Function of monocytes in chronic HCV infection: Role for IL-10 and interferon

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The studies presented in this dissertation were performed in the Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, The Netherlands.

Financial support for this publication was kindly provided by:

ISBN: 978-90-8570-850-6

Cover design, lay-out and illustrations: Bisheng Liu
Print: Wöhrmann Print Service, Zutphen

Function of monocytes in chronic HCV infection: Role for IL-10 and interferon

Functie van monocyten in chronische HCV infectie:

Rol voor IL-10 en interferon

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. H.G. Schmidt

and in accordance with the decision of the Doctorate Board
The public defence shall be held on

16th November 2011 at 9:30

by

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Born in Binhai, Jiangsu, China

ERASMUS UNIVERSITEIT ROTTERDAM

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Among any three people, I will find something to learn. Their good qualities are to be followed, and their shortcomings are to be avoided.

- Confucius Lunyun·Shu'er

Abbreviations

HCV	Hepatitis C virus
HBV	Hepatitis B virus
LCMV	Lymphocytic Choriomeningitis Virus
HIV	Human immunodeficiency virus
PBMC	Peripheral blood mononuclear cell
DC	Dendritic cell
pDC	Plasmacytoid dendritic cell
NK	Natural killer cell
Mφ	macrophages
KC	Kupffer cells
TLR	Toll-like receptor
R848	Resiquimod
LPS	Lipopolysaccharide
RIG-I	retinoic acid inducible gene I
IFN	Interferon
IL-29	Interleukin-29
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-28A	Interleukin-28A
IL-28B	Interleukin-28B
IL-4	Interleukin-4
TNF	Tumor necrosis factors
MCP-1	Monocyte chemotactic protein-1
MIP-1β	Macrophage inflammatory protein-1β
JAK	Janus kinase
STAT	Signal Transduction And transcription
PD-L1	Programmed cell death 1 ligand 1
IL-10R	Interleukin-10 receptor
IFNγR	Interferon-γ receptor
HLA-ABC	Human leukocyte antigen-ABC
HLA-DR	Human leukocyte antigen-DR
αIL-10R	Anti-interleukin-10 receptor
IL-28RA	Interleukin-28 receptor alpha chain
M-CSF	Macrophage colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
RT-PCR	Real-time polymerase chain reaction
mRNA	Messenger RNA
cDNA	Complementary DNA
ICS	Intracellular cytokine staining
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting

CONTENTS

Chapter 1

General Introduction and Outline of the Thesis

Hepatitis C virus and chronic infection

Hepatitis C virus (HCV) establishes persistent infection in about 80% of the infected individuals [1-2]. The symptoms are initially mild in those persistently infected patients, and it may take decades before the serious consequences of chronic HCV infection become apparent. Up to 20% of infected individuals may develop complications, including cirrhosis, liver failure, or hepatocellular carcinoma [3]. HCV infection is now the leading indication for liver transplantation in the United States and Europe [4-5]

HCV is an enveloped positive-stranded RNA virus in the family of *Flavivirida* [6-7]. The genome of HCV has a large-opening reading frame, which encodes a polyprotein precursor of approximately 3000 amino acids. The coding region is flanked by 5' and 3' noncoding regions, which are important for the regulation of genomic duplication as well as initiation of translation [8-9]. The single polyprotein is cleaved by host and viral proteases into individual structural and nonstructural (NS) proteins [8-13] **(Figure 1)**.

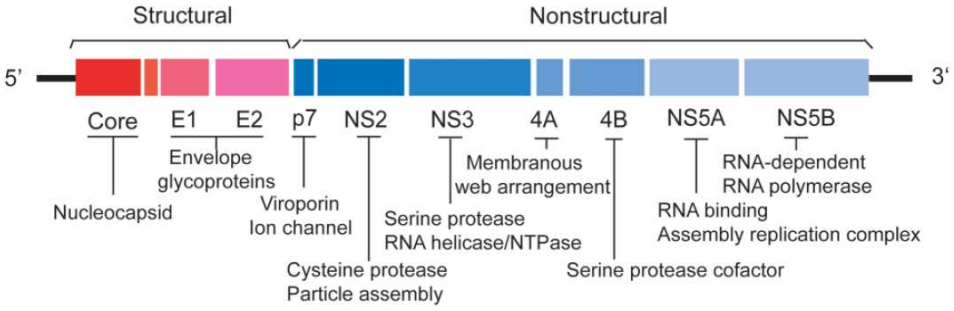

Figure 1. Genomic organization of wild-type HCV. The HCV RNA genome consists of a major open-reading frame, encoding a single polyprotein, and an alternative reading frame encoding F-proteins with unknown function. The cleavage of the polyprotein by viral and host cell proteases gives rise to the mature structural (core, envelope proteins E1 and E2, and p7) and nonstructural (NS) viral proteins (NS2 through NS5B). The putative activities and function of viral proteins are indicated. The internal ribosomal entry site (IRES) located in the 5' noncoding region initiates ribosome binding and translation. Both the 5' and 3' noncoding region are essential for viral RNA replication involving the RNA-dependent RNA polymerase NS5B. NTPase, nucleotide triphosphatase.

The major site of HCV replication is human liver. Remarkably, although HCV has been extensively studied at the molecular level, it is still unclear how many cells in the liver are infected or how much viral RNA and protein is associated with infected cells [14]. On the basis of measurement of viral RNA during acute infection, one study estimates that up to 10% of hepatocytes may support HCV replication [15].

After entry of HCV into the cells via several membrane receptors [16-17], HCV nucleocapsids are delivered to the cytoplasm, where the viral RNA functions directly as an mRNA for translation of a long polyprotein. Following synthesis and maturation, non-structural proteins and viral RNA form the membrane-associated replication complexes, which appear as a perinuclear membranous web [18]. These replication complexes then catalyse the transcription of negative-strand RNA intermediates from which, in turn, progeny positive-stranded RNA molecules are generated. Capsid proteins and genomic RNA assemble into a nucleocapsid and bud through intracellular membranes into cytoplasmic vesicles **(Figure 2)**. With the recent development of an *in vitro* model of HCV virion

production and release [10, 19], the analysis of this final part of the viral life cycle is an exciting area for future research.

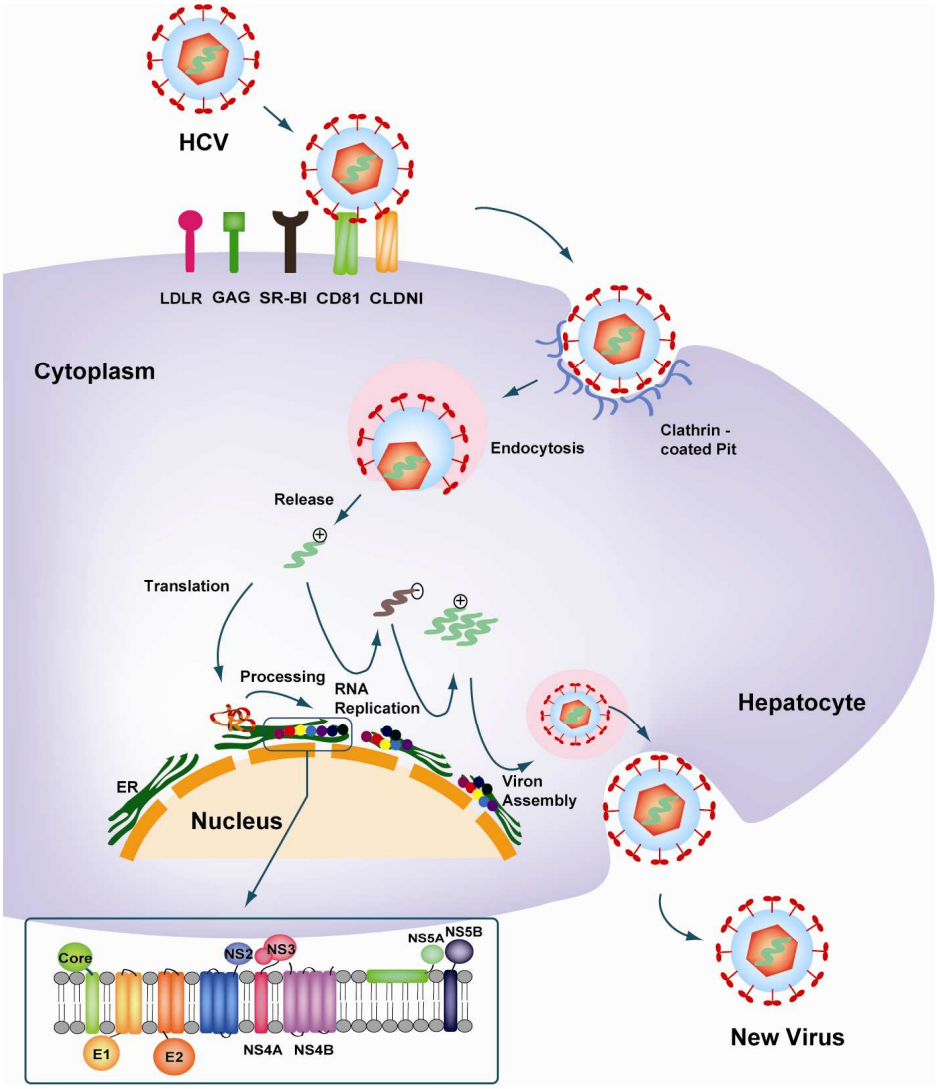

Figure 2. The life cycle of HCV infection. After entry to the cell, HCV nucleocapsids are delivered to the cytoplasm, where the viral RNA functions directly as an mRNA for translation of a long polyprotein. Replication occurs within cytoplasmic, membrane-associated replication complexes in a perinuclear membranous web. Genomic RNA containing plasmids bud through intracellular membranes into cytoplasmic vesicles, which fuse with the plasma membrane.

Viral production in the infected individuals is estimated at 10^{12} particles per day, and the half-life of HCV particles in serum is believed to be 3h [20-21]. There are six major HCV genotypes and HCV exists as a quasispecies, or swarm of several sequences, within the infected host [9]. HCV reaches high serum titres within 1 week of infection [22-23]. Both the adaptive cellular immune responses and the humoral immune responses are delayed by at least 1 month in both humans and chimpanzees, showing that virus replication 'outpaces' the adaptive immune response [12, 22, 24]. After the first week of infection, the increase in HCV viral titre slows down [23] **(Figure 3)**. Serum ALT levels peak, whereas HCV RNA titres decline after 2-3 months of infection **(Figure 3)**. Most patients develop chronic hepatitis with

relatively stable viral titres. Only a small proportion of patients recover and test negative for HCV RNA.

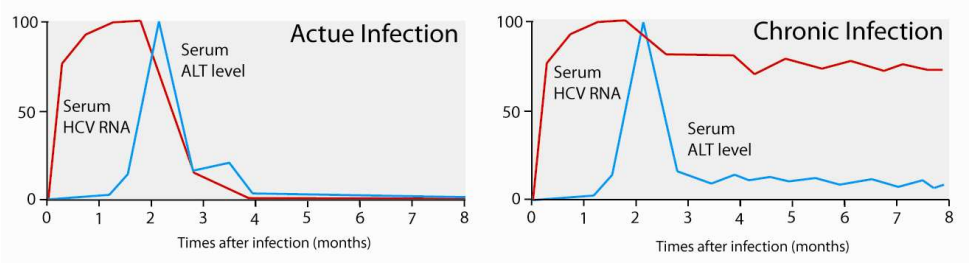

Figure 3. Clinical and virological courses of acute infection with HCV. A schematic presentation of serum HCV RNA and ALT (alanine transaminase), which is released by injured or dead hepatocytes in acute and chronic HCV infection.

Immunology of HCV infection

A characteristic feature of the immune status in chronically infected patients is a weak HCV-specific T cell response, which is short-lived, and targeted to a narrow range of epitopes [9, 12, 25]. There is a trend toward a decline in the number of epitopes recognized and in the number and function of responding T cells in peripheral blood of chronic HCV patients [26]. In infections that progress to chronicity, T cell responses seen in the acute stage seem to be lost [27], and specific loss of CD4+ T cell responses predicts recurrence of viremia and established of chronic infection [11, 28]. Also, the function of HCV-specific CD8+ T cells is affected in that they have impaired ability to produce IFNγ, to proliferate, or to kill cells presenting HCV antigen [29-31]. To explain these observations, many mechanisms have been proposed on the basis of *in vitro* studies, including viral mutation escape [32-36], functional impairment of DC [37-40] and macrophages [41-42], increase of peripheral CD4+CD25+ regulatory T cells [43-44] and intrahepatic IL-10-producing CD8+ T cells [45]. However, clinically, there is no global dysfunction of the immune system in patients chronically infected with HCV. The research on finding the mechanisms for chronic HCV infection is currently very active.

Monocytes and persistent viral infections

To explain the insufficient T cell responses, numerical and functional impairment of DC, natural killer (NK) cells and regulatory T cells have been reported in patients with chronic HCV [9, 37, 46]. However, monocytes have received relatively little attention in studies on the immune status of chronic HCV patients, despite the fact that monocytes comprise approximately 10% of circulating leukocytes, and play important roles in inflammatory responses. Furthermore, human monocytes are able to develop into DC or macrophages in the presence of certain cytokines **(Figure 4)**.

Figure 4. Human monocytes can develop into DC or macrophages. In the presence of certain cytokines, human monocytes can be derived into DC (GM-CSF plus IL-4) or macrophages (M-CSF).

Monocytes are important players in the first-line of defense against numerous pathogens, as well as in initiating and controlling adaptive immunity [47]. Human blood contains two distinct subpopulations of circulating monocytes, which can be distinguished on the basis of membrane expression of CD14 and CD16: $CD14^+CD16^-$ and $CD16^+CD14^-$ monocytes [47-50]. The majority of monocytes are the classical $CD14^+CD16^-$ monocytes. Compared to $CD14^+CD16^-$ monocytes, $CD16^+CD14^-$ monocytes are less frequent and comprise about 5~15% of the total monocytes. Functionally, both $CD16^+CD14^-$ and $CD14^+CD16^-$ monocytes express pathogen-recognition receptors, such as Toll-like receptors (TLR), which enable monocytes to respond to a broad range of bacterial and viral pathogens [51-52], resulting in the production of cytokines and chemokines [47, 53-55]. The responsiveness of both $CD16^+CD14^-$ and $CD14^+CD16^-$ monocytes to TLR4 agonists is well studied. Upon LPS stimulation, $CD14^+CD16^-$ monocytes produce relatively high levels of pro-inflammatory cytokines, TNF, IL-6 and IL-1β as well as the anti-inflammatory cytokine IL-10. Compared to $CD14^+CD16^-$ monocytes, $CD16^+CD14^-$ monocytes are thought to produce higher levels of pro-inflammatory cytokines, such as TNF and IL-1β in response to TLR stimulation [56], and lower levels of anti-inflammatory cytokines, such as IL-10 [56-57]. The differences of the two populations are further supported by a recent study, showing that the $CD14^+CD16^-$ and $CD16^+CD14^-$ monocytes have distinct gene expression profiles [58]. Interestingly, it was demonstrated that $CD16^+CD14^-$ monocytes are able to sense viruses via TLR7 or TLR8, resulting in the initiation of a pro-inflammatory response [59]. Also, we have shown that both $CD14^+CD16^-$ and $CD16^+CD14^-$ monocytes strongly respond to TLR8 agonists (Chapter 3 and Chapter 4). This is important, since HCV is a single-stranded RNA virus, which is a putative agonist for TLR8. The responsiveness of monocytes to the TLR4 agonist LPS has been extensively documented, however, how monocytes respond to TLR8 agonists is still much less known. Different from the TLR4 receptor, which is expressed on the membrane of monocytes, TLR8 is an intracellular receptor **(Figure 5)**. While both MyD88-dependent and TRIF-dependent signaling pathways are activated via triggering TLR4, TLR8 agonist only induces MyD88-dependent signaling pathway [60]. Currently, it is not known whether there is a different response of the two monocyte subpopulation to the TLR8 agonist.

The consequences of persistent infections on the functionality of monocytes have not been studied in detail. For HIV infections, it was shown that HIV-1 impairs innate immunity to bacteria by affecting the function of mononuclear phagocytic cells, and that HIV-infected individuals display an increased risk of bacterial infections [61]. For chronic HCV infections,

this is less clear, and conflicting data has been reported. It is generally accepted that $CD14^+CD16^-$ monocytes from HCV patients are more activated than their counterparts in healthy individuals, as shown by higher production of TNF, IL-12p40 and IL-10 in the absence of activating stimuli [62-68]. At present it is not entirely clear how monocytes respond to pathogen-derived products in chronic HCV patients as compared to healthy individuals. Stimulation of purified $CD14^+CD16^-$ monocytes from chronic HCV patients with the TLR4 ligand LPS resulted in higher TNF production as compared to healthy individuals by some groups [66-67], whereas others showed no difference [65]. Moreover, $CD14^+CD16^-$ monocyte-derived cytokine production induced by LPS was found to be reduced when assessing PBMC from HCV patients as compared to PBMC from healthy controls [69], but was enhanced when assessing the intracellular cytokine expression [62-63]. Furthermore, how $CD14^+CD16^-$ monocytes from chronic HCV patients respond to TLR8 agonist is currently not reported, despite the fact that HCV is a RNA virus. Also, the function of $CD16^+CD14^-$ monocytes in chronic HCV patients is still not clear, although it was demonstrated that $CD16^+CD14^-$ monocytes are able to sense viruses via TLR7 or TLR8, resulting in the initiation of a pro-inflammatory response [59].

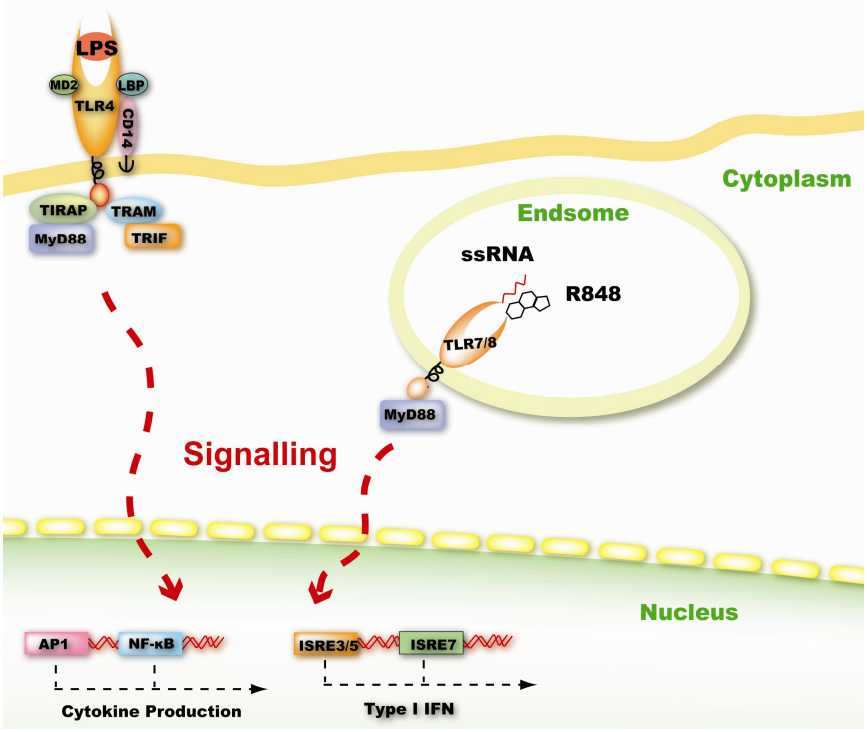

Figure 5. TLR4 and TLR8 signaling pathways. TLR4 is expressed on the cell membrane, whereas TLR8 is an intracellular receptor. Both MyD88-dependent and MyD88-independent pathways are induced by TLR4 triggering, whereas only the MyD88-dependent is induced by TLR8 agonists.

IL-10 as an anti-inflammatory cytokine

IL-10 is a well-studied immunosuppressive cytokine, which belongs to the IL-10 family of cytokines, including also IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B and IL-29 [70-71]. It inhibits pro-

inflammatory responses of both the innate and adaptive immunity, which prevents tissue lesions induced by otherwise over-reactivity of the host immune response, but also creates favourable conditions for the persistence of microbes and chronic infectious diseases. IL-10 is produced by numbrous cell types, including macrophages, monocytes, DC, B cells and CD4+ T cells [71-75]. Upon the recognition of pathogens, antigen-presenting cells (APC) are activated and cytokines, such as IL-12p70, IL-10, etc, are secreted. While IL-12p70 is important for the Th1 cells, IL-10 suppresses the activation of APC and thus represses or quenches Th1 responses (**Figure 6**).

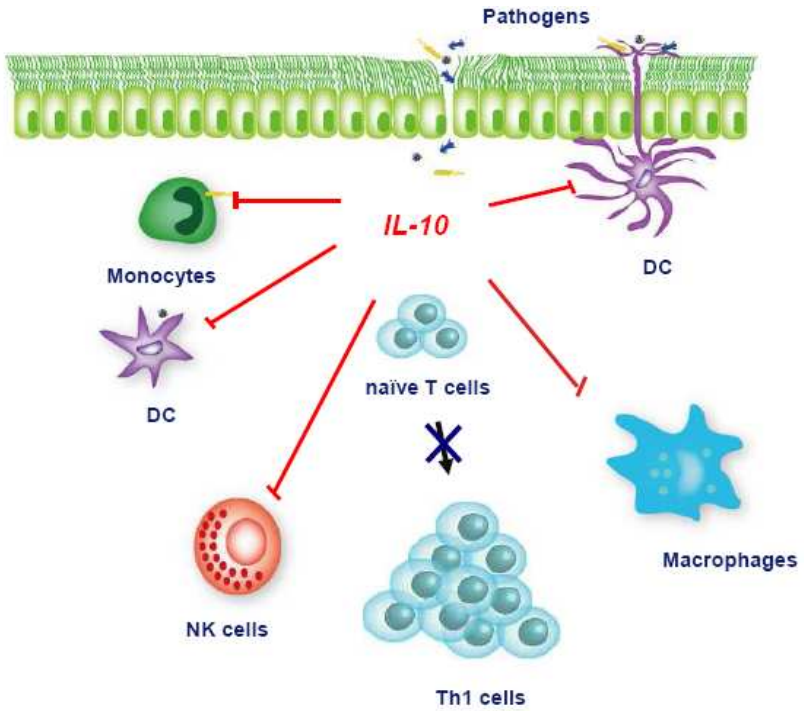

Figure 6. The suppressive effects of IL-10 on the immune system. Upon the recognition of pathogens, APC are activated and cytokines, such as IL-12p70, IL-10, etc, are secreted. While IL-12p70 is important for the Th1 cells, IL-10 suppresses the activation of APC and thus represses or quenches Th1 responses.

The receptor of IL-10 consists of IL-10R1 and IL-10R2 [71-72] **(Figure 7)**. IL-10R1 is expressed by most hemopoietic cells [72], whereas the expression of IL-10R2 is largely distributed [76-77]. The IL-10R complex is structurally analogous to the IFNγR complex. Moreover, the IL-10/IL-10R complex has similar quaternary structure to the IFNγ/IFNγR complex [78-79]. The best characterized IL-10 signaling pathway is the Jak/STAT system (**Figure 7**). Jak1 and Tyk2 are constitutively associated with IL-10R1 and IL-10R2 [71-72, 80]. Macrophages from $Jak^{-/-}$ mice do not respond to IL-10 [81], indicating thatJak1 is involved in the IL-10 signalling. Upon ligation of IL-10, Jak1 and Tyk2 induce tyrosine phosphorylation and activation of STAT3, STAT1 and STAT5 [82-84]. STAT3 is recruited directly to the IL-10/IL-10R complex and is required for IL-10 signaling [85-87]. In contrast to STAT3, the roles of STAT1 and STAT5 in IL-10 biology and signal transduction remain unclear. STAT1 and STAT5 do not appear to interact directly with IL-10/IL-10R complex [72, 84].

The activation of p-STAT3, while necessary for the anti-inflammatory effect of IL-10, is not sufficient [83, 86-87], indicating one or more additional pathway(s) must be involved in the anti-inflammatory action of IL-10. Although IL-10 activates PI-3-kinase and p70 S6 kinase, these pathways are not required for the anti-inflammatory action of IL-10 and are

involved for the proliferative effect of IL-10 [88]. Interestingly, it is shown that HO-1 (Heme oxygenase-1), a stress-inducible protein, induced by IL-10 in murine macrophages via a p38 MAP-kinase dependent pathway mediated the anti-inflammatory effect of IL-10 [89], indicating that the p38 MAP-kinase dependent pathway is also involved in the IL-10 signaling pathway **(Figure 7)**.

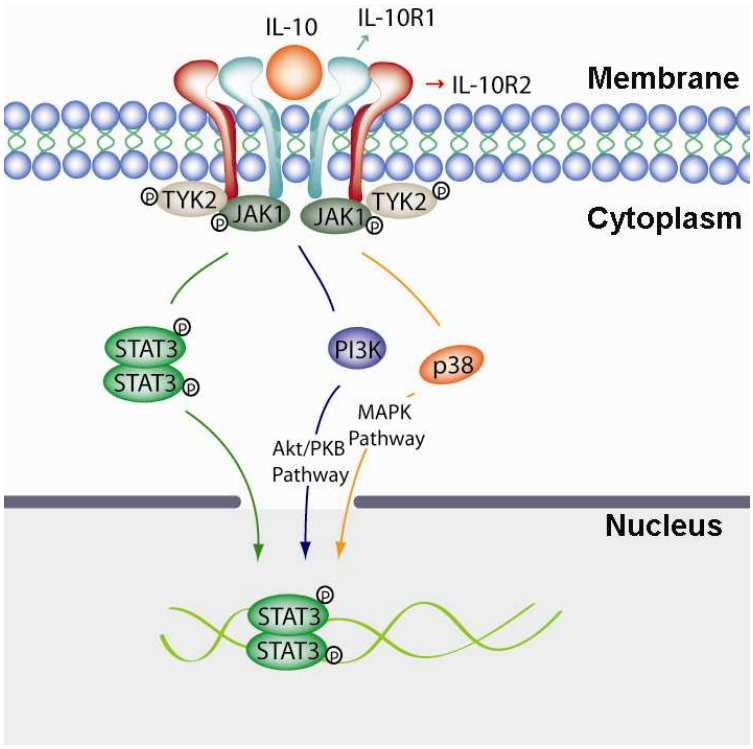

Figure 7. IL-10R complex and IL-10-induced signaling pathways. IL-10R comprises IL-10R1 and IL-10R2. Upon ligation of IL-10, JAK1 and TYK2 are activated. There are several signaling pathways induced by IL-10/IL-10R complex. The best characterized IL-10 signaling pathway is the Jak/STAT system [71-72]. In addition, PI3K and p38-MAPk pathways are also induced by IL-10 [88-89]. JAK/STAT and p38-MAPk pathways, but not PI3K pathway, are involved for the anti-inflammatory effect of IL-10 [72, 88-89].

Increased IL-10 expression and production have been associated with some chronic bacterial and viral infections [79]. The induction of IL-10 in APC represents a powerful mechanism of immune evasion used by various pathogens. It was shown that IL-10 itself impaired pathogen control and clearance in the infection models for lymphocytic choriomeningitis virus (LCMV) [90], *Schistosoma mansoni* [91], *Mycobacterium tuberculosis* [92], and *Candida albicans* [93]. An increase in systemic IL-10 production had been reported for several human chronic viral infections, such as HCV, HIV and EBV [45, 62, 94-97]. Patients with self-limiting HCV infection were reported with low levels of IL-10 production by monocytes [98]. Furthermore, Oleksyk et al. reported that single nucleotide polymorphisms in the *IL-10* gene region were associated with natural clearance of HCV in some infected individuals [99].

Interferon and signal transduction

Interferon (IFN) family cytokines, which have antiviral, antiproliferative and immunomodulatory effects, are recognized as the first line of defense against viral infection.

Three classes of IFN have been identified, designated type I, II and III, and are classified according to the receptor complex they signal through (**Figure 8**). Type I IFN, which comprise 13 IFNα subtypes and IFNβ, IFNκ, IFNε, IFNσ, IFNτ, and IFNδ in human, engage the same and ubiquitously expressed IFNα receptor complex that is composed of IFNαR1 and IFNαR2 chains. Type II IFN has only one cytokine, which utilizes the IFNγ receptor complex, a tetramer of two IFNγR1 and two IFNγR2. Type III IFN (also known as IFNλ) are comprised of IL-28A, IL-28B and IL-29, which signal through IL-28Rα and IL-10R2 complex. The receptor of type I IFN is expressed by most cell types, whereas the receptor of IFNγ is mainly expressed by APC, such as macrophages, DC and monocyte. The receptor of type III IFN is relatively restricted, and, in human, it is reported to be expressed by plasmacytoid DC (pDC), B cells, epithelial cells, and hepatocytes [100-104].

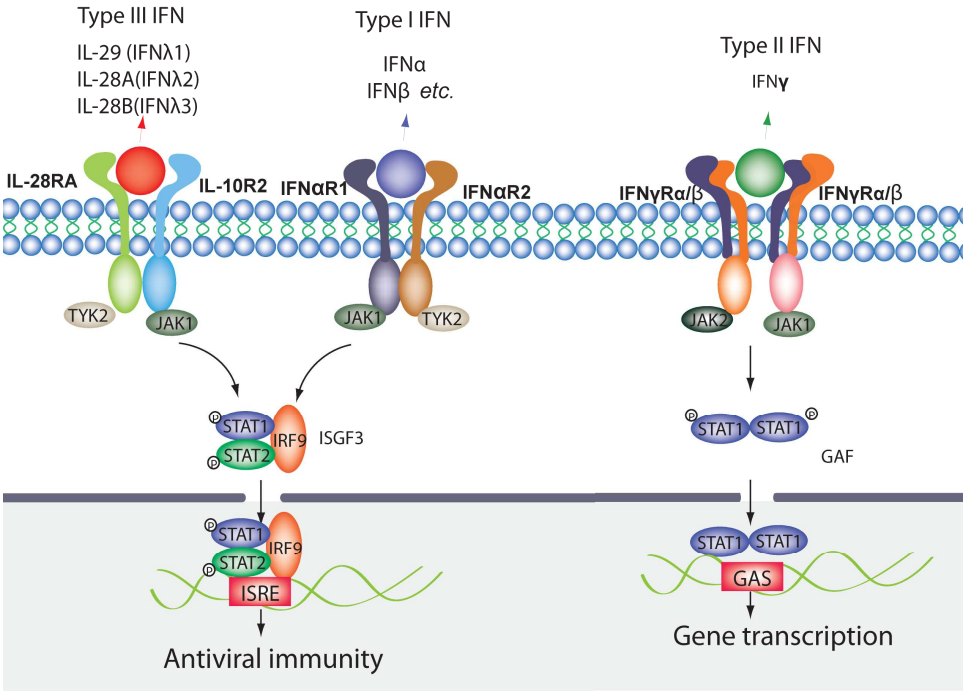

Figure 8. IFN family cytokines and IFN receptor signaling. IFN family cytokines are classified into 3 types based on the receptors they signal through. The receptor of type I IFN is a heterodimer of IFNα receptor 1 (IFNαR1) and IFNα receptor 2 (IFNαR2); IL-10R2 and IL-28Rα are associated with type III IFN, which have 3 members: IL-28A, IL-28B and IL-29; and a tetramer consisting of two IFNγ receptor 1(IFNγR1) chains and two IFNγ receptor 2(IFNγR2) chains are utilized by IFNγ. Upon the binding of IFN, the receptors of type I IFN and type III IFN first activate JAK1 and Tyk2, which result in the phosphorylation of STAT1 and STAT2. The STAT1-STAT2-IRF9 complex, known as ISG3, is subsequently formed. ISG3 further translocates to the nucleus and binds to ISRE region to initiate gene transcription. The receptor of IFNγ only activates STAT1 and the homodimer of STAT1 further translocates to the nucleus and binds to the GAS region of targeted genes.

The IFN receptors share a common manner of regulating gene expression by initiating JAK/STAT signal transduction **(Figure 8)**. It has been shown that type I IFN and type III IFN share the same signalling transduction mechanisms [100, 105-106]. Upon the binding of IFN, the receptors of type I IFN and type III IFN first activate JAK1 and Tyk2, which result in the phosphorylation of STAT1 and STAT2. The STAT1-STAT2-IRF9 complex, known as ISG3, is subsequently formed. ISG3 further translocates to the nucleus and binds to the IFN-sensitive response element (ISRE) region to initiate gene transcription **(Figure 8)**. Interestingly, other

STATs can also be recruited by the receptor of type I IFN [107], such as STAT3 and STAT5. However, currently, the roles of STAT3 and STAT5 induced by the receptor of type I IFN are still not completely understood. The receptor of IFNγ only activates STAT1 and the homodimer of STAT1 further translocates to the nucleus and binds to the GAS region of targeted genes **(Figure 8)**. Importantly, the STAT1 homodimer can also be induced by signalling via the receptor of type I IFN [107].

pDC are the main producer of both type I and III IFN [108], whereas NK cells and Th1 cells are the main producers of type II IFN [109]. The prominent activity of type I IFN is to induce antiviral immunity [107, 109-113] via inducing ISG15, Mx GTPases, OAS and PKB antiviral pathways [110]. Type III IFN have been shown to possess potent antiviral activities via the same mechanisms as type I IFN [100, 105-106]. IFNγ, on the other hand, functions predominantly on macrophages to induce a microbicidal state against ingested intracellular, non-viral pathogens. However, the complete picture of the function of all the three types of IFN is complex and therefore it is over-simplified to clearly separate the three types of IFN as antiviral or antibacterial factors.

Interferon as the immunoregulatory factor

APC, such as monocytes, DC and macrophages are the first line of defense of bacterial and viral infections and play important roles in initiating the adaptive T cell response. Upon recognition of pathogens, APC are activated and cytokines, such as IL12p40, IL-12p70 and IL-10. will be secreted. The production of IL-12p70, comprising IL-12p40 and IL-12p35, by pathogen-challenged APC has been shown to be one of the most crucial steps to initiate Th1 responses [114]. IL-10 will quench host immune responses after the clearance of pathogens [72].

In addition to the potent antiviral activities, type I IFN are also known to have strong modulatory effects on APC (**Figure 9**). IFNα has been shown to possess an inhibitory effect on TLR-stimulated IL-12p40 production by both human and murine APC [115-116]. However, the production of the biologically active IL-12p70 by APC upon TLR triggering is enhanced by IFNα [117], which indicates that IFNα favors Th1 response upon bacterial and viral infections, since the level of IL-12p70, but not IL-12p40, is important in promoting the Th1 response. IFNα is also able to regulate TLR-induced IL-10 by both human and mouse APC. However, conflicting data have been reported. Some groups [116] show that IFNα enhances IL-10 production by TLR-challenged macrophages, monocytes and DC, whereas these reports conflict with other publications showing that IFNα inhibits IL-10 production by APC [115, 118].

Although, much is known on the antiviral activities of type III IFN, little is reported about its immunoregulatory effects on APC. We have recently shown that IL-29, one member of type III IFN, enhances IL-12p40 and IL-23 production by TLR-stimulated human macrophages [119] (**Figure 9**). Type III IFN have no effect on DC and monocytes, and also they have no direct effect on Th1 responses, yet it has been reported that IL-29 is able to reduce IL-13 production by ConA-driven Th2 cells [120] and inhibit GATA3 expression in human naïve and memory T cells [121].

While the major producers of IFNγ are NK cells and Th1 cells, the main responders of type II IFN are APC. In contrast to IFNα, IFNγ potently enhances IL-12p40, IL-12p70 and IL-

23 production by monocytes, DC and macrophages in responses to TLR stimulation [115, 119, 122-123] (**Figure 9**).

