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Analysis of the transcriptome and immune function of monocytes during IFNα-based therapy in chronic HCV revealed induction of TLR7 responsiveness

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ABSTRACT

Although *in vitro* studies have been performed to dissect the mechanism of action of IFNα, detailed *in vivo* studies on the long-term effects of IFNα on monocytes have not been performed. Here we examined peripheral blood from 14 chronic HCV patients at baseline and 12 weeks after start of IFNα-based therapy. Monocytes were phenotyped by flow-cytometry and their function evaluated upon TLR stimulation and assessed by multiplex cytokine assays.

During therapy of HCV patients, monocytes displayed a hyperactive state as evidenced by increased TLR-induced pro-inflammatory cytokine levels, as well as enhanced CD69 and CD83 mRNA and protein expression. Moreover, monocytes from 8 patients at baseline and 12 weeks after start of IFNα-based therapy were transcriptomically profiled by high throughput RNA-sequencing. Detailed RNA-seq analysis of monocytes showed significant ISG mRNA induction during therapy. Importantly, IFNα-based therapy activated TLR7 signaling pathways, as demonstrated by up-regulated expression of TLR7, MyD88, and IRF7 mRNA, whereas other TLR family members as well as CD1c, CLEC4C, and CLEC9A were not induced. The induction of TLR7 responsiveness of monocytes by IFNα *in vivo* in HCV patients is relevant for the development of TLR7 agonists that are currently under development as a promising immunotherapeutic compounds to treat chronic viral hepatitis.

1. Introduction

Hepatitis C virus (HCV) infection results in chronic infection in approximately 80% of exposed individuals. In these individuals, the functionality of both innate and adaptive immune responses are compromised, including the antiviral interferon (IFN) pathways as well as the activity of natural killer cells, monocytes, dendritic cells (DC) and T cells (Claassen et al., 2013; Liu et al., 2009; Rehermann, 2009). Since chronic HCV infections are a major cause of liver cirrhosis and hepatocellular carcinoma, approaches to eliminate the virus and to restore the impaired immune response to the virus receive a lot of interest.

One of the compounds that is pivotal in antiviral immunity and is the backbone for therapy of chronic HCV patients is IFNα. In its pegylated form it is used for over a decade in combination with ribavirin. Evidence is accumulating that ribavirin – at least in part – acts by potentiating the antiviral response initiated by IFNα via several mechanisms (Feld et al., 2007; Thomas et al., 2011). Type I IFN, which includes IFNα and IFNβ, is known to prime immune responses by modulating the function of various leukocyte populations. It has broad effects on the immune system, since the receptor is ubiquitously expressed. The heterodimeric receptor is composed of two chains, IFNAR1 and IFNAR2, and can bind all type I IFN. Upon binding of the receptor, IFNα initiates a cascade of signaling events, which activates the transcription of IFN-inducible

Abbreviations: IFN, interferon; pegIFNα, pegylated IFNα; ISG, interferon stimulated gene; TLR, Toll-like receptor; IL, interleukin; PBMC, peripheral blood mononuclear cells; FPKM, fragments per kilobase per million; BP, biological process; FDR, false discovery rate; lncRNA, long non-coding RNA.

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genes. A large number of these gene products have direct antiviral effects (2,5-OAS, MxA, etc.), but also other genes are induced or modulated following exposure of cells to IFNα (Theofilopoulos et al., 2005; Trinchieri, 2010).

Most information on the direct effect of IFNα on immune cells is obtained by short-term *in vitro* exposure to IFNα. These experiments demonstrated that type I IFN regulate cytokine production by DC, macrophages and monocytes, and thereby modulate the type of immune response that is required under specific conditions (Hermann et al., 1998; Theofilopoulos et al., 2005; Waddell et al., 2010). In this respect, we reported before that short-term *in vitro* exposure to type I IFN can inhibit Toll-like receptor (TLR)-induced interleukin-10 (IL-10), IL-12p40, IL-23 and MIP-1β production by human monocytes (Liu et al., 2012). Monocytes are abundantly present in blood and play important roles in inflammatory responses. Monocytes from healthy individuals express mRNA for TLR1, TLR2, TLR4, TLR5, TLR6, and TLR8, while no TLR3, TLR7 and TLR9 mRNA was detected (Kadowaki et al., 2001). Functionally, we recently confirmed this by showing relatively weak responses of monocytes to ligands for TLR2 and TLR5, and relatively strong responses to TLR 4 and TLR8 agonists. As expected on the basis of lack of TLR mRNA expression, monocytes were unresponsive to TLR3, TLR7 and TLR9 ligation (Liu et al., 2011). Importantly, we showed that TLR4 and TLR8 mRNA expression was comparable between patients and healthy individuals, and that monocytes from patients responded to the TLR7/8-ligand R848, but not to a TLR7 specific agonist (Boonstra et al., 2012). Besides evasion mechanisms by HCV to escape TLR-induced responses in the liver (Liu et al., 2009), modulation of monocyte activity by HCV core protein and serum endotoxin and IFNγ was reported (Dolganiuc et al., 2007). The modulation of the activity of monocytes by type I IFN, induced by viral infection or administered as a component of antiviral treatment, may not only directly affect the response to pathogens, but also the ability of monocytes to differentiate into macrophages or DC, thereby further modulating the immune response to pathogens (Dauer et al., 2003; Ito et al., 2001; Mohty et al., 2003).

