Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Intrahepatic IL-8 producing Foxp3$^+$CD4$^+$ regulatory T cells and fibrogenesis in chronic hepatitis C

Bettina Langhans[1,*], Benjamin Krämer[1], Marcell Louis[1], Hans Dieter Nischalke[1], Robert Hüneburg[1], Andrea Staratschek-Jox[2], Margarethe Odenthal[3], Steffen Manekeller[4], Michael Schepke[5], Jörg Kalff[4], Hans-Peter Fischer[6], Joachim L. Schultze[2], Ulrich Spengler[1]

[1]*Department of Internal Medicine I, University of Bonn, Bonn, Germany;* [2]*LIMES-Institute, University of Bonn, Bonn, Germany;* [3]*Institute of Pathology, University of Cologne, Cologne, Germany;* [4]*Department of Surgery, University of Bonn, Bonn, Germany;* [5]*Helios-Kliniken, Siegburg, Germany;* [6]*Institute of Pathology, University of Bonn, Bonn, Germany*

Background & Aims: Regulatory CD4$^+$ T cells (Tregs) are considered to affect outcomes of HCV infection, because they increase in number during chronic hepatitis C and can suppress T-cell functions.

Methods: Using microarray analysis, *in situ* immunofluorescence, ELISA, and flowcytometry, we characterised functional differentiation and localisation of adaptive Tregs in patients with chronic hepatitis C.

Results: We found substantial upregulation of IL-8 in Foxp3$^+$CD4$^+$ Tregs from chronic hepatitis C. Activated GARP-positive IL-8$^+$ Tregs were particularly enriched in livers of patients with chronic hepatitis C in close proximity to areas of fibrosis and their numbers were correlated with the stage of fibrosis. Moreover, Tregs induced upregulation of profibrogenic markers TIMP1, MMP2, TGF-beta1, alpha-SMA, collagen, and CCL2 in primary human hepatic stellate cells (HSC). HSC activation, but not Treg suppressor function, was blocked by adding a neutralizing IL-8 antibody.

Conclusions: Our studies identified Foxp3$^+$CD4$^+$ Tregs as an additional intrahepatic source of IL-8 in chronic hepatitis C acting on HSC. Thus, Foxp3$^+$CD4$^+$ Tregs in chronic hepatitis C have acquired differentiation as regulators of fibrogenesis in addition to suppressing local immune responses.

Keywords: Hepatitis C virus; Regulatory T cells; IL-8; Fibrogenesis; Hepatic stellate cells.

Received 17 December 2012; received in revised form 4 April 2013; accepted 7 April 2013; available online 23 April 2013

* Corresponding author. Address: Department of Internal Medicine I, University of Bonn, Sigmund-Freud-Straße 25, 53105 Bonn, Germany.

E-mail address: bettina.langhans@ukb.uni-bonn.de (B. Langhans).

Abbreviations: alpha-SMA, alpha-smooth muscle actin; CCL2, chemokine ligand 2; Foxp3, forkhead box P3; GARP, glycoprotein A repetitions predimonant; HCV, hepatitis C virus; HSC, primary human hepatic stellate cells; MMP2, matrix metalloproteinases 2; mRNA, messenger RNA; RT-PCR, real-time polymerase chain reaction; Teffs, T effector cells; Tregs, regulatory CD4$^+$ T cells; TIMP1, tissue inhibitor of metalloproteinases 1.

Introduction

A majority of individuals infected with the hepatitis C virus (HCV) fail to eradicate the virus during the acute phase of infection and develop chronic persistent hepatitis, which is associated with relatively little inflammatory activity [1]. Nevertheless, chronic hepatitis C carries a substantial risk to progress towards liver fibrosis, cirrhosis, and hepatocellular carcinoma.

The mechanisms that favour HCV persistence are still uncompletely understood. However, previous studies have raised the possibility that regulatory CD4$^+$ T cells (Tregs) might play an important role in the evolution of chronic infections [2]. Tregs exhibit great heterogeneity with respect to their antigen-specificity, differentiation, mechanisms of action, and tissue distribution [3]. Tregs regulate local immune responses by contact-dependent mechanisms and release of cytokines such as IL-10. In addition, certain Treg subsets release TGF-beta1 [4], and thus have been proposed to also stimulate fibrosis.

