72-84) in CD4+ and CD8+TGF-β+ cells, respectively.

HCV-specific TGF-β production remains stable during follow-up:

We then evaluated TGF-β production in 22 patients at advanced time points of follow up, treated patients were examined post treatment at M12 and M15 (except for two patients at M1 and M5 of treatment), untreated patients were re-examined between M9 and M12 of follow-up.

We observed that anti-HCV TGF-β producing T cells remained undetectable in the 13 RE patients, except in patient 6006 with TGF-β producing cells maintained even after eradicating the virus. With other view, TGF-β producing T cells were undetectable in 14 patients at baseline and remained so within follow-up, except in two VI patients in whom these cells became detectable at M12 for 6204 and M1 of treatment for 6206.

In the 9 patients with detectable TGF-β producing T cells at baseline, 3 VI patients had lost TGF-β+ cells, in whom: one NR who had been tested TF at M5 of treatment, and two CH patients who were treated at the chronic phase and succeeded to eradicate the virus.

Anti-HCV TGF-β producing T cells declined in VI patients compared to that detected in the acute phase but always detectable, with median values 0.3 % (IQR: 0.24-0.67) of CD4+ TGF-β+ and 0.23 % (IQR: 0.21-0.57) of CD8+TGF-β+.

Concerning TGF-β+ cells phenotype, the profile did not change a lot. In CD4+ TGF-β+ population, the median expression was 39% (IQR: 24-50) for CM, and 39% (IQR: 29-62) for EM. In CD8+ TGF-β+ population, the median expression of EM cells was 58% (IQR: 34-66), with an augmentation of effector phenotype Eff (CD45RA+CCR7-), 24% (IQR: 16-31). TGF-β+ cells remained predominantly of CD161- phenotype.

Table-1: Patients' characteristics:

	HIV			HCV					
ID	CD4/mm3	HIV VL	ARV	HCV VL	HCV Genotype	Peak ALT	Acute HCV symptoms	HCV treatment	Outcome
6212	1032	500000	Yes	5.97	4	170	Yes	No	SR
4905	462	1300	Yes	1.08	na	70	No	No	SR
4906	540	75449	Yes	7.13	1a	193	No	No	SR
6009	480	400	Yes	6.47	4d	452	No	No	SR
6004	506	81000	No	2.78	4d	850	Yes	Yes	RVR/SVR
6006	410	39000	Yes	5.78	4d	148	Yes	Yes	RVR/SVR
6101	391	20400	Yes	6.19	4d	392	No	Yes	RVR/SVR
6201	628	400	Yes	6.65	4d	929	Yes	Yes	RVR/SVR
6203	403	400	Yes	2.78	4	728	Yes	Yes	RVR/SVR
6216	814	79357	No	7.07	3a	497	No	Yes	RVR/SVR
6001	374	400	Yes	6.89	4d	334	No	Yes	N RVR/SVR
6208	863	70036	No	5.24	4	494	No	Yes	N RVR/SVR
4904	684	21225	No	5	4d	541	No	Yes	RVR/reinfected
6012	511	418	Yes	6.71	4d	134	No	Yes	NR
6206	557	400	Yes	6.84	4	388	Yes	Yes	NR
6207	473	400	Yes	5.29	4d	653	No	Yes	NR
6210	439	400	No	6.91	4c4d	546	Yes	Yes	NR (relapse)
4903	558	400	Yes	5.62	3a	195	No	No	CH
6007	604	1850	Yes	6.89	1a	490	No	No	CH
6204	648	400	Yes	5.26	4c4d	531	No	No	CH
6014	505	400	Yes	3.06	4d	88	No	No	CH
6214	642	400	Yes	5.79	4	192	No	No	CH
6217	754	400	Yes	6.05	1a	159	Yes	No	CH
Median all	540	400		5.97		392			
min	374	400		1.08		70			
max	1032	500000		7.13		929			
median RE	506	21225		5.97		452			

min	374	400	1.08	70
max	1032	500000	7.13	929
median VI	557.5	400	5.92	291.5
min	439	400	3.06	88
max	754	1850	6.91	653

Table 2: Frequency of anti-HCV IFN-γ responses in HCV/HIV co-infected patients:

		HCV peptides pool								HIV	
		core		Env1		NS2		NS4		P24	
patient	**outcome**	**isotype**	**mAbs**	**isotype**	**mAbs**	**isotype**	**mAbs**	**isotype**	**mAbs**	**isotype**	**mAbs**
62 12	GS	0	0	0	33	0	0	9	24	NT	NT
60 06	RVR SVR	4	**122***	NT	NT	NT	NT	NT	NT	NT	NT
62 03	RVR SVR	0	30	26	26	0	28	34	**60**	40	**142***
49 04	RVR reinfected	0	3	0	5	13	8	0	3	**65**	**90**
6012	NR	10	23	7	0	3	0	0	33	**224**	**416**
62 07	NR	0	8	0	3	0	0	0	0	0	0
62 10	NR	0	0	0	0	0	0	0	13	0	0
49 03	Ch	**83**	**288***	0	**53***	8	**218***	**65**	43	**80**	**80**
62 04	Ch	0	0	0	0	0	0	0	0	**483**	**567**
62 14	Ch	NT	NT	20	**125***	NT	NT	**50**	**160***	NT	NT
62 17	CH	20	**63***	30	43	3	**66***	20	14	26	**53**
median		0	15.5	0	15.5	0	0	4.5	19	**52**	**85**
min		0	0	0	0	0	0	0	0	0	0
max		**83**	**288**	**30**	**125**	13	**218**	**65**	**160**	**483**	**567**

Result of anti-HCV IFN-γ producing cells measured by ELISpot assays, expressed as SFC/10^6 PBMC after HCV stimulation in the presence of mAbs against IL-10 and TGF-β or their isotypes controls (All results are expressed after subtraction of the number of SFC observed with no antigen stimulation).
*: The effect of adding mAbs was considered positive when the number of SFC in the presence of mAbs is 3X > the number of SFC in the presence of isotypes controls.

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Table-3: Frequency of CD4+ and CD8+TGF-β+ cells between diagnosis and option for therapy:

				Before treatment HCV-specific TGF-β+ cells		During Follow-up HCV-specific TGF-β+ cells		
Groupe 1:	ID	Treatment	Outcome	Time point	%CD4+TGF-β+	%CD8+TGF-β+	Time point	%CD4+TGF-β+	%CD8+TGF-β+
RE	6212	No	SR	M3	0.13	0	M12	<0.01	<0.01
	4905	No	SR	M1	0	0	M9	<0.01	<0.01
	4906	No	SR	M3	0	0	M9	<0.01	<0.01
	6009	No	SR	M3	0.03	0.05	M12	0.15	0.15
	6004	Yes	RVR/SVR	J0	0.06	0.1	PT	0.12	<0.01
	6006	Yes	RVR/SVR	M1	**0.22**	0.15	PT	**0.53**	**0.23**
	6101	Yes	RVR/SVR	J0	0	0	PT	0.02	0.1
	6201	Yes	RVR/SVR	M1	0	0	PT	0.09	0.1
	6203	Yes	RVR/SVR	M1	0.06	0.05	PT	0.06	0.02
	6216	Yes	RVR/SVR	M1	0.06	0.03	PT	<0.01	<0.01
	6001	Yes	N RVR/SVR	J0	0.03	0	PT	0.02	<0.01
	6208	Yes	N RVR/SVR	M1	0.03	0	PT	<0.01	<0.01
	4904	Yes	RVR/reinfected	M1	0.06	0.05	PT	<0.01	<0.01
Groupe 2:	6012	Yes	NR	J0	**0.58**	**0.57**	TF	0.09	0.05
VI	6206	Yes	NR	M1	0	0	TF	**0.31**	0.17
	6207	Yes	NR	M1	**0.81**	**0.27**	PT	**0.24**	0.17
	6210	Yes	NR (relapse)	M1	**0.7**	**0.73**	PT	**0.67**	**0.57**
	4903	No	CH	M4	**0.73**	**0.78**	M12	**0.27**	**0.24**
	6007	No	CH	M1	**0.36**	**0.34**	M15	**0.3**	**0.22**
	6204	No	CH	M3	0.04	0.1	M12	**0.28**	**0.22**
	6014*	No	CH	M1	**0.23**	0	M12	<0.01	<0.01
	6214	No	CH	M3	**0.2**	0.16	**NT**	NT	NT
	6217*	No	CH	M3	**0.71**	**0.71**	M21	0.04	0.11

ICS analysis after stimulation of PBMC from co-infected patients with HCV peptides pool. Percentages of CD4+ and CD8+TGF-β+ present the sum of all anti-HCV TGF-β producing T-cells after subtraction of background (medium).
NR: non responder to treatment.
PT: post treatment.
TF: through follow-up of treatment.
NT: none tested.
*: Patients 6014 and 6217 were treated at the chronic phase.