Although our previous studies provide insight into how monocytes are affected by short-term *in vitro* exposure to type I IFN, limited information is available on the long-term *in vivo* effects. However, it is important to get a better understanding on these long-term effects since IFNα is administered to chronic HCV patients over a prolonged period of 24–48 weeks. Therefore, in the present study, we investigate in detail the long-term effects of IFNα-based therapy on monocytes isolated from chronic HCV patients using profiling by RNA-sequencing. Most microarray studies that have been performed to dissect the effects of IFNα-based therapy in chronic viral hepatitis analyzed peripheral blood mononuclear cells (PBMC) and liver, and have identified numerous genes that were up-regulated, among which IFN-stimulated genes (ISG) were abundant (Helbig et al., 2005; Ji et al., 2003; Lanford et al., 2006). However, since IFNα is highly pleiotropic, the use of unseparated PBMC and liver cells complicates the interpretation of the results. Our present study focused on isolated monocytes, and examined in detail the effect of IFNα-based therapy on *in vivo* gene expression and immune function of monocytes during the course of IFNα-based therapy.

2. Materials and methods

2.1. Patients and antiviral treatment

Fourteen chronic HCV infected patients were included (Table 1 for patient details). All patients were between 18 and 70 years of age, infected with HCV genotype 1, and treatment-naive. Patients were excluded in case of decompensated liver disease, HBV- or HIV-coinfection. Patients were treated at the Erasmus MC for 48 weeks with pegIFNα-2a (Pegasys, 180 μg once weekly, Roche) and ribavirin (Copegus, 1200–2400 mg daily, Roche) according to a study protocol and were seen at our outpatient clinic (EudraCT 2007-005344-25). Peripheral blood samples were collected at baseline and 12 weeks after the start of therapy. The institutional review board of the Erasmus MC approved the protocols, and informed consent was obtained from all individuals. All clinical parameters were determined by routine diagnostics of the Erasmus MC. In addition, the IL-28B SNP rs12979860 was determined for all patients using competitive allele-specific PCR (KASP; KBioscience, Hoddesdon, UK).

2.2. Flow cytometric analysis

PBMC were isolated from peripheral blood by gradient-density centrifugation using Ficoll–Paque. For determination of the frequency of monocyte subpopulations, whole blood was lysed using ammonium-chloride and stained with antibodies against CD14-PE-Cy7 (61E3, eBioscience) and CD16-PerCP-Cy5.5 (3G8, BD Biosciences). For additional phenotyping, cells were stained with antibodies against CD69-APC (L78, BD), and CD83-APC (HB15e, BD Pharmingen). All staining intensities were evaluated by flow cytometry (Canto-II, BD). The data was analyzed using BD FACS Diva software.

2.3. Monocyte purification and stimulation

Monocytes were purified from PBMC of 14 patients at 2 different timepoints (baseline and at week 12 during therapy) using magnetic CD14-microbeads following the manufacturer's instructions (Miltenyi Biotec; purity: 94–99%), and stimulated in X-VIVO15 medium (BioWhittaker) in 96-well plates (5×10^5 cells/ml, 200 μl/well) for 24 h with the TLR7/8 agonist R848 (1 μg/ml; Alexis). After 24 h, supernatant was collected and cytokine production was determined using Multiplex MAP human cytokine/chemokines magnetic beads (Merck Millipore, Billerica MA, USA). The concentrations of 24 distinct analytes were measured using the microsphere-based multiplex LUMINEX-100.

2.4. Intracellular cytokine staining upon TLR ligation

PBMC from 8 patients at 2 different time points (baseline and at week 12 during therapy) were stimulated with the TLR7 agonist CL264 (1 μg/ml; Invivogen) in serum-free X-VIVO15 medium for 2 h, with brefeldin-A (10 μg/ml; Sigma) present for another 21 h. Samples were then fixed, permeabilized and stained with antibodies against CD14-Pacific Blue (M5E2, BD Pharmingen) and TNF-PE-Cy7 (MAb11, eBioscience). Cytokine-producing monocytes were detected by flow cytometry (Canto-II, BD). In some experiments, cryopreserved PBMC from patients were used.

2.5. RNA-seq analysis of purified monocytes

Total RNA from 8 patients at 2 different time points (baseline and at week 12 during therapy) were isolated from purified monocytes and converted into a template molecules library according to the Smarter RNA sample preparation kit (Clontech). The cluster generation, hybridization to a flow cell, amplification, linearization, denaturation, and sequencing were performed at the Erasmus Center for Biomics, on an Illumina HiSeq 2000 platform (Illumina Inc., San Diego, CA). Four samples were combined into one lane and a 36-cycle single-read (SR) sequencing strategy was used according to User Guide (Illumina, TruSeq V3 protocol). Quality values in FASTQ files were generated with the current Illumina pipeline.

Table 1
Patient characteristics.

	Week 0 (n = 14)	Week 12 (n = 14)	Paired t-test (sig. 2-tailed)	Wilcoxon matched-pair signed-rank test (sig. 2-tailed)
Age, years	51.4 (2.0)			
Gender (M/F)	2/12			
HCV–RNA (IU/ml)	1.2×10^6 (2.4×10^5)	615 (0)	0.00026	0.000
ALT (U/L)	82.4 (11.5)	37.9 (6.6)	0.00002	0.001
Leukocytes (*$10E^9$/L)	5.97 (0.51)	2.71 (0.25)	0.00000	0.001
Neutrophils (*$10E^9$/L)	2.88 (0.36)	1.19 (0.20)	0.00001	0.001
Monocytes (%)	7.94 (0.60)	10.03 (1.07)	0.51	0.054
rs12979860 (IL-28B: CC/CT/TT)	1/7/6			

All values are mean (±SEM).