Patients with chronic hepatitis C have been reported to have increased levels of natural and adaptive Tregs in peripheral blood [5–7]. Since depletion of Tregs enhanced antigen-specific CD4$^+$ and CD8$^+$ T-cell functions, Tregs are considered to contribute to the weak anti-HCV immune response in patients with persistent HCV infection [7–9]. Recently, we established HCV core-specific CD4$^+$ Treg clones from the blood of patients with chronic hepatitis C which (1) recognized distinct HCV epitopes in an HLA-DR-restricted manner and (2) inhibited HCV-specific T-effector cells (Teffs) via secretion of IL-10 and IL-35 [10]. However, production of TGF-beta1 was an infrequent finding in only few Treg clones from chronic hepatitis C. To further clarify their role in the pathogenesis of chronic hepatitis C, we characterized functional differentiation of adaptive CD4$^+$ Tregs by microarray analysis and functional *in vitro* studies, and determined their number and localization in liver tissue.

Materials and methods

Study groups

Liver tissue was obtained from explant livers (n = 5) and biopsies (n = 14) of patients with hepatitis C. Heparinized blood was collected prior to the biopsy

Research Article

or transplantation. All patients were treatment-naïve and had detectable HCV RNA (Table 1). Biopsies were performed with a 1.4 mm diameter disposable needle using the Menghini technique. Part of the samples was used for routine histopathology and the remaining tissue was split for immunofluorescence of cryostat sections and analysis by flowcytometry. Inflammatory activity and fibrosis stage were scored with the Metavir score. Biopsies from chronic hepatitis B (n = 3), alcoholic liver disease (n = 6), drug-induced liver injury (n = 2; anabolic steroid abuse, non-steroidal anti-inflammatory drugs) and primary biliary cirrhosis and primary sclerosing cholangitis (n = 1 each) served as controls. Ten healthy donors provided control samples to study Tregs in peripheral blood.

Informed consent was obtained from each subject prior to sample collection and the study protocol conformed to the guidelines of the 1975 Declaration of Helsinki as approved by the Bonn University Ethics Committee (decision #067/10).

$CD4^+$ T-cell clones from patients with chronic and self-limited HCV infection

In this study, we included 12, 11 and 7 Th1, Th2 and Treg clones from patients with chronic hepatitis C, and 10, 9 and 6 Th1, Th2 and Treg clones from subjects with self-limited HCV infection, respectively. Detailed characteristics of the clones are described by us elsewhere [10,11].

Human recombinant IL-2 was obtained from R&D Systems (Wiesbaden, Germany), anti-CD28 from BD Biosciences (Heidelberg, Germany), and OKT-3 (anti-CD3) from eBioscience (Frankfurt, Germany).

Transcriptomic profiling of $CD4^+$ Treg clones

One hundred ng of extracted total RNA from 3 randomly selected, stimulated Treg clones from each study group was transcribed and biotin-labeled using the Ambion® Illumina RNA amplification kit (Ambion Europe, Huntington, Cambridgeshire, UK). Complementary RNA was hybridized to Sentrix® whole genome bead chips 6V3 (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Expression data were exported from the BeadStudio 3.1.1.0 software and analysed using R Development Core Team 2011. Data were quality-weighed, background-corrected, quantile-normalized, log-transformed, and explored for differentially expressed genes, with a false discovery rate <0.05 using a paired comparison. The expression sets for all biological replicates were assessed for similarities and dissimilarities with principal component analysis using the "pcurve" package in R (data not shown). False discovery rate was corrected according to Benjamini and Hochberg [12].

Functional analysis of $CD4^+$ Treg clones

1×10^5 cells of all Treg clones from both study groups were stimulated with anti-CD3/anti-CD28 (1 μg/ml and 2.5 μg/ml). IL-4, IL-10, IFN-gamma, and TGF-beta1 were measured as described previously [10,11]. The CXCL8/IL-8 DuoSet® ELISA kit (R&D Systems) was used to determine IL-8. Cytokine values >50 pg/ml were considered positive.