Example 2: Lack of influence of the IL-28b gene polymorphism on the predictive value of the HCV-specific TGF-β production by T cells in acute hepatitis

We have investigated the frequency of rs12979860 SNP in IL-28B gene to compare its effect as a predictor of recovery from HCV infection (REF), with the present invention that shows the predictive effect of anti-HCV TGF-β production by T cells.

22 patients could be genotyped. 12 (54%) were C/C, 6 (27%) were C/T, and 4 (18%) were T/T. Comparing our two groups of patients RE and VI according to IL-28b genotype, we showed that recovery was not significantly associated with the C/C polymorphism as observed in 8/12 subjects of C/C genotype, in 4/6 subjects of C/T genotype, and in 1/4 subjects of T/T genotype (p=0.43).

A comparison of recovery between C/C and C/T + T/T group did not reveal more information: indeed recovery was observed in 8/12 subjects and in 5/10 subjects with C/C and C/T + T/T genotype, respectively, p=1. These results suggest that the IL-28B polymorphism is not significantly associated with recovery from HCV infection in patients co-infected with acute HCV and HIV in this study group. Moreover, we did not find a significant association between IL-28B polymorphism and TGF-β production, which was negative in 8/12 patients displaying the C/C genotype, in 4/6 patients having C/T genotype and in 2/4 having T/T genotype p= 0.85, suggesting that TGF-β production was independent of the IL-28B polymorphism.

In order to evaluate if the presence or absence of TGF-β production and C/C genotype could be more efficient than the effect of TGF-β alone at predicting recovery, we analyzed the recovery according to TGF-β production and IL-28B genotype together. We found that, in patients with the C/C polymorphism, recovery is achieved in 7/8 patients in the absence of TGF-β production, and in 1/4 patients in the presence of TGF-β production. In patients with the C/T and T/T polymorphisms, recovery was achieved in 5/6 patients in the absence of TGF-β, and in 0/4 patients in the presence of TGF-β production, p=0.007. In contrast, recovery was achieved in 12/14 of patients without TGF-β production, and in 1/8 patients with TGF-β production p=0.001.

Thus the predictive value of the TGF-β assay alone appears as the most potent predictor of recovery, whether it is spontaneous or treatment induced.

Example 3: TGFβ producing anti-HCV responses in Chronic Hepatitis C infected and HIV coinfected patients

The inventors have then analysed the frequency of TGF-β producing T cells in response to core, NS2 or NS4 during chronic phase of HCV infection in patients coinfected with HIV.

The inventors have further determined the impact of those cells on the progress of the infection, HCV viral load and liver disease stage. The same analysis was performed in monoinfected patients in HCV chronic phase to assess the impact of HIV on the frequency of TGF-β producing T cells.

Patients

A group of patients chronically infected with HCV have been studied (Table 3).

24 patients coinfected with HIV. Median CD4 T cell rate was 448/mm3 (IQR: 373-710). All these patients were treated for HIV infection. Median HIV viral load was 20 copies/mL (IQR: 20-20). Median ALAT rate was 51 UI/L (IQR: 36-109).

Median HCV viral load was 5,9 IU/mL log (IQR: 5,9-6,61).

HCV genotype was mainly genotype 1 (10 patients) and genotype 4 (10 patients). Genotype 3 was observed in 2 patients, genotype 2 in 1 patient and genotype 6 in a last patient.

The stage of the liver disease after a median infection period of 17 (IQR: 7-24) was mostly moderate fibrosis (11/24 patients) and cirrhosis (9/24 patients), 1 patient only had a minimal fibrosis and 3 patients had a severe fibrosis.

9 patients were treated (by PEG-IFN-alpha + Ribavirin) including 5 non-responders, and 4 responders/ relapsers. 3 patients had started treatment after joining the trial and were still under treatment. These 3 patients had an early viral response to treatment.