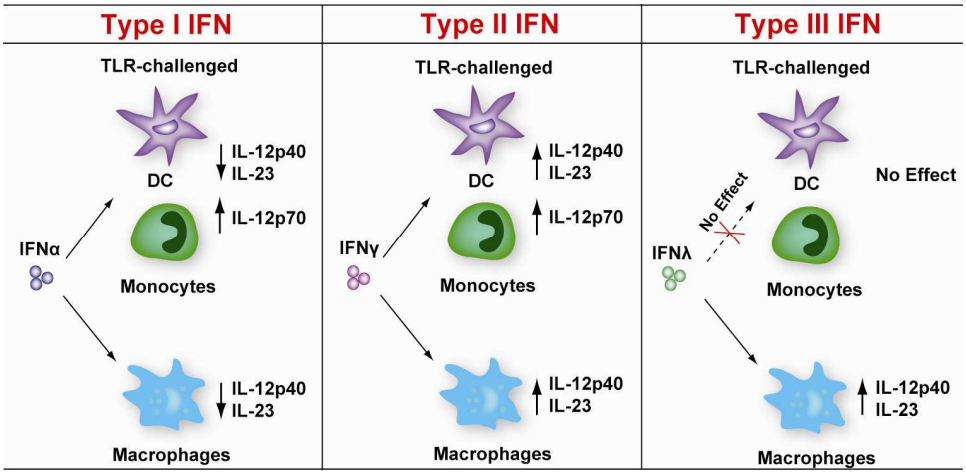

Figure 9. The regulatory effects of IFN on the production of IL-12 and IL-23 by APC. IFNα inhibits TLR-induced IL-12p40, IL-23 production by both human and murine APC, whereas it enhances TLR-induced IL-12p70 production. The knowledge of the immunoregulatory effect of IFNλ on APC is still largely unknown. IFNλ, in this thesis, is reported to enhance TLR-induced IL-12p40 and IL-23 production by human monocyte-derived macrophages, while they have no effect on human monocytes and DC. IFNγ potently enhances IL-12p40, IL-12p70 and IL-23 production by both human and murine APC.

IFNα-based therapy for chronic HCV patients

IFNα, due to its potent antiviral activities, was used for the first time in 1986 to treat patients chronically infected with HCV [124]. Currently, the sustained virological responses (SVR) of the therapy for HCV infection is dramatically improved by using pegylated IFNα plus the guanosine analog ribavirin [125-126]. Pegylated IFNα greatly improves the stability and half-life of non-pegylated IFNα, which therefore leads to the higher SVR [127-128]. The exact role of ribavirin in the therapy for chronic HCV patients is currently not clear, although, clinically, the addition of ribavirin alone with IFNα leads to the SVR rates far exceeded those obtained by IFNα monotherapy [125-126]. Currently, the standard therapy for chronic HCV patients, peyglated IFNα plus ribavirin, is able to achieve the SVR in about 80% of the treated patients with HCV genotype 1 or 3, however, in HCV genotype 1, only about 50% of the patients respond to IFNα-based therapy. Also, this combination therapy has many adverse effects. Thus, improved or alternative therapies are needed. Clinical studies are being conducted to examine the benefits of treatment with pegylated IL-29 [129], and importantly, polymorphisms close to the *IL-28B* gene that are associated with disease progression and the response to IFNα-based therapy have sparked interest in type III IFN [130-132]. In addition to the active research on different types of IFN, the roles of different TLR7/8 agonists, which are potent inducers of endogenous IFNα by pDC, in the treatment of chronic HCV patients are also under investigation [133-134].

Aims and outlines of the thesis

The underlying mechanisms of developing chronic HCV infection are currently not fully understood. To explain the impaired T cells responses in chronic HCV patients, the immune status and function of innate immune cells, such as myeloid DC (mDC), pDC and monocyte-derived DC from patients have been extensively studied, which is reviewed in **Chapter 2**. However, the function of monocytes in chronic HCV patients is still not clear. Human circulating monocytes are heterogeneous population, including at least $CD14^+CD16^-$ and $CD16^+CD14^-$ monocytes. Currently, conflicting data on the function of $CD14^+CD16^-$ monocytes isolated from chronic HCV patients are reported. Furthermore, the immune status of $CD16^+CD14^-$ monocytes is not known. Therefore, we examine the function $CD14^+CD16^-$ monocytes (**Chapter 3**) and $CD16^+CD14^-$ monocytes (**Chapter 4**) in response to bacterial- or viral-derived pathogens *in vitro*. We show in **Chapter 3** that $CD14^+CD16^-$ monocytes isolated from chronic HCV patients have impaired function of the TLR4 pathway, whereas an enhanced response to TLR8 agonists is observed in $CD14^+CD16^-$ monocytes isolated from chronic HCV patients. In contrast to $CD14^+CD16^-$ monocytes, the function of $CD16^+CD14^-$ monocytes isolated from chronic HCV patients is only mildly affected when assessing stimulation of the TLR4 pathway (**Chapter 4**).

The current standard care for chronic HCV patients is IFNα plus ribavirin, which can only cure about half of the treated patients. In addition to the potent antiviral activities, type I IFN are able to prime immune responses by modulating the function of various immune cells. However, type I IFN have also been shown to exert suppressive effects on monocytes and macrophages indirectly via the effect of interleukin-10 (IL-10). The interplay between type I IFN and IL-10 may dampen specific immunity to pathogens, which may result in failure to eliminate viral infection. In chronic HCV patients, systemic levels of IL-10 in serum are enhanced as compared with serum from healthy individuals (**Chapter 3**). It is therefore extremely interesting to study the effect of IFNα on the production and also the signalling pathway of IL-10. Since monocytes and macrophages are both important producers of IL-10 upon TLR stimulation (**Chapter 3 and Chapter 5**), we are interested to examine the effects of type I IFN on IL-10 production as well as IL-10 signalling in these cell types. We report in **Chapter 5** that despite IFNα-mediated inhibition of IL-10 production by human monocytes, TLR-induced IL-12p70 secretion by IFNα-primed cells is strongly controlled by IL-10. We observe that priming of monocytes with IFNα or IFNβ up-regulates membrane IL-10R1 expression, which may –at least partly- be responsible for enhanced IL-10 induced phosphorylation of STAT3. Moreover, type I and III IFN potentiate IL-10 signaling in a comparable manner in macrophages, indicating a more general effect of IFN on modulating the activity of IL-10 in APC. In this chapter, we demonstrate that one of the consequences of priming of human APC with type I and III IFN is to promote the cells' sensitivity to IL-10 rather than to promote IL-10 production.

Studies to find alternative therapy for chronic HCV patients are currently active, due to the severe side-effects and the relatively low response rates of the standard IFN-based therapy in some patients. In addition to the IFNα-based therapy, different TLR7/8 agonists, which are potent inducers of endogenous IFNα, for the treatment of chronic HCV patients are also under investigation. The effect of an oral TLR7 agonist, ANA773, in chronic HCV

patients is studied in **Chapter 6** in the thesis, which shows that the efficacy of viral load decline in chronic HCV patients treated with the TLR7 agonist ANA773 is likely due to intrinsic differences in the induction of endogenous IFN and ISG products (IFNα and IP-10) upon TLR7 ligation.

Furthermore, polymorphisms close to the *IL-28B* gene that are associated with disease progression and the response to IFNα-based therapy have sparked interest in type III IFN. Clinical studies are being conducted to examine the benefit of treatment with pegylated-IL-29 in chronic HCV patients. However, the immunoregulatory effect of type III IFN is currently not clear. **Chapter 7** shows for the first time that IL-29 is able to enhance IL-12p40 and IL-23 by human macrophages in response to TLR stimulation.

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Chapter 2

Modulation of Dendritic Cell Function by Persistent Viruses

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Journal of Leukocyte Biology 2009; 85(2):205-214.

SUMMARY

Worldwide, chronic viral infections cause major health problems with severe morbidity and mortality. HIV and HCV both manifest themselves as persistent infections, but they are entirely distinct viruses with distinct replication mechanisms, tropism and kinetics. Co-infections with HCV among people with HIV is emerging as a growing problem. Cellular immune responses play an important role in viral clearance and disease pathogenesis. However, cellular immunity to HIV and HCV is severely affected in chronic patients. Various hypotheses have been proposed to explain the dysfunctional T cell response, including viral escape mutations, exhaustion of the T cell compartment and the activity of regulatory T cells. Also, modulation of the function of DC has been suggested as one of the mechanisms used by persistent viruses to evade the immune system. In this review, we will focus on DC interactions with one murine persistent virus (LCMV clone 13) and two human persistent viruses (HIV-1 and HCV), intending to examine if general strategies are employed by persistent viruses to modulate the function of DC in order to improve our understanding of the mechanisms underlying the development and maintenance of viral persistence.

INTRODUCTION

Viral infection initiates a series of events that may culminate in the generation of an effective immune response capable of eliminating the virus. The immune response to viral infection relies on the combined action of both the innate and adaptive immune system. The innate immune system, which involves dendritic cells (DC), natural killer cells, complement and cytokines, is the first response to various viral infections prior to the appearance of the adaptive or virus-specific immune response, mediated by T or B cells. Because of their extraordinary features, DC fulfill a special role in the immune system [1-3]. They originate from the bone marrow, and migrate through blood to secondary lymphoid organs and tissue. DC operate at the interface between the innate and adaptive immune response by their ability to sample their environment for pathogenic products, to process them, and to present viral antigens to T cells [4]. This results in T cell proliferation, and the induction of virus-specific adaptive immune responses.

T helper 1 (Th1) cells produce IFN-γ and play a central role in cell-mediated immunity [5-7]. The development of Th1 cells can be promoted through the activation of distinct populations of DC via the production of IL-12p70 and in some cases IFN-α [8-13]. The activation of DC relies on its expression of numerous pathogen recognition receptors, like C-type lectins and Toll-like receptors (TLR) that recognize molecular patterns expressed by pathogens, such as lipopolysaccharides (LPS), RNA or DNA sequences [14-16]. These microbial stimuli induce significant morphological and biochemical changes in DC, such as enhanced secretion of TNF, IL-6, IL-12, IL-10 and IFN-α, and increased expression on DC of MHC and costimulators, including CD80, CD86 and CD40. This activation of DC is required for efficient priming of pathogen-specific T cells.

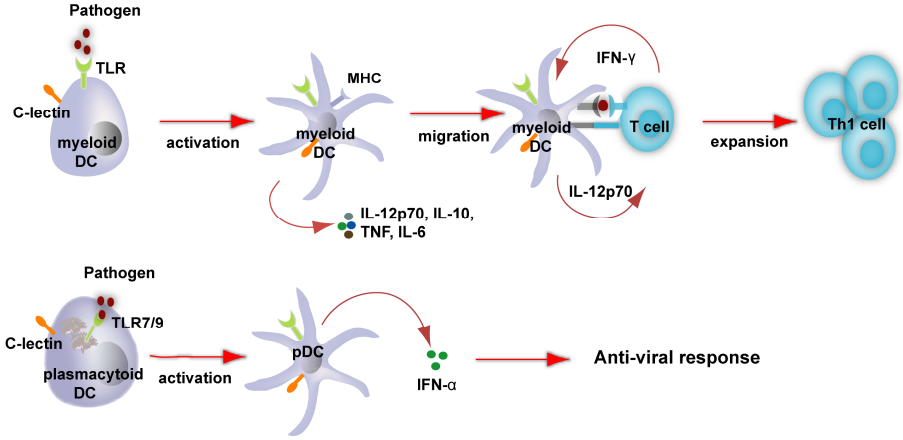

Figure 1. Human myeloid DC and plasmacytoid DC express different TLR, and consequently respond to distinct microbial stimuli. IL-12 production by myeloid DC can be stimulated by a large range of microbial products and augmented by CD40 ligation, or cytokines such as IFN-γ, resulting in the development of Th1 cells. Cytokines, such as IL-10, negatively regulate the production of IL-12. Activated plasmacytoid DC produce large amounts of IFN-α, which has potent anti-viral activity.

Human DC have been categorized into two major subsets, CD11c+ myeloid DC and CD11c- CD123+ plasmacytoid DC [17-20]. They have been shown to express different TLR, and consequently respond to distinct microbial products. For example, human myeloid DC express TLR3, and respond to the TLR ligand poly-I:C by producing IL-12p70, which

promotes Th1 cell development [21]. Myeloid DC are considered classical antigen-presenting cells, since they are able to initiate the activation of naïve and effector T cells. Because of the low numbers of myeloid DC in blood, DC generated from peripheral blood monocytes, in the presence of IL-4 and granulocyte-monocyte colony stimulating factor (GM-CSF), have been used extensively [22]. These cells share some, but not all features of blood myeloid DC. It is unclear whether a counterpart of monocyte-derived DC is circulating in the body, but it has been suggested that these cells represent inflammation-induced tissue mDC [23]. Plasmacytoid DC, on the other hand, are best known for their extraordinary ability to secrete high levels of IFN-α in response to ligation of TLR7 and TLR9, and by bacterial and viral RNA or DNA [21, 24]. Plasmacytoid DC exert strong antiviral effects mediated via IFN-α, as has been reported in a number of viral infections [24, 25]. However, they are poor inducers of T cell proliferation, due to their low efficiency in capturing, processing and loading antigen onto MHC molecules, and their weak expression of costimulators [26].

The activation of DC and subsequent cytokine production, such as IL-12p70 and IFN-α, are highly regulated by both positive and negative feedback mechanisms. For instance, positive regulation is achieved by additional CD40 ligation and the presence of IFN-γ, which are signals normally provided by T cells [27]. On the other hand, anti-inflammatory cytokines, such as IL-10, strongly inhibit the expression of IL-12p70 and IFN-α, as well as suppress the production of other pro-inflammatory cytokines [27, 28]. This negative regulation may be important to prevent excessive DC and T cell activation, which might result in pathology, but at the same time may limit the efficacy of the ongoing immune response against pathogens, thereby allowing pathogen survival.

When antiviral responses are insufficient, the host and the virus may establish some form of long-term relationship, i.e. viral persistence, as observed following infections with HIV and HCV. Since DC bridge innate and adaptive responses, exploitation of DC by the virus is an effective strategy to disrupt the host immune response by impairing DC function, and as a consequence achieve persistent infection. In this review, we will discuss if distinct persistent viruses indeed exploit DC to promote the development of chronic infections. Integration of our knowledge on the immune evasion mechanisms used by a murine persistent virus (LCMV variant clone 13) and two distinct human persistent viruses (HIV-1 and HCV) indicates that a number of similar strategies are employed by these unrelated viruses to modulate the function of DC.

Lymphocytic choriomeningitis virus, a murine RNA virus

Over the last decade, detailed insight has been obtained in the immunological mechanisms that are involved in the establishment and maintenance of various persistent viral infections. Most of this knowledge is derived from studies in mice, in which infections with lymphocytic choriomeningitis virus (LCMV), a murine, ambisense RNA virus, are considered the prototype for viral persistent infections [29]. The outcome of infection of mice with the LCMV Armstrong strain is resolution of the infection. Following inoculation, a sharp increase in viral levels is observed for 3-4 days, which declines soon afterwards until the virus is cleared completely. Clearance of the LCMV Armstrong strain is mediated by a strong adaptive immune response characterized by proliferation and activation of highly effective LCMV-

specific $CD4^+$ and $CD8^+$ T cells [30-32]. In contrast, infection with LCMV variant clone 13 in mice displays all the features of a persistent viral disease, and high viral titers are observed months after inoculation [33, 34]. Importantly, in mice persistently infected with LCMV clone 13, a generalized immunosuppression is observed characterized by ablation of specific T cell responses to multiple viruses, as well as antibody responses to many different antigens [34, 35]. Immunosuppression in mice infected by LCMV clone 13 appears to result from a defect in antigen presentation, rather than from a direct effect on T cells and B cells as demonstrated by adoptive transfer experiments [34-36].

Macrophages, stromal cells and DC can all be infected by LCMV. The receptor for LCMV is alpha-dystroglycan [37], which is a cellular receptor for extracellular matrix proteins. Importantly, it was found that persistent LCMV strains, such as LCMV clone 13, bind alpha-dystroglycan with higher affinity than LCMV Armstrong [37]. A 2-3 log difference in binding affinity was observed for LCMV strains that caused a persistent infection as compared to strains that did not [38], which could be mapped to a single amino acid change in the viral glycolipid-1 ligand that binds alpha-dystroglycan [38]. The high dependency of persistent LCMV strains on alpha-dystroglycan most likely leads to their preferential infection of splenic $CD11c^+$ and $DEC205^+$ DC in the marginal zone and white pulp of the spleen [34, 38]. Three weeks following LCMV infection, the majority of splenic DC are infected. On the other hand, LCMV strains that do not cause persistent infections mainly infect macrophages and few DC in the red pulp, most likely because they are less dependent on alpha-dystroglycan for infection [34, 38, 39]. In addition, LCMV clone 13, but not LCMV Armstrong, can infect the majority of hematopoietic progenitors from bone marrow, rendering them unresponsive to Flt3-ligand and GM-CSF *in vivo* and *in vitro.* As a consequence of LCMV clone 13 infection of hematopoietic progenitors, the development of $CD8\alpha^+$ and $CD8\alpha^-$ DC is impaired [40], which was found to require IFN-α/β, but was not via induction of apoptosis of DC [38]. Interestingly, it was previously reported that LCMV clone 13-infected DC, but not Armstrong-infected DC, induced the secretion of IFN-α/β [41]. Thus, infection with LCMV clone 13, but not LCMV Armstrong, leads to reduced numbers of DC within the host, which may explain the difference in disease outcome. In addition, infection of DC with LCMV clone 13, but not LCMV Armstrong, renders LCMV clone 13 infected DC as targets for the cytotoxic activity of LCMV-specific $CD8^+$ T lymphocytes resulting in a further reduction of DC numbers [34].

Splenic $CD11c^+$ DC isolated from mice infected with LCMV clone 13 demonstrated markedly inhibited expression of MHC class I, MHC class II, CD40, CD86, and CD80 molecules, which was not observed for DC from LCMV Armstrong infected mice [40]. Interestingly, reduced expression of the costimulators MHC class II, CD80 and CD86 was still observed at day 120 when LCMV clone 13-infected mice had controlled the infection, whereas the expression of CD40 and MHC class I had recovered completely at that time. Therefore, control of the infection and the recovery of the costimulatory ability of DC do not correlate in time. At day 360 after infection, the expression of all costimulators had recovered completely. As a result of the reduced expression of costimulators during clone 13 infection, DC were unable to induce T cell proliferation efficiently in a primary allogeneic MLR [34, 38]. These results indicate that LCMV clone 13 specifically targets DC, which may render them ineffective to stimulate T cells, and ultimately lead to immunosuppression.

Besides an effect on the levels of costimulators, LCMV also modulates cytokine production by DC. IL-12, produced by DC, is a key factor in promoting the development of IFN-γ producing Th1 cells. However, in LCMV infections, the development of Th1 responses appears to be independent of IL-12, since the cytokine secretion profile of LCMV-specific $CD4^+$ T cells in IL-12-deficient mice was identical to normal mice [42, 43]. Instead, under normal conditions, LCMV-specific IFN-γ responses by $CD8^+$ T cells were mediated via IFN-α/β, as demonstrated using IFN-α/β-receptor knock-out mice [42]. Lack of IFN-α/β resulted in enhanced IL-12 production, demonstrating negative feedback mechanisms controlling IL-12 production. In this situation, the LCMV viral titers were increased, demonstrating the superior effect of type I IFN over IL-12 in inhibiting viral replication [42]. The source of IFN-α/β in response to LCMV remains controversial. Following LCMV Armstrong infection, a rapid increase of the numbers of plasmacytoid DC as well as upregulation of IFN-α expression were observed in the spleen [44]. Using IFN-α-GFP-reporter mice, it was recently reported that plasmacytoid DC are indeed responsible for high MyD88-dependent IFN-α production following infection [45]. However, depletion of plasmacytoid DC *in vivo* did not affect IFN-α/β levels in serum during LCMV Armstrong infection [46]. Moreover, non-plasmacytoid DC from mice infected with LCMV have also been shown to produce high IFN-α levels [41].

Distinct production of IFN-α/β has been implicated in the establishment of persistence to LCMV. Mice infected with LCMV clone 13 demonstrated sustained production of IFN-α/β by both immature and mature DC from the spleen and bone marrow for about 2 months, which was not observed in mice infected with LCMV Armstrong [47]. However, LCMV clone 13 infections in mice were less sensitive to IFN-α/β and IFN-γ, as compared to LCMV Armstrong [48]. Studies with mice deficient in the IFN-α/β pathway revealed that a “resolving” LCMV variant (LCMV-WE) was able to initiate a persistent infection due to the absence of virus specific $CD8^+$ T cells, while clearance of LCMV Armstrong proceeded but with slower kinetics [42, 49, 50]. Thus, these findings indicate that infection with persistent LCMV strains can subvert the antiviral effect of type I IFN to benefit its own survival, which was, at least in part, by inhibition of the development of the DC compartment in infected mice.

Another important level of regulation of immune responses is mediated by immunosuppressive cytokines, such as IL-10. IL-10 suppresses the function of APC and T cells, mainly via inhibition of pro-inflammatory cytokine production, costimulation and MHC class II expression [28]. In recent years, it was reported that IL-10 production is dramatically increased in mice persistently infected with LCMV clone 13 as compared to LCMV Armstrong [51, 52]. Brooks et al. suggested that DC are the source of IL-10 in persistently infected mice, whereas Ejrnaes et al. demonstrated that modulation of the DC compartment results in enhanced IL-10 production by $CD4^+$ T cells. Importantly, both studies showed that neutralization of the activity of IL-10 in mice chronically infected with LCMV clone 13, resulted in restoration of the impaired T cell response, and clearance of the virus. The induction of IL-10 by specific strains of the virus, or the tendency of the host to produce more IL-10 may contribute to the inability to clear the virus, and the development of viral persistence.

Thus, persistent strains of LCMV have evolved multiple strategies for suppressing and altering DC function, thereby reducing the host's ability to induce adaptive immune responses.

Human immunodeficiency virus, a retrovirus

Infection with the HIV-1 virus continues to develop as a global pandemic with an estimated 33 million infected individuals and 2.1 million people dying from AIDS in 2007 [http://www.who.int/mediacentre/news/releases/2007/pr61/en/index.html]. The hallmark of HIV-1 pathogenesis is the gradual loss of $CD4^+$ T cells throughout chronic disease, ultimately resulting in enhanced susceptibility to opportunistic infections. The progressive depletion of $CD4^+$ T cell during the chronic stage of infection is most likely due to direct HIV infection and subsequent cell deletion, as well as activation induced cell death, as reviewed in [53].

At the mucosal site, DC may capture HIV, and promote spreading and transmission of the virus. This may lead to delivery of the virus to the lymph nodes, where infection of $CD4^+$ T cells may occur [54-56]. HIV also infects DC via specific receptors on DC, such as CC-chemokine receptor 5 (CCR5), and CXC-chemokine receptor 4 (CXCR4) [57-59]. However, compared to $CD4^+$ T cells, HIV replication in DC is less productive, and the frequency of HIV-infected DC is very low due to the low level of CXCR4 and CCR5 expression, and the activity of fusion restriction factors in DC [60, 61]. Mucosal DC are among the first cellular targets for HIV-1 during sexual transmission [62-67]. *In vitro*, Langerhans cells from the skin, vaginal DC, blood myeloid DC, and DC generated from monocytes or $CD34^+$ progenitors can all be infected with HIV [58, 68-76]. The maturation status of DC is thought to affect the susceptibility of DC to become infected with HIV. Immature DC are more susceptible to infection, whereas mature DC are more difficult or even resistant to become infected [77, 78].

Numerous studies have examined the role of blood myeloid and plasmacytoid DC in HIV-1 immunopathogenesis. A decrease in the absolute numbers of both myeloid DC and plasmacytoid DC in blood of HIV-1 infected donors is observed in most studies [79-84]. It was suggested that loss of DC in HIV infection may contribute to disease progression, since the depletion is progressive and correlates with HIV-1 plasma viral load [80, 85]. Importantly, asymptomatic long-term survivors had increased numbers of plasmacytoid DC relative to individuals with progressive disease or uninfected controls, suggesting that plasmacytoid DC can protect against disease progression, although the increased numbers can also be the consequence of lower levels of viral replication [85]. Moreover, patients undergoing anti-retroviral therapy show a recovery of the numbers of plasmacytoid DC [86], further suggesting a role for plasmacytoid DC in HIV pathogenesis. There are no indications that HIV-1 inhibits DC progenitor expansion, but depletion of plasmacytoid DC via apoptosis and necrosis *in vitro* has been reported [87]. In addition, disappearance of DC from the circulation has been suggested to be due to recruitment of cells to lymphoid tissue, as demonstrated on the basis of expression of the CCR7 and CXCR3 migration markers [84, 88-90]. In addition, reduced numbers of plasmacytoid DC as observed in AIDS patients might also be the consequence of opportunistic infections [85].

Functionally, less efficient stimulation by DC of allogeneic T cells was observed when comparing peripheral blood DC of HIV-infected individuals at different stages of infection with DC from healthy donors [82, 91, 92]. Interestingly, DC infected with HIV-1 *in vitro* induced IL-

10 secretion by T cells, which may explain, at least in part, the reduced T cell response [93]. Whereas viruses can generally activate DC by inducing HLA-DR and costimulators, such as CD80, CD86 and CD40, HIV infection of DC does neither lead to activation of immature monocyte-derived DC [93] nor plasmacytoid DC *in vitro* [94], except when large amounts of virus are added [74, 93, 95]. Also when exposed to different maturation stimuli, DC infected with HIV-1 failed to become activated. In contrast, HIV viral protein R (vpr) and Nef protein expressed in DC using vaccinia or adenovirus have been found to reduce the levels of CD86, CD80 and HLA-DR on monocyte-derived DC *in vitro* [96-99]. Coinciding with this reduced expression of co-stimulators, these DC were impaired in their ability to activate $CD8^+$ T cells.

Modulation of DC-derived cytokine production by HIV, which was observed in most studies, may further contribute to evasion of host immune responses by HIV. Stimulation of PBMC or whole blood from HIV infected individuals showed reduced production of IL-12 as compared to controls [100-103]. In agreement with this, upon stimulation with a HIV-1 isolate, p24-expressing DC failed to produce IL-12p70 in response to CD40 ligation [104]. This may be mediated via the HIV vpr protein as inhibition of the production of IL-12 and upregulation IL-10 production was observed in monocyte-derived DC stimulated in the presence of vpr protein, whereas IL-6 and IL-1β levels were not affected [96]. However, adenoviral encoded Nef in immature DC induced IL-6, IL-12 and TNF production [99, 105], and monocyte-derived DC stimulated *in vitro* with gp120 from the HIV-1 strain JR-FL induced IL-10 secretion in the majority of donors [106]. These distinct, and in some cases, opposing effects on modulation of DC-derived cytokine production by HIV, are likely due to the use of distinct HIV isolates or HIV components, and the use of different sources of DC.

In addition to the reduced numbers of plasmacytoid DC during chronic HIV infection, also the capacity to produce IFN-α was found to be reduced by plasmacytoid DC of these patients [83, 85, 86, 103]. This is important since HIV-induced IFN-α contributes, at least in part, to the restriction of viral replication in plasmacytoid DC [107, 108] and $CD4^+$ T cells [87, 109], as well as to bystander activation of myeloid DC [89]. Plasmacytoid DC directly recognize and respond to HIV-1 infection by inducing maturation, and the production of large quantities of IFN-α [74, 89, 107, 109-111]. This is in contrast to myeloid DC, which do not mature upon incubation with HIV. There is general consensus that gp120 is required for IFN-α induction by plasmacytoid DC, which is mediated through its interaction with CD4 [89, 109, 111, 112]. However, recently it was shown that gp120 suppresses CpG-induced activation of plasmacytoid DC, including the production of IFN-α. This effect was only observed when plasmacytoid DC were stimulated via TLR9, but not via TLR7 [113]. On the other hand, it was found that IFN-α produced by plasmacytoid DC after HIV-1 exposure regulates the expression of TRAIL on $CD4^+$ T cells, resulting in apoptosis of these T cells [114].

Similar to persistent LCMV, HIV-1 also inhibits DC activation and modulates the cytokine expression by DC, thereby evading the host immune response. Both HIV and LCMV have evolved strategies to subvert the function of type I IFN for their own benefits.

The hepatitis C virus, an RNA virus

Worldwide, another viral pathogen that causes major health problems is the hepatitis C virus (HCV). The HCV virus is not a retrovirus, but an enveloped, positive single-stranded RNA

virus. It is estimated that 80-90% of individuals infected with HCV become chronically infected, and these patients are at increased risk of developing cirrhosis and hepatocellular carcinoma, which may take decades to develop. At present it is still unclear why some individuals are able to clear the infection spontaneously whereas others do not. Weak and functionally impaired HCV-specific T cells responses are a characteristic feature of chronic HCV infection, in common with persistent HBV and LCMV infections [115-117].

Binding of HCV to hepatocytes involves many receptors that were identified by screening for surface markers that bind the envelope proteins E1 and E2. In this way, CD81, scavenger receptor class B, DC-SIGN, L-SIGN and the asialoglycoprotein receptor were identified [118-121]. In addition, the LDL receptor, by binding HCV particles associated with lipoprotein, and claudin-1, a tight-junction component highly expressed in the liver, are involved in viral binding and/or entry [122]. The involvement of these diverse proteins in HCV cell entry either suggests multiple pathways, or a complex series of sequential steps for viral entry. The primary site of infection of HCV is the liver, and replication can take place in hepatocytes. However, HCV RNA has also been detected in extrahepatic locations, including cells of the lymphatic system (PBMC), bone marrow and the central nervous system [123, 124]. Although there is still debate, numerous studies have reported the presence of positive-strand HCV RNA, and importantly its replicative intermediate- negative strand HCV RNA, in peripheral blood DC following infection *in vitro* or directly *ex vivo* [125-129]. However, the frequency of DC containing HCV RNA, and the levels of the virus in DC are extremely low [125, 126]. The scavenger receptor B1 has recently been show to be required for not only binding, but also the uptake of HCV and cross-presentation by human DC [130]. At present, no information is available whether other receptors on DC can perform similar activities.

Similar to LCMV and HIV-1 infection, in patients with chronic HCV infections decreased frequencies of peripheral myeloid DC and plasmacytoid DC have been demonstrated in the majority of studies [127, 131-139]. However, similar as described for HIV, it is possible that altered frequencies of peripheral DC may be a consequence of migration towards the site of infection, and therefore peripheral numbers do not necessarily mirror the capacity of the DC compartment in chronic HCV patients. For this it is preferable to monitor DC numbers and their function in the liver, but to date only few studies have examined intrahepatic DC in HCV infections [139]. Using immunohistochemistry it was shown that the numbers of myeloid and plasmacytoid DC in the livers of patients with chronic HCV were markedly increased, as compared to normal control specimens [139]. However, it is difficult to determine if accumulation of DC in the liver is causally related to the decrease of DC numbers in peripheral blood. Another possibility is that HCV targets DC precursors as reported by Sansonno et al [140], or that HCV directly targets DC to reduce their numbers. In this regard, it has been shown that HCV core, NS3 and NS5 proteins all induce apoptosis in mature DC *in vitro* [141].

Besides reduced numbers of myeloid DC, it was found that myeloid DC from chronic HCV patients showed a decrease in their capacity to stimulate allogeneic T cells [131, 142, 143]. Also, with respect to the levels of co-stimulators expressed on peripheral blood myeloid DC from chronic HCV patients as compared to healthy controls, reduced expression of HLA-DR and CD86 was observed by some [142], but not all studies [134, 144]. Interestingly, Tsubouchi et al found that successful therapy with IFN-α and ribavirin increased the

expression of costimulators on myeloid DC, and increased their allostimulatory capacity when DC were examined before and 4 weeks after therapy [127]. Clearly, more studies with larger cohorts of patients need to be performed to resolve this issue.

In chronic HCV patients, myeloid DC were found to produce less IL-12 in response to stimuli, such as poly-I:C or CD40-ligand, whereas the production of the anti-inflammatory cytokine IL-10 was enhanced [103, 131, 142, 143, 145-147]. The reduced IL-12p70 production by DC could be restored following successful antiviral therapy of chronic HCV patients, suggesting that the presence of HCV specifically inhibits the activity of DC [127]. Although Longman et al. reported normal phenotypic characteristics and allogeneic functions in monocyte-derived DC [148], the majority of researchers found that monocyte-derived DC of patients with chronic HCV infections displayed a less mature phenotype and had an impaired allostimulatory capacity [125, 135, 149, 150].

To date, the mechanisms whereby HCV affects DC function remain largely elusive. It is possible that HCV proteins play a role in suppressing protective immunity through interactions with host immune cells, such as DC. Indeed, the HCV core protein has been reported to impair the function of DC [151-155]. Mouse myeloid DC treated with HCV core-expressing plasmid had a reduced surface expression of MHC I, MHC II, CD80, CD86 and PD-L1, and associated with this was an impaired in vitro priming of CD4+ T cells [155]. HCV core protein was also able to selectively inhibit TLR4-induced IL-12 production after interacting with the gC1q-receptor on the surface of monocyte-derived DC by activating the PI3K pathway, leading to reduced Th1 cell development [151, 154]. Besides the HCV core protein, also suppressed T cell responses were described due to the effect of NS3 and NS4 on monocytes or DC [156, 157].

Further indications that HCV directly affects DC function came from studies using the recently described cell-culture grown HCV (HCVcc). Culture with HCVcc demonstrated inhibition of maturation of monocyte-derived DC induced by a cocktail of cytokines (IL-1β, TNF, IL-6, prostaglandin-E_2), while enhancing the production of IL-10. In addition, DC exposed to HCVcc were impaired in their ability to stimulate antigen-specific T cell responses [158]. In contrast, similar experiments performed by Shiina et al., found no inhibition of maturation induced by LPS or poly-I:C, nor affected cytokine production of blood myeloid DC and monocyte-derived DC, or T cell proliferation in an MLR response [159]. The distinct maturation stimuli used, different doses of HCVcc, or differences in the HCVcc itself might explain the conflicting findings reported by these studies. Thus, although individual HCV proteins have been shown to modulate the function of DC *in vitro*, more studies need to be conducted to determine the immunomodulatory effect of the complete HCV virus on DC function.

Numerous studies have also reported on an impairment of the function of plasmacytoid DC from blood of HCV patients as compared to healthy controls, as demonstrated by reduced production of IFN-α upon stimulation with herpes simplex virus or TLR ligands [103, 131, 132, 137, 142, 159-161]. Importantly, patients who spontaneously resolved their HCV infection, and patients who responded to therapy showed similar numbers of plasmacytoid DC and IFN-α production as healthy control individuals [132, 138]. Interestingly, Dolganiuc et al. demonstrated *in vitro* that in response to HCV core protein, monocyte-derived TNF and IL-10 were responsible for the reduction of IFN-α production by plasmacytoid DC [138]. The

limited number of studies that examined the consequence of HCV infection on the ability of plasmacytoid DC to stimulate T cells found reduced activation of $CD4^+$ T cells [131, 161]. Although the majority of studies support the observations that the plasmacytoid DC are impaired, others demonstrated that on a per-cell basis, IFN-α production by pDC is similar to healthy controls [132, 134]. Also, the effect of exposure of plasmacytoid DC to cell culture-produced HCVcc is still unclear, since inhibition of IFN-α production in a dose-dependent manner was reported [159], as well as no effect on plasmacytoid DC as determined by a broad array of cytokines and chemokines (Decalf, 2007}.

In recent years it has been shown that HCV is very efficient in interfering with the IFN signaling at multiple levels. Multiple HCV proteins were capable of selectively degrading STAT-1 and to reduce accumulation of phosphorylated STAT-1 in the nucleus [162], resulting in a reduced capacity to stimulate IFN-target genes. In addition, specific molecules of signaling pathways activated upon recognition of viral RNA, such as TRIF and Cardif are targeted by the HCV NS3 and NS4 proteins (reviewed in [163]). Disruption of these signaling pathways may be a critical mechanism of HCV to reduce type I IFN responses, and thus potently disrupt the antiviral response.