Treg clones from chronic hepatitis C inhibit proliferation of autologous Teffs via secretion of soluble factors such as IL-10 [10]. To check whether IL-8 produced by the HCV-specific Treg clones altered proliferation of autologous Teffs, cells (5×10^4 cells/well) were stimulated with anti-CD3/anti-CD28 in the presence of autologous Treg at a 1:1 ratio. Blocking experiments were performed in the co-cultures by adding neutralizing IL-8 and IL-10 antibodies (AF-208-NA; AF-217-NA) or isotype control antibody AB-108-C (10 μg/ml each; all R&D Systems), respectively. After 5 days, proliferation of T cells was assessed by [^{3}H] thymidine incorporation [10].

In situ-detection of $IL\text{-}8^+$ and $Foxp3^+CD4^+$Tregs

Shock-frozen liver specimens were cut into 6 μm slices on a Leica microtome (Microsystems GmbH, Wetzlar, Germany). After drying overnight, sections were fixed with acetone and blocked with human AB-serum (10% in PBS). The following anti-human antibodies were used: anti-CD4 (clone 34930¸ R&D Systems), anti-CD8 (clone M7103; DAKO, Hamburg, Germany), anti-CD3 (clone NCL-L-CD3; Leica Biosystems, Wetzlar, Germany), anti-GARP (LRRC32; Enzo Life Sciences, NY, US), anti-alpha-SMA (clone 1A4, Dako, Hamburg, Germany), anti-CD68 (clone PG-M1, Dako), and anti-CXCL8/IL-8 (AF-208-NA; R&D Systems). Double-staining was performed using NL-637 and NL-557 (R&D Systems). Nuclei were counterstained with DAPI (Life Technologies-Invitrogen, Darmstadt, Germany). Antibodies MAB002 and AB-108-C (both R&D Systems) were used as isotype controls. After mounting with Aqueous Mounting Medium (R&D Systems), sections were scanned with a Zeiss Axiovert 200M Apotome using the AxioVision Software (Zeiss, Jena, Germany).

Table 1. Characteristics of HCV-infected patients.

Patient	Age (yr)	Sex	Duration of infection	Source of infection	Genotype	ALT (IU/L)	Viral load (U/ml)	Metavir fibrosis score	Metavir inflammatory activity score	*IL28B* rs12979860 C/T genotype
#1	32	M	4	IVDA*	3a	134	2,137,889	0	1	CT
#2	58	M	18	unknown	4	47	1,800,126	4	1	CC
#3	24	M	1	IVDA	1a	456	1,190,714	0	0	TT
#4	62	F	>10	IVDA	1	61	308,000	0	0	CT
#5	40	M	2	unknown	1b	92	20,500,000	2	2	CC
#6	49	F	1	unknown	2a	352	183,530	3	2	CT
#7	19	F	>10	unknown	3a	33	26,426	0	0	CT
#8	54	F	15	unknown	2b	284	243,000	2	3	CC
#9	50	M	11	unknown	1b	118	1,496,043	4	2	TT
#10	59	M	>10	unknown	1b	104	3,315,927	4	1	CT
#11	33	M	10	IVDA	1b	84	976,984	1	1	CT
#12	52	M	35	blood transfusion	1b	150	1,733,213	1	0	CT
#13	64	M	>10	unknown	1b	176	25,711	4	2	CC
#14	49	M	>10	unknown	1b	32	194,201	4	1	CT
#15	53	M	6	IVDA	1a	53	2,078,636	2	0	CT
#16	50	F	>10	unknown	1	162	36,529	3	2	CT
#17	34	M	>10	IVDA	1b	99	245,000	1	1	CT
#18	66	F	>10	unknown	1b	204	134,121	4	1	CC
#19	48	M	>10	unknown	1b	170	4,928,786	1	2	CT

*IVDA, intravenous drug abuse.