Table 3: patients' characteristics

		patients HCV/HIV
characteristics		**n = 24**
Age (years)	median	48 (IQR:45-51)
Period of infection (years)	median	17 (IQR: 6-24)
CD4/mm3	median	448 (IQR: 373-710)

VL HIV copies/mL	median	20 (IQR: 20-20)
VL HCV IU/mL log	median	5,9 (IQR: 5,9-6,61)
ALAT UI/L	median	51 (IQR: 36-109)
Anti HIV-treatment	N°	24 patients
Anti-HCV treatment	N°	12/24 (50%)
HCV genotype		HCV genotype
1	N°	10 (41%)
2	N°	1
3	N°	2
4	N°	10 (41%)
6	N°	1
Inflammation stage (Metavir)		
normal	N°	aucun
Light fibrosis	N°	1
Moderate fibrosis	N°	11/24 p (45%)
Severe fibrosis	N°	3
cirrhosis	N°	9/24 p (37%)

Results

The inventors have analysed the frequency of TGF-β producing CD4 and CD8 T cells in response to a stimulation with 3 HCV peptide pools (core, NS2, and NS4). Anti HCV T cells were detected at rate in the group of patients (frequency was considered positive when superior to 0.1 for CD4+TGF-β+, and superior to 0.12 for CD8+TGF β+).

Anti-HCV T cells with production of TGF-β were detected against at least 1 peptide pool peptidique in 7/24 patients coinfected (29%) (Table 4).

These responses were directed against Core in 1/4 patients, against NS2 in 3/3 patients, against Core+NS2 in 1/3 patients wherein T cells stimulated with Core and NS2 were pooled after stimulation and before labelling. Yet the inventors have detected TGF-β producing T cells against NS4 in 4/6 patients. The anti-HCV TGF-β producing T cells were CD4+ and/or CD8+. Medians of percentages in anti-NS4 T cells are : 0.2 (Range: 0.04-0.27) for

CD4+TGF-β+ T cells, and 0.1 (Range: 0-0.22) for T CD8+TGF-β+T cells . All medians are shown in Table 4.

Frequency of anti-HCV TGF-β producing T cells was independent of CD4 T cellcounts , HIV viral load or HCV viral load, and HCV genotype.

The inventors have observed that the patients with anti-HCV TGF-β producing T cells tend to be at a more advanced stage of the liver disease since Cirrhosis and severe fibrosis were observed in 6/7 (86%) patients with TGF-β+ cells, versus 6/17 (35%) in patients with no such cells (**Figure 2**).

The inventors considered as responders the patients who respond to the treatment at least once, even if they have relapsed afterwards, and the patients who showed an early virological response after initiation of the treatment.

There were then 7/12 patients considered responders to the first treatment, and 4 non responder patients.

Further to this classification, a response to the treatment was reached in 5/7 treated patients among those who did not have anti-HCV TGF-β+ T cells and in 2/5 patients who had anti-HCV TGF-β+ T cells.

Table 4: anti-HCV TGF-β producing T cells in patients coinfected with HIV and HCV, (after substracting background noise).

								% cellules T CD4+ et CD8+ TGF-β+							
	HIV Infection			HCV Infection				stimulation core		stimulation NS2		stimulation core+NS2		stimulation NS4	
N° patient	CV HIV cp/ml	CD4 /mm3	CV HCV ui/ml log 10	Génotype HCV	ALAT	Score fibrosis METAVIR	IL-28B polymorphism	%CD4+TGF-β+	%CD8+TGF-β+	%CD4+TGF-β+	%CD8+TGF-β+	%CD4+TGF-β+	%CD8+TGF-β+	%CD4+TGF-β+	%CD8+TGF-β+
1	20	217	1,08	4	27	cirrhosis		0,02	0,00	0,00	0,00			0,00	0,00
2	20	698	6,61	4	19	moderate fibrosis		0,00	0,00	0,00	0,00			0	0
3	20	890	5,94	4	36	moderate fibrosis		0,04	0,05	0,02	0,02			0,00	0,00
4	20	196	6,57	4	50	moderate fibrosis	c/c	0,05	0,04	0,03	0,04			0,00	0,00
5	20	115	6,18	2	52	moderate fibrosis		NT	NT	NT	NT	0,00	0,00	NT	NT
6	20	409	5,73	1	45	moderate fibrosis	c/t	0,05	0,03	0,10	0,11			0,03	0,04
7	20	399	6,09	4	74	severe fibrosis	c/t	0,05	0,06	NT	NT			0,03	0,03
8	20	375	5,77	1	51	moderate fibrosis		0,04	0,04	NT	NT			0,05	0,08
9	22	447	6,59	4	35	light fibrosis		0,02	0,00	0,05	0,04			0,02	0,05
10	501	801	6,83	1	28	moderate fibrosis		0,08	0,03	NT	NT			0,10	0,06
11	20	87	6,88	3	145	cirrhosis		0,00	0,02	0,05	0,09			0,09	0,06
12	20	389	6,50	4	66	moderate fibrosis	c/c	0,02	0,02	0,04	0,07			0,03	0,03
13	20	910	6,61	1	86	moderate fibrosis	c/t	0,02	0,04	0,01	0,01			0,05	0,03