Together, the reduced frequency of both myeloid DC and plasmacytoid DC, reduced IL-12, IFN-α and increased IL-10 production, accompanied by an impaired capacity to prime naïve T cells, may contribute to the insufficient immune response to HCV in chronic HCV patients. Different from infections with LCMV or HIV-1, viral proteins seem to play a more important role in evading the host immune response to HCV.

CONCLUDING REMARKS

LCMV, HIV and HCV are highly distinct viruses. From a clinical point of view, the only feature these viruses have in common is their ability to establish persistent infections in the host. Infections with LCMV, HIV and HCV all demonstrate that DC can not stimulate T cell responses as efficiently as DC from healthy control individuals. As described in this review, there are many indications that these viruses modulate DC frequencies or function, but the molecular and viral factors responsible are still poorly defined. Although difficult to prove, especially for human viruses, it is highly likely that reduced numbers of DC, or altered DC function, contributes to the development of weaker antiviral T cell responses. Moreover, to determine if this in turn leads to viral persistence is even more difficult to prove. Reversal of virus-induced modulation of the DC compartment by therapeutic intervention is the only way to determine a causal role for DC in the induction and maintenance of viral persistence. However, at present no such approaches have been tested in patients.

LCMV clone 13 infection in mice leads to infection and subsequent deletion of DC progenitors, resulting in reduced number of peripheral DC. For HIV and HCV infection, also reduced numbers of peripheral DC have been described, but this is most likely due to altered migration of DC from peripheral blood to lymphoid organs, as has been described for plasmacytoid DC during HIV infection [84, 89, 90], or tissue.

Most studies demonstrate that infection with LCMV, HCV and HIV *in vivo* “produces” a DC with a diminished capacity to activate T cells. Besides affecting activation and maturation of DC, also altered cytokine production might underlie the limited ability to stimulate the adaptive immune response. In this, all three viruses have evolved ways to undermine the potent anti-viral type I IFN response, either by disturbing intracellular signaling downstream of the IFN-receptor and pattern recognition receptors, or by affecting survival of DC and $CD8^+$ T cells. In addition, the recent finding that neutralization of DC-derived IL-10 was able to resolve persistent LCMV infection, leading to complete cure of the infected mice [51, 52] demonstrates another important role for DC-derived cytokines in the establishment of persistence. Although enhanced IL-10 production has been described by DC stimulated with viral products from HIV and HCV, and blockade of IL-10/IL-10-receptor pathway *in vitro* enhanced $CD4^+$ T cell responses in samples from chronic HIV or HCV patients [164, 165], no *in vivo* trials to block the activity of IL-10 have been conducted in human.

These findings showing that the DC compartment is functionally affected in chronic viral infections, as discussed in this review, support the rationale for the development of DC-based strategies for the prevention and treatment of chronic virus infections. In a preliminary study, therapeutic DC vaccination for chronic HIV-1 infection using monocyte-derived DC loaded with inactivated HIV-1 has been shown to reduce the viral load, while enhancing the HIV-specific T cell response [166]. Also, numerous groups are currently exploring the use of therapeutic manipulation of the innate immune system using TLR agonists for treatment of chronic HIV and HCV infections (reviewed in [167]). Restoring the impaired DC compartment may represent a powerful strategy for the treatment of chronic HIV and HCV infection.

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Chapter 3

Role for IL-10 in inducing functional impairment of monocytes upon TLR4 ligation in patients with chronic HCV infections

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Journal of Leukocyte Biology 2011;89(6):981-988.

SUMMARY

The consequences of chronic infection with the hepatitis C virus (HCV) on immunity to distinct pathogens are not fully appreciated despite the potent modulatory effects of HCV on the immune system. We observed that upon TLR4 ligation, monocytes from chronic HCV patients demonstrated 3-5 times lower TNF and IL-12p40 production as compared to healthy individuals. However, augmented production of TNF, IL-12p40 and IL-12p70 by monocytes was observed upon stimulation with R848. Importantly, we observed that the levels of IL-10 in chronic HCV patients are higher in serum and that more IL-10 is produced by monocytes as compared to healthy individuals. The inhibitory effect of IL-10 on the production of pro-inflammatory cytokines by monocytes was only observed upon LPS stimulation, but not upon R848 stimulation, showing that only the TLR4 pathway in monocytes is sensitive to the suppressive effects of IL-10. And interestingly, monocytes stimulated with the TLR4 agonist, but not TLR8 agonist, produced higher levels of IL-10, when exposed to patient serum as compared to serum from healthy individuals. Our results indicate that, by differentially affecting TLR4 and TLR8 pathways, IL-10 may mediate highly selective modulation of the function of monocytes observed in chronic HCV patients. This suggests that there is no overall increased susceptibility to pathogens, but a specific suppression of the functionality of TLR4 signaling pathway in monocytes, which is, at least partly, mediated via IL-10.

INTRODUCTION

The majority of individuals infected with the hepatitis C virus (HCV) develop a chronic infection. A characteristic feature of the immune status in these chronically infected patients is a weak HCV-specific T cell response, which is short-lived, and targeted to a narrow range of epitopes [1-3]. To explain the insufficient T cell responses, numerical and functional impairment of dendritic cells (DC), natural killer (NK) cells and regulatory T cells have been reported in patients with chronic HCV patients [3-5]. Monocytes have received relatively little attention in studies on the immune status of chronic HCV patients, despite the fact that monocytes comprise approximately 10% of circulating leukocytes, and play important roles in inflammatory responses. Monocytes express a specific panel of receptors for diverse pathogen-derived products, including Toll-like receptors (TLR), which enable them to respond to a broad range of bacterial and viral pathogens [6-7]. In response to TLR ligation, monocytes can produce large quantities of pro-inflammatory and anti-inflammatory cytokines, which are important in the eradication of the pathogen, but may also lead to immunopathology [8-10]. As important producers of IL-10 in the periphery, monocytes can also act as important negative immune regulators, to limit the excessive immune response to pathogens and thereby preventing damage to the host [11-12].

The consequences of persistent infections on the functionality of monocytes have not been studied in detail. For HIV infections, it was shown that HIV-1 impairs innate immunity to bacteria by affecting the function of mononuclear phagocytic cells, and that HIV-infected individuals display an increased risk of bacterial infections [13]. For chronic HCV infections, this is less clear, and conflicting data has been reported. It is generally accepted that monocytes from HCV patients are more activated than their counterparts in healthy individuals, as shown by higher production of TNF, IL-12p40 and IL-10 in the absence of activating stimuli [14-20]. The mechanisms underlying this enhanced activation state of monocytes in chronic HCV patients are not clear, but IFN-γ, IFN-α, as well as HCV core protein have been detected in serum of chronic HCV patients, and may prime circulating monocytes [18, 21-23]. At present it is not entirely clear how monocytes respond to pathogen-derived products in chronic HCV patients as compared to healthy individuals. Stimulation of purified monocytes from chronic HCV patients with the TLR4 ligand LPS resulted in higher TNF production as compared to healthy individuals by some groups [18-19], whereas others showed no difference [17]. Moreover, monocyte-derived cytokine production induced by LPS was found to be reduced when assessing PBMC from HCV patients as compared to PBMC from healthy controls [24], but was enhanced when assessing the intracellular cytokine expression [14-15].

Currently, the functionality of monocytes from chronic HCV patients is not fully understood. This is important since an altered monocyte function may compromise the immune status of the patient and consequently their susceptibility to pathogens. In this study we show that monocytes from chronic HCV patients show an impaired response to TLR4 ligation, but not TLR8 ligation. The selective impairment of the TLR4 –but not the TLR8-signalling pathway, appears to be the consequence of a higher sensitivity of the TLR4 signalling pathway to suppression by IL-10, which is present at relatively high levels during persistence of HCV infections.

MATERIALS AND METHODS

Patients

Blood samples were obtained from patients with chronic HCV infection visiting our outpatient clinic and from healthy individuals. Patients co-infected with human immunodeficiency virus, hepatitis A virus, hepatitis B virus or hepatitis D viruses were excluded from the study. Monocytes were isolated from 23 naïve chronic HCV patients (male=15, female=8; age=51±10; ALT=105±90) and 23 healthy individuals (male=12, female=11; age=36±11). The HCV genotype was determined in all 23 patients: genotype 1=13; genotype 2=4; genotype 3=4; genotype 4=2 patients. In 9 out 23 patients, the fibrosis grade was lower than F2, and cirrhosis was diagnosed in 3 patients. The levels of fibrosis or cirrhosis are unknown in 11 patients. Serum was obtained from a different cohort consisting of 58 chronic HCV patients (male=41, female=17; age=47±7; ALT=88±57) and 20 healthy individuals (male=14, female=6; age=49±6). The protocol conformed with ethical guidelines of the Erasmus Medical Center, and all patients gave their informed consent.

Intracellular cytokine staining

PBMC were isolated from peripheral blood by gradient-density centrifugation. PBMC were stimulated with ultrapure LPS *S. minnesota* (100 ng/ml; InvivoGen) or R848 (1 μg/ml; Alexis) in serum-free X-VIVO15 medium (BioWhittaker) for 5h, with brefeldin-A (10 μg/ml; Sigma) present for the last 3h. Samples were then fixed, permeabilized and stained with antibodies against CD14-Pacific Blue (M5E2, BD Pharmingen), TNF-PE-Cy7 (MAb11, eBioscience) and IL-10-APC (JES3-9D7, BD Pharmingen). Cytokine producing monocytes were detected by flow cytometry (Canto-II, BD). In some experiments, frozen PBMC from both chronic HCV patients and healthy individuals were used.

Monocyte purification and stimulation

Monocytes were purified from PBMC using magnetic CD14-microbeads (Miltenyi Biotec; purity: 95-99%), and stimulated in X-VIVO15 medium in 96-well plates ($5x10^5$ cells/ml, 200 μl/well) for 24h with ultrapure LPS *S. minnesota* (100 ng/ml, TLR4), Pam3CSK4 (100 ng/ml, TLR2), polyIC (25 μg/ml, TLR3/mda-5), flagellin (50 ng/ml, TLR5), loxoribine (0.4 μM, TLR7), R848 (1 μg/ml, TLR7/8; Alexis) and CpG2216 (5 μg/ml, TLR9; Coley-Pharma). In order to compare the response of monocytes to different LPS preparations, LPS *E. coli* 055:B5 (Sigma-Aldrich) and LPS *S. minnesota* (Sigma-Aldrich) were compared to ultrapure LPS *S. minnesota* (InvivoGen). All TLR agonists used for stimulations were from InvivoGen, unless indicated otherwise. To determine how IL-10 modulates TLR4 and TLR8 signaling pathways, monocytes from healthy individuals were challenged with various concentration of IL-10 (R&D) in the presence of TLR ligands for 24h.

Serum IL-10 concentration determination and monocyte stimulation

The concentrations of IL-10 in serum were measured using the IL-10 Quantikine ELISA Kit (R&D). To determine the effects of serum on cytokine production, monocytes were isolated from buffycoats and cultured in X-VIVO15 medium supplemented with 20% serum from chronic HCV patients or from healthy individuals in the presence or absence of ultrapure LPS

S. minnesota or R848 for 24h. To determine a role for IL-10, 10 μg/ml anti-IL-10 receptor antibody (3F9, Biolegend) was added in some experiments.

Quantification of TLR mRNA expression in monocytes

Total RNA from monocytes was extracted using the RNeasy kit (Qiagen), and cDNA prepared using the iScript cDNA Synthesis Kit (Bio-Rad). All real-time PCR reactions were performed using a MyIQ5 detection system (Bio-Rad). Primers for GAPDH (forward 5'-TGCACCACCAACTGCTTAGC-3' and reverse 5'-GGCATGGACTGTGGTCATGAG-3') and TLR4 (forward 5'-TCTACAAAATCCCCGACA-3' and reverse 5'-AGGTGGCTTAGGCTCTGA-3') were used. Furthermore, primer-probes for GAPDH (Hs00266705_g1), TLR8 (Hs00152972_m1), and IL-10 (Hs00174086_m1) were purchased from Applied Biosystems. The expression of target genes was normalized to GAPDH using the formula: $2^{-\Delta Ct}$, $\Delta Ct = Ct_{TLR} - Ct_{GAPDH}$.

Immunoassay for detection of cytokines in supernatant

The concentration of cytokines in supernatant were determined using sandwich ELISA specific for IL-10 (eBioscience), IL-12p40 (C8.6 and C8.3 antibody pairs, Biolegend), IL-12p70 (eBioscience) and TNF (eBioscience). The detection limits for IL-10, IL-12p70 and TNF were 15 pg/ml and for IL-12p40 30 pg/ml.

Statistics

Values are expressed as mean values, unless indicated otherwise. Data was analyzed using the Mann-Whitney t-test to compare variables between two independent groups. Two-tailed p-values of less than 0.05 were considered statistically significant.

Results

Serum IL-10 levels are enhanced in chronic HCV patients as compared to healthy individuals.

Since HCV only infects liver hepatocytes, the putative effects of chronic HCV infection on the peripheral immune system are likely via an indirect way. We postulated that components present in serum from chronic HCV patients, but not in serum from healthy individuals, may be able to modulate the activity of the peripheral immune system. To examine this, we focused on the involvement of IL-10 as a possible candidate because of its potent immunomodulatory activities [11-12]. As shown in Figure 1A, serum from chronic HCV patients has a significant higher level of IL-10 as compared to serum from healthy individuals, which was not associated with age, ALT or viral load of chronic HCV patients (data not shown). Next, we determined whether monocytes obtained from patients differed from healthy individuals with respect to their ability to express IL-10 mRNA and to produce IL-10 protein without stimulation. Indeed, we observed that monocytes from chronic HCV patients have significantly higher levels of IL-10 mRNA expression (Figure 1B) as well as enhanced IL-10 production *ex vivo* when compared to monocytes from healthy individuals (Figure 1C).

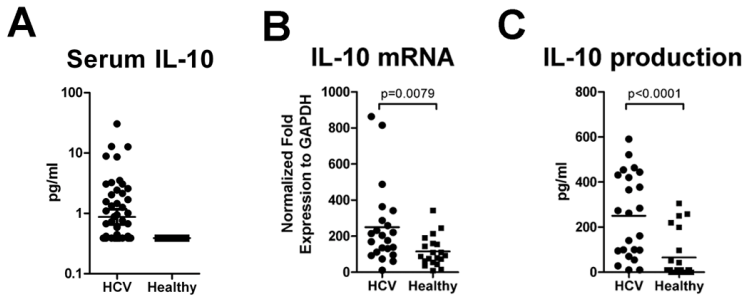


Figure 1. Higher serum IL-10 levels and higher spontaneous monocyte-derived IL-10 production in chronic HCV patients as compared to healthy individuals. **(A)** Serum levels of IL-10 were determined in patients (n=58) and healthy individuals (n=20). Threshold level is 0.4pg/ml. **(B)** Levels of IL-10 mRNA expression **(C)** and spontaneous IL-10 production by monocytes from chronic HCV patients and healthy individuals were measured.

Serum from chronic HCV patients increased higher LPS-induced IL-10 production by healthy monocytes when compared to serum from healthy individuals.

To determine the production of IL-10 by monocytes in response to distinct TLR ligands, purified monocytes from healthy individuals were purified and stimulated with various TLR agonists. As shown in Figure 2A, LPS, Pam3CSK4 and R848 are more potent to induce IL-10 and/or TNF by monocytes than flagellin, PolyIC, loxoribin and CpG. The level of IL-10 was highest upon stimulation of monocytes with LPS. Furthermore, upon LPS stimulation monocytes were shown to be an important source of IL-10 in the peripheral leukocytes as demonstrated by intracellular cytokine staining (Figure 2B). Since we previously observed that the function of monocytes was strongly affected by the choice of serum used for cell culture, we examined whether serum from chronic HCV patients has the ability to modulate the activity of monocytes. As shown in Figure 2C, purified monocytes from healthy individuals stimulated with LPS produced higher levels of IL-10, but not TNF and IL-12p40

(Figure 2D), when exposed to patient serum as compared to serum from healthy individuals (average IL-10 levels of 1507 pg/ml and 408 pg/ml, respectively). To gain insight into the role of IL-10 produced by LPS-stimulated monocytes from healthy individuals exposed to chronic HCV serum, we blocked the IL-10 receptor using an anti-IL10R antibody. In the absence of TLR ligation, cultures with medium alone and with anti-IL-10R antibody did not induce TNF or IL-12p40 production by monocytes exposed to either patient serum or serum from healthy individuals (data not shown). In the absence of anti-IL-10R antibodies, LPS stimulated monocytes exposed to patient serum or healthy serum produced similar amounts of TNF and IL-12p40 (Figure 2D). Importantly, blocking of the IL-10R enhanced the production of LPS-induced pro-inflammatory cytokines when monocytes were exposed to patient serum, while IL-10R blockade only weakly enhanced cytokine production when monocytes were cultured in serum from healthy individuals (Figure 2D). These findings suggest that serum from chronic HCV patients enhances LPS-induced IL-10 production by monocytes, which in turn strongly inhibits TNF and IL-12p40 production.

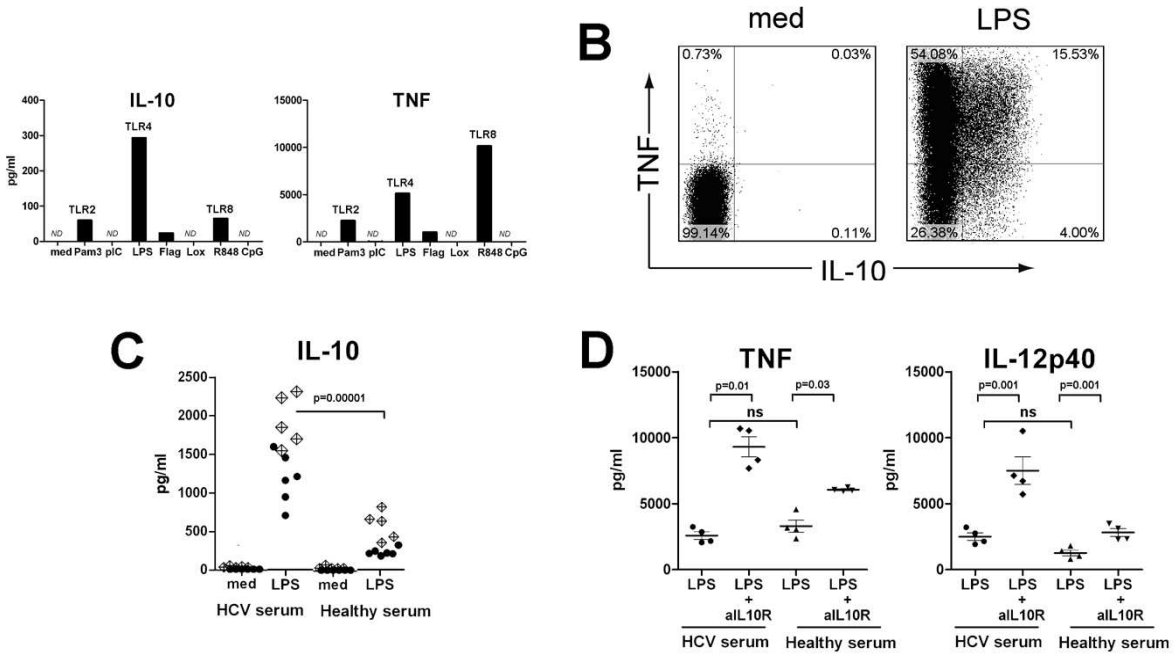


Figure 2. Serum from chronic HCV patients enhances IL-10 production by monocytes upon LPS stimulation when compared to serum from healthy individuals. **(A)** Monocytes from healthy individuals were stimulated with various TLR agonists to determine the IL-10 and TNF production. **(B)** PBMC were stimulated with LPS or medium, and intracellular cytokine production gated on CD14 positive monocytes were analyzed. Representative dot plots show the intracellular TNF and IL-10 upon stimulation of CD14 positive monocytes. **(C)** Monocytes purified from buffycoats ("●" and "⌖" represents two different donors) were incubated in serum-free medium supplemented with 20% serum from different chronic HCV patients (n=11) or healthy individuals (n=11) in the presence of LPS for 24h. **(D)** Using anti-IL-10 receptor antibody (aIL10R), TNF and IL-12p40 production by LPS stimulated monocytes, which were exposed to serum from chronic HCV patients or healthy individuals were examined. Medium and antiIL-10R antibody alone did not induce TNF or IL-12p40 production by monocytes exposed to either patient serum or serum from healthy individuals (data not shown).

TLR4 signaling pathway is highly sensitive to the suppressive effects of IL-10.

In order to further address the sensitivity of the TLR4 pathway to IL-10, we stimulated monocytes from healthy individuals with LPS in the presence of different concentrations of IL-10. As expected, the levels of TNF and IL-12p40 produced by monocytes were suppressed by IL-10, which occurred at relatively low concentrations (0.1-1 ng/ml; Figure 3A), whereas blocking of IL-10 signaling restored the production of TNF and IL-12p40 to normal levels. The inhibitory effect of IL-10 on the TLR4 pathway was also observed when different doses of LPS were examined (Supplementary Figure S1A). Importantly, the inhibitory effect of IL-10 on TNF and IL-12p40 production was not via down regulation of TLR4 levels, since no reduction of TLR4 mRNA was detected (Figure 3B) and also no down-regulation of TLR4 protein levels on the surface of monocytes were observed (Supplementary Figure S2).

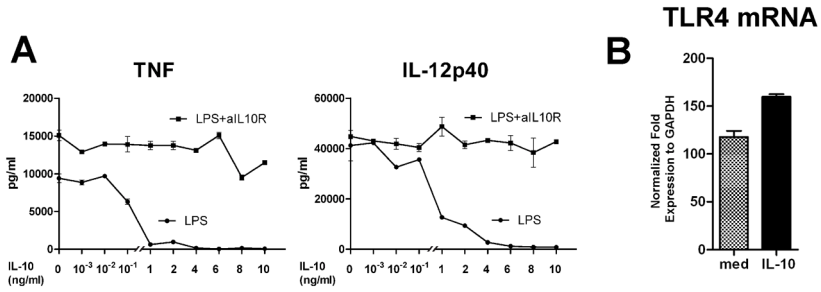


Figure 3. The TLR4 signaling pathway is very sensitive to the suppressive effects of IL-10. **(A)** Monocytes from healthy individuals were stimulated for 24h with different concentrations of IL-10 in the presence of LPS, and TNF and IL-12p40 levels were measured by ELISA. Anti-IL-10 receptor antibody (aIL10R) was used to block IL-10 signaling. **(B)** Monocytes from healthy individuals (n=3) were stimulated with medium and IL-10 (10 ng/ml) for 5h and TLR4 mRNA expression was measured.

Monocytes purified from chronic HCV patients exhibit impaired TNF and IL-12p40 in response to TLR4 ligation, but with normal TLR4 mRNA expression.

Since the response to LPS can be easily inhibited by IL-10, and also monocytes exposed to patient serum produce relatively high levels of IL-10 in response to TLR4 stimulation, we hypothesized that monocytes isolated from chronic HCV patients exhibit an impaired response to LPS. As shown in Figure 4A, stimulation of highly purified monocytes with LPS resulted in 3-5 times lower levels of TNF and IL-12p40 in chronic HCV patients as compared to healthy individuals, whereas similar levels of IL-10 were detected by LPS-stimulated monocytes from patients and healthy individuals (average: 373 and 375 pg/ml), indicating that IL-10 is regulated via a distinct mechanism in monocytes as compared with TNF and IL-12p40. Despite lower production, the percentage of TNF-producing monocytes was similar as shown by intracellular TNF staining (p=0.169; Figure 4B and 4C). This indicates that monocytes from chronic HCV patients produce less TNF and IL-12p40 per cell, as compared to cells from healthy individuals. We further observed that the level of TLR4 mRNA expression in monocytes was similar for patients and controls (Figure 4D), which suggest that the impaired TNF and IL-12p40 production by LPS stimulated monocytes from chronic HCV patients is likely due to alterations in TLR4 signaling pathways.

Previous studies have reported that LPS induced higher TNF production by monocytes from chronic HCV patients than from healthy individuals [18, 20]. To examine if different LPS preparations could explain these conflicting findings, monocytes were stimulated with ultrapure LPS *S. minnesota*, LPS *E. coli* 055:B5 and “regular” LPS *S. minnesota* (purchased from Sigma-Aldrich) for cytokine production. Ultrapure LPS *S. minnesota,* as used in our study, induced 2-3 times lower levels of TNF

in purified monocytes (Table 1), whereas 2-3 times higher levels of IL-12p40 were observed when compared to activation with LPS *E.coli* 055:B5 and LPS *S. minnesota* (data not shown). In line with previous reports, we found that LPS *E.coli* 055:B5 and LPS *S. minnesota*, but not ultrapure LPS, induced higher levels of TNF secretion by monocytes from chronic HCV patients than from healthy individuals (Table 1).

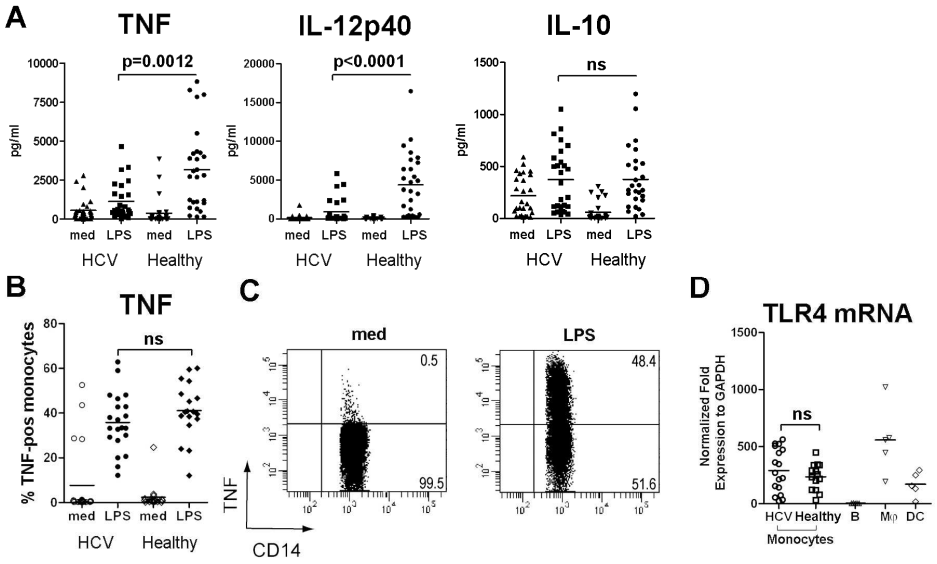


Figure 4. LPS challenged monocytes from chronic HCV patients exhibited impaired TNF and IL-12p40 production as compared to healthy individuals. **(A)** Monocytes isolated from HCV patients (n=23) and healthy individuals (n=23) were stimulated with LPS for 24h. **(B)** PBMC from chronic HCV patients (n=21) and healthy individuals (n=19) were stimulated with LPS and intracellular TNF production by monocytes was determined. **(C)** Representative dot plots showing TNF-positive monocytes upon stimulation of PBMC from a chronic HCV patient. (**D**) TLR4 mRNA expression in monocytes isolated from HCV patients (n=17) and healthy individuals (n=14) was quantified by real-time PCR. TLR4 mRNA expression in B cells, monocyte-derived macrophages and monocyte-derived dendritic cells were included as controls.

Table 1. Analysis of TNF production by monocytes isolated from chronic HCV patients and healthy individuals (n=4/group) in response to different LPS preparations

LPS preparations	HCV patients	Healthy individuals
S.minnesota ultrapure LPS	1822 (902-2476)*	3786 (3091-4204)*
E.coli 055:B5 LPS	17480 (15243-21996)	9630 (5159-13765)
S.minnesota LPS	16352 (12870-25007)	8967 (3735-11066)

** the range of TNF production is shown in brackets.*

Monocytes isolated from chronic HCV patients demonstrate elevated responses to R848.

To examine whether the impairment of monocyte responsiveness in patients with chronic HCV infections is limited to TLR4 ligation, we performed similar experiments in the presence of the TLR7/8 agonist R848. In contrast to LPS stimulation, R848-stimulated monocytes from chronic HCV patients secreted significantly higher levels of TNF and IL-12p40 as compared to healthy individuals (average values: 11.8 to 2.7 ng/ml TNF, and 3.7 vs 2.0 ng/ml IL-12p40, respectively; Figure 5A). R848-induced IL-12p70 production by monocytes was detected in

the majority of patients (average: 254 pg/ml), whereas IL-12p70 by monocytes was undetectable in 15 out of 23 healthy individuals (average: 90 pg/ml). Again, the percentage of TNF-producing monocytes in R848-stimulated PBMC was similar between patients and healthy individuals (p=0.5; Figure 5B and 5C). This indicates that individual monocytes from chronic HCV patients produce more TNF and IL-12p40 as compared to healthy individuals. The increased TLR8-induced TNF and IL-12p40 production by monocytes from chronic HCV patients could not be fully explained by an up-regulation of TLR8 mRNA expression, since we observed a weak, but not significant, up-regulation of TLR8 mRNA expression in chronic HCV patients (Figure 5D). These data suggest that activation via TLR8 leads to more potent signaling downstream of TLR8 in monocytes from chronic HCV patients as compared to healthy individuals.

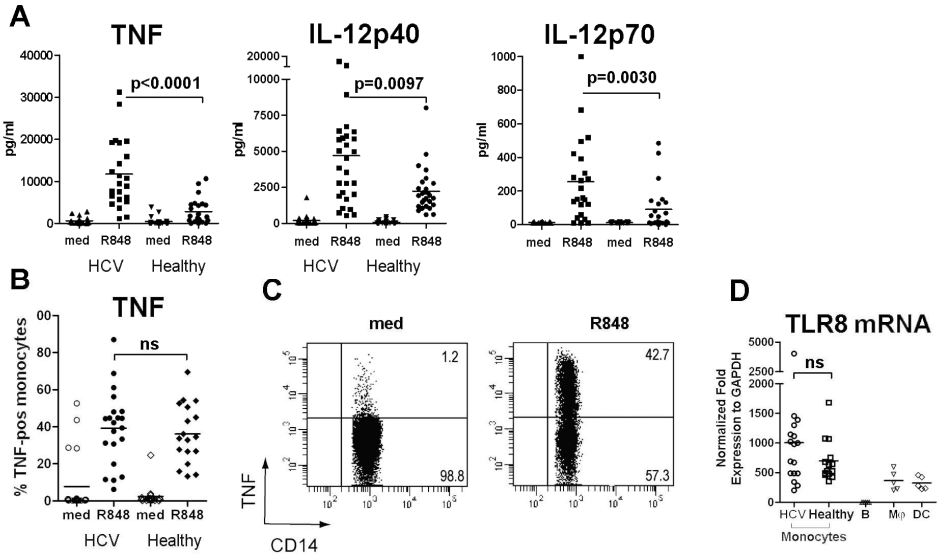


Figure 5. Monocytes from chronic HCV patients demonstrate elevated responses to R848. **(A)** Monocytes isolated from HCV patients (n=23) and healthy individuals (n=23) were stimulated with R848 for 24h. **(B)** PBMC from chronic HCV patients (n=21) and healthy individuals (n=19) were stimulated with LPS and intracellular TNF production by monocytes was determined. **(C)** Representative dot plots showing TNF-positive monocytes upon stimulation of PBMC from a chronic HCV patient. (**D**) TLR8 mRNA expression in monocytes isolated from both HCV patients (n=17) and healthy individuals (n=14) was quantified by real-time PCR. TLR8 mRNA expression in B cells, monocyte-derived macrophages and monocyte-derived dendritic cells were included as controls.

Compared to the response to LPS, the TLR8 signaling pathway is less sensitive to the suppressive effects of IL-10.

We next determined whether the response to TLR8 ligation of monocytes is also suppressed by IL-10, as was observed for the response to LPS. To our surprise, we observed that in contrast to TLR4 responses, the TLR8 signaling pathway is considerably less sensitive to the suppressive effects of IL-10 (Figure 6A). Even at relatively high IL-10 concentrations (10 ng/ml), the production of TNF and IL-12p40 was only weakly inhibited. Furthermore, upon R848 stimulation, monocytes exposed to chronic HCV serum produce similar levels of IL-10 as monocytes exposed to healthy serum (Figure 6B). Combined, these findings indicate that

chronic HCV serum has no suppressive effect on cytokine responses induced by TLR8 ligation as was observed upon TLR4 ligation.

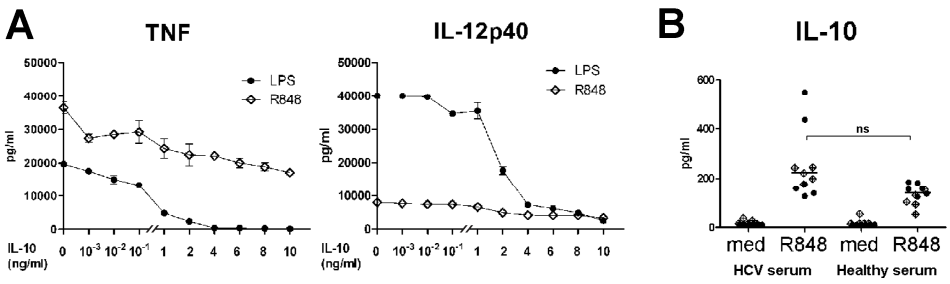


Figure 6. In contrast to the TLR4 signaling pathway, TLR8 signaling is not sensitive to the suppressive effects of IL-10. **(A)** Monocytes from healthy individuals were stimulated with different concentrations of IL-10 in the presence of LPS or R848, and the levels of TNF and IL-12p40 were measured by ELISA. **(B)** Monocytes purified from buffycoats ("●" and "❖" represents two different donors) were incubated in serum-free medium supplemented with 20% serum from different chronic HCV patients (n=11) or healthy individuals (n=11) in the presence of R848 for 24h.

Discussion

In this study we examined whether persistent infections with HCV influences the functionality of monocytes in patients. We demonstrate that the response of highly purified, circulating monocytes to distinct TLR agonists is differentially affected between chronic HCV patients and healthy individuals. Reduced production of pro-inflammatory cytokines in response to TLR4 ligation, and augmented production upon TLR8 ligation of monocytes from chronic HCV patients demonstrates specific modulation of the function of monocytes in patients with chronic HCV infection. We further show that the differences in suppression of TLR4- and TLR8-induced activation as observed in chronically infected HCV patients, was likely due to differential responsiveness to IL-10.

Monocytes are important players in the first-line of defense against numerous pathogens, as well as in initiating and controlling adaptive immunity [25]. Indeed, in HIV-1 infection, reduced function of mononuclear phagocytic cells results in the weaker innate immunity to bacterial infection [13]. In persistent HCV infections the numbers of studies examining this issue are limited, and the conclusions on the functionality of monocytes in patients are conflicting.

We demonstrate that, in contrast to TLR8 ligation, triggering of monocytes from chronic HCV patients with TLR4 ligands resulted in lower levels of the pro-inflammatory cytokines TNF and IL-12p40. Interestingly, exposure of human monocytes to recombinant HIV Tat or Vpr proteins lead to defective responses to LPS as shown by TNF and IL-12p40 production [26-27], whereas no information is available on the effect of exposure to these antigens upon TLR8 ligation. The reduced TLR4-induced responses were not simply due to lower TLR4 levels, since TLR4 mRNA levels in monocytes were similar in chronic HCV patients as compared to control individuals. Importantly, our data further shows that monocytes from chronic HCV patients spontaneously produce higher level of IL-10 as compared with monocytes from healthy controls, and that serum from chronic HCV patients contains higher IL-10 levels than control serum. Furthermore, since also cytokine production of monocytes induced by TLR4 ligation is suppressed by IL-10 very efficiently, whereas this is more modest upon TLR8 ligation, IL-10 is a likely candidate to explain the reduced LPS responses of monocytes from chronic HCV patients. At present, the cell types responsible for the relatively high serum IL-10 levels in these patients are not known. However, some studies have shown that HCV encoded proteins, such as HCV core, NS3 and NS4 proteins have the ability to induce IL-10 production by monocytes isolated from both patients and healthy individuals [19, 28-33]. The importance of monocyte-derived IL-10 was further highlighted in a study that demonstrated that patients with self-limiting HCV infections produced significantly less IL-10 than chronic HCV patients [28]. Moreover, in the chronic LCMV model in mice, the role for IL-10 in preventing viral clearance was demonstrated in which therapeutic administration of an antibody that blocks the IL-10R restored T-cell function and eliminated LCMV infection [34].