2.5.1. Alignment of short reads to genome

The quality check on sequencing data was performed using FASTQC. Each sample had over ~92% reads with a quality score over 36 (Phred score). The generated single-end reads were independently aligned to the human reference genome (assembly build UCSC hg19) in Bowtie with no mismatch allowed, and subsequently annotated with human genome track on hg19. In the following analyses, only reads mapped to the protein coding transcripts were used.

2.5.2. Estimation of the abundance of each gene/transcript

To determine the alterations of gene expression in monocytes during the course of antiviral therapy, the abundance of read counts for each annotated transcript was quantified by assessing the total number of reads for the entire transcript using the program Cufflinks and was then normalized per kilobase of genes/transcripts per million mapped reads (FPKM). The expression values at the gene level were determined by calculating the average of the abundances from all transcripts overlapping the same gene.

2.5.3. Testing differential expression of genes/transcripts

Differential expression analysis was performed using Significant Analysis of sequencing data for two classes of paired samples in R as described by Li and Tibshirani (2013). The two classes were defined as before treatment (week 0) and during treatment (week 12) with 8 paired samples within each class. The ratio of gene expression over 1.5-fold and q-values less than 0.01 indicated highly significant differences.

2.5.4. Pathway enrichment analysis

Functional pathways overrepresented by genes modulated by the IFNα-based therapy were identified using the previously described methodology (Hou et al., 2012). The functional annotation was retrieved from Gene Ontology in terms of biological process (BP) for identified differentially expressed genes. The enrichment of biological processes was statistically determined by comparing the co-occurrence of gene members associated with a certain BP from the differentially expressed genes to a reference background of the human genome. The ratio of two occurrences was used to generate an enrichment score for each BP, and subjected to Fisher's exact test to determine the probability of random assignment. Multiple test correction was controlled using false discovery rate (FDR) from the Benjamini–Hochberg method.

2.6. Statistical analysis

Continuous variables were represented as mean ± standard deviation, unless indicated otherwise. Paired-sample t-test and Wilcoxon signed-rank non-parametric test for matched samples were used to compare variables between two groups. In all analyses, a two-tailed p-value of less than 0.05 (confidence internal 95%) from non-parametric test was considered statistically significant. IBM SPSS Statistics (v21, Armonk, NY, USA) was used to perform all analyses.

3. Results

3.1. The effect of IFNα-based antiviral therapy on monocytes

Fourteen treatment-naïve patients chronically infected with HCV genotype 1 were treated with pegIFNα and ribavirin. At week 12 of antiviral therapy, all patients achieved an early viral response with undetectable HCV RNA in serum (<615 IU/ml; Table 1). Ten out of 14 patients showed normalization of ALT levels at week 12 (<40 U/l; Table 1). Chronic HCV patients presented a significant decrease of the absolute number of monocytes in blood on week 12 compared to baseline (Fig. 1A). Monocyte subpopulations in PBMC were identified on the basis of their FSC/SSC profile, and further characterized by flow-cytometry using antibodies specific for CD14 and CD16. Within the monocyte compartment, no significant differences were observed in the ratio of $CD14^+CD16^-$ monocytes, while the $CD16^+CD14^{+/-}$ subpopulation was significantly increased when comparing monocytes before and during the course of antiviral therapy (Fig. 1B).

3.2. Modulation of the function of monocytes from chronic HCV patients during IFNα-based therapy

To examine whether the function of monocytes was altered in chronic HCV patients undergoing IFNα-based therapy, purified monocytes at baseline and at week 12 were stimulated *in vitro* with the TLR7/8 agonist R848. As shown in Fig. 2, R848-induced monocytes produced the pro-inflammatory cytokines G-CSF, GM-CSF, IL-12p40, IL-1α, as well as the anti-inflammatory mediators IL-10 and IL-1R1 at relatively low levels, while IL-1β, IL-6 and TNF were detected at relatively high levels (>1 ng/ml). During the course of antiviral therapy, monocytes became more responsive to R848 as evidenced by secretion of significantly higher levels of most cytokines examined (Fig. 2A). In contrast to the higher cytokine levels, the R848-induced production of chemokines by purified monocytes from chronic HCV patients was not modulated as a consequence of IFNα-based therapy (Fig. 2B). Besides R848, purified monocytes were also stimulated with the TLR4 agonist LPS. Importantly, also upon ligation of TLR4 higher levels of cytokines and chemokines were detected at week 12 as compared to baseline (Supplementary Fig. 1). The augmented cytokine production of monocytes upon TLR4 ligation was comparable to stimulation with TLR7/8 agonists.

3.3. Gene expression profiling of monocytes from chronic HCV patients

To examine the effect of IFNα-based therapy on purified monocytes from chronic HCV patients in more detail, RNA-sequencing was performed to analyze changes in the transcriptomic profiles