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14	64	540	6,45	1	107	cirrhosis		0,00	0,00	0,00	0,00			0,00	0,00
15	26	747	6,34	4	320	cirrhosis	c/t	NT	NT	NT	NT	0,00	0,00	0,08	0,04
16	20	564	6,71	6	71	moderate fibrosis		NT	NT	NT	NT	0,03	0,06	0,05	0,05
17	20	581	6,11	1	36	cirrhosis		0,07	0,04	NT	NT			0,09	0,07
median	20	447	6,45		51			0,03	0,03	0,02	0,03	0	0	0,03	0,04
Min	20	87	1		19			0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Max	501	910	7		320			0,08	0,06	0,10	0,11	0,03	0,06	0,10	0,08
18	26	449	5,71	1	26	severe fibrosis		NT	NT	NT	NT	**0,14**	**0,14**	**0,27**	**0,22**
19	20	262	6,34	1	116	moderate fibrosis		**0,14**	0,11	NT	NT	NT	NT	NT	NT
20	20	370	5,95	3	154	cirrhosis	c/c	0,03	0,00	0,08	**0,13**	NT	NT	0,05	0,07
21	20	844	7,47	1	46	cirrhosis	c/t	NT	NT	NT	NT	0,03	0,03	**0,25**	0,10
22	20	512	6,75	4	45	severe fibrosis		NT	NT	NT	NT	0,04	0,04	**0,26**	**0,18**
23	23	832	5,67	4	190	cirrhosis		0,05	0,02	**0,19**	**0,18**	NT	NT	**0,15**	0,11
24	20	440	5,80	1	123	cirrhosis		0,06	0,01	**0,17**	0,09	NT	NT	0,04	0,00
mediane	20	449	5,95		116			0,06	0,01	0,17	0,13	0,04	0,04	0,20	0,10
Min	20	262	6		26			0,03	0,00	0,08	0,09	0,03	0,03	0,04	0,00
Max	26	844	7		190			0,14	0,11	0,19	0,18	0,14	0,14	0,27	0,22

NT: non tested. Percentages in bold are considered positive.

CLAIMS

1. An *in vitro* method for predicting whether a patient affected with a viral hepatitis will cure spontaneously, which method comprises determining the level of TGF-β produced by T cells that are specific for the hepatitis virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-β, in a biological sample from the patient, wherein the patient is predicted to cure spontaneously when said level is not significantly higher, compared to a control value.

2. An *in vitro* method for predicting whether a patient affected with a viral hepatitis will be responsive to an antiviral hepatitis treatment, which method comprises determining the level of TGF-β produced by T cells that are specific for the hepatitis virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-β, in a biological sample from the patient, wherein the patient is predicted to be responsive to said treatment when said level is not significantly higher compared to a control value.

3. The method of claim 2, wherein the treatment includes a treatment with an interferon and/or an anti-viral nucleoside, preferably ribavirin.

4. The method of any of claims 1 to 3, which further comprises determining polymorphisms in the IL-28b gene.

5. An *in vitro* method for assessing the severity or progress of a liver fibrosis in a patient infected with a hepatitis virus, which method comprises determining the level of TGF-β produced by T cells that are specific for the hepatitis virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-β, in a biological sample from the patient, wherein the patient is likely to have or to progress toward a severe liver fibrosis or a cirrhosis, when said level is significantly higher, compared to a control value.