In contrast to activation via TLR4, we demonstrated that monocytes from chronic HCV patients are more responsive to TLR8 ligation than monocytes from healthy individuals by producing cytokines. Similar to healthy individuals, also monocytes from chronic HCV patients were unresponsive to pure TLR7 ligands (data not shown). The enhanced response to TLR8 ligation could not be fully explained by elevated TLR8 mRNA expression in

monocytes from chronic HCV patients in our patient group, as has been reported before [35-36]. Interestingly IL-10 has less effect on TLR8 signalling in suppressing TNF and IL-12p40 production, since the TLR8 pathway is only weakly sensitive to the suppressive effects of IL-10, even at high concentrations. Also, serum from chronic HCV patients has no effect in increasing IL-10 production by monocytes after triggering of TLR8 signalling pathway. At present, it is unknown why IL-10 is able to inhibit the TLR4, but not the TLR8-induced responses. One possibility is that IL-10 signalling events may differentially affect the MyD88 and TRIF signalling pathways, since the MyD88-independent TRIF pathway, is activated upon TLR4 ligation, but not TLR8 ligation [37]. To further add to the complexity, we observed that upon combined triggering of TLR4 and TLR8, TLR8 ligation was able to overcome the inhibitory effect of IL-10 on TLR4 stimulation (Supplementary Figure S1B). Detailed signalling studies need to be conducted in order to delineate the underlying mechanisms. However, the specific inhibition by IL-10 of responses induced by LPS, but not R848 *in vitro*, is reflected by the selective inhibition of the TLR4 pathway as observed in chronic HCV patients.

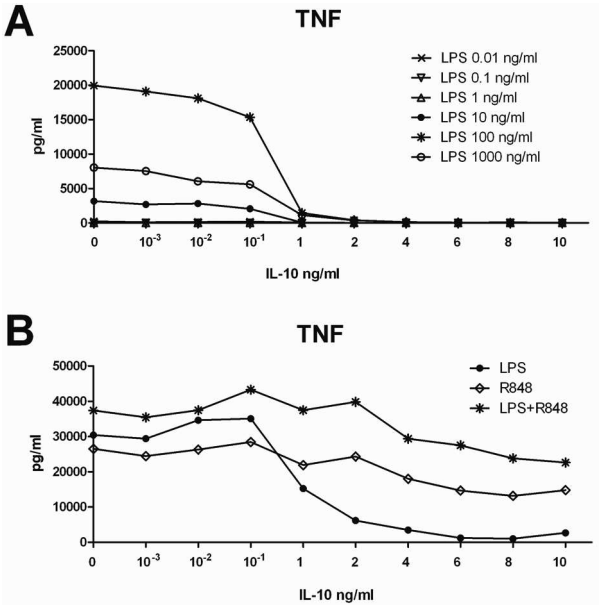
In contrast to our findings, enhanced LPS-induced TNF production by monocytes from chronic HCV patients was observed by some groups [14, 18, 20], whereas others - similar to our findings - did not [17, 24]. Besides the method of purification, the choice of medium and serum, and the read-out assay, also the specific LPS preparation used to stimulate monocytes is important as we demonstrate in this study. Great differences in TNF and IL-12p40 production by healthy monocytes stimulated with ultrapure LPS and the commonly used LPS preparations suggest that contaminants present in some LPS preparations activate monocytes, which may explain, at least in part, the opposing findings in literature. Although it is well-known that many preparations of LPS contain low amounts of TLR2 ligands [38], we observed that stimulation with Pam3CSK4 resulted in similar levels of TNF produced by monocytes from patients and healthy controls (data not shown). However, triggering by a different TLR2 ligand present in the LPS preparations, or synergistic triggering of the TLR2 and TLR4 pathways may be important in this.

Together, our results indicate that, by differentially affecting TLR4 and TLR8 pathways, IL-10 may mediate highly selective modulation of the function of monocytes observed in chronic HCV patients. This suggests that there is no overall increased susceptibility to pathogens, but a specific inhibition of the functionality of TLR4 signaling pathway in monocytes, which is likely mediated by IL-10.

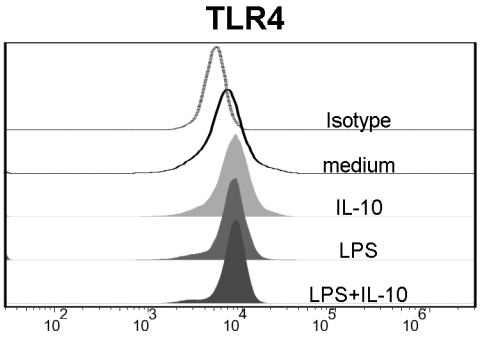
Acknowledgment

We would like to thank Robert de Knegt, Robert Roomer and Daphne Hotho for their help with collecting blood samples from chronic HCV patients visiting our outpatient clinic, and Andrea Woltman and Mark Claassen for critically reading the manuscript. We also want to thank Cheng Peng for performing the experiments on intracellular TNF staining on PBMC.

Supplementary Figures



Supplementary Figure S1. TLR4 signaling is sensitive to the suppressive effect of IL-10, but TLR8 signaling appears to overcome the effect of IL-10 on TLR4 signaling. **(A)** Highly purified monocytes from healthy individuals were stimulated with IL-10 and LPS at the indicated concentrations for 24h. The level of TNF in supernatant was determined by ELISA. Note that 1000 ng/ml LPS yields lower TNF levels than 100 ng/ml LPS. The values depicted show representative data from 3 independent experiments. **(B)** Highly purified monocytes from healthy individuals were stimulated with LPS (100 ng/ml), R848 (1 ug/ml) or LPS plus R848 for 24h. The level of TNF in supernatant was determined by ELISA. The values depicted show representative data from 3 independent experiments.



Supplementary Figure S2. IL-10 does not down-regulate TLR4 protein levels on the surface of human monocytes. Highly purified monocytes from healthy individuals were stimulated IL-10 (1 ng/ml), LPS (100 ng/ml) or LPS plus IL-10 for 5h. Samples were then stained with TLR4-PE (HTA125, Biolegend) or proper isotype control antibody. The TLR4 protein expression on monocytes was examined by flow cytometry (Canto-II, BD). The values depicted show representative data from 5 independent experiments.

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Chapter 4

The response to TLR ligation of human $CD16^{+}CD14^{-}$ monocytes is weakly modulated as a consequence of persistent infection with the hepatitis C virus

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Molecular Immunology 2011;48(12-13):1505-1511.

SUMMARY

Little is known about the frequency and function of CD16+CD14- monocytes from chronic HCV patients. We observed that the absolute numbers and ratio of CD16+CD14- to CD14+CD16- monocytes were similar between chronic HCV patients and healthy individuals. Functionally, we found that CD16+CD14- monocytes are more responsive to TLR8-ligation and only weakly responsive to LPS stimulation in producing TNF as compared to CD14+CD16- monocytes. We found no overt impairment of the function of CD16+CD14- monocytes from patients, except for an augmented induction of MIP-1β-producing CD16+CD14- monocytes upon TLR4-ligation. However, the increased frequency of MIP-1β-producing CD16+CD14- monocytes was not associated with viral load, ALT or fibrosis level. Our findings indicate that, different from other infectious diseases, the frequency and function of CD16+CD14- monocytes are only minimally altered as a consequence of the persistent state of HCV infections, and our findings therefore do not suggest a role for CD16+CD14- monocytes in HCV pathogenesis.

INTRODUCTION

The hepatitis C virus (HCV) is a major cause of chronic liver disease that can result in cirrhosis of the liver and hepatocellular carcinoma. In the majority of infected individuals, the immune response against HCV is insufficient to eradicate the virus. A weak HCV-specific T cell response is generally observed in patients chronically infected HCV. In addition, numerical or functional impairment of dendritic cells (DC), natural killer (NK) cells, regulatory T cells have been reported in patients with chronic HCV [1-4]. Although monocytes are important cells in initiating and maintaining immune responses, and comprise about 10% of circulating leukocytes, relatively little is known on the effect of chronic HCV infection on the functionality of monocytes.

Human blood contains two distinct subpopulations of circulating monocytes, which can be distinguished on the basis of membrane expression of CD16 and CD14: CD16+CD14- and CD14+CD16- monocytes [5-8]. Compared to CD14+CD16- monocytes, CD16+CD14- monocytes are less frequent and comprise about 5~15% of the total monocytes. Furthermore, CD16+CD14- monocytes are thought to produce higher levels of pro-inflammatory cytokines, such as TNF and IL-1β in response to TLR stimulation [9], and lower levels of anti-inflammatory cytokines, such as IL-10 [9-10]. Recently, it was demonstrated that CD16+CD14- monocytes are able to sense viruses via TLR7 or TLR8, resulting in the initiation of a pro-inflammatory response [11]. Several studies have reported that patients with various infections have increased numbers of CD16+CD14- monocytes in blood [12-18]. Also, in a heterogeneous patient cohort of individuals with chronic liver disease (as a result of autoimmunity, alcohol toxicity, viral infections or unspecified etiology) increased numbers of circulating CD16+CD14- monocytes were observed [19]. The importance of CD16+CD14- monocytes in the pathology of liver diseases was suggested since increased numbers of CD16+ cells were observed in the liver at areas of inflammation [19-20].

Previously, we showed that CD14+CD16- monocytes from chronic HCV patients produce lower level of TNF and IL-12p40 as compare to healthy individuals upon TLR4 ligation and augmented production of TNF, IL-12p40 and IL-12p70 was observed upon stimulation via TLR8 [21]. Since at present no information is available whether the function of CD16+CD14- monocytes is affected as a consequence of chronic infection with HCV, we examined in detail the frequency and function of circulating CD16+CD14- monocytes in patients with chronic HCV infections.

MATERIALS AND METHODS

Patients

Peripheral blood was collected from patients with chronic HCV infection visiting our outpatient clinic and from healthy individuals. Patients co-infected with human immunodeficiency virus, hepatitis A virus, hepatitis B virus or hepatitis D viruses were excluded from the study. All characteristics of HCV patients are presented in Table 1. In addition, blood from 19 healthy individuals was examined in this study (age=33±8; Male/Female: 11/8). The institutional review board of the Erasmus MC approved the protocols, and informed consent was obtained from all individuals.

Table 1. Characteristics of chronic HCV patients

Chronic HCV patients					
Age (years)	Gender	ALT	Genotype	Viral loads (IU/ml)	Fibrosis
48 (32-61) *	Male=11 Female=8	125 (10-375) *	genotype 1: 15 genotype 3: 3 genotype 4: 1	$2.0x10^6$ ($3.3x10^4$-$7.0x10^6$)*	F0: 3 F1: 1 F2: 5 F3: 3 F4: 1

* the range is shown in brackets

Enumeration of monocytes and leukocytes in whole blood

Absolute numbers of monocytes and leukocytes in whole blood were determined by an automated impedance hematology analyzer (ABX Micros-60, Horiba Medical). To determine the frequency of distinct monocyte subpopulations, whole blood was stained with antibodies against CD14-PE-Cy7 (61D3, eBioscience) and CD16-PerCP-Cy5.5 (3G8, BD Biosciences), and evaluated by flow cytometry (Canto-II, BD). The data was analyzed using BD FACS Diva software.

Stimulation of $CD16^+CD14^-$ and $CD14^+CD16^-$ monocytes

PBMC of healthy volunteers or patients with chronic HCV infections were isolated from fresh blood by Ficoll-Paque gradient centrifugation. PBMC were incubated with CD3-PE (UCHT1, Biolegend) and $CD3^+$ cells were magnetically depleted with anti-PE microbeads (Miltenyi Biotec) following the manufacturer's instructions. The CD3-depleted PBMC were further incubated with antibodies against CD14-Pacific Blue (M5E2, BD Pharmingen), CD16-PerCP-Cy5.5 (3G8, BD Biosciences) and CD56-APC (N901, Beckman). $CD16^+CD14^-$ and $CD14^+CD16^-$ monocytes were sorted on a BD FACS Aria SORP. The purity of sorted $CD16^+CD14^-$ and $CD14^+CD16^-$ monocytes was 98.3 ± 0.6%, 97.2 ±2.4%, respectively. $CD16^+CD14^-$ and $CD14^+CD16^-$ monocytes sorted from both HCV patients and healthy individuals were cultured with serum-free X-VIVO15 medium (BioWhittaker) at $2.5x10^5$ cells/ml in 96-well flat bottom plates (200 µl/well) for 24h stimulated with ultra pure LPS *S.minnesota* (100 ng/ml, TLR4 agonist) or R848 (1 µg/ml, TLR7/8 agonist; Alexis). In some experiments, $CD16^+CD14^-$ and $CD14^+CD16^-$ monocytes sorted from healthy individuals were stimulated with Pam3CSK4 (100 ng/ml, TLR2 agonist), polyIC (25 µg/ml, TLR3/Mda-5 agonist), flagellin (50 ng/ml, TLR5 agonist), CL264 (2.5 µg/ml, TLR7 agonist) and CpG-2216 (5 µg/ml, TLR9 agonist, type A; Coley Pharma). Supernatants from cultures were collected

and stored at -20□. All TLR agonists used for stimulations were from InvivoGen, unless indicated otherwise.

Intracellular detection of cytokine production

PBMC from HCV infected patients or healthy individuals were stimulated with ultra pure LPS *S.minnesota* (100 ng/ml; InvivoGen) or R848 (1 μg/ml; Alexis) in serum-free X-VIVO15 medium (BioWhittaker) for 5h, with brefeldin-A (10 μg/ml; Sigma) present for the last 3h. Samples were fixed with 2% formaldehyde, permeabilized with 0.5% saponin, and stained with antibodies against CD14-Pacific Blue (M5E2, BD Phamingen), HLA-DR-PerCP-Cy5.5 (LN3, ebioscience), TNF-PE-cy7 (MAb11, eBioscience), CD3-AmCyan (SK7, BD Biosciences), CD19-APC-H7 (SJ25C1, BD Biosciences), IL-8-FITC (6217, R&D), MCP-1-APC (5D3-F7, eBioscience) and MIP-1β-PE (D21-1351, BD Pharmingen). The frequency of cytokine producing cells was determined by flow cytometry (Canto-II, BD).

Immunoassay for detection of cytokines

The levels of TNF in supernatant were determined using sandwich ELISA (eBioscience) according to the manufacturer's instructions. The detection limit for TNF was 15 pg/ml.

Statistics

Continuous variables were represented as mean ± standard deviation, unless indicated otherwise. Mann-Whitney t-test was used to compare variables between two independent groups. In all analyses, a two-tailed p-value of less than 0.05 (confidence internal 95%) was considered statistically significant. GraphPad Prism V5.0 (GraphPad Software Inc, San Diego, CA, USA) was used to perform all analyses.

Results

The frequency of total monocytes and the ratio of $CD16^+CD14^-$ monocytes in total monocytes were not altered in chronic HCV patients

We first examined whether the contribution of monocytes in peripheral blood of chronic HCV patients differs from healthy individuals. We observed that the absolute numbers of peripheral leukocytes and monocytes were similar between patients and healthy individuals (Figure 1A). Total monocytes were further divided into $CD16^+CD14^-$, $CD14^+CD16^-$ and $CD16^+CD14^+$ monocytes using antibodies against CD14 and CD16 [9-12, 28]. As shown in Figure 1B, within the monocyte compartment, no differences were observed in the ratio of $CD16^+CD14^-$, $CD14^+CD16^-$ and $CD16^+CD14^+$ subpopulations when comparing blood from chronic HCV patients with healthy individuals.

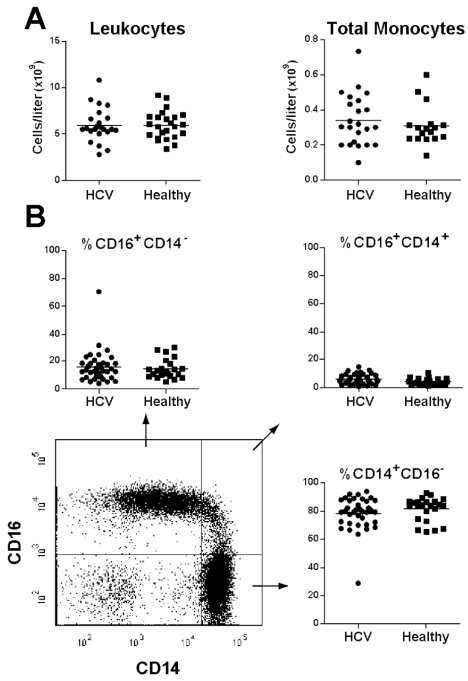


Figure 1. The numbers and composition of the circulating monocyte compartment were not affected by chronic HCV infection. (A) The absolute number of leukocytes and monocytes in peripheral blood of chronic HCV patients and healthy individuals is shown. (B) Monocytes were identified on the basis of their FSC/SSC profile, and further characterized by flowcytometry using CD14 and CD16 specific antibodies. The contribution of the specific subpopulation within the total monocyte pool is shown.

In contrast to $CD14^+CD16^-$ monocytes, $CD16^+CD14^-$ monocytes isolated from healthy individuals highly respond to R848 but only weakly respond to LPS in producing TNF

Having demonstrated that the number of CD16+CD14- monocytes was not affected in chronic HCV patients, we examined if functional differences exist between patients and healthy individuals.

CD16+CD14- and CD14+CD16- monocytes were sorted by flow cytometry from PBMC on the basis of FSC/SSC profile, and exclusion of CD3+ and CD56+ cells (Figure 2A). Morphological evaluation of CD16+CD14- and CD14+CD16- monocytes after cell sorting demonstrated that both subpopulations showed a typical monocytic morphology (data not shown). Functionally, however, the TLR7/8 agonist R848 induced about 8-10 times higher levels of TNF by CD16+CD14- monocytes than by CD14+CD16- monocytes, whereas LPS-challenged CD16+CD14- monocytes produced around 8-10 times lower levels of TNF when compared with CD14+CD16- monocytes in response to LPS (Figure 2B and 2C).

Both CD16+CD14- and CD14+CD16- monocytes did not respond to the TLR7 agonist CL264 (Figure 2B), indicating that in human monocytes R848 triggers TLR8, but not TLR7. At the

concentrations tested, Pam3CSK4 (TLR2 agonist), polyIC (Mda5/TLR3 agonist), flagellin (TLR5 agonist) and CpG (TLR9 agonist) induced relatively low or undetectable cytokines by bothCD16+CD14- and CD14+CD16- monocytes (Figure 2B). Together, CD16+CD14- and CD14+CD16- monocytes differ in their response to TLR4 and TLR8 stimulation.

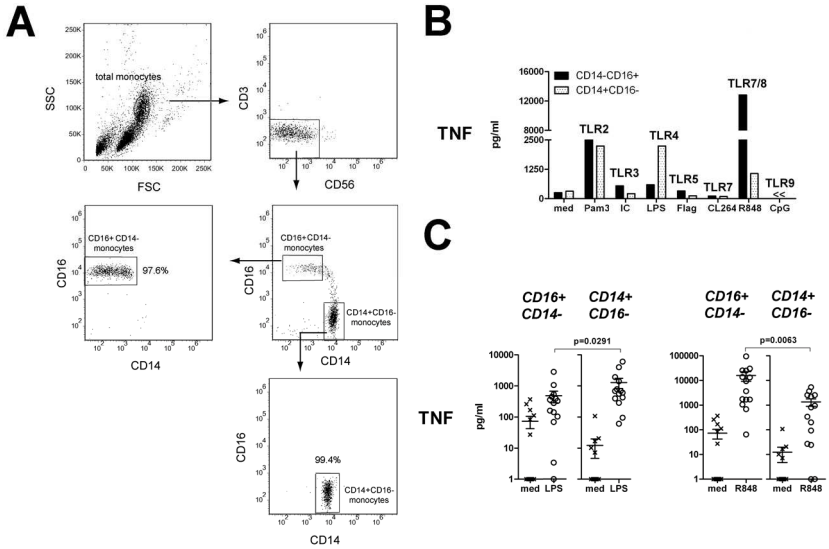


Figure 2. CD16+CD14- and CD14+CD16- monocytes sorted from healthy individuals differ in their response to LPS and R848 stimulation in producing TNF. (A) CD3+ T cells were first depleted from PBMC of healthy individuals, followed by identification of total monocytes on the basis of their FSC/SSC profile. Monocytes were sorted using antibodies against CD14 and CD16. (B) Sorted monocytes were stimulated with various TLR agonists for 24h and TNF production was evaluated. The values depicted show representative data of 2 independent experiments. (C) CD16+CD14- and CD14+CD16- monocytes obtained from 14 healthy individuals were stimulated with LPS or R848 and TNF production was determined.

TLR-induced TNF production by CD16$^+$CD14$^-$ monocytes from chronic HCV patients is not affected as compared to healthy individuals

Previously, we showed that the function of CD14$^+$ monocytes from chronic HCV patients was modulated in response to TLR stimulation as compared to healthy individuals. Yet, the response of CD16$^+$CD14$^-$ monocytes from chronic HCV patients to TLR agonists is still not clear. To examine this we stimulated CD16$^+$CD14$^-$ monocytes from PBMC of chronic HCV patients and healthy individuals, which were purified by cell sorting. As shown in Figure 3, no differences were observed in the levels of TNF produced by LPS- or R848-challenged CD16$^+$CD14$^-$ monocytes from chronic HCV patients versus healthy individuals.

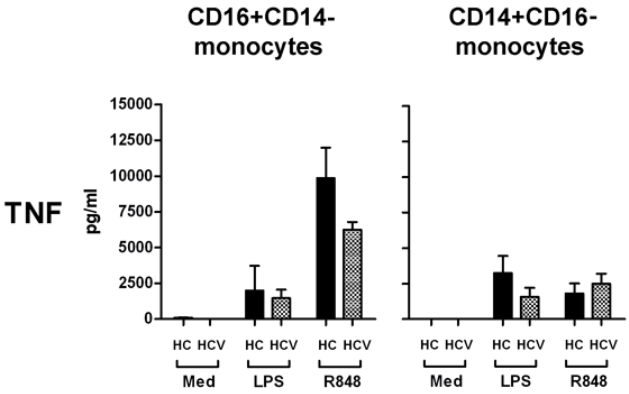


Figure 3. TLR-induced TNF production is comparable between CD16$^+$CD14$^-$ monocytes from chronic HCV patients and from healthy individuals. CD16$^+$CD14$^-$ and CD14$^+$CD16$^-$ monocytes purified from chronic HCV patients (n=4) and healthy individuals (n=5) were stimulated with medium, LPS or R848 for 24h. TNF production was measured by ELISA.

The percentage of MIP-1β producing CD16^{+}CD14^{-} monocytes induced by LPS is increased in chronic HCV patients as compared to healthy individuals

Next, we determined TLR-induced cytokine responses by CD16+CD14- monocytes in a larger cohort of chronic patients versus healthy individuals. For this, flow cytometric analysis of cytokine producing cells is preferred due to the low percentages of CD16+CD14- monocytes in peripheral blood. Antibodies against HLA-DR and CD14 were used to identify CD16+CD14- and CD14+CD16- monocytes, since the CD16 molecule on monocytes is down-regulated upon stimulation (Supplementary Figure S1). As shown in Figure 4 and 5, CD16+CD14- monocytes from healthy individuals cultured without specific stimuli have lower percentages of spontaneous MCP-1- and MIP-1β-producing cells as compared to CD14+CD16- monocytes from healthy individuals. Without stimulation, both monocyte subpopulations from healthy individuals exhibit a low percentage of TNF-producing cells (Figure 4 and 5). Comparison of CD16+CD14- monocytes from chronic HCV patients and healthy individuals showed similar percentages of cells producing TNF, MCP-1, or MIP-1β in the absence of stimuli (Figure 5).

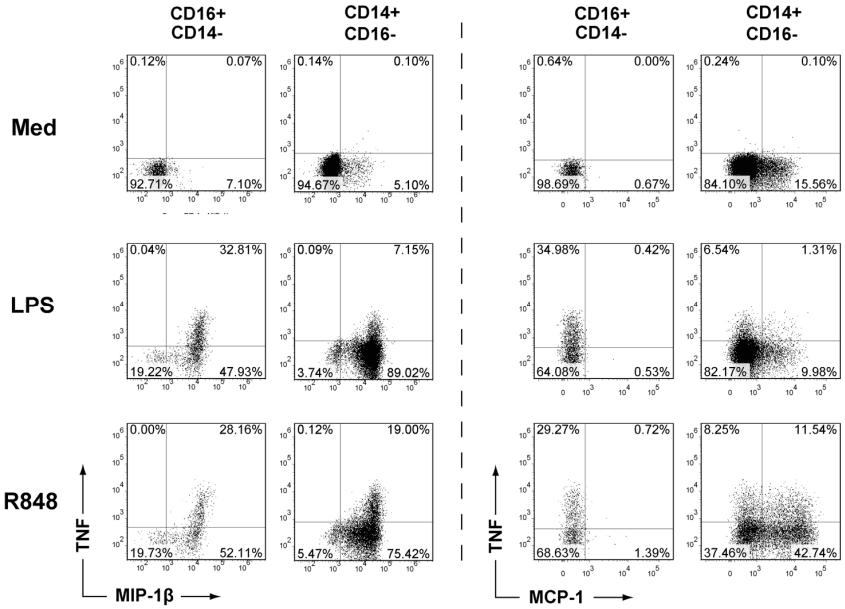


Figure 4. The intracellular cytokine profiles by PBMC from healthy individuals. PBMC were stimulated with medium, LPS or R848 for 5h, and intracellular cytokine production was determined by flow cytometry. Representative dot plots show CD16+CD14- and CD14+CD16- monocytes (identified on the basis of HLA-DR and CD14 expression) producing TNF and MIP-1β (left), or TNF and MCP-1 (right).

As expected, stimulation with LPS or R848 led to augmented percentages of TNF-, MCP-1- or MIP-1β-producing CD16+CD14- and CD14+CD16- monocytes obtained from patients and healthy individuals (Figure 4 and 5). Upon LPS or R848 stimulation of PBMC from healthy individuals we observed that CD16+CD14- and CD14+CD16- monocytes did not differ in the frequency of cells producing TNF. However, the percentages of TLR-induced MCP-1- and MIP-1β-producing monocytes are significantly lower in CD16+CD14- monocytes as compared to CD14+CD16- populations (Figure 5), which is in line with previous reports [22].

Compared to healthy individuals, TLR-challenged CD16+CD14- monocytes from chronic HCV patients show a similar frequency of cells producing TNF or MCP-1 (Figure 5).

However, the number of LPS-induced MIP-1β-producing CD16+CD14- monocytes is higher in PBMC from chronic HCV patients; with an average of 70% in healthy CD16+CD14- monocytes and 84% in CD16+CD14- monocytes from chronic HCV patients (Figure 5). Importantly, no differences were observed in the level of LPS-induced MIP-1β production by sorted CD16+CD14- monocytes from chronic HCV patients and healthy individuals (data not shown), suggesting that on a per-cell basis the MIP-1β levels are reduced, whereas their frequency is increased. Upon LPS stimulation, increased percentages of MIP-1β-producing cells are only observed for CD16+CD14- monocytes from chronic HCV patients, but not for CD14+CD16- monocytes (Figure 5). Also, there is no difference in the percentages of MIP-1β-producing CD14+CD16- monocytes in response to R848 between chronic HCV patients and healthy individuals (Figure 5), indicating that the modulation of MIP-1β by CD16+CD14- monocytes from chronic HCV patients is TLR4 pathway dependent. The elevated MIP-1β producing CD16+CD14- monocytes from chronic HCV patients in response to LPS were not associated with age, viral load, ALT or fibrosis level of chronic HCV patients studied (data not shown). Interestingly, TLR4-induced TNF-, MCP-1- and IL-8-producing CD16+CD14- monocytes were not altered in chronic HCV patients when compared with healthy individuals (Figure 5 and data not shown). Together, our findings show CD16+CD14- monocytes from chronic HCV patients are affected in their ability to induce MIP-1β upon TLR4 ligation, whereas the production of MCP-1 and TNF is not affected. The mild but specific changes in the functionality of CD16+CD14- monocytes from chronic HCV patients may play a role in HCV disease or pathogenesis.

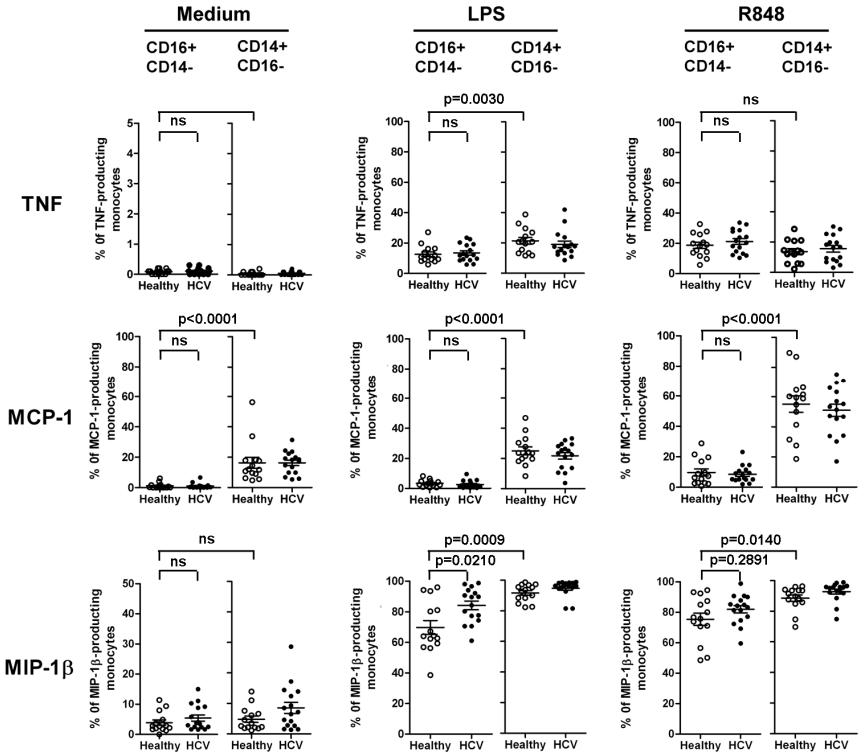


Figure 5. The frequency of MIP-1β-producing CD16+CD14- monocytes upon TLR4 stimulation is enhanced in chronic HCV patients as compared to healthy individuals. The frequencies of the monocyte subpopulations producing TNF, MCP-1 or MIP-1β are presented upon stimulation with LPS or R848. The data show the results from 17 chronic HCV patients and 14 healthy individuals.

Discussion

In this study, we evaluated the responses of CD16+CD14- monocytes to several TLR agonists as well as the function of CD16+CD14- monocytes from chronic HCV patients. We report here that CD16+CD14- monocytes isolated from healthy individuals are more responsive to TLR8 ligation by their production of TNF as compared to CD14+CD16- monocytes. In contrast, CD16+CD14- monocytes are less responsive to TLR4 ligation than CD14+CD16- monocytes. Comparison of chronic HCV patients and healthy individuals showed that the absolute numbers of monocytes and the ratio of CD16+CD14- cells to other monocyte populations in peripheral blood were similar. A detailed analysis of the functionality of CD16+CD14- monocytes in blood from chronic HCV patients showed no overt modulation as compared to healthy individuals, except for an augmented induction of MIP-1β producing CD16+CD14- monocytes upon TLR4 ligation in monocytes from chronic HCV patients as compared to healthy individuals.

In our patient cohort, we observed no differences between the absolute numbers of monocytes in peripheral blood from chronic HCV patients and healthy individuals. Also, the relative contribution of CD16+CD14- and CD14+CD16- monocytes in the circulation was similar in chronic HCV patients as compared with healthy individuals in our cohort. Our observations are in line with a recent study in chronic HCV patients in which the ratio of CD16+CD14- and CD14+CD16- monocytes was comparable with healthy subjects [23]. Although the composition of monocyte subpopulations does not change in as a consequence of infection with HCV, CD16+CD14- monocytes have been reported to be recruited to diseased human liver mediated via vascular adhesion protein-1 and CX3CL1 [20]. The preferential recruitment of the CD16+ monocyte population may induce differentiation into dendritic cells or Kupffer cells in the liver, thereby leading to augmented intrahepatic inflammation. However, in this study no livers from chronic HCV patients were examined for infiltration of monocytes, and we now show that the frequency of CD16+CD14- monocytes in blood of chronic HCV patients is not affected.

Although the frequency of CD16+CD14- monocytes is not affected as a consequence of chronic HCV infection, the function of this populated may be altered. In this study, we first examine the function of CD16+CD14- monocytes isolated from healthy subjects. We report here that CD16+CD14- monocytes stimulated with the TLR7/8 agonist R848 produce significantly higher levels of TNF as compared to CD14+CD16- monocytes from healthy individuals. It has been reported that TLR8 mRNA expression in CD16+CD14- and CD14+CD16- monocytes is similar [11, 24], which makes it plausible that the number of R848-responsive monocytes is comparable between CD16+CD14- and CD14+CD16- monocytes, This is confirmed by our findings that the percentages of TNF-producing CD16+CD14- and CD14+CD16- monocytes are similar (Figure 5B). The fact that CD16+CD14- monocytes produced higher levels of TNF than CD14+CD16- monocytes on a per-cell basis suggests that the signaling pathways downstream of TLR8 may differ in these two monocyte subpopulations. Indeed, in line with our findings, it was recently found that signaling cascades differ between both monocyte populations [11].