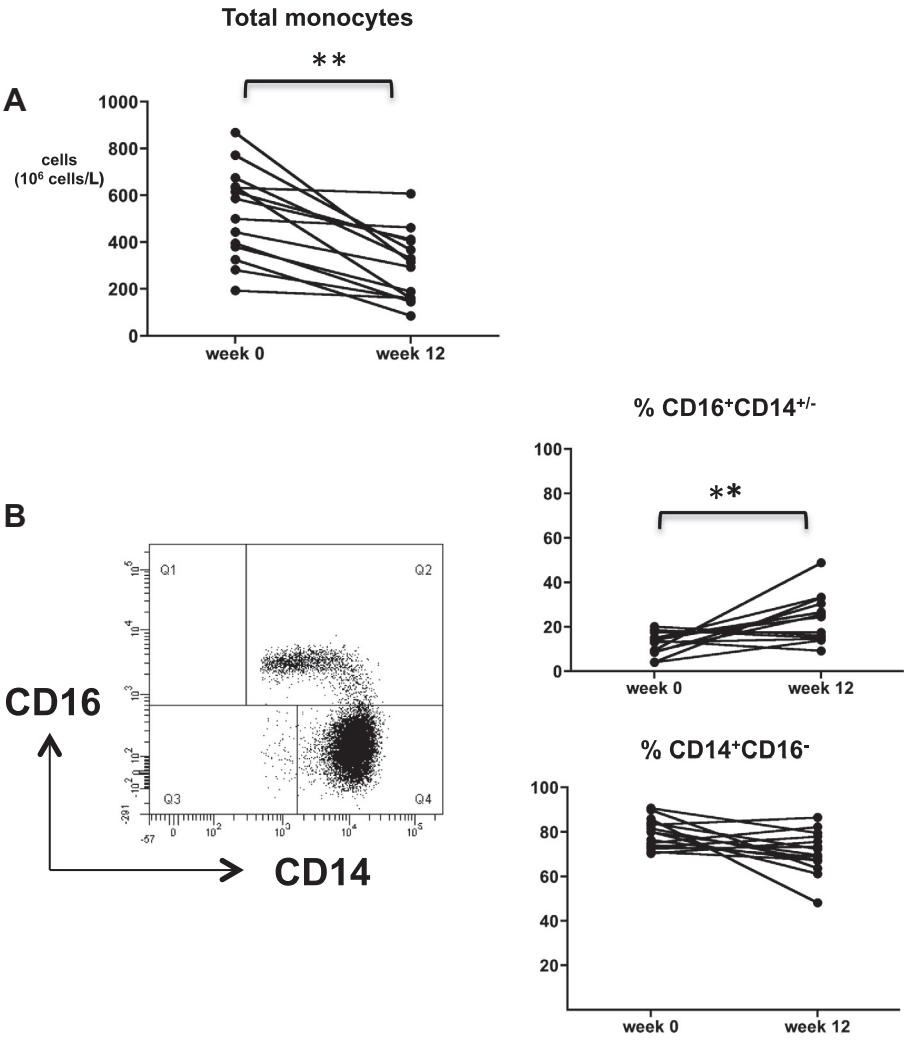

Fig. 1. The numbers and composition the monocyte compartment are affected by IFNα-based therapy in chronic HCV patients. (A) The absolute numbers of monocytes in peripheral blood of chronic HCV patients ($n = 14$) is shown at week 0 and at week 12 during IFNα-based therapy. **denotes $p < 0.05$ calculated from Wilcoxon signed-rank non-parametric test for paired samples. (B) Monocytes from chronic HCV patients ($n = 10$) were identified on the basis of their FSC/SSC profile, and further characterized by flow-cytometry using CD14 and CD16 specific antibodies. The contribution of the specific subpopulation within the total monocyte pool is shown. **denotes $p < 0.05$ calculated from Wilcoxon signed-rank non-parametric test for paired samples.

of monocytes at week 12 of therapy. Aligned reads were annotated with the UCSC genome track on hg19, which resulted in 24,045 annotated tracks. Of these tracks, 78.6% corresponded to protein-coding genes, while the others included long non-coding RNA (lncRNA), miRNA, and other non-protein-coding genes (Fig. 3A). Only annotated protein-coding genes ($n = 18,904$) were included in the subsequent analyses. We identified 928 genes presenting a significant response to IFNα-based therapy in monocytes. Of these, 418 were up-regulated and 510 were down-regulated (Fig. 3B and Table S1).

3.4. Global changes in mRNA expression to IFNα-based therapy

A large number of interferon stimulated genes (ISG) responded to IFNα-based therapy, with induced higher expression at week 12 (Fig. 3C). These ISG include DDX58, DDX60, EIF2AK2, IFI44(L), IFI6, ISG15, OAS, SP100, TRIM5 and TRIM22 (Table 2). In addition, genes encoding cytokines or other molecules known to be produced by monocytes were found up-regulated by the IFNα-based regimen. For instance, genes encoding IL-10 and TNF showed augmented mRNA expression at week 12 of 8.47-fold and 1.16-fold, respectively (q-value < 0.01). The cytokine protein levels presented in Fig. 2 as well as the gene expression profiles indicated that as a consequence of antiviral therapy, monocytes from chronic HCV patients on therapy became more activated as compared to monocytes collected at the start of therapy. Indeed, validation of the activation state of monocytes by flow-cytometry demonstrated that these cells displayed higher expression of the activation marker CD69 and the maturation marker CD83 on the surface of monocytes during therapy (Fig. 3D), which was confirmed by the RNAseq data (Table S1).

Next, to get a global insight into the modulation of the function of monocytes from chronic HCV patients by IFNα-based treatment, we performed gene set enrichment analysis. Specific classes of signaling pathways were significantly activated in monocytes by antiviral therapy, such as the IL-10-, TREM1-, and TLR-signaling pathways (Table S2). Other functional pathways affected in monocytes by the IFNα-based treatment included "pattern recognition receptors", "communication between innate and adaptive immune cells", and "dendritic cell maturation".