6. The method of any of claims 1 to 5, wherein the patient is infected with hepatitis C virus (HCV).

7. The method of claim 6, wherein the patient is co-infected with HIV virus.

8. The method of any of claims 1 to 4, and 6 or 7, wherein the patient is affected with an acute viral hepatitis.

9. The method of claim 5, wherein the patient is affected with a chronic viral hepatitis.

10. The method of any of claims 1 to 9, wherein TGF-β is TGF-β1.

11. The method of any of claims 1 to 10, wherein the level of TGF-β and/or of T cells, that are specific for the hepatitis virus and produce TGF-β, is determined in a biological sample taken from the patient within three months after an acute viral hepatitis is diagnosed.

12. The method of any of claims 1 to 11, wherein the biological sample is blood.

13. The method of any of claims 1 to 12, which method comprises determining the level of TGF-β produced by T cells, upon stimulation with a hepatitis virus antigen.

14. The method of claim 13, which comprises stimulating Peripheral Blood Mononuclear Cells (PBMC) obtained from the biological sample of the patient with a hepatitis virus antigen, detecting TGF-β in the culture supernatant, and comparing with the level of TGF-β produced in the absence of hepatitis virus antigen.

15. The method of any of claims 1 to 12, which method comprises determining the level of T cells that produce TGF-β, upon stimulation with a hepatitis virus antigen.

16. The method of claim 15, which comprises stimulating Peripheral Blood Mononuclear Cells (PBMC) obtained from the biological sample of the patient with a hepatitis virus antigen, and quantifying the subset of cells that produce TGF-β, *e.g.* by flow cytometry.

17. A kit for predicting whether a patient affected with a viral hepatitis will cure spontaneously, or whether a patient affected with a viral hepatitis will be responsive to an antiviral hepatitis treatment, of for assessing the severity or progress of a liver fibrosis in a patient infected with a hepatitis virus comprising (i) means for determining the level of TGF-β produced by T cells that are specific for the hepatitis C virus and/or the level of T cells that are specific for the hepatitis virus and produce TGF-β, in a biological sample from the patient, and optionally (ii) instructions for use.