Using intracellular cytokine staining, we observed that the frequency of MIP-1β producing CD16+CD14- monocytes was significantly increased upon TLR4 ligation in chronic

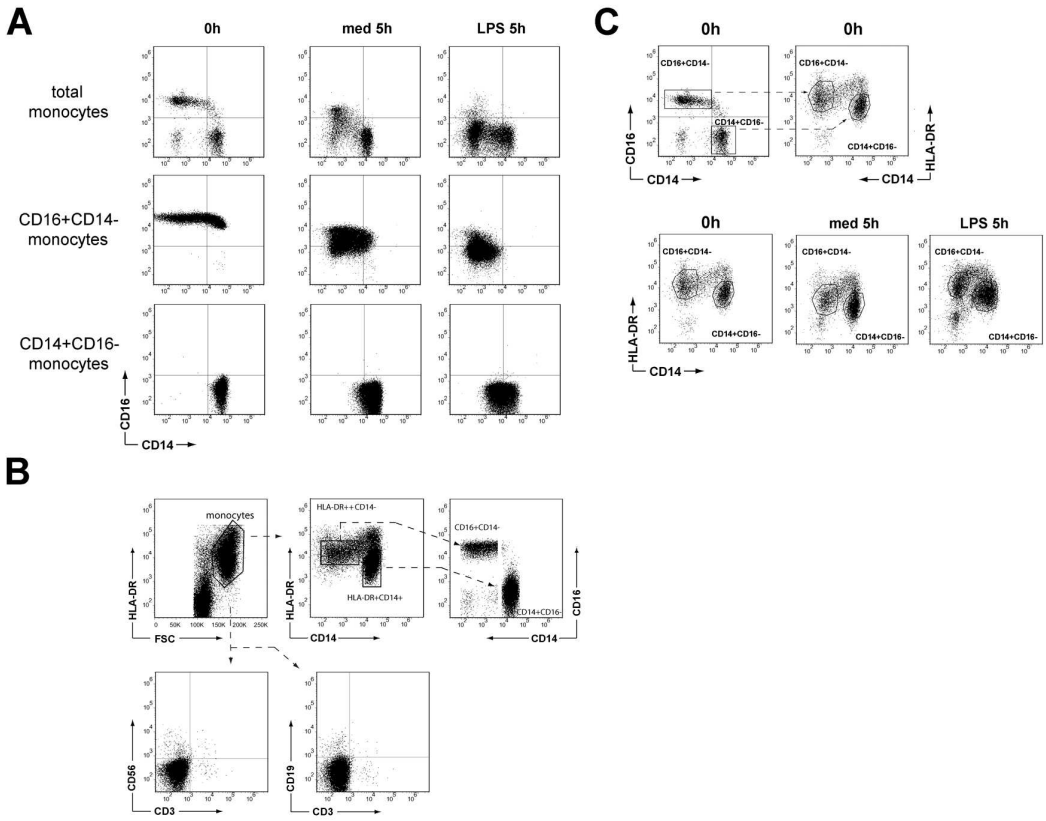
HCV patients. MIP-1β is an important chemokine in the pathogenesis of some inflammatory conditions and diseases [25]. For example, MIP-1β is one of the predictors for the severity of dengue patients [26] and induces inflammatory responses against pathogens such as influenza or parasites [27]. However, we find that the increased percentage of MIP-1β producing CD16+CD14- monocytes is not associated with age, viral load, and ALT and fibrosis level (data not shown). The immunomodulatory effects of MIP-1β are currently not completely known. Interestingly, it has been suggested that CD14+CD16- monocytes from HCV patients spontaneously secrete higher levels of MIP-1β, and that MIP-1β modulates the differentiation of monocyte-derived dendritic cells, resulting in reduced IFN-gamma production by allogeneic T cells [28]. Moreover, A number of studies have demonstrated higher MIP-1β levels in serum of chronic HCV patients as compared to healthy individuals, and importantly, MIP-1β levels are reduced upon therapy-induced viral load reduction in chronic HCV patients. Additionally, liver tissue obtained from chronic HCV patients also exhibit enhanced MIP-1β mRNA expression as compared to control liver tissue (Larrubia et al., 2008).

In summary, our findings show that CD16+CD14- monocytes are highly responsive to TLR8 ligation. We demonstrate that the frequency and function of CD16+CD14- monocytes are only minimally altered as a consequence of the persistent state of HCV infections, and our findings therefore do not suggest a role for CD16+CD14- monocytes in HCV pathogenesis.

Acknowledgements

We would like to thank Heleen van Santen for collecting blood samples from chronic HCV patients visiting our outpatient clinic, and Bettina Hanssen for statistical analyses. Furthermore, we are grateful to Jin Liu and Wenda Schoordijk for their excellent assistance in obtaining sorted monocytes.

Supplementary Figures



Supplementary Figure S1. $CD16^{+}CD14^{-}$ and $CD14^{+}CD16^{-}$ monocytes can be identified following in vitro stimulation by distinctive HLA-DR and CD14 expression. (A) The surface expression of CD16 and CD14 molecules was compared between freshly isolated monocytes (total monocytes, $CD16^{+}CD14^{-}$ and $CD14^{+}CD16^{-}$ monocytes) before and after culture with LPS for 5h. (B) Monocytes were identified on the basis of their FSC/SSC and the expression of HLA-DR, while antibodies against CD3, CD56 and CD19 were used to exclude contaminating cells. $HLA\text{-}DR^{+}CD14^{-}$ monocytes correspond to $CD16^{+}CD14^{-}$ monocytes, while $HLA\text{-}DR^{+}CD16^{-}$ monocytes correspond to $CD14^{+}CD16^{-}$ monocytes. (C) In vitro stimulation with LPS for 5h retained HLA-DR expression on monocytes, allowing identification of distinctive $CD16^{+}CD14^{-}$ and $CD14^{+}CD16^{-}$ monocytes using antibodies against HLA-DR and CD14 after in vitro stimulation.

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Chapter 5

Type I and III interferons increase the sensitivity of human monocytes and macrophages to IL-10 via enhanced IL10R expression and STAT3 phosphorylation

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Submitted

SUMMARY

Type I interferons (IFN) form the backbone of current therapy for chronic HCV patients, and to a lesser extent for chronic HBV infection. However, only about 30-50% of chronic HCV and HBV patients respond to IFN-based therapy. It has been suggested that the activity of type I IFN on antigen-presenting cells (APC) is weakened via modulation of the immunosuppressive cytokine interleukin-10 (IL-10). However, the effect of type I IFN on IL-10 production by immune cells is still under debate, and how IL-10 signaling is affected by type I IFN is not clear. Here we report that upon priming of human monocytes with type I IFN, the production of IL-10 is inhibited, whereas, unexpectedly, IL-10 still strongly controls TLR-induced IL-12p70 secretion. Furthermore, type I IFN pretreatment increases the sensitivity of monocytes to exogenous IL-10. These observations are explained by our findings that priming of monocytes with type I IFN augments membrane IL-10 receptor 1 expression, which may –at least partly- be responsible for enhanced IL-10-induced p-STAT3. Moreover, type I IFN as well as IL29, a member of the type III IFN family, comparably potentiate IL-10 signaling in macrophages, indicating a more general effect of IFN on modulating the activity of IL-10 in APC. In summary, we demonstrated that one of the consequences of priming of human APC with type I and III IFN is to promote the cells' sensitivity to IL-10. These findings are highly relevant to further improve IFN-based therapy for patients with multiple sclerosis or viral hepatitis.

INTRODUCTION

The human immune system is highly efficient to fight viral infections. However, a number of viruses, including human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV), have evolved mechanisms to escape eradication by the immune system. In these chronic viral infections, the functionality of both innate and adaptive immunity is compromised, including the antiviral interferon (IFN) pathways as well as the activity of dendritic cells (DC), NK cells and virus-specific T cells (1-3). Furthermore, during persistent viral infections elevated levels in serum of the immunosuppressive cytokine IL-10 and enhanced production of IL-10 by immune cells have been reported (4-7). The inhibitory effect of IL-10 on the development of an effective antiviral immune response and the maintenance of persistence has been demonstrated in mice where chronic LCMV infection was resolved by blockade of the IL-10 receptor (IL-10R) (7-8).

IL-10 inhibits pro-inflammatory responses by strongly suppressing a broad spectrum of activities of the innate immune system, which indirectly affects adaptive immunity. As a consequence of the IL-10 mediated suppression, IL-10 may prevent immunopathology, whereas it creates favorable conditions for the persistence of pathogens. IL-10 can be produced by a wide variety of cells (9-12). and the receptor for IL-10 is a heterodimeric complex composed of IL-10R1 and IL-10R2. (9-10). The IL-10R1 chain is unique for IL-10, and has a more restricted expression than IL-10R2, which is also part of the receptor for IL-22 and IL-26 (9, 13). Upon ligation of IL-10 to the IL-10R, Jak1 and Tyk2 induce tyrosine phosphorylation and activation of STAT3, STAT1 and STAT5 (14-16). While STAT1 and STAT5 do not appear to interact directly with IL-10/IL-10R complex (9), STAT3 is recruited directly to the IL-10/IL-10R complex and is required for IL-10 signaling (17-19).

Type I IFN play a crucial role in the defense against viral infections via the induction of the expression of IFN-stimulated genes (ISG), such as 2,5-OAS and MxA (20). In addition, type I IFN are also known to regulate the production of IL-12: IFNα has an inhibitory effect on IL-12p40 production by both mice and human monocytes, DC and macrophages (21-22). In contrast to the effect on IL-12p40, the production of IL-12p70 is enhanced by exposure to IFNα in monocytes and DC (23). Less is known on the interaction of type I IFN and IL-10, although this is highly relevant with respect to the outcome of infection with potentially persistent viruses as well as to further improve the efficacy of treatment with type I IFN in for instance patients chronically infected with HCV. It has been reported that IFNα or IFNβ present during the differentiation or activation of human monocyte-derived DC and macrophages enhance the production of IL-10 (21-25). However, the effects of type I IFN on IL-10 production by human monocytes are still under debate. While some studies showed that either pretreatment or direct stimulation of IFNα enhanced IL-10 production by monocytes in response to TLR stimulation (26-27), other groups showed the contrary (28-29).

In this study, we investigate in detail the effect of type I IFN on TLR-induced IL-10 production by monocytes as well as the effect of type I IFN on IL-10 signaling events. We demonstrate that IFNα pretreatment inhibits TLR-induced IL-10 production by human monocytes. Despite a significant decrease in production, IL-10 is still able to potently control TLR-induced IL-12p70 secretion by IFNα-primed monocytes. Furthermore, we observed that type I IFN exposure resulted in up-regulation of IL-10R1 expression on the surface of

monocytes, and consequently an increased IL-10-induced p-STAT3. Interestingly, potentiation of IL-10 signaling in IFNα-pretreated monocytes was also observed following IFNβ priming. Type I IFN and type III IFN (IL-29) similarly enhanced IL-10-induced p-STAT3 in human monocytes and macrophages, suggesting a general function of type I and III IFN on IL-10 signaling in APC.

MATERIALS AND METHODS

Cell culture and purification

PBMC were isolated from peripheral blood of buffycoats (Sanquin) by gradient-density centrifugation. Monocytes were purified from PBMC with magnetic CD14-microbeads (Miltenyi Biotec) following the manufacturer's instructions. The purity of the monocytes used in the study was always above 97%. Macrophages were generated from purified monocytes with 10 ng/ml M-CSF (R&D) in 6-well plates (Costar) at a density of $1.5x10^6$ cells/well in 2 ml RPMI1640 supplemented with 8% FCS**.** On day 2 and day 5, half of the medium was refreshed and on day 6 monocyte-derived macrophages were harvested, and used for various purposes.

PBMC stimulation and intracellular cytokine staining

PBMC were cultured with serum-free X-VIVO15 medium (BioWhittaker) in 24-well plates (Greiner Bio-one BV), and stimulated overnight with LPS (100 ng/ml, InvivoGen) or R848 (1 μg/ml, Alexis), with brefeldin A (10 μg/ml; Sigma) added 2h after the addition of TLR agonists. In some experiments, PBMC were pretreated with IFNα (10 ng/ml, Intron; Schering Plough) for 5h and then further with medium, LPS or R848 overnight, and brefeldin A added together with TLR agonists. Samples were then fixed, permeabilized and stained with IL-10-APC (JES3-9D7, BD Pharmingen) and TNF-PE-Cy7 (MAb11, eBioscience). Cytokine producing cells were detected by flow cytometry (Canto-II, BD).

Stimulation of monocytes and monocyte-derived macrophages

Monocytes were pretreated with IFNα (10 ng/ml) for 5h and then further cultured with medium, LPS, R848 or LPS plus R848 for another 24h at a density of $0.2x10^6$ cells/well in a volume of 200 µl. Monocyte-derived macrophages were pretreated with IFNα (10 ng/ml), IFNβ (10 ng/ml, PeproTech), or IL-29 (100 ng/ml, PeproTech) for 5h and then further cultured with medium, LPS, R848 or LPS plus R848 for another 24h at a density of $0.2x10^6$ cells/well in 200 µl medium. In order to investigate the role of IL-10 in IFN-pretreated cells, anti-IL-10R antibody (αIL-10R, 10 µg/ml, 3F9, Biolegend) was added to some cultures. In addition, IFNα-pretreated monocytes were exposed to exogenous IL-10 (R&D) at different concentrations in some experiments. Cytokine levels were determined by ELISA.

Flow cytometric analysis of the expression of IL-10R1 and IL-10R2

Monocytes were pretreated with IFNα (10 ng/ml) for 5h. Monocytes were then stained with antibodies against IL-10R1-PE (3F9, Biolegend) and IL-10R2-Biotin (R&D). Streptavidin-PerCP (BD Pharmingen) was used to visualize the IL-10R2. The specificity of the stainings was controlled with appropriate isotype antibodies.

Flow cytometric analysis of p-STAT3 staining

Monocytes were pretreated with IFNα (10 ng/ml) for 5h and monocyte-derived macrophages were pretreated with IFNα (10 ng/ml), IFNβ (10 ng/ml), or IL-29 (100 ng/ml) for 5h, with the last hour on ice. Monocytes and monocyte-derived macrophages were then incubated with IL-6 (10 ng/ml, R&D), IL-27 (10 ng/ml, R&D) or IL-10 (R&D) at the indicated concentrations for the indicated periods. Stimulated cells were immediately fixed with BD Phosflow Lyse/Fix (BD Bioscience) and then permeabilized with BD Phosflow Perm Buffy III (BD Bioscience). Cells were then incubated with anti-p-STAT3-PE (pY705, 4/P-STAT3, BD Bioscience) and the phosphorylation state of STAT3 was measured by flow cytometry (Canto-II, BD).

Immunoassay for detection of cytokines in supernatant

The concentrations of cytokines in supernatant were determined using sandwich ELISA specific for IL-12p40 (C8.6 and C8.3 antibody pairs, Biolegend), and Ready-Set-Go kits for IL-12p70, IL-23, IL-10 and TNF (all from eBioscience). The detection limits for IL-10, IL-12p70, IL-23 and TNF were 15 pg/ml and for IL-12p40 30 pg/ml.

Statistics

Data was analyzed with Prism 5.0 (Graphpad software) using the Mann-Whitney t-test to compare variables between two paired groups. In all analyses, a two-tailed p-value of less than 0.05 was considered statistically significant.

Results

IFNα priming decreases the percentage of IL-10-producing monocytes in response to LPS or R848 stimulation

TLR stimulation of monocytes leads to relatively high production of IL-10 and TNF as compared to other blood leukocytes (data not shown). To examine the immunomodulatory effects of IFNα on cytokine production by monocytes, PBMC were pretreated with IFNα and further stimulated with TLR agonists. As shown in Figure 1A, IFNα pretreatment decreased the percentage of IL-10-producing monocytes in response to LPS stimulation from 11.9% to 4.4%, and from 13.2% to 3.9% in response to TLR8 ligation using R848 (Figure 1A). The inhibitory effect of IFNα on TLR-induced IL-10-producing monocytes was observed in the majority of individuals, whereas the percentages of TNF-producing monocytes upon TLR ligation were not or only mildly affected by IFNα (Figure 1B). The observed effect of IFNα on TNF production indicates that the inhibitory effect on IL-10 is not the consequence of reduced monocyte viability.

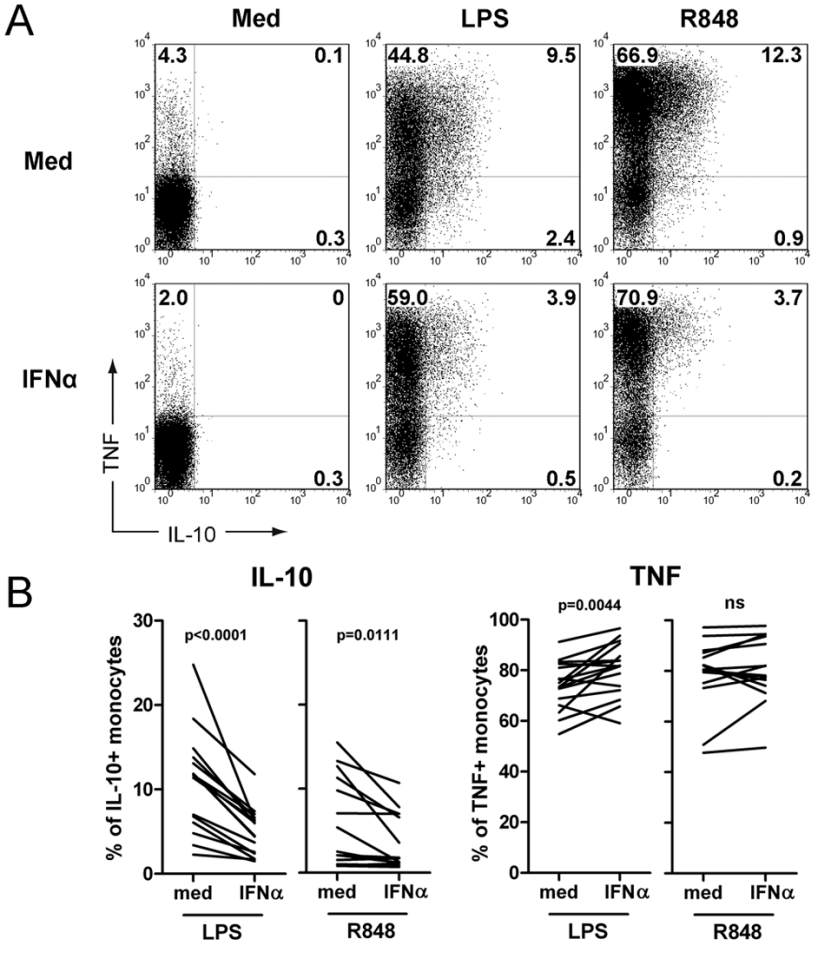

Figure 1. IFNα priming decreases the percentage of IL-10-producing monocytes in response to LPS or R848. PBMC were pretreated with IFNα for 5h and further stimulated overnight with medium, LPS (100 ng/ml) or R848 (1 μg/ml), and brefeldin A was added together with TLR agonists. Representative plots **(A)** and the results of 11 independent experiments are presented **(B)** showing IL-10- and TNF-producing cells gated on total monocytes.

IFNα pretreatment inhibits IL-10, whereas it enhances IL-12p70 production by purified monocytes upon R848 ligation

To further examine whether IFNα pretreatment has a direct inhibitory effect on IL-10 production by monocytes in response to TLR triggering, we examined the effect of IFNα on highly purified human monocytes. As shown in Figure 2A and 2B, IFNα pretreatment significantly inhibited LPS- and R848-induced IL-10 production by purified monocytes in the majority of healthy individuals, whereas LPS- and R848-induced TNF production by monocytes was not affected by IFNα (Figure 2B). IFNα only has a mild or no effect on TLR4 or TLR8 mRNA expression in monocytes (Figure S1A), indicating that the inhibitory effect of IFNα on TLR-induced IL-10 production by monocytes is not likely due to modulation of TLR mRNA expression. Importantly, IFNβ, which uses the same receptor as IFNα, also inhibits TLR-induced IL-10 production by human monocytes (Figure S1B).

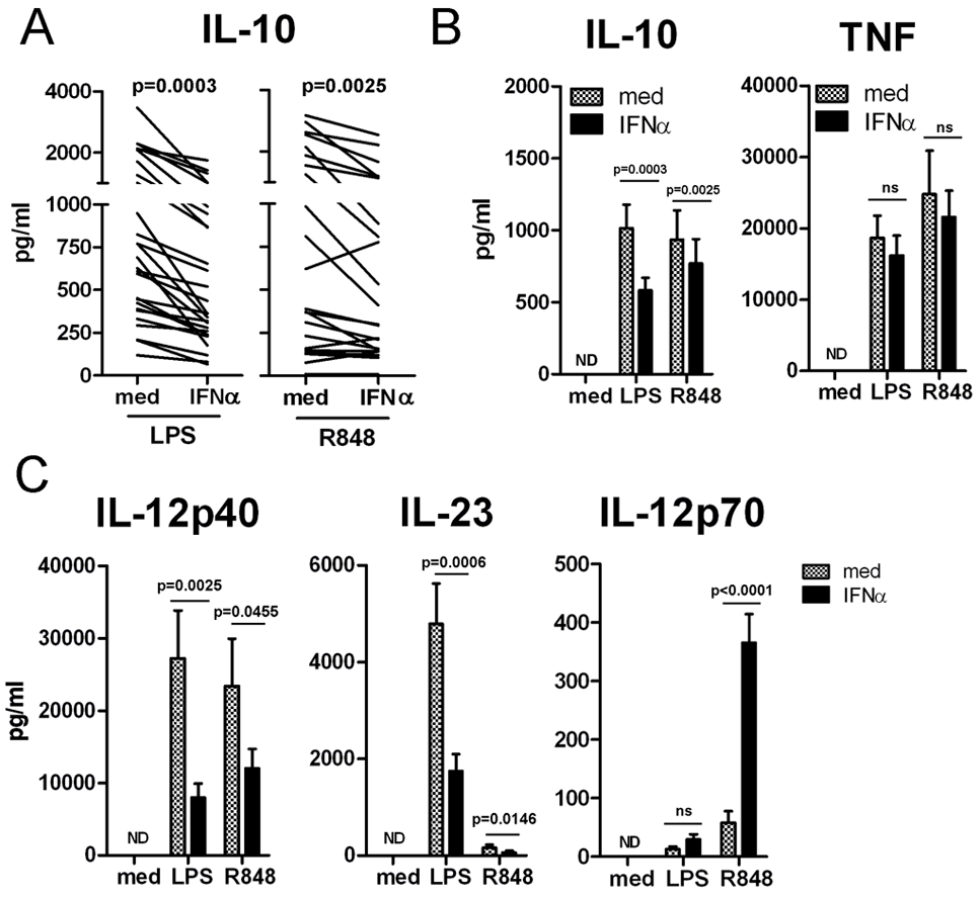

Figure 2. IFNα pretreatment inhibits IL-10 whereas it enhances IL-12p70 production by monocytes upon R848 ligation. Human purified monocytes were pretreated with IFNα for 5h and then further stimulated for 24h with medium, LPS (100 ng/ml) or R848 (1 μg/ml). IL-10 **(A, B)**, TNF **(B)**, IL-12p40, IL-23 and IL-12p70 **(C)** production were measured by ELISA. The values depicted in **(B, C)** show the mean +/- SEM of 27 independent experiments. LPS-induced IL-12p70 production by monocytes was detected in 3 out of 27 independent experiments and the levels of IL-12p70 was not higher than 50 pg/ml.

Consistent with previous reports (21, 27, 29), we found that IFNα inhibits IL-12p40 production by monocytes in response to TLR stimulation, which was accompanied by reduced IL-23 levels (Figure 2C). However in contrast to IL-23, IFNα increased IL-12p70 production by monocytes upon R848 stimulation (Figure 2C). In the majority of the individuals, no IL-12p70 production by LPS-challenged monocytes was observed. Together these findings indicate that IFNα inhibits the production of IL-10, IL-12p40 and IL-23 by TLR-stimulated monocytes, while IFNα pretreatment promotes the production of IL-12p70.

Despite reduced IL-10 levels, R848-induced IL-12p70 secretion by IFNα-pretreated monocytes is still controlled by IL-10

To examine whether enhanced TLR-induced IL-12p70 production by IFNα-pretreated monocytes can be explained by the effect of IFNα on IL-10 production, we performed experiments in which IL-10 signaling was blocked using antibodies to the IL-10R. Similar as presented in Figure 2C, we observed that stimulation of monocytes with LPS induced no or low levels of IL-12p70, which was only weakly enhanced by blocking the IL-10R or by pretreatment with IFNα (Figure 3A). In contrast, although R848 induced low levels of IL-12p70 by monocytes, blocking the IL-10R resulted in increased IL-12p70 production upon R848 stimulation (Figure 3A; from 51 pg/ml to 370 pg/ml). As shown before, pretreatment of monocytes with IFNα resulted in enhanced R848-induced IL-12p70 production (Figure 2C and 3A). However, in these cultures, additional blocking of the IL-10R during R848 stimulation potently enhanced the production of IL-12p70 (Figure 3A; from 385 pg/ml to 1365 pg/ml). Regulation by IL-10 of R848-induced IL-12p70 production by IFNα-pretreated monocytes was observed in the majority of individuals examined (Figure 3B). In contrast to IL-12p70 production, R848-induced IL-12p40 and IL-23 production by both untreated monocytes and IFNα-pretreated monocytes were only minimally affected by IL-10 (Figure 3A). Moreover, although IL-10 suppressed LPS-induced IL-12p40 and IL-23 production by untreated monocytes, the inhibitory effect of IFNα on LPS-induced IL-12p40, IL-23 and TNF levels were independent on IL-10 signaling (Figure 3A and data not shown).

Our findings indicate that, despite IFNα-mediated inhibition of IL-10 levels, IL-12p70 production by IFNα pretreated monocytes is IL-10-dependent, which suggests that IFNα-pretreated monocytes have enhanced responsiveness to IL-10. To further investigate this, we examined the sensitivity of IFNα-pretreated monocytes to exogenous IL-10. As shown in Figure 3C and 3D, upon exposure to IL-10, the production of IL-12p70 by monocytes in response to R848, or LPS plus R848 was suppressed by exogenous IL-10 at concentrations higher than 0.1 ng/ml. Interestingly, TLR-induced IL-12p70 levels by IFNα-pretreated monocytes were suppressed by exogenous IL-10 at concentrations lower than 0.1 ng/ml (Figure 3C and 3D). In summary, these data suggest that IFNα priming of monocytes alters

the responsiveness of monocytes to IL-10, which controls their TLR8-induced IL-12p70 production.

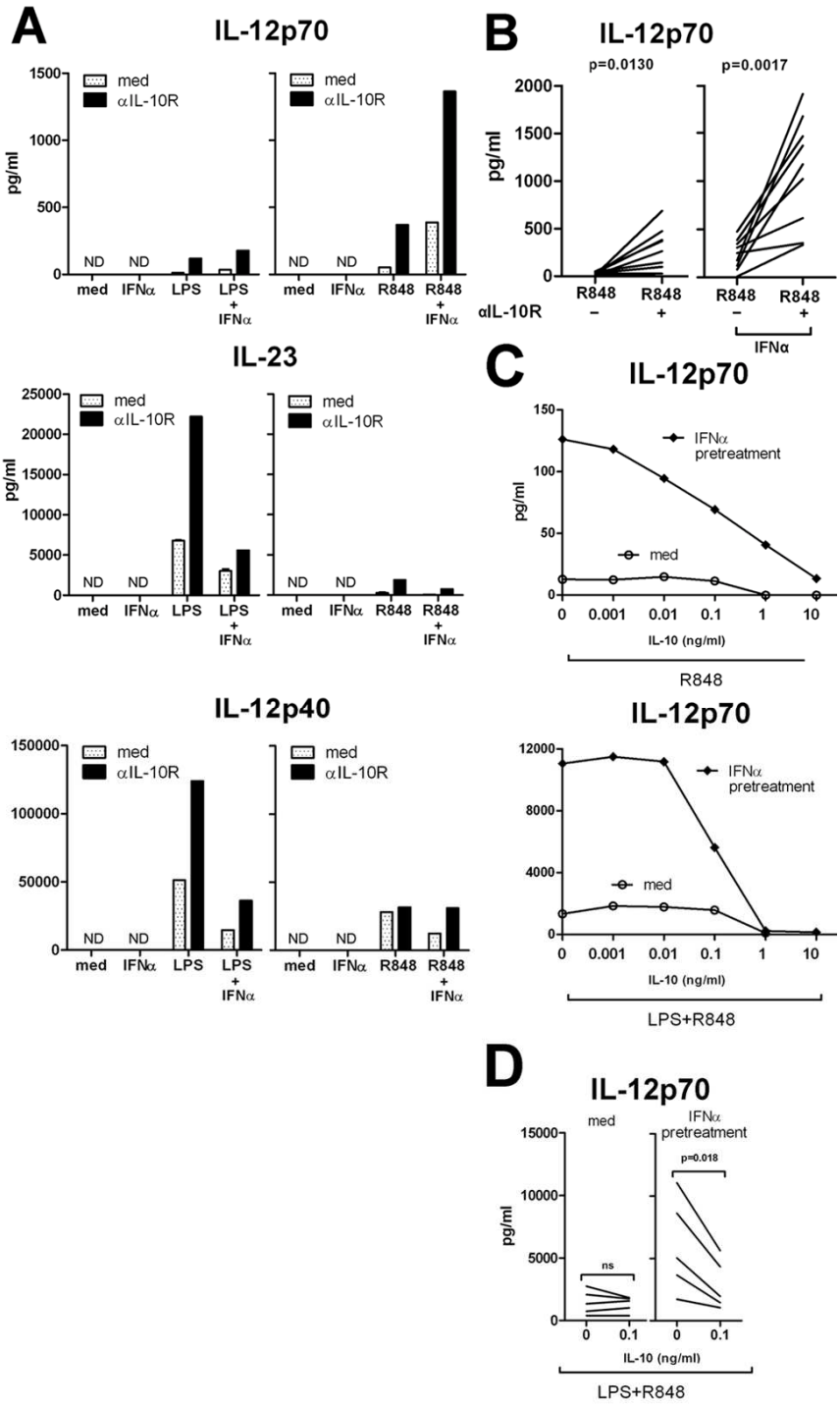

Figure 3. Despite a significant decrease in production, IL-10 strongly controls R848-induced IL-12p70 secretion by IFNα-pretreated monocytes. **(A, B)** Purified monocytes were pretreated with IFNα for 5h and then further stimulated for 24h with medium, LPS (100 ng/ml) or R848 (1 μg/ml) in the presence or absence of αIL-10R. IL-12p70**,** IL-12p40 and IL-23 production were measured by ELISA. The values of IL-23 and IL-12p40 depicted in **(A)** show representative data from **(B)** 4 independent experiments. **(C, D)** Monocytes were pretreated with IFNα for 5h and then further stimulated for 24h with medium, LPS (100 ng/ml), R848 (1 μg/ml) or the combination of LPS and R848 in the presence of indicated concentrations of IL-10.

IFNα up-regulates IL-10R1 expression on the surface of human monocytes

Since IFNα-primed monocytes show enhanced responsiveness to exogenous IL-10 as indicated by R848-induced IL-12p70 production, we examined the effect of IFNα on IL-10R expression on the surface of monocytes. As shown in Figure 4, both IL-10R1 and IL-10R2 are expressed on the surface of monocytes. Upon exposure of monocytes to IFNα, we observed that the IL-10R1 was up-regulated on the surface of monocytes in 10 out of 14 independent experiments, whereas IL-10R2 was not affected.

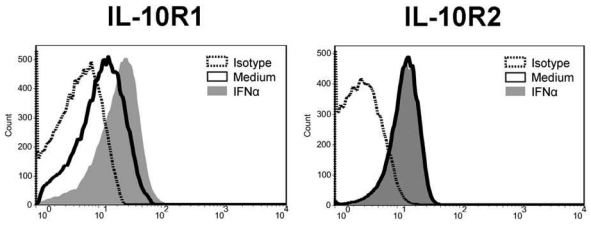

Figure 4. IFNα up-regulates IL-10R1 expression on the surface of monocytes. Monocytes were pretreated with IFNα for 5h and IL-10R1 and IL-10R2 expression on the surface of monocytes were assessed by flow cytometry. The histograms depict representative data from 14 independent experiments.

IFNα treatment enhances IL-10-induced STAT3 phosphorylation in human monocytes

To further examine whether IL-10 signaling in monocytes is enhanced by pretreatment with IFNα, we evaluated the effect of IFNα on the levels of phosphorylated STAT3 in monocytes upon IL-10 challenge. As expected, IL-10 induced phosphorylation of STAT3 in monocytes: the percentage of p-STAT3 positive monocytes increased from 2.1% to 23.1% (Figure 5A). Interestingly, IL-10-induced p-STAT3 in monocytes was further enhanced by pretreatment of IFNα, which was observed in the majority of individuals (Figure 5B). The levels of p-STAT3 by IFNα-treated monocytes increased in a dose-dependent manner with increasing concentrations of IL-10 (Figure S2A and S2B). Next, we investigated whether the augmented IL-10/p-STAT3 signaling was sustained in IFNα-primed monocytes as compared to untreated monocytes. As shown in Figure S2C and S2D, p-STAT3 induced by IL-10 in unprimed monocytes peaked at 30min and decreased to base-line levels at 120min. Although higher p-STAT3 levels were observed in IFNα-primed monocytes, the kinetics of STAT3 phosphorylation was similar between IFNα-primed and untreated monocytes, indicating that IL-10 signaling is not sustained in monocytes upon IFNα priming. To determine whether the effects of IFNα on the JAK/STAT3 signaling are IL-10 specific or more general, we examined the response to IL-6 and IL-27, which also signal via JAK/STAT3. We observed that in contrast to the effect of IFNα on IL-10 signaling, pretreatment of IFNα resulted in inhibition of IL-6- or IL-27-induced p-STAT3 in monocytes (Figure 5C and 5D). Therefore, IFNα pretreatment enhances IL-10-derived STAT3 phosphorylation in human monocytes, which provides an explanation of the above findings that IFNα priming renders monocytes more sensitive to the suppressive effect of IL-10 on TLR-induced IL-12p70 production.

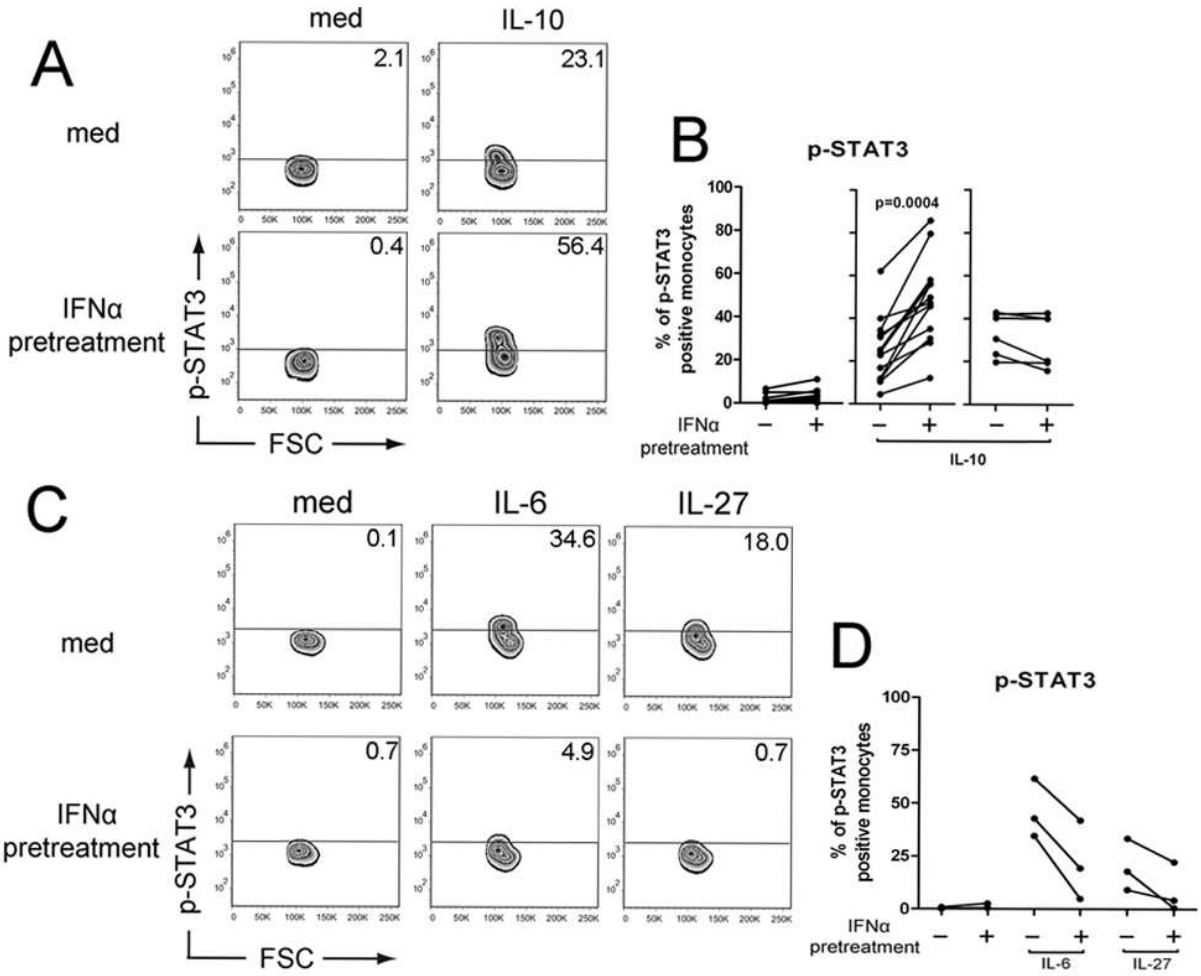

Figure 5. IFNα treatment enhances IL-10-induced STAT3 phosphorylation in human monocytes. Monocytes were pretreated with IFNα for 5h, with the last 1h on ice. Monocytes were then incubated with IL-10 (100 ng/ml) for 30min **(A, B)**, or with IL-6 (10 ng/ml) or IL-27 (10 ng/ml) for 30min **(C, D)**. STAT3 phosphorylation (p-STAT3) was evaluated by flow cytometry.