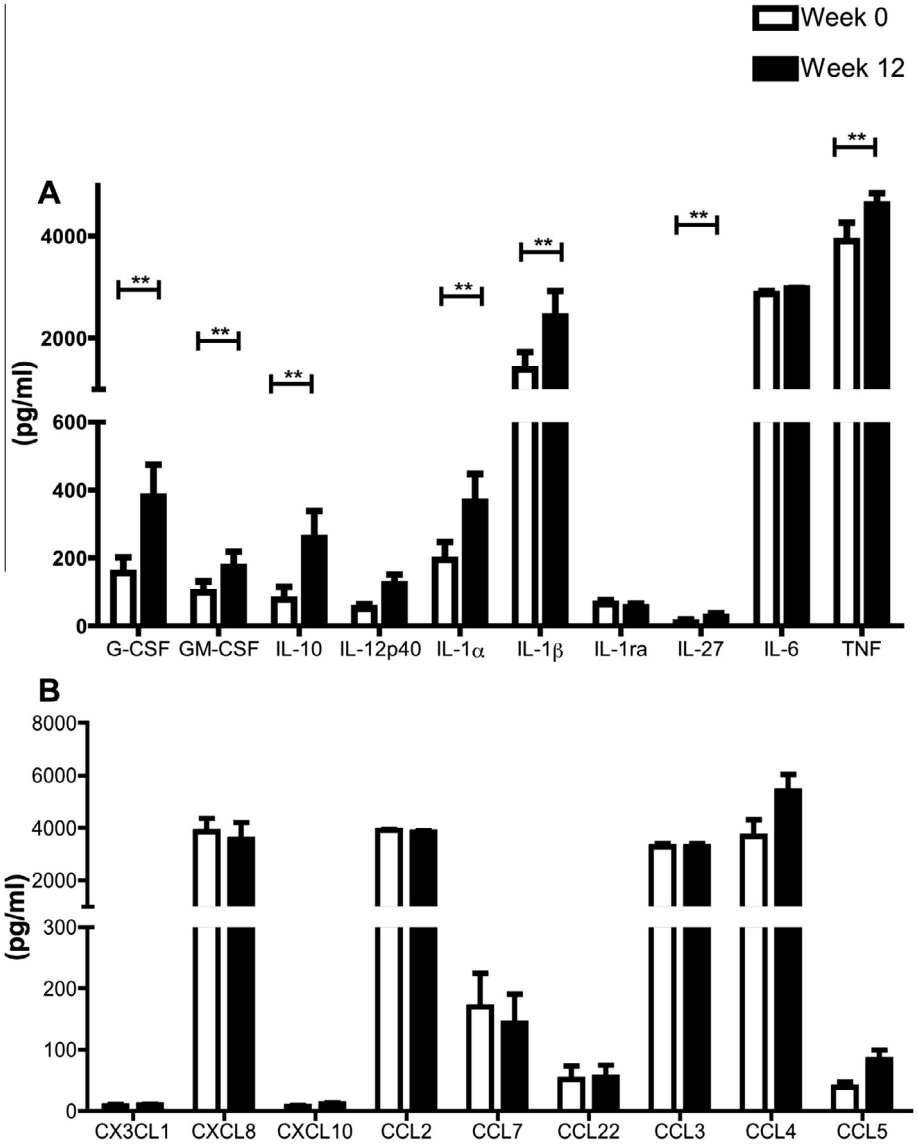

Fig. 2. R848-induced cytokine production by monocytes from IFNα-treated chronic HCV patients is enhanced during therapy. Monocytes purified from peripheral blood of 14 chronic HCV patients at baseline and at week 12 during IFNα-based therapy were stimulated with R848 for 24 h. The levels of cytokines (A) and chemokines (B) were determined in supernatant. The background values (i.e. cells cultured in medium) were subtracted. The levels of eotaxin and IL-23 were below the detection limit of the assay in cultures obtained from both time-points. ** denotes $p < 0.05$ calculated from paired t-test. Wilcoxon signed-rank non-parametric test for paired samples was performed in addition to compare the median difference of molecule production by monocytes during IFNα-based therapy (at baseline vs at week 12): the levels of G-CSF, GM-CSF, IL-10, IL-12p40, IL-1α, and CCL2, CCL4, CCL5 were significantly increased during therapy (at significance level 0.05).

3.5. IFNα-based therapy modulated mRNA expression of TLR7 signaling molecules, but not other TLR signaling

The expression of diverse TLR on monocytes enables them to respond to a broad range of bacterial and viral pathogens. We demonstrated that at baseline purified monocytes from patients expressed relatively high mRNA levels of TLR1, TLR2 and TLR4, and relatively low levels of TLR5, TLR6, TLR7 and TLR8 (Fig. 4A). As a consequence of IFNα-based therapy, an increase of TLR2 mRNA was observed, albeit not significant, and interestingly, TLR7 mRNA expression was significantly augmented at week 12 during antiviral therapy as compared to baseline expression. The expression levels of all other TLR were not affected. Paired evaluation at week 0 and week 12 showed that TLR7 mRNA levels from purified monocytes were enhanced in 6 out of 8 patients (Fig. 4B). Also, MyD88 and IRF7 mRNA were sensitive to IFNα-based treatment, showing a 1.4-fold and 3.0-fold increase of FPKM, respectively, as compared to expression at baseline (q-value < 0.01) (Table S1).

Interestingly, since monocytes from healthy individuals and chronic HCV patients are responsive to the TLR7/8 agonist R848, but unresponsive to specific TLR7 agonists (Boonstra et al., 2012), we examined whether monocytes from patients undergoing therapy responded to these TLR agonists by conducting an intracellular cytokine staining for TNF upon stimulation with a highly specific TLR7 agonist, the adenine analog CL264. As shown in Fig. 4C, an increase of the frequency of TNF-producing monocytes was observed upon stimulation with the TLR7 agonist as a consequence of IFNα-based therapy. Importantly, TLR7-induced TNF was only observed at week 12 during the course of antiviral therapy, but not at week 0, prior to antiviral therapy of chronic HCV patients.