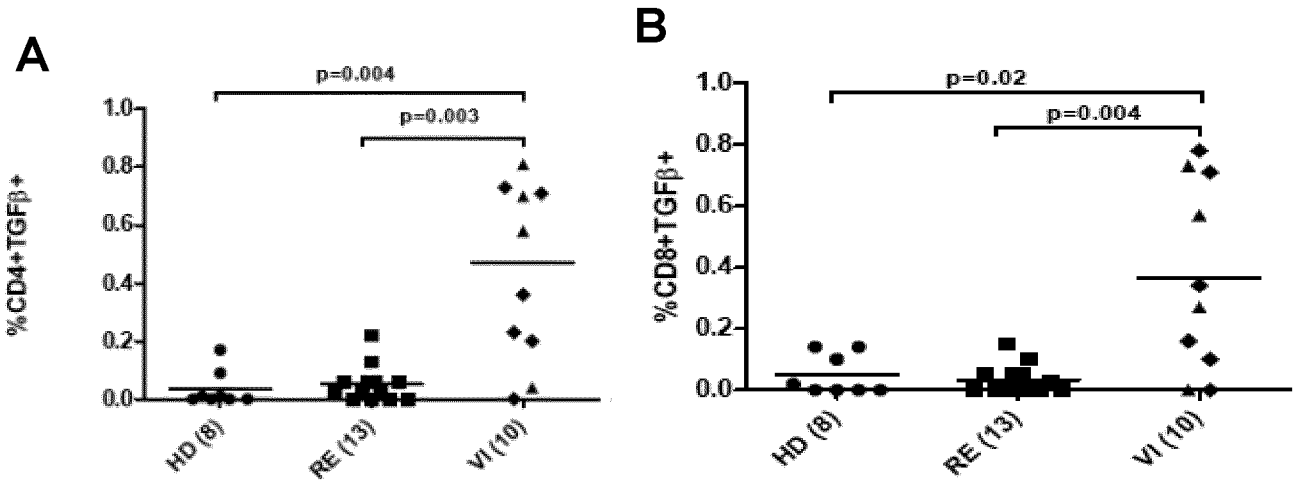

Figure 1

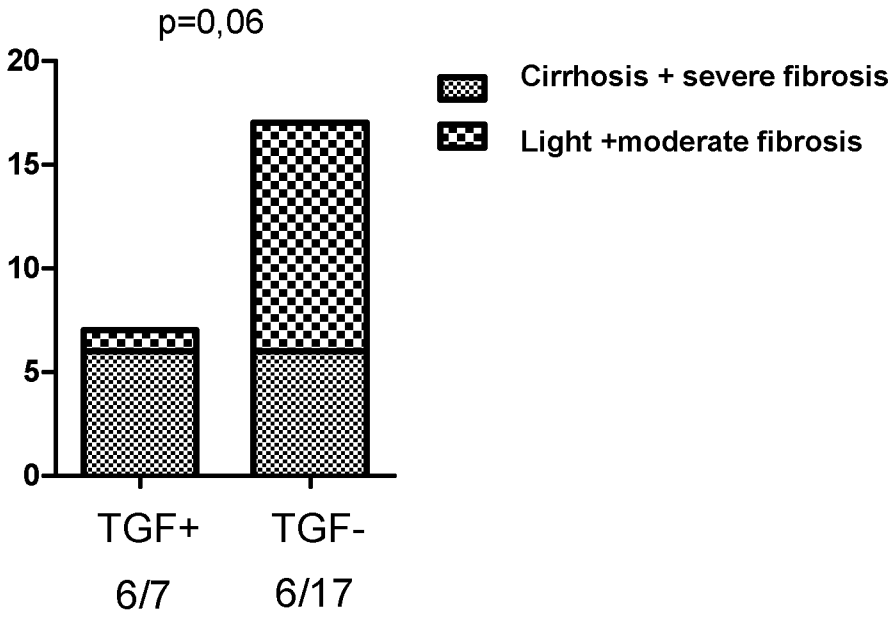

Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/052359

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/576
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALATRAKCHI NADIA ET AL: "Hepatitis C virus (HCV)-specific CD8(+) cells produce transforming growth factor beta that can suppress HCV-specific T-cell responses", JOURNAL OF VIROLOGY, vol. 81, no. 11, June 2007 (2007-06), pages 5882-5892, XP002635131, ISSN: 0022-538X see discussion ----- -/--	1-17

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 2012	06/03/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hinchliffe, Philippe

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/052359

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HALL CAROLINE H T ET AL: "HCV+ Hepatocytes Induce Human Regulatory CD4(+) T Cells through the Production of TGF-beta", PLOS ONE, vol. 5, no. 8, August 2010 (2010-08), XP002635132, ISSN: 1932-6203 page 4, column 2	1-9, 12-15,17
X	CABRERA ET AL: "An immunomodulatory role for CD4+CD25+ regulatory T lymphocytes in hepatitis C virus infection", HEPATOLOGY, WILLIAMS AND WILKINS, BALTIMORE, MD, US, vol. 40, no. 5, 1 November 2004 (2004-11-01), pages 1062-1071, XP008103184, ISSN: 0270-9139, DOI: DOI:10.1002/HEP.20454 see discussion	1-17
A	MAREK B ET AL: "TGF-beta 1 mRNA expression in liver biopsy specimens and TGF-beta 1 serum levels in patients with chronic hepatitis C before and after antiviral therapy", JOURNAL OF CLINICAL PHARMACY AND THERAPEUTICS, vol. 30, no. 3, June 2005 (2005-06), pages 271-277, XP002635133, ISSN: 0269-4727 abstract	1-17
X	DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; September 2008 (2008-09), BARBOZA LUISA ET AL: "[Impaired activation and costimulation of T CD4+ lymphocytes during chronic hepatitis C infection].", XP002635134, Database accession no. NLM18846776 abstract & INVESTIGACIÓN CLÍNICA SEP 2008 LNKD-PUBMED:18846776, vol. 49, no. 3, September 2008 (2008-09), pages 353-367, ISSN: 0535-5133	1-17

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GE DONGLIANG ET AL: "Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance", NATURE, NATURE PUBLISHING GROUP, LONDON, GB, vol. 461, no. 7262, 17 September 2009 (2009-09-17), pages 399-401, XP002602519, ISSN: 0028-0836, DOI: DOI:10.1038/NATURE08309 [retrieved on 2009-08-16] abstract ----- ROSÂNGELA TEIXEIRA ET AL: "Immunopathogenesis of hepatitis C virus infection and hepatic fibrosis: New insights into antifibrotic therapy in chronic hepatitis C", HEPATOLOGY RESEARCH, vol. 37, no. 8, 1 August 2007 (2007-08-01) , pages 579-595, XP55020336, ISSN: 1386-6346, DOI: 10.1111/j.1872-034X.2007.00085.x abstract -----	4 1-17