IFNβ and IL-29, similar as IFNα, increase IL-10-induced p-STAT3 in human monocytes and macrophages

To examine whether the augmented IL-10-induced p-STAT3 in monocytes is unique for IFNα, we investigated whether pretreatment of monocytes with IFNβ resulted in comparable effects. Similar to IFNα, IFNβ up-regulated surface expression of IL-10R1 on monocytes (Figure S3A). In addition, as shown in Figure 6A and Figure S4A, higher IL-10-induced percentages of p-STAT3 positive monocytes were observed following priming with IFNβ, as compared with unprimed monocytes. These findings indicate that IFNβ and IFNα regulate IL-10/STAT3 signaling events in a similar manner in monocytes.

Besides monocytes, also macrophages are major IL-10 producers, and are also responsive to IL-10 (9, 30) as well as to IFNα and IFNβ. Moreover, we recently showed that a member of the type III IFN family, IL-29, modulates cytokine production of human macrophages (21). Therefore, we decided to examine the effect of type I and III IFN on IL-10-induced p-STAT3 in human monocyte-derived macrophages. As shown in Figure 6B and

Figure S4B, similar to monocytes, high levels of p-STAT3 were induced in macrophages by IL-10. Importantly, in the majority of individuals, priming of macrophages with IFNα, IFNβ and IL-29 resulted in enhanced IL-10-induced p-STAT3. In line with the findings presented for monocytes, also priming of macrophages with IFNα, IFNβ and IL-29 resulted in up-regulation of the surface expression of IL-10R1 on human monocyte-derived macrophages (Figure S3B), which indicates that type I and III IFN are able to enhance IL-10 signaling events in macrophages. To further examine IL-12p70 production by TLR-challenged macrophages, we blocked the IL-10R using antibodies. Relatively high levels of IL-12p70 were only found in LPS plus R848 stimulated macrophages, but not in LPS- or R848-stimulated macrophages, and the production of IL-12p70 was only moderately enhanced by IFN priming or by blocking the IL-10R (Figure 6C). However, the levels of IL-12p70 were substantially increased only when macrophages were primed with type I IFN and stimulated in the absence of IL-10 signaling, indicating that, upon IFNα or IFNβ pretreatment, IL-10 strongly controls TLR-induced IL-12p70 production by human macrophages. However, interestingly, in the absence of IL-10 signaling, IL-29 priming did not enhance IL-12p70 production by macrophages in response to LPS plus R848 when compared to pretreatment of IFNα or IFNβ (Figure 6C). These data show that upon pretreatment with type I and III IFN, IL-10-induced STAT3 phosphorylation is enhanced in human monocytes and macrophages, demonstrating that IL-10 signaling in these cells is potentiated by IFN priming, most likely as a consequence of enhanced IL-10R expression.

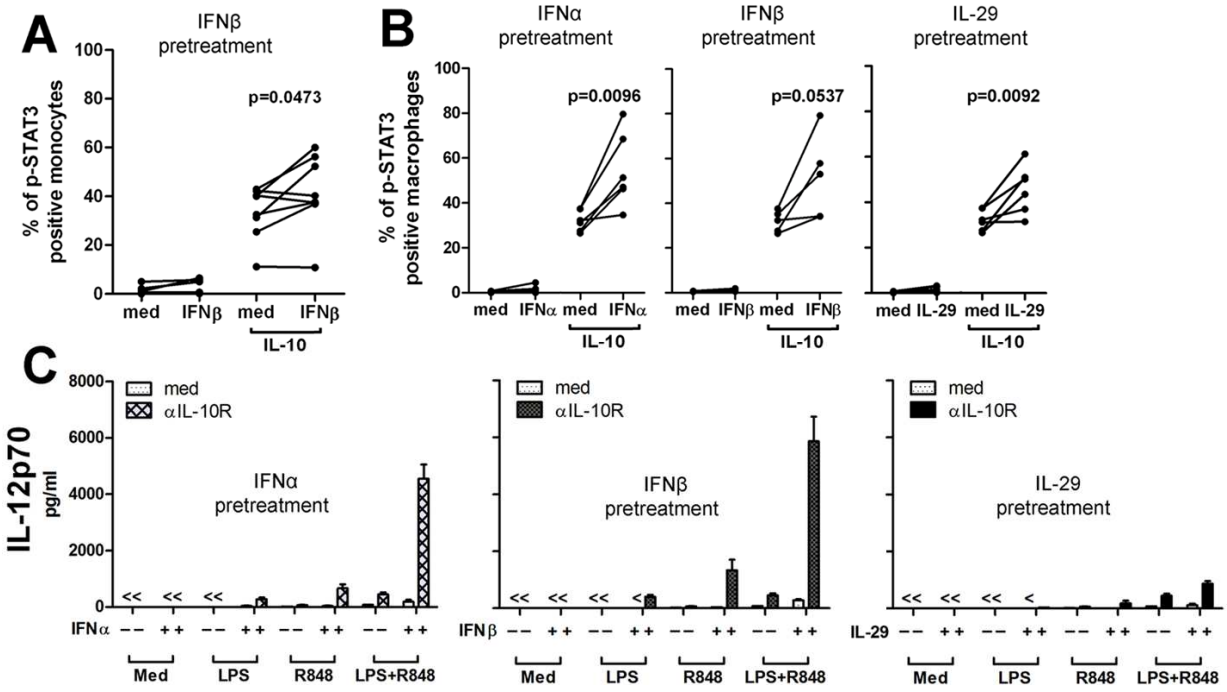

Figure 6. Similar to IFNα, IFNβ and IL-29 pretreatment enhance IL-10-induced STAT3 phosphorylation in monocytes and macrophages. **(A)** Human monocytes were pretreated with IFNβ for 5h with the last 1h on ice. Monocytes were then incubated with IL-10 (100 ng/ml) for 30min. p-STAT3 levels were examined by flow cytometry. **(B)** Monocyte-derived macrophages were

pretreated with IFNα, IFNβ and IL-29 for 5h, with the last 1h on ice. Macrophages were then stimulated by IL-10 (100 ng/ml) for 30min. **(C)** Monocyte-derived macrophages were pretreated with IFNα, IFNβ and IL-29 for 5h and then further stimulated for 24h with medium, LPS (100 ng/ml), R848 (1 μg/ml) or LPS plus R848 in the presence or absence of αIL-10R. IL-12p70 levels were measured by ELISA. The values depicted in **(C)** show the mean +/- SEM from 3 independent experiments.

Discussion

In this paper, we examine the effect of type I IFN on TLR-induced IL-10 production by human monocytes as well as the effect of type I IFN on IL-10-induced signal events. We find that type I IFN inhibit IL-10 production by human monocytes in response to TLR ligation. However, despite a significant decrease in production, IL-10 is still able to potently control TLR-induced IL-12p70 secretion by type I IFN-primed monocytes. This is explained by our finding that type I IFN priming strengthens IL-10 signaling in monocytes as demonstrated by up-regulation of IL-10R1 on type I IFN-primed monocytes, which results in enhanced IL-10-induced phosphorylation of STAT3. Interestingly, type I IFN and type III IFN (IL-29) similarly enhance IL-10-induced p-STAT3 in human monocytes and macrophages, whereas IL-6- and IL-27-induced p-STAT3 in monocytes are decreased upon treatment with type I IFN.

Type I IFN are known to prime immune response by modulating the function of immune cells. In this study, we show that type I IFN inhibit TLR-induced IL-10, IL-12p40, IL-23 and MIP-1β production by human monocytes (Figure 2 and data not shown), which is in line with previous reports showing that type I IFN inhibit TLR-induced IL-10 (28-29), IL-12p40 (21, 27, 29), and IL-8 (31-32) production by APC. Currently, the mechanism by which TLR-induced cytokines are inhibited by type I IFN is not understood. The inhibitory effect of IFNα on TLR-induced cytokine production by monocytes is unlikely due to the regulation of TLR mRNA expression, since we only observed a mild effect of IFNα on TLR mRNA expression in monocytes. Furthermore, TNF production by IFNα-primed monocytes was not affected and IL-12p70 production was enhanced, indicating that the inhibitory effect of IFNα is not the consequence of reduced cell viability. Interestingly, interferon-stimulated responsive elements (ISREs) are reported to be present in the promotors of the *IL-8*, *IL-12p40* and *IL-23p19* genes (27, 31-33) and ISRE in the promotor region of the *IL-8* gene are required for type I IFN-mediated inhibition of IL-8 production (31-32). Therefore, it is possible that type I IFN inhibit TLR-induced IL-10 production by monocytes via similar mechanisms. With two different methods, we observed an inhibitory effect of IFNα on TLR-induced IL-10 production. Previously, Byrnes *et al.* reported that IFNα priming up-regulated IL-10 mRNA expression in human monocytes in response to *Staphylococcus aureus* Cowan strain (SAC) combined with IFNγ (27). However, in that study, the multiple TLR signaling pathways induced by SAC stimulation as well as the combined effect of IFNα and IFNγ on IL-10 production by monocytes make it difficult to make a direct comparison with the current study.

Despite a significant inhibition of IL-10, TLR-induced IL-12p70 production is potently controlled by IL-10 in IFNα-pretreated monocytes. Surprisingly, IFNα priming renders monocytes more sensitive to IL-10 as evidenced by the inhibition of TLR-induced IL-12p70 secretion. Indeed, we find that both IFNα and IFNβ up-regulate membrane IL-10R1

expression on monocytes. It is reported that up-regulation of IL-10R1 is crucial for human neutrophils to become fully responsive to IL-10 (34). Also, it has been suggested that any stimulus activating IL-10R1 expression renders the targeted cells responsive to IL-10 (9). These reports suggest that the up-regulation of the membrane expression of IL-10R1 is an important mechanism to modulate IL-10 signaling pathway. In line with IL-10R1 expression, we further observe that IL-10 induces a higher level of p-STAT3 in type I IFN-primed monocytes from the majority of individuals examined, while the kinetics of IL-10-induced p-STAT3 is not affected. The enhanced IL-10-induced p-STAT3 by type I IFN could be explained by the up-regulation of IL-10R1 on the membrane of monocytes, but it is also possible that type I IFN directly affects downstream IL-10 signaling, since we and others found an induction of p-STAT1 by IL-10 in IFNα-primed monocytes (35). Previously, it was reported that IFNα priming has no effect on IL-10 induced p-STAT3 but results in STAT1 phosphorylation by IL-10 in monocytes (35). However, in that study, human monocytes were primed with IFNα in the presence of M-CSF for 2 days allowing the cells to differentiate, while in our study, the cells were primed with type I IFN for 5h in the absence of M-CSF. It is important to mention that it has been suggested that M-CSF is able to enhance LPS-induced p-STAT1 in murine bone marrow-derived macrophages and also affects IFN signaling in these cells (36).

The effect of type I IFN on IL-10 signaling is a general effect on APC, since, similar to monocytes, type I IFN pretreatment also strengthens IL-10 signaling in human macrophages via up-regulating membrane IL-10R1 expression, and enhancing IL-10-induced p-STAT3 in macrophages, which results in a potent inhibition of TLR-induced IL-12p70 production by IL-10 in those macrophages. Moreover, pretreatment of IL-29, a member of type III IFN, also results in enhanced IL-10 signaling in macrophages, indicating that the effects of type I IFN on IL-10 signaling seem to be a common activity of type I and III IFN. However, type I IFN appear to have different effects on TLR-induced cytokine profiles by APC. We report here that type I IFN inhibit TLR-induced IL-10 production by monocytes, whereas its production is increased by monocyte-derived DC and macrophages (21-25), as well as by mouse bone marrow-derived macrophages (37). In line with this, we also observed that IFNα has a different effect in human monocytes, monocyte-derived DC and macrophages when TLR-induced TNF, IL-12p40 and IL-23 production are evaluated (data not shown). Furthermore, TLR mRNA expression in monocytes and monocyte-derived macrophages are also differentially regulated by type I IFN. IFNα has no or only mild effects on TLR8 mRNA expression in monocytes, whereas it potently up-regulates TLR8 mRNA expression in monocyte-derived macrophages (21). Combined, these findings suggest that type I IFN stimulate distinct signaling pathways in monocytes as compared to DC or macrophages. It was reported that the concentration of type I IFN affects the biological outcome of triggering

of the receptor on specific leukocyte populations. Low levels of IFNα promote the cellular responses to IFNγ, whereas relatively high levels inhibit these responses (21, 38-39). It is possible that the effective doses for type I IFN differ among distinct APC due to variations in receptor density, and consequently the signaling upon type I IFN is not identical in these cells.

Our findings are relevant in light of the use of type I IFN in the standard of care therapy to treat chronic HCV patients as well as in patients suffering from multiple sclerosis (40-41). It will be important to examine whether enhanced IL-10R expression and consequently augmented responsiveness to IL-10 following exposure to type I IFN is also observed in patients during the course of therapy. Furthermore, our findings warrant further studies to examine if enhanced IL-10 sensitivity of leukocytes can explain the limited clinical benefit of IFN-based treatment in a large number of patients.

In summary, we find that type I IFN inhibit TLR-induced IL-10 production but strengthen IL-10 signaling in monocytes via up-regulating membrane IL-10R1 expression and enhancing IL-10-induced p-STAT3 in human monocytes. We further demonstrate that the effect of type I IFN on IL-10 signaling among APC is a general function of type I and III IFN. The findings of this study have uncovered an important indirect suppressive effect of type I and III IFN on APC via strengthening IL-10 signaling, and are thus important for the understanding and improvement of type I or III IFN-based therapies in patients suffering from multiple sclerosis or viral hepatitis.

Supplementary Figures

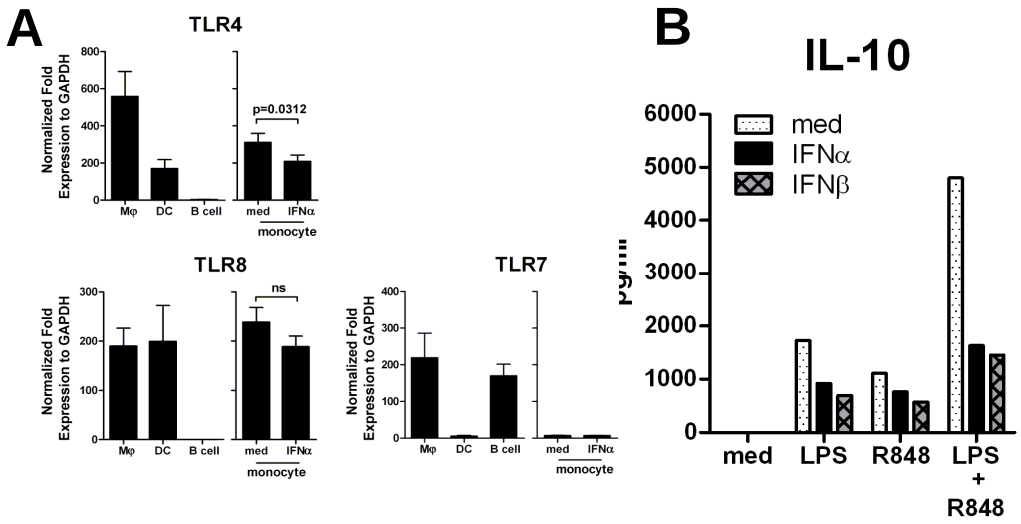

Figure S1. The effect of type I interferon on TLR mRNA expression and IL-10 production by human monocytes. **(A)** Human monocytes were purified from healthy individuals (n=6), with purity above 97%, and then stimulated with IFNα (10 ng/ml) for 5h. Total RNA from monocytes was extracted using NucleoSpin RNA II kit (Macherey-

Nagel) according to the manufacturer's instructions. RNA was quantified using a Nanodrop ND-1000 (Thermo). cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). All real-time PCR reactions were performed in Bio-Rad optical 96-well plates using a MyIQ5 detection system (Bio-Rad Laboratories). SYBR-Green present in the MasterMix Plus (Eurogentec) was used for quantification in real-time PCR reactions for TLR4 and TLR7. Primers for GAPDH (forward 5'-TGCACCACCAACTGCTTAGC-3' and reverse 5'-GGCATGGACTGTGGTCATGAG-3'), TLR4 (forward 5'-TCTACAAAATCCCCGACA-3' and reverse 5'-AGGTGGCTTAGGCTCTGA-3') and TLR7 (forward 5'-AATGTCACAGCCGTCCCTAC-3' and reverse 5'-GCGCATCAAAAGCATTTACA-3') were designed to determine the TLR4 and TLR7 mRNA expression. Furthermore, primer-probes for GAPDH (Hs00266705_g1) and TLR8 (Hs00152972_m1) were purchased from Applied Biosystems. The expression of target genes was normalized to GAPDH using the formula: $2^{-\Delta Ct}$, $\Delta Ct = Ct_{TLR} - Ct_{GAPDH}$. **(B)** Human purified monocytes were pretreated with IFNα (10 ng/ml) or IFNβ (10 ng/ml) for 5h and then further stimulated with LPS (100 ng/ml), R848 (1 ug/ml) or LPS plus R848 for another 24h. IL-10 production was measured by ELISA. The values depicted in **(B)** show the representative data from 3 independent experiments.

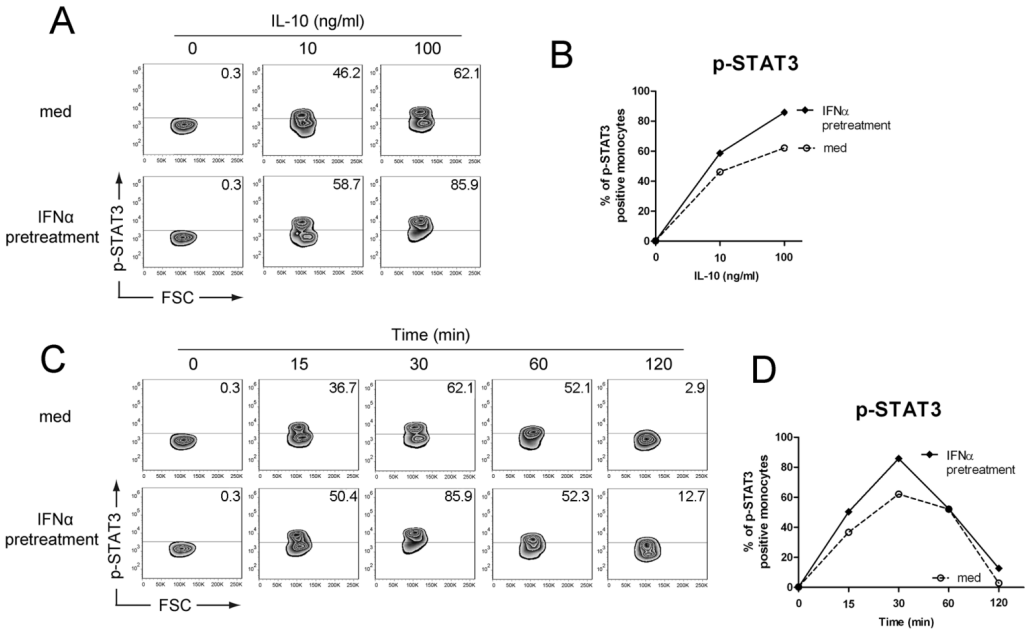

Figure S2. The levels of p-STAT3 by IFNα-treated monocytes increased in the IL-10 dose-dependent while the kinetics of IL-10-induced p-STAT3 is not affected. Monocytes were pretreated with IFNα for 5h, with the last 1h on ice. Monocytes were then incubated with various concentrations of IL-10 for 30min **(A, B)** or with IL-10 (100 ng/ml) at the indicated time-points **(C, D)**. STAT3 phosphorylation (p-STAT3) was evaluated by flow cytometry.

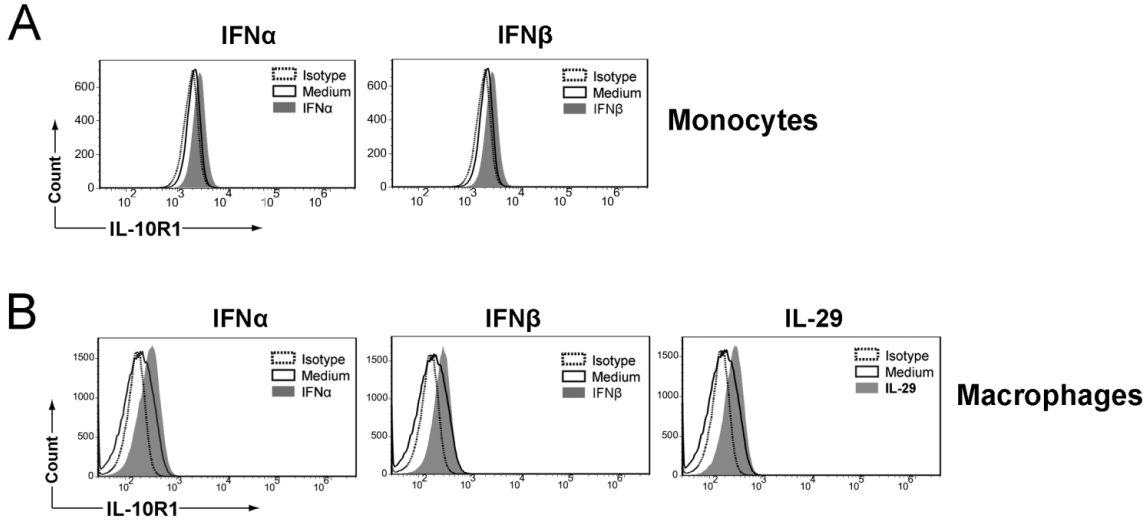

Figure S3. Type I and III IFN up-regulate IL-10R1 expression on human monocytes and macrophages**.** Monocytes or monocyte-derived macrophages were pretreated with IFNα, IFNβ or IL-29 for 5h and IL-10R1 expression on the surface of monocytes **(A)** and macrophages **(B)** was assessed by flow cytometry. The histograms depict representative data from 3 independent experiments.

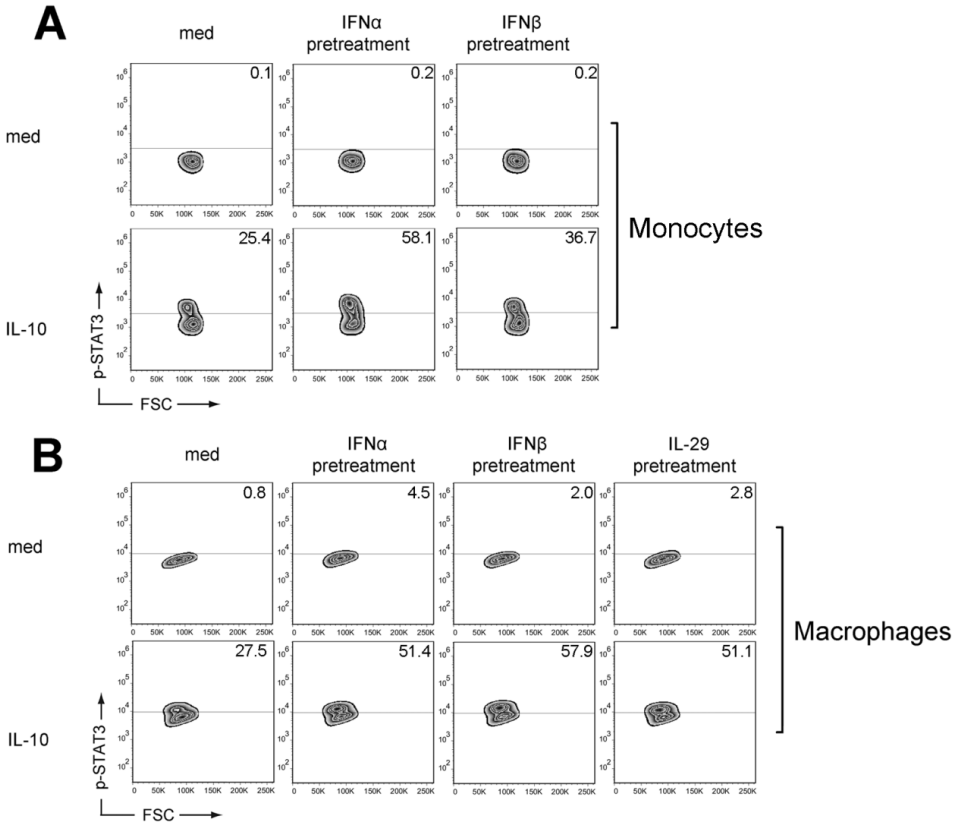

Figure S5. Type I and III IFN enhance IL-10-indiced pSTAT3 in human monocytes and macrophages**. (A)** Human monocytes were pretreated with IFNα or IFNβ for 5h with the last 1h on ice. Monocytes were then incubated with IL-10 (100 ng/ml) for 30min. **(B)** Monocyte-derived macrophages were pretreated with IFNα, IFNβ and IL-29 for 5h, with the last 1h on ice. Macrophages were then stimulated by IL-10 (100 ng/ml) for 30min. The levels of p-STAT3 levels examined by flow cytometry.

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Chapter 6

Potent immune activation in chronic hepatitis C patients upon administration of an oral inducer of endogenous interferon that acts via TLR7

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Antiviral Therapy, in press.

SUMMARY

Background ANA773, an oral prodrug of a small-molecule TLR7 agonist, induces a dose-related decrease in serum HCV RNA levels in chronic hepatitis C patients.

Methods The prodrug ANA773 was administered to healthy individuals and chronic hepatitis C patients. At different time-points during the course of treatment, modulation of the phenotype and function of peripheral leukocytes were evaluated to determine the role of distinct immune cells on the clinical outcome of therapy.

Results Early after administration of the TLR7 agonist, a mild, transient reduction of the number of lymphocytes was observed in both healthy individuals and chronic hepatitis C patients. Moreover, repeated administration of ANA773 resulted in transiently reduced numbers of myeloid and plasmacytoid dendritic cells (DC) in blood. Interestingly, reduced plasmacytoid DC numbers as well as increased serum IFN-α and IP-10 levels were observed only in virological responders (≥1 $\log_{10}$ IU/mL reduction of HCV RNA levels upon ANA773 treatment), but were absent in virological non-responders. *In vitro* stimulation of peripheral blood mononuclear cells from virologic responders showed a high frequency of IFN-α-producing plasmacytoid DC upon stimulation *in vitro* with ANA773, whereas no IFN-α was induced in non-responders.

Conclusions These findings indicate that the viral load decline in chronic hepatitis C patients treated with the TLR7 agonist ANA773 is likely due to intrinsic differences in the induction of endogenous interferons and interferon-stimulated gene products (IFN-α and IP-10) upon TLR7 ligation.

INTRODUCTION

The hepatitis C virus (HCV) is a major cause of chronic liver disease, affecting more than 170 million individuals globally. In about 80% of individuals infected with HCV, the infection does not resolve spontaneously, resulting in persistent infection. Chronic HCV infected patients are at increased risk for developing liver fibrosis, cirrhosis and/or hepatocellular carcinoma, which may take decades to become apparent. The long-term complications of liver failure, as a result of chronic HCV infection, are worldwide the most common causes for liver transplantation [1, 2]. At present, no vaccine to prevent persistent HCV infection is available. The standard treatment for chronic HCV infection is pegylated IFN-α plus ribavirin. This combination therapy has many adverse effects, and a sustained viral response is only observed in about 50% of HCV genotype 1 infected patients. Thus, improved therapies are urgently needed.

Patients who eventually develop chronic hepatitis C, initially have a strong T cell response, but this response is not sustained. In fact, during chronic infections HCV-specific $CD4^+$ and $CD8^+$ T cell responses are difficult to detect in blood and liver, and are functionally impaired, which may be a direct consequence of high viral load, viral escape mutations, or due to active suppression mediated by immunoregulatory mechanisms [3-6].

Stimulation of the immune system in order to boost antiviral immunity is the basis for research in search of effective T cell vaccines against HCV [7]. However, an alternative approach is to activate the innate immune system making use of its ability to respond to pathogen-derived products. Activation of DC and macrophages by pathogens can be achieved by the specific interaction between pattern recognition receptors, such as the members of the TLR family, and pathogen-derived products [8, 9]. Distinct leukocyte populations in both mice and humans have been shown to express different TLRs, and consequently to respond to distinct microbial products [10, 11]. For example, human plasmacytoid DC express TLR7 mRNA, and respond to specific TLR7 agonists, such as single-stranded RNA and R848, to produce type I interferons [12].

Activation of the innate immune system by intravenous administration of a TLR7 agonist isatoribine [13] and oral administration of the TLR7/8 agonist resiquimod [14] have been previously described for the treatment of chronic hepatitis C patients. However, the latter compound interacts with TLR7 and TLR8 and therefore activates not only plasmacytoid DC but also other leukocytes such as monocytes [15], leading to more severe adverse effects. We recently reported the first results of the clinical study in which the TLR7 agonist ANA773 was administered to chronic HCV infected patients via oral administration [16]. In this trial, we observed a significant treatment-induced viral decline of serum HCV RNA levels (range 0.14 to -3.10 log at the highest dosing group receiving 2000 mg), which was observed in some, but not all patients. In the current study, we examined the immunological effects following oral administration of the TLR7 agonist ANA773 in patients, and evaluated the immunological differences between responders and non-responders.

MATERIALS AND METHODS

Study design

The characteristics of the chronic hepatitis C patients and healthy individuals who participated in this study have been described in detail before [16]. This study was a phase 1 study, which was conducted at the Erasmus Medical Center (Rotterdam), Academic Medical Center (Amsterdam) and PRA International (Zuidlaren), the Netherlands, in accordance with Good Clinical Practice and the World Medical Association Declaration of Helsinki, after approval by the institutional review board. All patients and healthy individuals provided written informed consent before participating in any study-related activity. For the ancillary study the cohorts of chronic HCV infected patients receiving a dose of 1600 mg or 2000 mg ANA773 were evaluated for immune status, as well as a cohort of healthy controls receiving 1600 mg ANA773. The highest dose cohorts were examined since considerable reductions of serum HCV RNA load were observed in these cohorts. In the 1600 mg group, 6 chronic HCV infected patients received oral ANA773 and 2 received placebo. In the 2000 mg group, 8 patients received ANA773 and 2 received placebo. Blood samples of the 1600 mg group were drawn on day 0, 5, 13, 27, and 41; the blood samples of the 2000 mg group were drawn on day 0, 5, 9 and 18. No blood was collected from one patient in each dosing group, and therefore immunological assays were performed on PBMC from 5 patients in the 1600 mg group and from 7 patients in the 2000 mg group. The patient details are described before [16]. In addition, the *IL28B* SNP rs12979860 was determined for all patients using competitive allele-specific PCR (KASP; KBioscience, Hoddesdon, UK). In the 1600 mg group, the patients unresponsive to ANA773 had the TC, TC and CC genotype, while the responsive patients both had the CC genotype. In the 2000 mg group, both non-responders to ANA773 were TC, while in patients responding to ANA773 2 individuals had the CC genotype and 3 individuals the TC genotype. Patients were dosed with oral ANA773 every-other-day for either 28 days (1600 mg group) or 10 days (2000 mg group). Study medication (100 mg capsules) and placebo capsules were supplied by Anadys Pharmaceuticals, Inc., San Diego, USA.

Patients

Key inclusion criteria included male and female chronic HCV patients between 18 to 65 years, with body mass indexes of 18 to 35 kg/m^2, treatment-naive or relapse from prior IFN-based therapies (defined as recurrence of HCV RNA following a full course of treatment and having achieved an undetectable HCV RNA during treatment), and an HCV RNA level ≥ 75 x 10^3 IU/mL. Key exclusion criteria included decompensated liver disease, findings consistent with Child Pugh B/C liver cirrhosis, and co-infection with HIV or HBV. Patients receiving antiviral therapy or immunomodulatory therapy within 90 days prior to administration of the first dose of ANA773 were excluded.

Enumeration of monocytes and leukocytes in whole blood, and quantitation of lymphocyte subpopulations

Absolute numbers of leukocytes, lymphocytes, monocytes and granulocytes in whole blood were measured by an automated impedance hematology analyzer (ABX Micros-60, Horiba

Medical). To determine the frequency of distinct leukocyte subpopulations, whole blood was lysed using ammoniumchloride, stained with antibodies against CD4 (SK3, BD), CD8 (RPA-T8, BD), CD56 (MY31, BD), CD19 (SJ25C1, BD), CD14 (61D3, eBioscience), BDCA1 and BDCA4 (both from Miltenyi Biotech). NK cells were defined as CD3-negative lymphocytes that expressed CD56. This population included both $CD56^{dim}$ and $CD56^{bright}$ NK cells. In addition, the expression of CD69 on NK cells was assessed using CD56-PE (MY31, BD) and CD69-APC (L78, BD). All events were evaluated by flow cytometry (Canto-II, BD), and the data was analyzed using BD FACS Diva software. All assays were performed on the day of blood collection.

Intracellular cytokine staining

PBMC were isolated from peripheral blood of patients prior to treatment with ANA773 (2000 mg-group only). Cells were isolated from peripheral blood by density centrifugation on Ficoll-Hypaque (GE healthcare). PBMC were stimulated on the day of blood collection with medium, ANA773 (300 μM) or R848 (1 μg/ml; Alexis) in RPMI-1640 medium (BioWhittaker) supplemented with 10% human serum for 5h, with brefeldin-A (10 μg/ml; Sigma) present for the last 4h. Samples were then fixed with 2% formaldehyde, permeabilized with 0.5% saponin and stained with antibodies against CD14-Pacific Blue (M5E2, BD Pharmingen), BDCA4-APC (AD5-17F6, Miltenyi Biotech), TNF-PE-Cy7 (MAb11, eBioscience), and IFN-α-FITC (MMHA-1, PBL). Cytokine-producing plasmacytoid DC and monocytes were detected by flow cytometry (Canto-II, BD).

Immunoassay for detection of cytokines

The levels in serum of IFN-α and IP-10 during the course of treatment with ANA773 were detected by enzyme-linked immunosorbent assays by Alta Analytical Laboratory, San Diego, USA. 2,5-OAS was analyzed by radio-immunoassay at PRA International, Assen, The Netherlands.

Statistics

Values are expressed as mean values, unless indicated otherwise. Data was analyzed with Prism 5.0 (Graphpad software) using the Mann-Whitney t-test to compare variables between two independent groups. In all analyses, a two-tailed p-value of less than 0.05 (confidence internal 95%) was considered statistically significant.

Results

Administration of TLR7 agonist ANA773 leads to a transient reduction of the absolute number of lymphocytes in blood of healthy individuals and HCV infected patients

To examine the consequence of administration of the TLR7 agonist ANA773 on immune parameters, we first assessed the effect of treatment on the absolute numbers of various leukocyte subpopulations prior to treatment and 6 hours after the first administration by comparing paired blood samples. As shown in Figure 1, treatment of healthy individuals with a dose of 1600 mg ANA773 every other day did not affect the absolute numbers of peripheral leukocytes, monocytes or neutrophils. Comparable findings were observed when chronic HCV infected patients were treated with a dose of 1600 mg or 2000 mg ANA773 every other day, except for the number of monocytes, which declined within 6 hours following administration of 2000 mg TLR7 agonist. The absolute numbers of lymphocytes was significantly reduced 6 hours after start of treatment in both healthy individuals (dose 1600 mg) and chronic HCV patients (dose 1600 and 2000 mg).