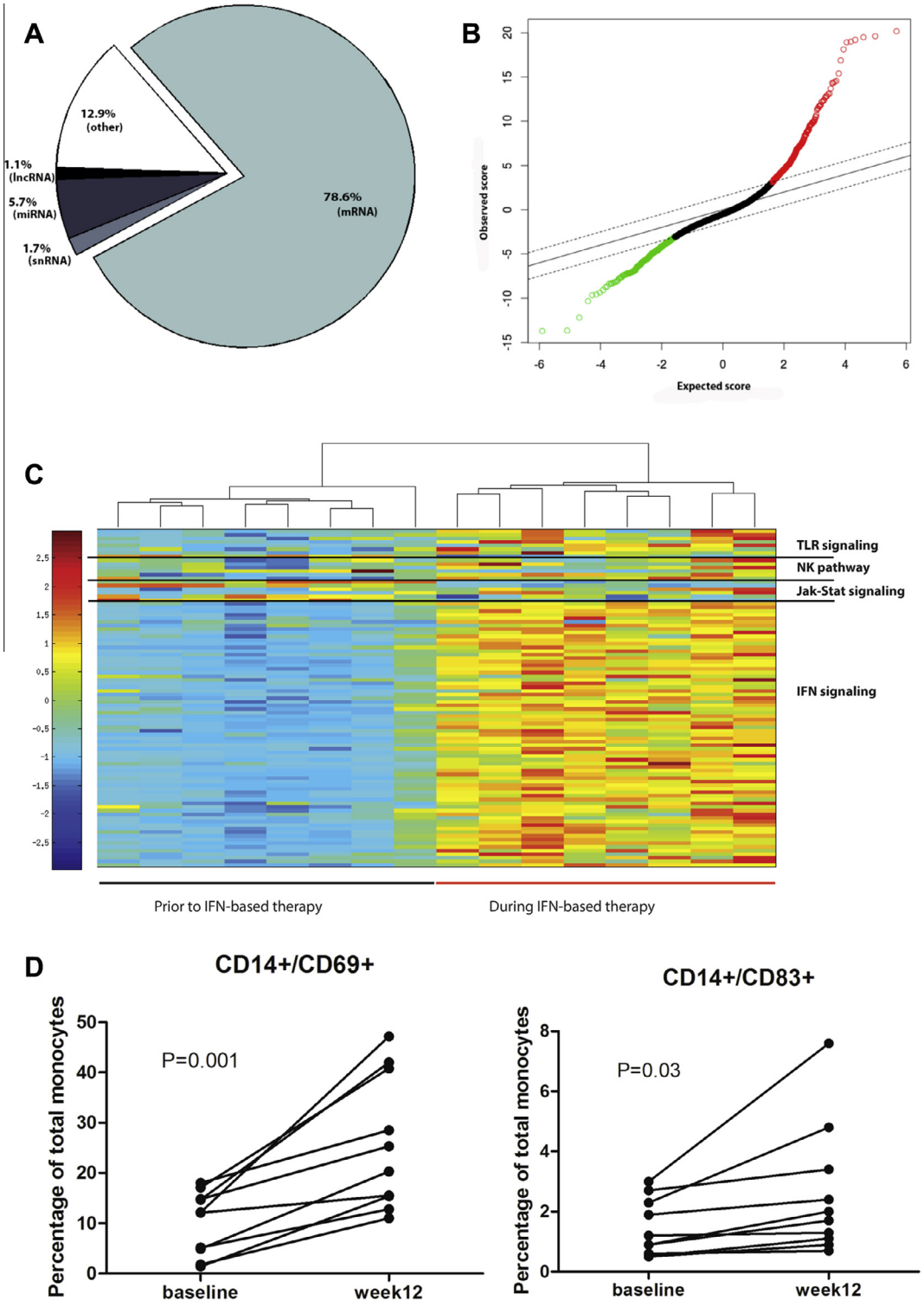

Fig. 3. Differentially expressed genes modulated by IFNα-based therapy in monocytes identified by RNA-seq analysis. (A) Pie chart demonstrating the proportion of RNA-seq reads assigned to human reference genomic features (GRCh37.p11, Ensembl). Of 24,045 reads, 78.6% was assigned to protein-coding genes and these were used for the following analyses, 1.1% to genes encoding long non-coding RNA (lncRNA); 1.7% to genes encoding small nucleolar RNA (snRNA); 5.7% to genes encoding microRNA (miRNA); and 12.9% to other non-protein coding genes. (B) Significant analysis of sequencing data (SAMseq) plot of differentially expressed genes in paired monocytes from 8 chronic HCV patients at week 12 of therapy compared with baseline controls. The central solid black line indicates equal expression between baseline and week 12 (Expected score = Observed score). 928 Probe sets were called significant with a False Discovery Rate (FDR) of 20%. Genes with expression levels that are significantly modulated are plotted either above (up-regulated, red) or below (down-regulated, green) the dashed lines. (C) The heat map shows the relative expression levels of 94 genes (row) from the selected pathways in paired samples (column) from 8 patients (baseline and week 12). Expression values (FPKM) were log2-transformed and subsequently median-centered by gene. Rows were hierarchically clustered based on complete linkage using Spearman correlation coefficients as the distance measure. The color scale indicates the degree of relative expression of immune genes (deep red, high expression; deep blue, low expression). (D) Paired samples from chronic HCV patients ($n = 10$) were evaluated by flow-cytometry for surface expression of CD69 and CD83 on monocytes at baseline and at week 12 during antiviral therapy. *P*-values in the figure were calculated from paired *t*-tests. In addition, Wilcoxon signed-rank non-parametric test for paired samples was performed to compare expression of surface markers on monocytes during IFNα-based therapy: CD69 ($p = 0.005$) and CD83 ($p = 0.005$) were significantly higher expressed at week 12 compared to baseline.