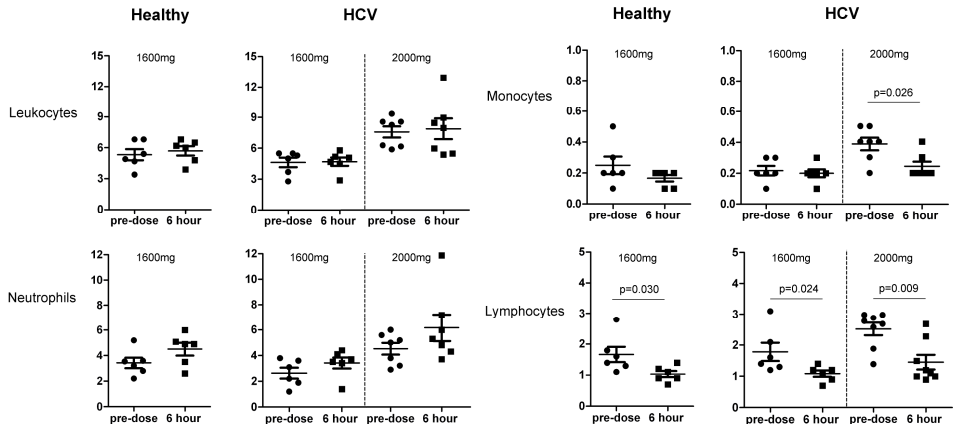

Figure 1. The effect of ANA773 on the numbers of blood leukocytes early after administration. Healthy individuals and chronic hepatitis C patients were administered a single dose of 1600 mg or 2000 mg ANA773. Blood was collected before and 6h after administration. The absolute numbers of cells were determined and shown for individual patients.

The reduction in the number of lymphocytes 6 hours after administration of ANA773 was transient, since the number of leukocytes, lymphocytes and monocytes was similar as their pre-treatment numbers after day 5 (Figure 2A). Further phenotyping of the lymphocytes in $CD4^+$ T cells, $CD8^+$ T cells, $CD3^-CD56^+$ NK cells and $CD19^+$ B cells did not demonstrate any significant shifts in cell numbers during the treatment period.

Administration of ANA773 leads to a transient reduction of the number of plasmacytoid DC only in virologic responders

Since ANA773 interacts with the TLR7, which is expressed at high levels by plasmacytoid DC, we determined the numbers of plasmacytoid DC and myeloid DC, in blood of chronic hepatitis C patients during treatment. As shown in Figure 2B, repeated administration of 1600 mg ANA773 showed a reduction of plasmacytoid DC numbers in blood and myeloid DC

which was most prominent on day 13 ($1.5x10^6$ to $0.8x10^6$ cells/l and $9.4x10^6$ to $3x10^6$ cells/l, respectively), and returned to baseline levels thereafter. Similar to the 1600 mg group, multiple dosing of 2000 mg ANA773 showed the same trend with respect to the decline of the numbers of DC, which was not significant.

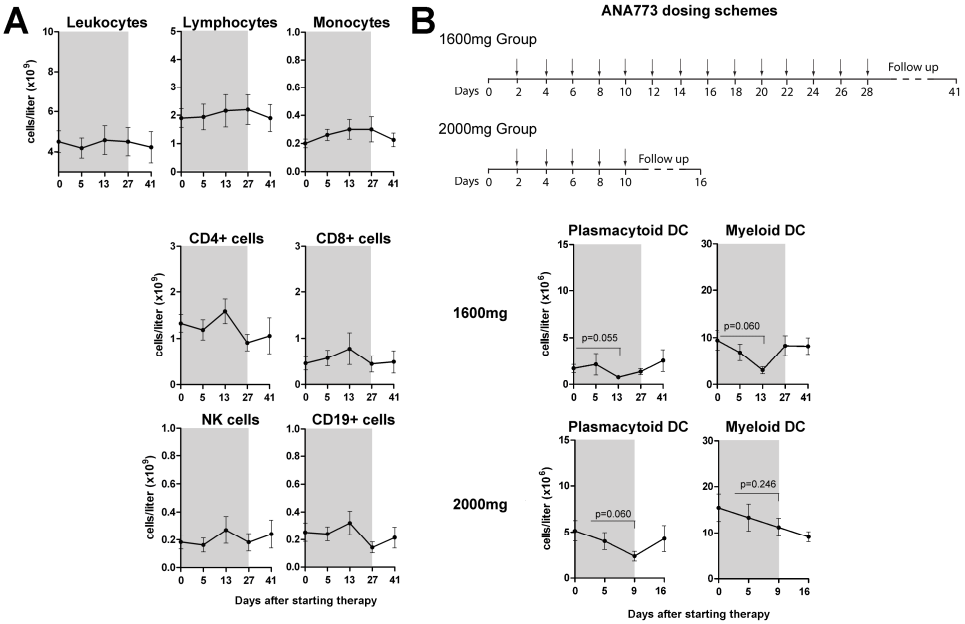

Figure 2. Repeated administration of ANA773 does not influence the number of leukocyte subpopulations over a period of 4 weeks. (A) Chronic hepatitis C patients were treated with ANA773 every 48h for a period of 28 days (1600 mg), and blood was collected at the indicated time-points. Leukocyte subpopulations were determined in whole blood by automated analyses and flowcytometry as described in the material and methods. (B) The effect of ANA773 on DC populations was determined in whole blood of patients treated with 1600 mg ANA773 (as described in above) or 2000 mg, which was administered every 48h for 10 days.

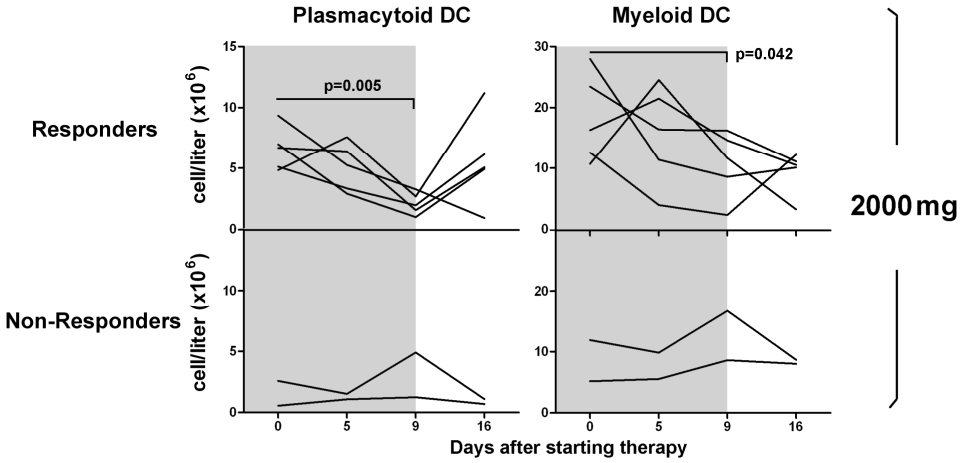

Figure 3. Reduced plasmacytoid DC numbers in chronic HCV infected patients with a decline of HCV RNA levels upon treatment with ANA773, but not in non-responders. Chronic HCV patients were treated with ANA773 at a dose of 2000 mg. The absolute numbers of plasmacytoid DC and myeloid DC are presented at different time points after start of treatment, and displayed separately for patients with a viral decline of more than one log (responders) or less than one log (non-responders).

Administration with 2000 mg ANA773 resulted in a viral load reduction of more than one log in 5 out of 7 patients. We determined whether the differential clinical responsiveness was reflected by a differential effect on the numbers of plasmacytoid DC. Indeed, as shown in Figure 3, all patients who were considered responders to treatment with TLR7 agonists showed a significant reduction of circulating plasmacytoid DC and myeloid DC numbers at day 9, which was not observed in patients who did not respond to TLR7 ligation. Shortly after ending treatment at day 10, plasmacytoid DC numbers recovered in responders, whereas the number of myeloid DC were still reduced in some, but not all, patients. It is interesting to note that the baseline plasmacytoid DC frequency is lower in the 2 non-responder patients as compared to the responder patients, which was also observed when examining the non-responder patients of the 1600 mg group (Supplementary Figure S1).

Differential effects of TLR7-induced responses in virologic responders versus non-responders

To explore the differences between the observed effects of TLR7 ligation in chronic hepatitis C patients who responded and patients who were non-responders, we examined the serum levels of interferon stimulated genes IFN-α, IFN-induced protein IP-10 and mRNA levels for 2,5-OAS. As presented in Figure 4A, IFN-α and IP-10 were detectable in serum from most responders, but undetectable in patients who did not respond to ANA773 as defined by no reduction of serum HCV RNA levels. However, in both responder and non-responders to TLR7 ligation, the levels of 2,5-OAS mRNA in serum were induced 6 hours after start of treatment.

In addition, we examined the activation status of NK cells in treated patients. By performing flow-cytometry, we observed that 6h after the first administration, the expression of the early activation marker CD69 was increased on the majority of $CD3^{-}CD56^{dim}$ NK cells in responding patients, but not non-responding patients (Figure 4B). We did not observe TLR7-induced changes of activation markers expressed on plasmacytoid DC or myeloid DC, such as CD80, CD86 or CD40, at different time-points following ANA773 administration (data not shown).

Finally, we compared the *in vitro* response of PBMC to ANA773 and R848 (a TLR7/8 agonist) with the patient's subsequent virologic response to ANA773 treatment. As shown in Figure 5, a high frequency of IFN-α-producing plasmacytoid DC upon stimulation *in vitro* was detected in PBMC from patients who were subsequently virologic responders, whereas no IFN-α was induced in cells from non-responders. As a control experiment, we observed that monocytes were unresponsive to ANA773, whereas activation by R848 induced a high frequency of TNF-producing monocytes. These findings suggest that the *in vitro* assay may be used as a screening tool for the expected efficacy of antiviral activity of TLR agonists such as ANA773, and that intrinsic properties of plasmacytoid DC may determine the efficacy of treatment with TLR7 agonist of patients with chronic HCV infections.

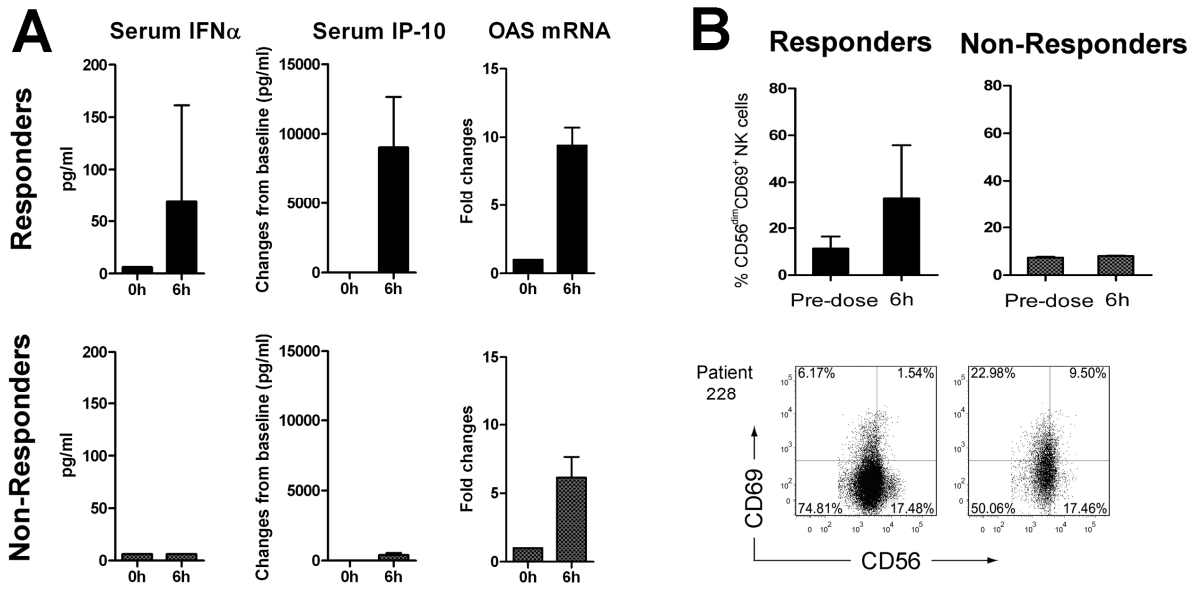

Figure 4. *Ex vivo analysis demonstrates stronger activation of immunity in virologic responders to ANA773 as compared to non-responders.* (A). The serum levels of IFN-α and IP-10 were determined by ELISA, and the 2,5-OAS levels in serum by RIA before and 6h after start of treatment. (B). The expression of CD69 on CD56-expressing cells is determined in whole blood before and 6h after start of treatment.

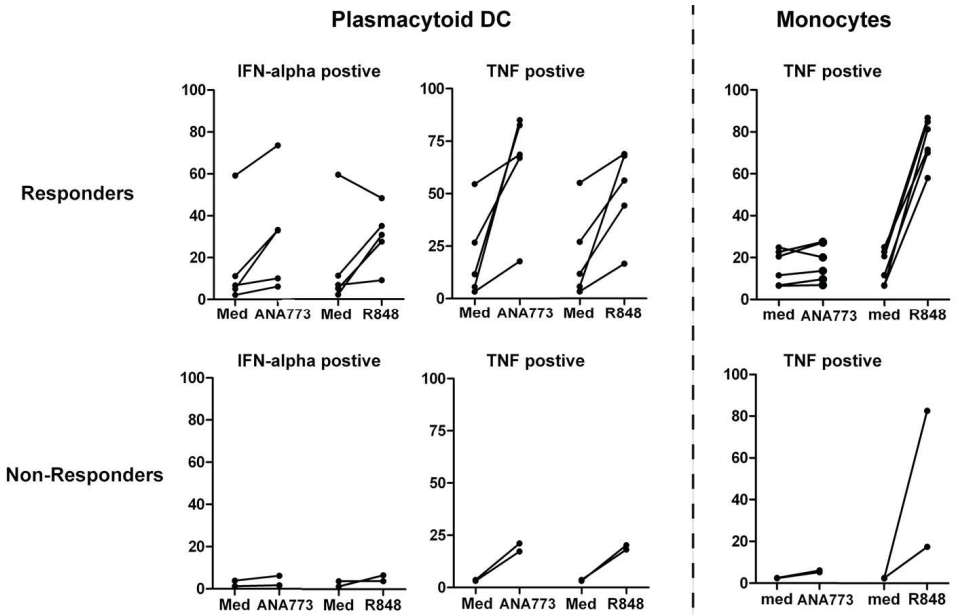

Figure 5. The frequency of IFN-α-producing plasmacytoid DC in vitro was higher in PBMCs from patients that were subsequently virologic responders, whereas no IFN-α was induced in non-responders. PBMC, collected prior to treatment with 2000mg ANA773, were stimulated in vitro with medium, ANA773 or R848. The percentage of cytokine producing plasmacytoid DC and monocytes was determined by intracellular cytokine staining for IFN-α and TNF.

Discussion

At present, TLR7 agonists to treat HCV infection are not used in clinical practice. These compounds act by specifically inducing antiviral activity initiated by the induction of endogenous IFN-α as well as by specific TLR7-induced activation of various leukocyte populations, such as plasmacytoid DC. Direct stimulation of the immune system may be an important advantage over the use of exogenous IFN-based antiviral therapy, which does not lead to activation of leukocyte populations. Previously, intravenous administration of a TLR7 agonist isatoribine [13] and oral administration of the TLR7/8 agonist resiquimod [14] has been described in the treatment of chronic hepatitis C patients. The disadvantage of the combined TLR7/8 agonist resiquimod over specific TLR7 agonists is that TLR8 is also expressed on monocytes, and will thus induce pro-inflammatory cytokines other than IFN-α (Figure 5). The consequence of this is a higher chance of adverse effects [17], and this was indeed observed in the clinical study with resiquimod [14].

The present study demonstrates that treatment of chronic hepatitis C patients with the TLR7 agonist ANA773 activates the immune system by the release of IFN-α and IFN-α-induced molecules as well as the NK cell compartment. We demonstrate that oral administration of TLR7 agonists leads to a mild and transient reduction of circulating lymphocytes, plasmacytoid DC and myeloid DC in viral responders to ANA773 treatment. As a direct consequence of TLR7 ligation, or indirectly as a result of enhanced IFN-α activity, viral responders exhibited increased IP-10 and 2',5'-OAS. Together with activated NK cell activity, this illustrated that important components of the antiviral immune responses were activated upon ANA773 administration. In addition, elevated levels of circulating IFN-α and IP-10, as well as TLR7-induced activation of NK cells, were only demonstrated in patients with a significant drop in HCV RNA levels upon treatment with TLR7 agonists. Differential responsiveness to TLR7 ligation upon treatment could be reproduced *in vitro*, suggesting that intrinsic differences between patients accounted for the different efficacy of ANA773. Interestingly, also evaluation of the effect of ANA773 on PBMC from healthy individuals showed induction of IFN-α by plasmacytoid DC in the majority of individuals (8 out of 10 individuals; data not shown).

Despite activation of various components of the innate antiviral immune response, the decline of serum HCV RNA levels was mild. To explain this, we can not exclude that the highest dose of ANA773 administered in this study was still suboptimal with respect to viral decline. As an alternative explanation, it has been described that the TLR7 signaling pathway is selectively impaired in plasmacytoid DC [18] and monocyte-derived DC [19] from chronic HCV infected patients, as well as in hepatoma cell lines [20]. However, we show that upon oral administration of ANA773, no differences were observed between healthy individuals and chronic hepatitis C patients in the immune parameters examined, which were mainly focused on shifts in leukocyte populations and the expression of activation markers. Moreover, functionally, plasmacytoid DC from chronic HCV infected patients were still capable of responding to TLR7 ligation using either ANA773 or R848, indicating that plasmacytoid DC were not completely inert to stimulation via TLR7. Another possible explanation for the modest viral decline observed after ANA773 administration is the

reduction of the number of circulating plasmacytoid DC, which may affect the IFN-α levels that are induced during therapy. TLR ligation as well as exogenous administration of IFN-α in mice also showed a transient lymphopenia which was the result of redistribution rather than deletion of lymphocytes [21]. At present, the transient nature of the response is not clear. However, tight regulation of TLR7 expression may lead to lower responsiveness of cells to TLR ligation upon repeated exposure to the ANA773.

We observed that not all patients responded to ANA773 administration with regard to a decline in viral load. Interestingly, we showed that the responsiveness to ANA773 during the course of treatment was determined by intrinsic characteristics of the individual's leukocytes, since the ability to respond *in vivo* was paralleled by the *in vitro* stimulation of the cells with ANA773 prior to treatment. These differences in responsiveness may be influenced by TLR7 polymorphisms which were found to correlate with the response to interferon-based therapy in chronic HCV infected patients [22], and also gender differences are known to influence the levels of IFN-α produced upon TLR7 ligation [23]. Furthermore, although the clinical outcome of IFN-based therapy is strongly dependent on specific *IL-28B* gene polymorphisms, our study cohort was too small to draw firm conclusions on the importance of the *IL28B* SNP in the response to ANA773. Among individuals responsive to ANA773, both the CC and TC rs12979860 genotypes were found (see Methods section). Another mechanism that may limit the efficacy of treatment with TLR agonists is elicitation of compensatory mechanisms that regulate and prevent excessive inflammation [24, 25]. In mice, it was shown that $CD4^+CD25^+FoxP3^+$ regulatory T cells were induced upon topical administration of imiquimod in a model of human breast cancer, and also serum levels of the immunosuppressive cytokine IL-10 were elevated following treatment with imiquimod [26]. In our study, we did not find any shifts in the number of $CD4^+CD25^+FoxP3^+$ regulatory T cells during the course of treatment with ANA773 (data not shown), thereby limiting the possibility that the induction of $FoxP3^+$ regulatory T cells underlies the weak antiviral activity.

The effect of TLR7 agonist therapy on the immune system of patients with chronic HCV infections was evaluated in this phase 1 study. The conclusions drawn from this study have to be considered in light of the limited number of patients per dosing group. Despite the small group size, our findings demonstrate that the treatment of chronic hepatitis C patients with the TLR7 agonist ANA773 resulted in a decrease of serum HCV RNA levels, and that this treatment strategy activates parts of the innate immune system. Importantly, those patients that display potent induction of endogenous interferons and interferon-stimulated gene products, most likely via an effect on plasmacytoid DC, also show a therapy-induced decline of viral load. To further improve strategies to develop ANA773 as an approach for HCV treatment, it will be important to examine the mechanism underlying the observation that certain patients are responsive and others are unresponsive to treatment.

In conclusion, the fact that administration of TLR7 agonists lead to a significant viral load reduction in chronic hepatitis C patients, combined with clearly detectable activation of the components of the anti-viral immune response, make these novel immunomodulatory compounds promising for further development. Combined or sequential treatment regimens of direct antiviral agents or standard of care to reduce the viral load with the use of TLR7 agonists, as immunomodulators to stimulate the immune system, may well be efficient to

eradicate the virus, and simultaneously allow the development of effective HCV-specific T cell memory responses to prevent relapses and re-infection.

Acknowledgements

We would like to thank the patients who agreed to participate in the clinical study. We also acknowledge the contribution of Cokki van der Ent and Irene Brings (Clinical Research Bureau, Erasmus MC) and Martine Peters (AMC, Amsterdam). Furthermore, we would like to thank B. Eam, M.V. Sergeeva, and T.W. Harding with their help during various stages of this project.

Supplementary Figure S1

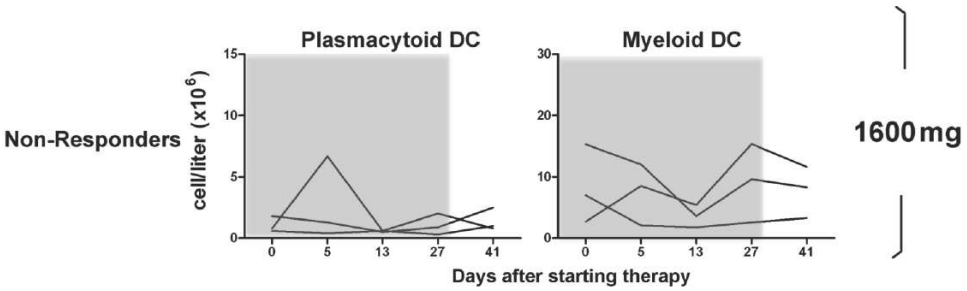

Figure S1. Chronic HCV patients were treated with ANA773 at a dose of 1600 mg. The absolute numbers of plasmacytoid DC and myeloid DC are presented at different time points after start of treatment, and displayed for patients who were categorized as non-responders.

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Chapter 7

IL-29 and IFNα differ in their ability to modulate IL-12 production by TLR-activated human macrophages, and exhibit differential regulation of the IFNγ receptor expression

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Blood 2011;117(8):2385-2395

SUMMARY

The interferon-lambda (IFNλ) family of cytokines, consisting of IL-28A, IL-28B and IL-29, has been extensively studied for their antiviral activities. However, little is known about the effect of IFNλ on antigen-presenting cells. Here, we show for the first time that IL-29 can increase TLR-induced IL-12p40 production by human monocyte-derived macrophages. In contrast, IL-29 did not affect monocytes or monocyte-derived DC due to the restricted IL-28RA expression by macrophages. Furthermore, IL-29-treated macrophages were more responsive to IFNγ, since IL-29 enhanced IFNγ-induced IL-12p40 and TNF production by macrophages upon R848 stimulation. However, IFNα suppressed IFNγ-induced IL-12p40 and TNF production by human macrophages. The differential effects of IL-29 and IFNα on the responsiveness of macrophages to IFNγ could not be explained by an effect on TLR7 or TLR8 mRNA expression, or by altered IL-10 signaling. However, we demonstrated that IL-29 up-regulated, whereas IFNα down-regulated, the surface expression of the IFNγ receptor 1 chain on macrophages, thereby resulting in differential responsiveness of TLR-challenged macrophages to IFNγ. Our findings on the differences between IFNα and IL-29 in modulating TLR-induced cytokine production by macrophages may further contribute to understand the role of interferons in regulating immunity to pathogens.

INTRODUCTION

Over the last years, the interferon-lambda (IFNλ) family members have been extensively studied for their antiviral activities. IL-28A (IFNλ2), IL-28B (IFNλ3) and IL-29 (IFNλ1) have been shown to possess potent antiviral activity via mechanisms similar to IFNα despite triggering of a unique IL-28 receptor pair which is distinct from the IFNα receptor.[1-3] At present, IFNα combined with ribavirin is the most efficient therapy to treat patients chronically infected with the hepatitis C virus (HCV). However, many side-effects and a limited effectiveness in a large group of patients make that alternatives to the standard of care treatment are needed. Clinical studies are being conducted to examine whether pegylated-IL-29 holds promise for future therapeutic use in the treatment of chronic HCV patients.[4] Interestingly, polymorphisms close to the IL-28B gene have been reported that are associated with disease progression and response to therapy, and have sparked interest in the IFNλ family members.[5-8] Numerous studies have examined the antiviral activity of IFNλ,[9-13] however, little is known about the effect of IFNλ on innate immune cells and their immunoregulatory activity.

Macrophages are crucial innate immune cells to eliminate pathogens and apoptotic cells.[14-16] Upon stimulation of specific pathogen-recognition receptors, such as Toll-like receptors (TLR), macrophages produce inflammatory mediators and cytokines, such as IL-1β, IL-6, TNF, and IL-12. IL-12p70, consisting of IL-12p40 and IL-12p35, plays an important role in the development of Th1-type responses, which are essential for the clearance of many infections.[17] TLR ligands derived from microorganisms are strong inducers of IL-12,[18-19] however, additional signals from cytokines, such as IFNγ and IL-4, as well as the interaction between CD40L and CD40 are necessary for the optimal production of IL-12 by monocytes, dendritic cells (DC) and macrophages. [20-21] Both IFNα and IFNγ are able to regulate the production of IL-12. While the production of both IL-12p70 and IL-12p40 are strongly enhanced by IFNγ in antigen-presenting cells,[20,22] IFNα has an inhibitory effect on IL-12p40 production by both mice and human monocytes, DC and macrophages.[22-23] In contrast to the effect on IL-12p40, the production of IL-12p70 is enhanced by exposure to IFNα in monocytes and DC.[24]

IFNλ and IFNα interact with different receptors.[25-27] The receptor of IFNα is composed of 2 unique receptor chains, IFNαR1 and IFNαR2, whereas the receptor of IFNλ comprises the IL-28 receptor alpha chain (IL-28RA) and the IL-10 receptor 2 chain (IL-10R2). Importantly, while IFNαR1, IFNαR2 and IL-10R2 are ubiquitously expressed, IL-28RA appears to be more restricted, and expression of this receptor chain has been reported by plasmacytoid DC, B cells, epithelial cells, and hepatocytes.[1,27-30] The more restricted receptor expression of IL-28RA makes it likely that IL-29 would lead to less adverse effects as compared to IFNα if used therapeutically, for instance to treated chronic HCV patients. However, at present, little is known about the effect of IFNλ on innate immune cells, and their immunoregulatory activity.

In this paper, we show that although IL-28RA is not expressed by human primary monocytes and, monocyte-derived DC, it is expressed by monocyte-derived macrophages. As a consequence, IL-29 enhances TLR-induced cytokine production of human monocyte-derived macrophages, but does not affect monocytes or monocyte-derived DC. Unlike the reported similarities in antiviral activity, we show for the first time that IL-29 and IFNα differ in

their ability to modulate TLR-induced IL-12p40 production, especially in combination with IFNγ. In addition, IL-29 up-regulates the IFNγR1 chain, whereas IFNα down-regulates this receptor chain, which explains the differential responsiveness of human macrophage to IFNγ. Thus, our findings demonstrate that besides its potent antiviral activity, IL-29 plays an important role in modulating cytokine production by macrophages, which may enhance immune responses to pathogens.

MATERIALS AND METHODS

Cell culture and purification

Monocytes were purified from PBMC obtained from buffycoats (Sanquin) with magnetic CD14-microbeads (Miltenyi Biotec) following the manufacturer's instructions. The purity was always more than 97%. Macrophages were generated from purified monocytes with 10 ng/ml M-CSF (R&D) in 6-well plates (Costar) at a density of $1.5x10^6$ cells/well in 2 ml RPMI1640 supplemented with 8% research-grade FCS**.** On day 2 and day 5, half of the medium was refreshed and on day 6 monocyte-derived macrophages were harvested, and used for various purposes. Monocyte-derived DC were generated from monocytes with 10 ng/ml IL-4 (eBioscience) and 10 ng/ml GM-CSF (Leukine, Bayer Healthcare Pharmaceuticals) in 6-well plates (Costar) at a density of $1.5x10^6$ cells/well in 2 ml RPMI1640 supplemented with 8% research-grade FCS**.**

Stimulation of monocyte-derived macrophages, monocytes, and monocyte-derived DC

In order to determine which cells respond to IL-29, monocyte-derived macrophages, monocytes and monocyte-derived DC were pretreated with IL-29 (100 ng/ml, R&D) for 5h and further stimulated for 24h with LPS (100 ng/ml, InvivoGen) or R848 (1 ug/ml, Alexis) without removing the supernatant. To compare the effects of distinct IFNλ family members and IFNα, monocyte-derived macrophages were pre-exposed to IL-29 (100 ng/ml, R&D), IL-28A (100 ng/ml, R&D), IL-28B (100 ng/ml, R&D) or IFNα (10 ng/ml, IntronA, Schering-Plough) for 5h and further stimulated for 24h with LPS (100 ng/ml) or R848 (1 ug/ml). To check the response to IFNγ, monocyte-derived macrophages pretreated with IFNα or IL-29 were further stimulated with IFNγ (10 ng/ml, Miltenyi Biotec) and R848. In some experiments, anti-human IL-10R antibody (anti-IL-10R, clone: 3F9, 5 ug/ml, Biolegend) was used to block IL-10 signaling in the cultures. Cytokine production was determined by ELISA.

Flow cytometric analysis of the expression of interferon receptors, HLA-DR and HLA-ABC

Monocyte-derived macrophages, monocytes and monocyte-derived DC were stained with the antibodies IL-28RA-PE (Biolegend) and IL-10R2-Biotin (R&D) to evaluate the expression of the IFNλ receptor. Prior to the addition of antibodies, Fc-receptors were blocked to prevent non-specific staining. Streptavidin-APC (BD Pharmingen) was used to visualize the IL-10R2. IFNγR1-PE and IFNγR2-PE (Biolegend) were used to determine the IFNγ receptor expression on monocyte-derived macrophages pretreated with IL-29 (100 ng/ml, R&D), IL-28A (100 ng/ml, R&D), IL-28B (100 ng/ml, R&D) or IFNα (10 ng/ml, IntronA). The specificity of the stainings was controlled with appropriate isotype antibodies.

To examine the surface expression of HLA-DR and HLA-ABC on the surface of monocyte-derived macrophages, cells were first exposed to IL-29 (100 ng/ml, R&D) or IFNα (10 ng/ml, IntronA) for 5h and then further stimulated with IFNγ (10 ng/ml) for another 20h. HLA-DR and HLA-ABC expression on macrophages were determined by flow cytometry using antibodies against HLA-DR-PerCP-Cy5.5 (eBioscience, LN3) and HLA-ABC-FITC (Biolegend, W6/32). The specificity of the stainings was controlled with appropriate isotype antibodies.

Flow cytometric analysis of pSTAT-1 staining

Monocyte-derived macrophages, monocytes and monocyte-derived DC were stimulated with IL-29 or IFNα for 20 min. Stimulated cells were immediately fixed with BD Phosflow Lyse/Fix (BD Bioscience) and then permeabilized with BD Phosflow Perm Buffy III (BD Bioscience). Cells were then incubated with mouse anti-pSTAT1-Alexa Fluor488 (eBioscience) and the phosphorylation state of STAT-1 were measured by flow cytometry (Canto-II, BD).

Quantification gene expression by RT-PCR

To determine the effect of IL-29 and IFNα on TLR7 and TLR8 mRNA expression, monocyte-derived macrophages were stimulated with IL-29 (100 ng/ml, R&D) or IFNα (10 ng/ml, IntronA) for 5h and then lysed using TRI® Reagent (Sigma-Aldrich) and stored at -80°C. To determine the IL-12p40, IL-12p35 and IL-12p19 mRNA expression in macrophages, monocyte-derived macrophages were stimulated with IL-29 (100 ng/ml, R&D) or IFNα (10 ng/ml, IntronA) for 5h and then further stimulated for 5h with R848 (1 μg/ml) plus IFNγ (10 ng/ml, Miltenyi Biotec). The cells were lysed using TRI® Reagent (Sigma-Aldrich) and stored at -80°C.

Total RNA from monocyte-derived macrophages was extracted using NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. RNA was quantified using a Nanodrop ND-1000 (Thermo). cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). All real-time PCR reactions were performed in Bio-Rad optical 96-well plates using a MyIQ5 detection system (Bio-Rad Laboratories). SYBR-Green present in the MasterMix Plus (Eurogentec) was used for quantification. Primers for GAPDH (forward 5'-TGCACCACCAACTGCTTAGC-3' and reverse 5'-GGCATGGACTGTGGTCATG AG-3') and TLR7 (forward 5'-AATGTCACAGCCGTCCCTAC-3' and reverse 5'-GCGCATCAAAAGCATTTACA-3') were designed to determine the TLR7 mRNA expression. Furthermore, primer-probes for GAPDH (Hs00266705_g1), TLR8 (Hs00152972_m1), IL-12p40 (Hs01011518_m1), IL-12p35 (Hs01073447_m1) and IL-23p19 (Hs00372324_m1) were purchased from Applied Biosystems. The expression of target genes was normalized to GAPDH using the formula: $2^{-\Delta Ct}$, $\Delta Ct = Ct_{TLR} - Ct_{GAPDH}$.

Immunoassay for detection of cytokines in supernatant

The concentrations of cytokines in supernatant were determined using sandwich ELISA specific for IL-12p40 (C8.6 and C8.3 antibody pairs, Biolegend), and Ready-Set-Go kits for IL-12p70, IL-23, IL-10 and TNF (all from eBioscience). The detection limits for IL-10, IL-12p70, IL-23 and TNF were 15 pg/ml and for IL-12p40 30 pg/ml.

Statistics

Values are expressed as mean values, unless indicated otherwise. Data was analyzed with Prism 5.0 (Graphpad software) using the Mann-Whitney t-test to compare variables between two independent groups. In all analyses, a two-tailed p-value of less than 0.05 (confidence internal 95%) was considered statistically significant.

Results

Monocyte-derived macrophages, but not monocytes or monocyte-derived DC, respond to IL-29 due to the restricted IL-28RA expression by monocyte-derived macrophages

To examine the role of IL-29 in modulating immune responses, we first compared the responsiveness of different innate target cells to IL-29. For this purpose, human circulating monocytes, monocyte-derived DC and monocyte-derived macrophages were challenged with IL-29 in combination with LPS or R848. As shown in Figure 1A, IL-29 enhanced the levels of TNF produced by LPS-stimulated monocyte-derived macrophages by 2-fold, and R848-induced responses were enhanced 5-fold by IL-29. However, neither TLR-stimulated monocytes nor monocyte-derived DC responded to IL-29, as indicated by their TNF production. Both monocytes and monocyte-derived DC did not respond to increasing doses of IL-29 (Supplementary Figure S1A), or when the cells were exposed to IL-29 for different pretreatment periods (Supplementary Figure S1B). Importantly, monocytes remained unresponsive to IL-29 when cultured in the presence of M-CSF for 24h (data not shown).

The receptor of IL-29, consisting of IL-28RA and IL-10R2, is expressed by a limited number of cells types, including plasmacytoid DC, B cells, epithelial cells, and hepatocytes.[1,27-30] To examine whether the differential responsiveness of human monocyte-derived macrophages, monocytes and DC to IL-29 can be explained by their receptor expression, flowcytometric analysis for IL-28RA and IL-10R2 expression was performed. Although IL-10R2 is expressed by monocyte-derived macrophages, monocytes and weakly by DC, IL-28RA expression is only observed by monocyte-derived macrophages, but not by monocytes and monocyte-derived DC (Figure 1B). In addition, phosphorylation of STAT-1 upon exposure to IL-29 was only observed by monocyte-derived macrophages, but not by monocytes or monocyte-derived DC (Figure 1C). These data indicate that differential responsiveness of human monocyte-derived macrophages, monocytes and DC to IL-29 is likely due to restricted IL-28RA expression by monocyte-derived macrophages.