Table 2
Genes identified by RNAseq of monocytes that were modulated by IFNα-based therapy.

Gene name	Week12-to-baseline ratio	
A. Top differentially expressed ISG		
RSAD2	3.710	
IFI44L	3.285	
IFIT3	3.090	
ISG15	2.905	
MX1	2.690	
IFI6	2.742	
OASL	2.519	
IFIT2	2.475	
IFI44	2.240	
OAS3	2.261	
OAS2	2.153	
EIF2AK2	1.938	
MX2	1.964	
DDX60	1.789	
DDX58	1.853	
OAS1	1.871	
XAF1	1.787	
TRIM22	1.710	
IRF7	1.547	
PLSCR1	1.531	
SP100	0.719	
		Pathway involved
B. Differentially expressed genes from other pathways		
IL10	2.140	Jak Stat signaling
IL2RG	0.686	Jak Stat signaling
CD244	−0.605	NK pathway
CD48	0.352	NK pathway
FAS	0.912	NK pathway
ICAM2	0.622	NK pathway
IFNGR1	0.527	NK pathway
CD80	1.843	TLR signaling
CD86	0.418	TLR signaling
IRF7	1.547	TLR signaling
MAP2K6	1.354	TLR signaling
MYD88	0.473	TLR signaling
STAT1	1.004	TLR signaling
TLR7	0.987	TLR signaling
CD69	2.008	T cell and NK cell signaling
CD83	0.737	TREM1 signaling & DC maturation
CD163	0.535	Acute-phase signaling

4. Discussion

In this study we showed that global gene expression of monocytes from chronic HCV patients was greatly affected during the course of by 12 weeks of IFNα-based antiviral therapy. Specifically, 928 genes were significantly modulated as a consequence of therapy. One of the most striking observations in our study was the induction of TLR7 mRNA in monocytes during the course of therapy. Importantly, the enhanced TLR7 mRNA expression also had functional consequences since we showed that monocytes from chronic HCV patients during IFNα-based therapy were responsive to specific TLR7 ligands, which was not observed prior to therapy. Furthermore, we observed up-regulation of mRNA expression of downstream molecules participating in the TLR7 signaling pathways, including MyD88, IRF7 and STAT1, as well as some ISG, including ISG15 and IFIs. This demonstrates that during IFNα-based antiviral treatment monocytes of chronic HCV patients acquired an active transcription of genes involved in TLR7 signaling and its downstream pathways (Liu et al., 2012). The appearance of TLR7-expressing monocytes was unexpected since it has been reported by numerous groups that in blood only plasmacytoid DC, B cells, and granulocytes express TLR7 mRNA, but not monocytes (Shortman and Liu, 2002). Previously, we reported that upon 5 h-exposure of monocytes to IFNα *in vitro*, the TLR7 mRNA levels remained unaffected (Liu et al., 2012), whereas in the present study, we observed that the TLR7 mRNA expression levels of purified monocytes obtained from patients was significantly increased at week 12 of IFNα-based therapy. Although this is not in line with our earlier *in vitro* observations, the present findings are reminiscent of studies in which monocyte-derived DC generated in the presence of GM-CSF and IFNα during a 7 days culture period, were found to express TLR7 mRNA (Mohty et al., 2003).

As a consequence of IFNα-based antiviral therapy, we also observed that monocytes were more activated as reflected by higher expression of CD69 and CD83, and higher cytokine production upon *in vitro* TLR stimulation. These findings are in line with a study by Cheng et al. who also showed higher expression of HLA–ABC and CD86 on the surface of monocytes during IFNα-based therapy (Cheng et al., 2008). However, it is important to mention that despite their augmented functionality, the absolute numbers of circulating monocytes were lower during therapy as compared to baseline levels, which is a well-known adverse effect of IFNα administration. The status of the monocyte compartment (i.e. less monocytes, yet more active) may be important for the ability of the host to respond to anti-microbial challenges. However, also other responses may be modulated since differentiation of monocytes into macrophages or DC is a crucial step that determines the efficacy of the immune response to pathogens or other immune triggers. All patients in our study responded to the antiviral therapy, and were negative for HCV–RNA at week 12 (<615 IU/ml), and over 70% of patients restored ALT levels to normal. This made that we were unable to correlate modulation of gene expression in monocytes to treatment outcome.

Although the biological effects of IFNα have been studied for many years, our understanding of the effects of IFNα in humans is still incomplete since most studies were conducted *in vitro* or using mouse models. In *in vitro* experiments, we previously demonstrated that one of the consequences of priming monocytes for 5 h with IFNα is to promote the cells' sensitivity to IL-10 by up-regulating the IL-10R1 expression, leading to the inhibition of TLR-induced IL-12p70 production (Liu et al., 2012). In the present study, we show at the mRNA level an opposite effect of IFNα-based therapy on the IL-10R1-encoding gene: at week 12 of therapy, the IL-10RA gene expression of monocytes was 1.4-~2.0-fold lower than the expression level observed prior to therapy in 4 out of 8 patients. In the remaining patients no significant changes of IL-10RA gene expression were observed during 12 weeks of IFNα-based therapy. The discrepancy between our *in vitro* findings on monocytes (Liu et al., 2012) and our *in vivo* findings in the current study shows that it is important to put more emphasis on human studies. Our findings were obtained during treatment in chronic HCV patients. However, it is important to examine whether the same phenomena are also observed during IFNα-based treatment of HCV patients infected with other HCV genotypes, patients with chronic HBV, as well as non-viral autoimmune diseases, such as multiple sclerosis. Moreover, future clinical studies using IFN-free regimens with direct acting antivirals to treat chronic HCV patients are needed to formally determine whether the enhanced TLR7 responses and the described modulation of other genes during IFN-based therapy are due to the activity of IFN or to the decrease in viremia.

Our findings on monocytes that TLR7 expression and responsiveness can be induced during the course of IFNα-based therapy, and that monocytes become more activated is important for the assessment of adverse effects observed during current pre-clinical and clinical testing of various TLR compounds (Bergmann et al., 2011; Boonstra et al., 2012; Lopatin et al., 2013). Alternatively, it is conceivable that beneficial effects of the induction of TLR7 may occur, which may be used in approaches in which a lead-in phase with pegylated IFNα may sensitize monocytes, and maybe other immune cells, to become more responsive to subsequent treatment with TLR7 agonists.

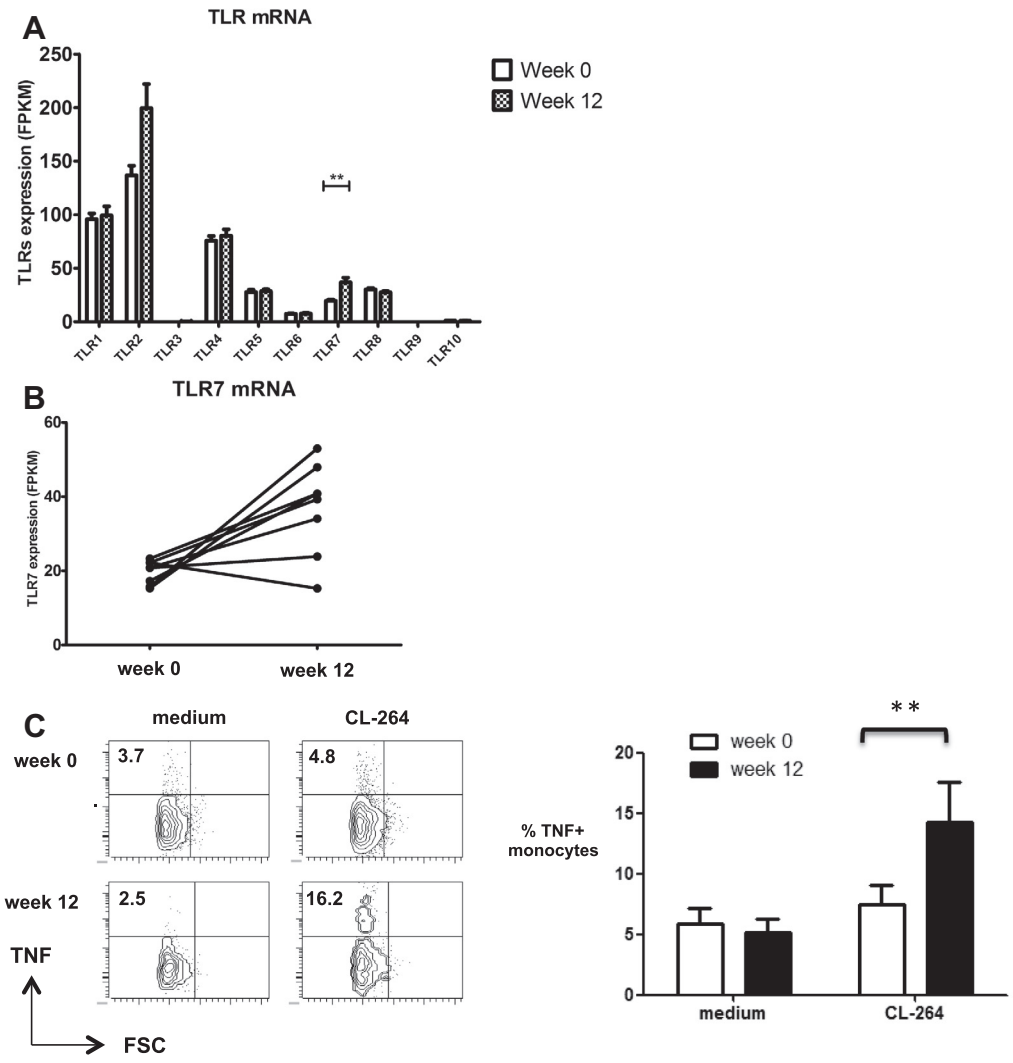

Fig. 4. As a consequence of IFNα-based therapy, monocytes express TLR7 mRNA and become responsive to TLR7 ligation in chronic HCV patients. (A) TLR1–10 mRNA expression in purified monocytes of chronic HCV patients ($n = 8$). The expression values (FPKM) of TLR mRNAs were calculated from the abundance of RNA-seq reads mapped to the corresponding genes, then normalized by kilobase of transcript per million mapped reads. (B) TLR7 mRNA expression determined from RNA-seq data of individual patients. Paired data is presented of TLR7 mRNA expression by purified monocytes obtained at baseline and at week 12 during therapy. (C) The intracellular cytokine profiles of PBMC prior to and during IFNα-based therapy. PBMC from chronic HCV patients were stimulated *in vitro* with medium or the TLR7-specific agonist CL264, and intracellular TNF was detected in monocytes by flow-cytometry. Left panel: representative dot plots showing the monocytes producing TNF. Right panel: The frequencies of monocytes producing TNF are presented upon stimulation with medium or CL264. As positive controls, monocytes were stimulated with the TLR7/8 agonist R848, resulting in a frequencies of monocytes producing TNF of 28.3 and 31.8% at week 0 and week 12, respectively. ** denotes $p < 0.05$.

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Conflict of interest

H.L.A.J., R.J.D.K. and A.B. received grants from Bristol Myers Squibb, Gilead Sciences, Novartis, Roche, Janssen, and Merck.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.06.020.

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