IL-29 enhances TLR-induced IL-12p40 production by human monocyte-derived macrophages

Although much is known about the antiviral activity of IFNλ, their immunoregulatory activity on immune cells is still poorly understood. To examine this, we determined whether IL-29 affects the cytokine production by TLR-stimulated monocyte-derived macrophages. We compared the effect of IL-29 on macrophages with IFNα to determine whether these cytokines modulate macrophage responses in a similar manner. This is especially relevant since IL-29 and type I interferon induce antiviral activity via similar mechanisms.[1-3] Furthermore, since macrophages are highly responsive to IFNγ, we also included IFNγ in our analysis.

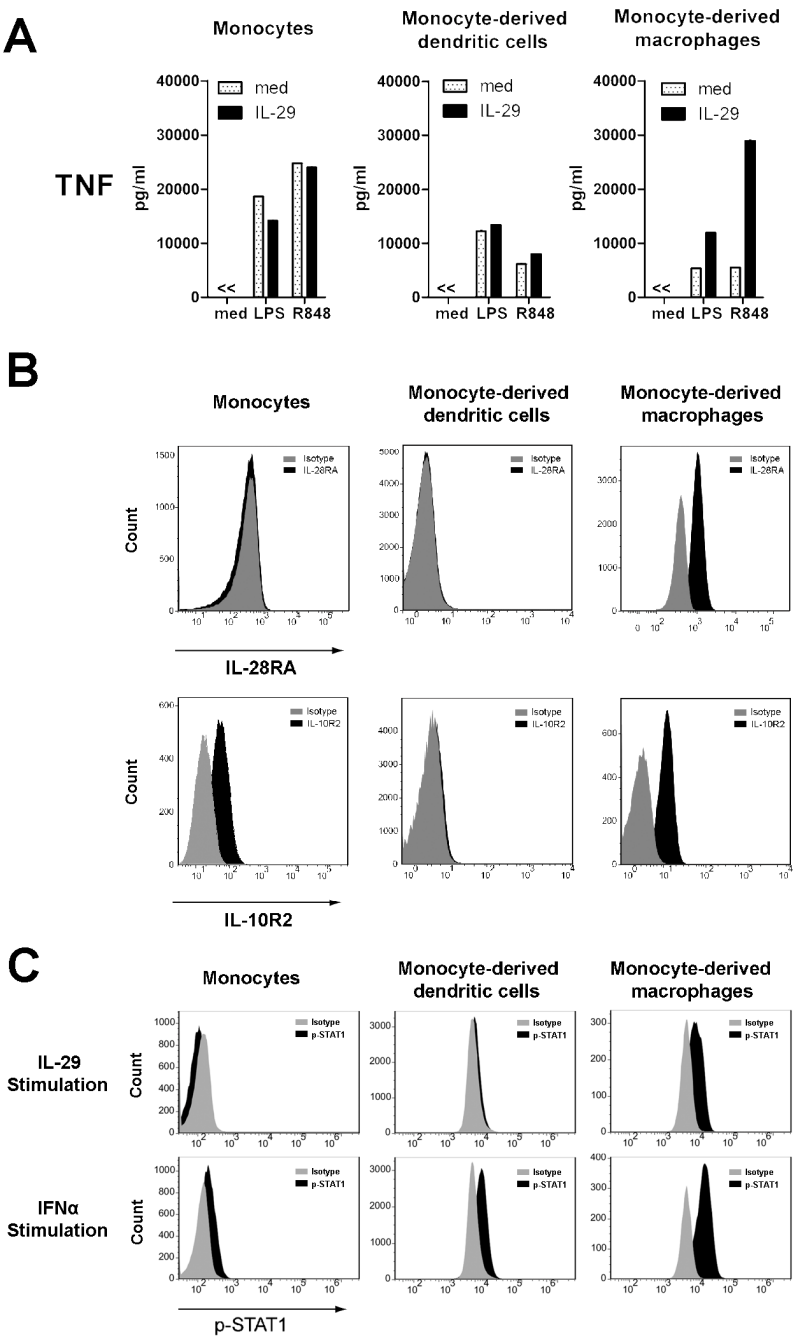


Figure 1. Monocyte-derived macrophages, but not monocytes nor monocyte-derived DC, respond to IL-29 due to the restricted IL-28RA expression by monocyte-derived macrophages. **(A)** Monocytes, monocyte-derived DC and monocyte-derived macrophages were pretreated with IL-29 for 5h and then further stimulated with LPS or R848 for 24h. TNF production was determined by ELISA. The values depicted show representative data from 11 independent experiments. **(B)** Monocytes, monocyte-derived DC and monocyte-derived macrophages were stained with antibodies against IL-28RA and IL-10R2 to evaluate the expression of the IFNλ receptor by flow cytometry. The specificity of the staining was controlled with the appropriate isotype antibodies. The histograms depicted show representative data from 10 independent experiments. **(C)** Monocytes, monocyte-derived DC and monocyte-derived macrophages were stimulated with IL-29 or IFNα for 20min, and then cells were fixed and permeabilized. The phosphorylation of STAT-1 was measured by flow cytometry (Canto-II, BD).

As shown in Figure 2A, low or undetectable IL-12p40 was produced by monocyte-derived macrophages in response to LPS irrespective of the addition of IL-29. However, upon R848 stimulation, IL-29 increased the IL-12p40 production by monocyte-derived macrophages by about 2-fold, which was observed in the majority of donors (Figure 2A).

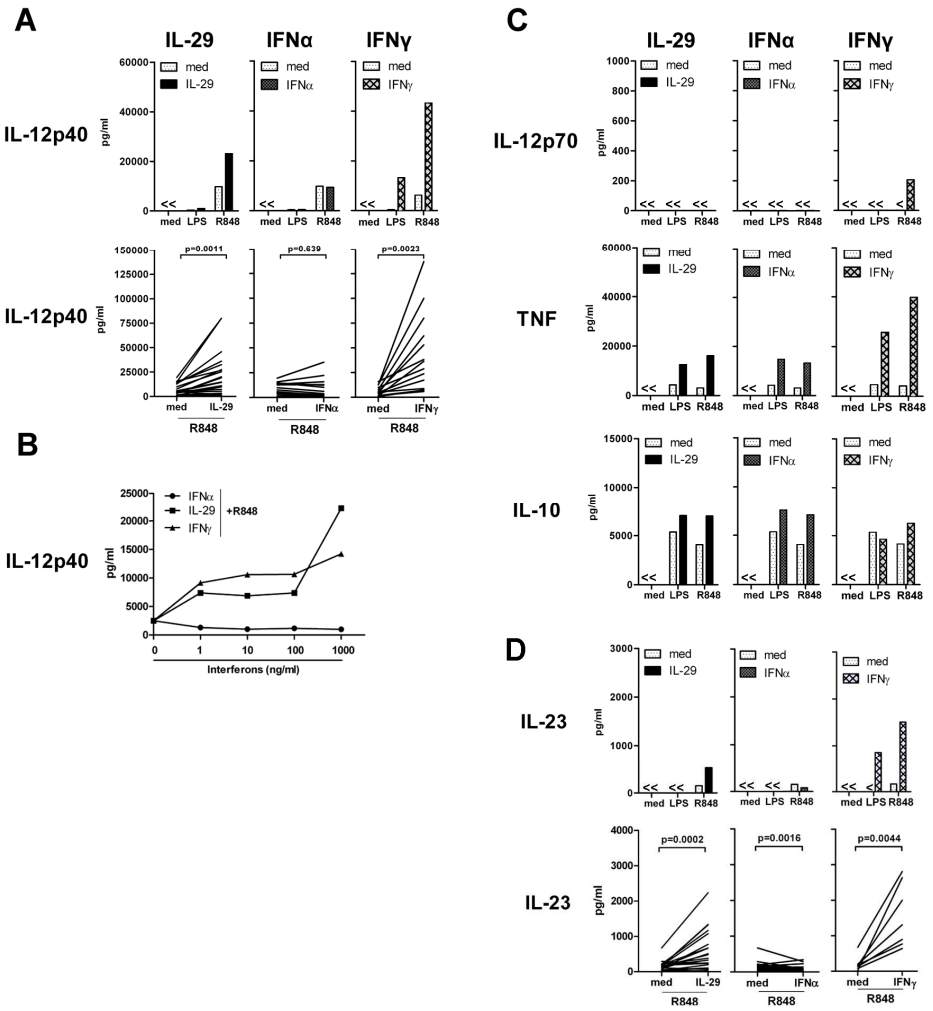


Figure 2. IL-29 enhances TLR-induced IL-12p40 production by human monocyte-derived macrophages. **(A)** Monocyte-derived macrophages were pretreated with IL-29 (100 ng/ml), IFNα (10 ng/ml), or IFNγ (10 ng/ml) for 5h and then further stimulated with LPS or R848. The levels of IL-12p40 in supernatant were determined by ELISA. The values depicted show representative data from 27 independent experiments. The increase of IL-12p40 by IL-29 in monocyte-derived macrophages was observed in 19 out of 27 healthy individuals. **(B)** Monocyte-derived macrophages were pretreated with IL-29, IFNα or IFNγ at the indicated concentrations for 5h and then further stimulated with R848. The levels of IL-12p40 in the supernatants were determined by ELISA. The values depicted show representative data from 3 independent experiments. Medium, IL-29, IFNα or IFNγ alone did not induce IL-12p40 production by R848-stimulated monocyte-derived macrophages**. (C)** Monocyte-derived macrophages were stimulated as described for Figure 2A. The concentrations of IL-12p70, TNF and IL-10 were determined in supernatant by ELISA. Macrophages from 10 out of 15 healthy individuals showed undetectable levels of IL-12p70 in response to IFNγ and R848 stimulation. **(D)** Monocyte-derived macrophages were pretreated with IL-29 (100 ng/ml), IFNα (10 ng/ml), or IFNγ (10 ng/ml) for 5h and then further stimulated with LPS or R848. The levels of IL-23 in supernatant were determined by ELISA.

In line with previous reports,[20,22-23] no modulation of the levels of R848-induced IL-12p40 was observed by IFNα-treated monocyte-derived macrophages, whereas IFNγ strongly enhanced IL-12p40 levels by macrophages upon TLR ligation (Figure 2A). Importantly, the enhancement of R848-induced IL-12p40 production by IL-29 was dose-dependent, which

was also observed for IFNγ (Figure 2B). However, even at relatively high IFNα concentrations no increase of IL-12p40 production was observed, but a mild decrease instead (Figure 2B). Reduced IL-12p40 production by IFNα was not due to IFNα-induced cytotoxicity (Supplementary Figure S2A). This demonstrates that the distinct ability of IL-29 and IFNα to modulate the IL-12p40 levels is not the result of sub-optimal dosing of IFNα. Although IL-29 and IFNα regulate IL-12p40 production differently, IL-29 has similar effects as IFNα or IFNγ in enhancing TNF and IL-10 production by monocyte-derived macrophages in response to R848 stimulation (Figure 2C). Furthermore, upon R848 stimulation, the production of IL-12p70 was only observed by IFNγ-treated monocyte-derived macrophages in 5 out of 15 experiments, but not by macrophages treated with IL-29 or IFNα (Figure 2C). However, the production of bioactive IL-23 (heterodimer consisting of a IL-12p40 and IL-23p19 chain) following stimulation in the presence of IL-29 reflected the observed effect on IL-12p40 production, which suggests that IL-12p40 production contributes to the levels of bioactive IL-23 (Figure 2D). In summary, IL-29 has similar effects as IFNγ, but not as IFNα, in augmenting IL-12p40 production by human monocyte-derived macrophages upon TLR ligation.

IL-28A and IL-28B also enhance R848-induced IL-12p40 production by human monocyte-derived macrophages

We next examined whether IL-28A and IL-28B also affected TLR-induced cytokine production by human monocyte-derived macrophages. As shown in Figure 3, both IL-28A and IL-28B were able to increase IL-12p40, TNF and IL-10 production by monocyte-derived macrophages in response to R848 stimulation, although the effects of IL-28A and IL-28B were weaker than that of IL-29. Similarly, also upon LPS stimulation, both IL-28A and IL-28B enhanced the TNF and IL-10 production by monocyte-derived macrophages in response (data not shown). These data indicate that the effects of IL-28A and IL-28B are similar as IL-29 in monocyte-derived macrophages upon R848 stimulation, and all lead to an enhanced production of IL-12p40, TNF and IL-10.

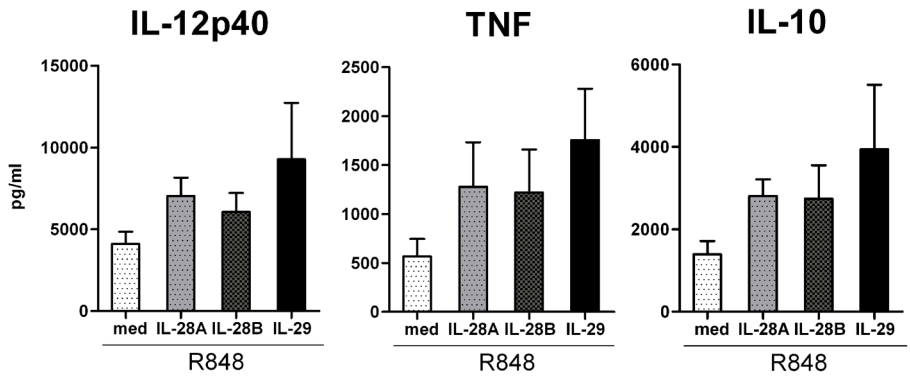


Figure 3. Similar to IL-29, IL-28A and IL-28B enhance R848-induced IL-12p40, TNF and IL-10 production by human monocyte-derived macrophages. Monocyte-derived macrophages were pretreated with IL-28A, IL-28B and IL-29 (all 100 ng/ml) for 5h and then further stimulated with R848. The levels of IL-12p40, TNF and IL-10 in supernatants were determined by ELISA. The values depicted show the mean +/- SE from 7 independent experiments.

IL-29 enhances IFNγ-induced IL-12p40 production by monocyte-derived macrophages in response to R848 stimulation, whereas IFNγ-induced IL-12p40 production is suppressed by IFNα

Previously, it has been reported that the combination of IL-29 and IFNγ synergistically inhibited HCV replication in Huh7 by inducing the expression of multiple genes, and to exert stronger antiviral activity than the combination of IL-29 and IFNα or the combination of IFNα and IFNγ.[31] Based on these findings, we further examined the effect of IL-29 on IL-12p40 production by TLR-stimulated monocyte-derived macrophages by combining IL-29 with IFNγ, which is also known to enhance IL-12p40 production.[32]

Both IL-29 and IFNγ augmented IL-12p40 production by R848-challenged monocyte-derived macrophages (Figure 2A, Figure 4B). However, upon R848 stimulation, IFNγ induced higher levels of IL-12p40 production in IL-29 pretreated monocyte-derived macrophages than in macrophages without IL-29 pretreatment (61 ng/ml and 35 ng/ml, respectively; Figure 4A). This effect was observed in IL-29 pretreated macrophages from the majority of healthy donors (Figure 4B). The additive effect of IL-29 and IFNγ was also observed for TNF production in IL-29 pretreated monocyte-derived macrophages (Figure 4A). In addition, IL-29 and IFNγ synergistically up-regulated IL-12p40 mRNA expression in R848-challenged monocyte-derived macrophages (Figure 4C). Although IL-12p70 production was not detectable in the majority of the experiments, we found that IL-29 increased the level of IFNγ-induced IL-12p35 mRNA expression in monocyte-derived macrophages upon R848 stimulation (Figure 4C). Importantly, IL-29-treated macrophages had higher levels of IL-23p19 mRNA expression upon IFNγ plus R848 stimulation compared to macrophages that were not treated with IL-29 (Figure 4C). These data indicate that IL-29 can cooperate with IFNγ in inducing high levels of IL-12p40 by monocyte-derived macrophages. Although IFNγ potently induced IL-12p40 production by monocyte-derived macrophages upon R848 stimulation, this induction of IL-12p40 by IFNγ was suppressed by IFNα pretreatment (from 35 ng/ml to 5 ng/ml, respectively; Figure 4A), which was in line with the finding that IFNα-pretreated macrophages had lower fold expression of IL-12p40 mRNA in response to IFNγ plus R848 stimulation (Figure 4C). Failure to induce IL-12p40 production by IFNγ was observed in IFNα-pretreated monocyte-derived macrophages in all the experiments performed (Figure 4B), which could not be explained by enhanced cytotoxicity after exposure to IFNα (Supplementary Figure S2B). This inhibitory effect of IFNα was also observed for TNF production (Figure 4A). These data demonstrate that IFNα potently suppress the responsiveness of monocyte-derived macrophages to subsequent stimulation with IFNγ upon TLR ligation.

Our findings thus demonstrate that IL-29 pretreatment renders monocyte-derived macrophages more responsive to IFNγ stimulation, as indicated by their IL-12p40 production in response to R848, whereas IFNα suppresses IFNγ-induced IL-12p40 production by human macrophages upon TLR ligation.

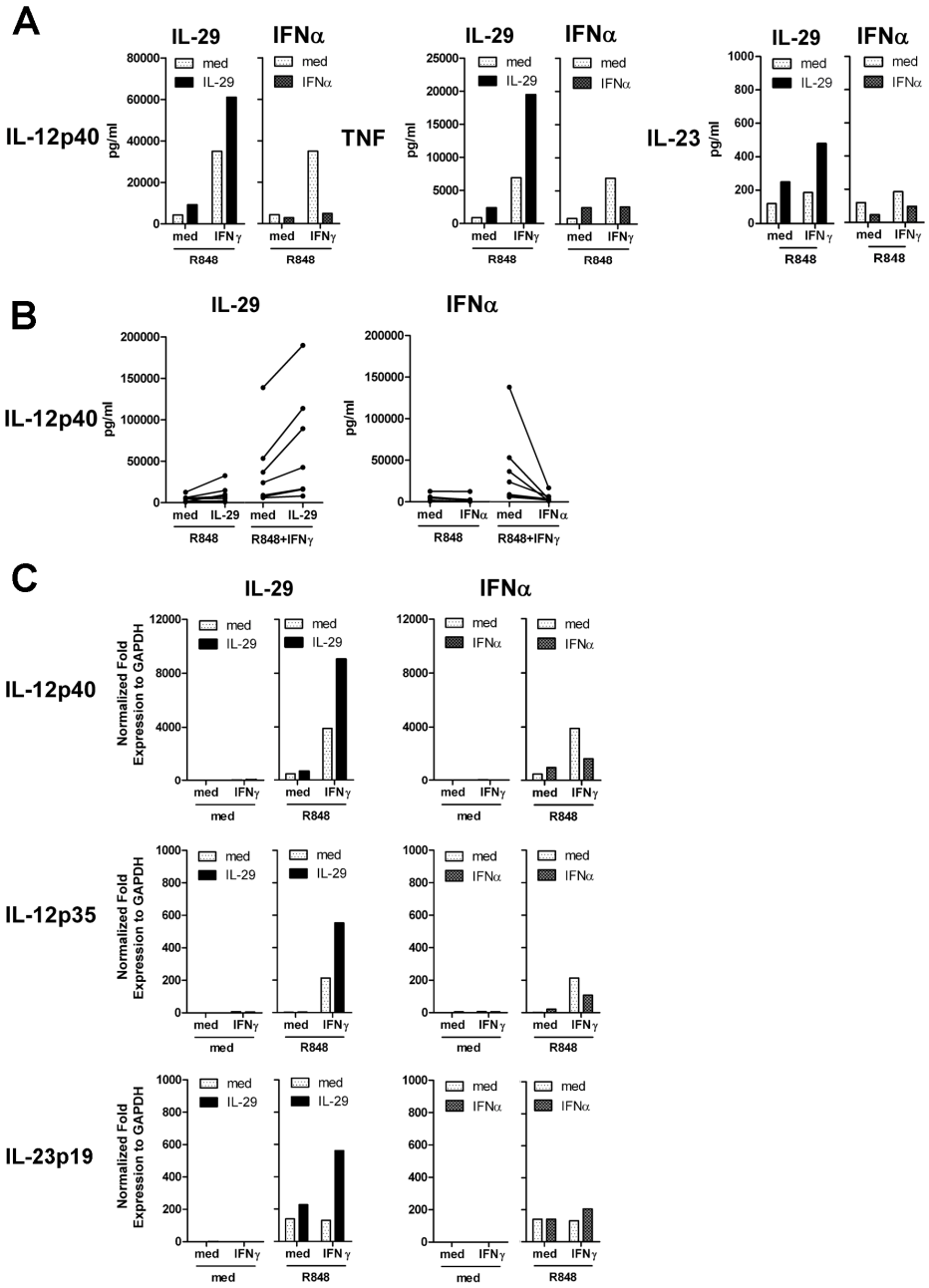


Figure 4. IL-29 enhances, but IFNα suppresses, IFNγ-induced IL-12p40 production by human monocyte-derived macrophages in response to R848 stimulation. **(A, B)** Monocyte-derived macrophages were pretreated with IL-29 or IFNα for 5h and then further stimulated with IFNγ in combination with R848. IL-12p40, TNF and IL-23 production were determined by ELISA. The values depicted show representative data from 7 independent experiments. **(C)** Monocyte-derived macrophages were pretreated with IL-29 or IFNα for 5h and then further stimulated with IFNγ in combination with R848 for another 5h. IL-12p40, IL-12p35 and IL-23p19 mRNA expression in macrophages were quantified by RT-PCR. The values depicted show representative data from 2 independent experiments.

IL-10 is not involved in the differential regulation of IFNγ-induced IL-12p40 production by IL-29 and IFNα in human monocyte-derived macrophages upon R848 stimulation

We previously showed that endogenous IL-10 produced by murine macrophages upon TLR ligation suppresses the induction of IL-12 and TNF.[33] To examine whether the activity of IL-10 is involved in the distinct regulation of IFNγ-induced IL-12p40 production by IL-29 or IFNα

pretreated human macrophages upon TLR ligation, we blocked the IL-10 receptor with antibodies.

As shown in Figure 5, IFNγ-induced IL-12p40 levels were increased about 20-fold by blocking the IL-10 receptor in monocyte-derived macrophages upon R848 stimulation (from 23 ng/ml to 471 ng/ml), indicating that IFNγ-induced IL-12p40 by R848-challenged monocyte-derived macrophages was strongly suppressed by endogenous IL-10. We showed that IL-29 treated macrophages were more responsive to IFNγ as evident by IL-12p40 production upon R848 stimulation (Figure 4A and Figure 5 upper panel). Interestingly, the additive effect of IL-29 and IFNγ on human macrophages was still observed in the absence of IL-10 signaling as shown by IL-12p40 production (Figure 5, upper panel). IFNα-pretreated monocyte-derived macrophages have impaired IFNγ-induced IL-12p40 production in response to R848 stimulation (Figure 4 and Figure 5, lower panel). Upon IL-10 receptor blockade, IFNα pretreatment did not restore IFNγ-induced IL-12p40 production by monocyte-derived macrophages (Figure 5, lower panel). These results show that IL-10 is not involved in the differential regulation of IFNγ-induced IL-12p40 production by IL-29 and IFNα in human macrophages upon R848 stimulation.

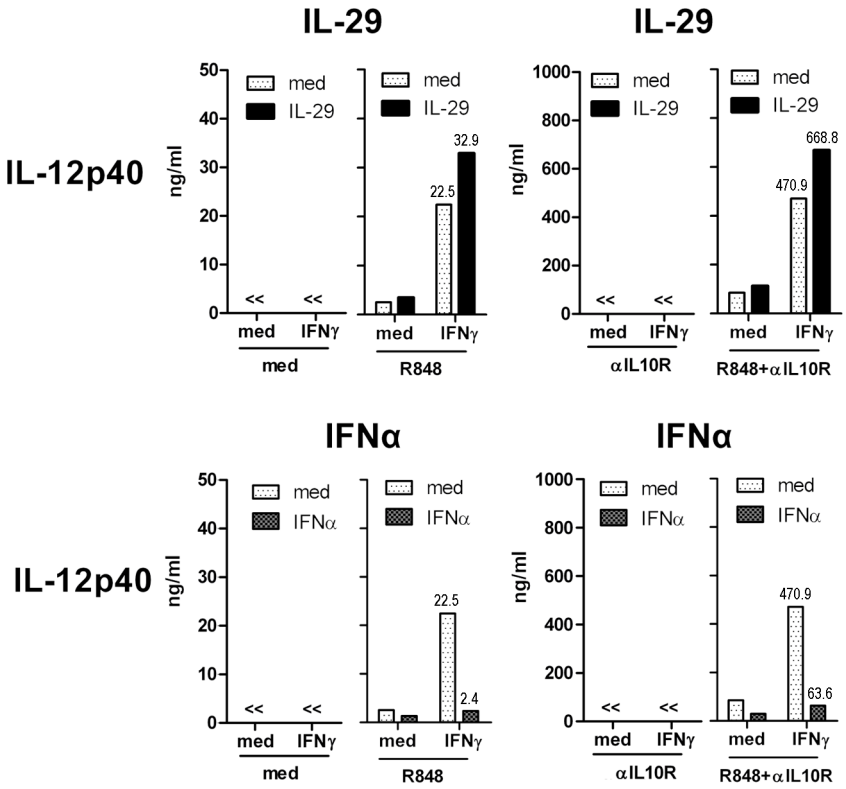


Figure 5. IL-10 is not involved in the differential regulation of IFNγ-induced IL-12p40 production by IL-29 and IFNα in human monocyte-derived macrophages upon R848 stimulation. Monocyte-derived macrophages were pretreated with IL-29 or IFNα for 5h and then further stimulated with IFNγ and R848. Anti-human IL-10 receptor antibody (αIL10R, 5 μg/ml) was added to some conditions to block the IL-10 receptor. The IL-12p40 concentrations in supernatant were determined by ELISA. The values depicted show representative data from 3 independent experiments.

Both IL-29 and IFNα up-regulate TLR8 mRNA expression in human monocyte-derived macrophages

A possible explanation for the distinct regulation of IFNγ-induced IL-12p40 production by IL-29 and IFNα in human macrophages is a different effect of these two cytokines on TLR mRNA expression. As shown in Figure 6, upon incubation with IL-29 or IFNα for 5h, a 3-4 fold increase of TLR8 mRNA expression was observed in monocyte-derived macrophages. The regulation of TLR7 by IL-29 or IFNα in macrophages was next investigated, since TLR7 is also the receptor for R848. While IL-29 did not increase TLR7 mRNA expression, IFNα up-regulated TLR7 mRNA expression in monocyte-derived macrophages by 3-fold (Figure 6). Since both IL-29 and IFNα up-regulated TLR8 mRNA expression, TLR expression does not explain our findings on the different IFNγ-induced IL-12p40 production by IL-29 and IFNα in macrophages.

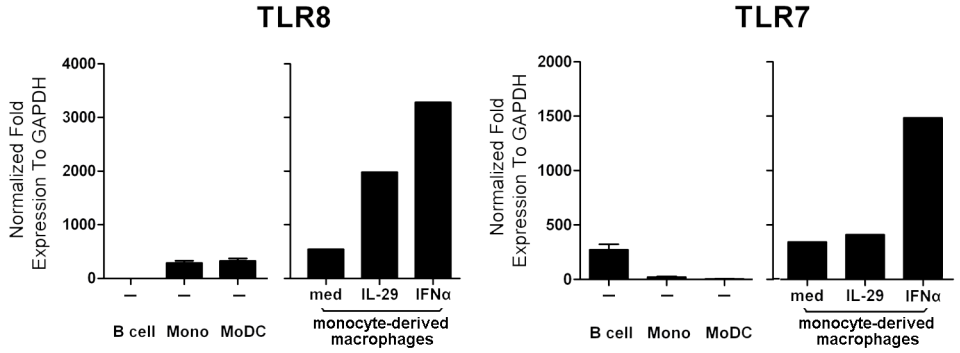


Figure 6. Both IL-29 and IFNα up-regulate TLR8 mRNA expression. Monocyte-derived macrophages were stimulated with medium, IL-29 or IFNα for 5h, and the mRNA expression of TLR7 and TLR8 were measured by real-time PCR. B cells, monocytes, and monocyte-derived DC were included as controls. The values depicted show representative data from 5 independent experiments.

IL-29 enhances, but IFNα suppresses, IFNγ-induced IL-12p40 production by human monocyte-derived macrophages via differential regulation of IFNγR1 expression

The regulation of TLR mRNA expression by IL-29 and IFNα could not explain that IL-29 and IFNα differentially affect IFNγ-induced IL-12p40 production by human macrophages. We next investigated whether the receptor for IFNγ (IFNγR) is differentially regulated by IL-29 and IFNα.

As shown in Figure 7A, IFNγR1 expression on the membrane of monocyte-derived macrophages was up-regulated after incubation with IL-29 for 5h, whereas no up-regulation of IFNγR1 was observed in macrophages incubated with IL-28A or IL-28B for 5h. In contrast, down-regulation of IFNγR1 on the membrane of macrophages was observed upon IFNα treatment (Figure 7A). However, both IL-29 and IFNα did not affect the expression of IFNγR2 on monocyte-derived macrophages (data not shown). These data strongly suggest that IL-29 and IFNα alter the response of macrophages to IFNγ via distinct regulation of the expression of surface IFNγR1 on monocyte-derived macrophages.

To further examine whether the additive effect between IL-29 and IFNγ also occurs in the absence of TLR-signaling, we investigated the effect of exposure to IL-29, IFNα and IFNγ on the surface expression of HLA-DR and HLA-ABC on monocyte-derived macrophages. As shown in Figure 7B, pre-treatment of macrophages with IL-29 did not affect the HLA-DR and HLA-ABC expression. However, pre-treatment of macrophages with IL-29 resulted in a strong upregulation of HLA-DR and HLA-ABC in response to IFNγ. These findings indicate

that the synergistic effect between IL-29 and IFNγ also occurs in the absence of TLR triggering.

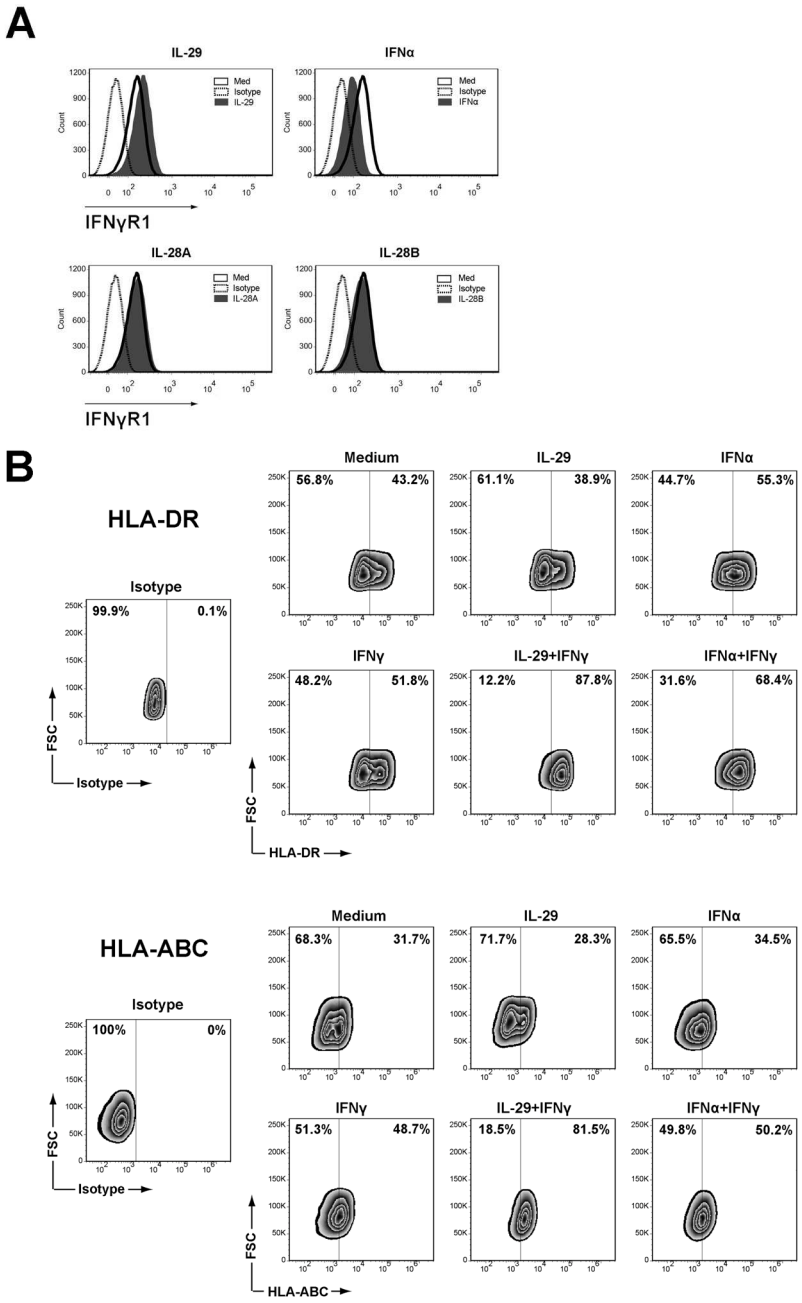


Figure 7. IL-29 and IFNα differentially regulate the surface IFNγR1 expression on monocyte-derived macrophages. **(A)** Monocyte-derived macrophages were treated with IL-29, IL-28A, IL-28B, or IFNα for 5h and then stained with antibodies against IFNγR1. The specificity of the stainings was controlled with appropriate isotype antibodies. **(B)** Monocyte-derived macrophages were treated with IL-29 or IFNα for 5h and then further stimulated with IFNγ (10 ng/ml) for another 20h. Cells were then harvested and stained with antibodies against HLA-DR and HLA-ABC. The specificity of the staining was controlled with appropriate isotype antibodies.

Discussion

In this study we examined whether persistent infections with HCV influences the functionality of monocytes in patients. We demonstrate that the response of highly purified, circulating monocytes to distinct TLR agonists is differentially affected between chronic HCV patients and healthy individuals. Reduced production of pro-inflammatory cytokines in response to TLR4 ligation, and augmented production upon TLR8 ligation of monocytes from chronic HCV patients demonstrates specific modulation of the function of monocytes in patients with chronic HCV infection. We further show that the differences in suppression of TLR4- and TLR8-induced activation as observed in chronically infected HCV patients, was likely due to differential responsiveness to IL-10.

Monocytes are important players in the first-line of defense against numerous pathogens, as well as in initiating and controlling adaptive immunity [25]. Indeed, in HIV-1 infection, reduced function of mononuclear phagocytic cells results in the weaker innate immunity to bacterial infection [13]. In persistent HCV infections the numbers of studies examining this issue are limited, and the conclusions on the functionality of monocytes in patients are conflicting.

We demonstrate that, in contrast to TLR8 ligation, triggering of monocytes from chronic HCV patients with TLR4 ligands resulted in lower levels of the pro-inflammatory cytokines TNF and IL-12p40. Interestingly, exposure of human monocytes to recombinant HIV Tat or Vpr proteins lead to defective responses to LPS as shown by TNF and IL-12p40 production [26-27], whereas no information is available on the effect of exposure to these antigens upon TLR8 ligation. The reduced TLR4-induced responses were not simply due to lower TLR4 levels, since TLR4 mRNA levels in monocytes were similar in chronic HCV patients as compared to control individuals. Importantly, our data further shows that monocytes from chronic HCV patients spontaneously produce higher level of IL-10 as compared with monocytes from healthy controls, and that serum from chronic HCV patients contains higher IL-10 levels than control serum. Furthermore, since also cytokine production of monocytes induced by TLR4 ligation is suppressed by IL-10 very efficiently, whereas this is more modest upon TLR8 ligation, IL-10 is a likely candidate to explain the reduced LPS responses of monocytes from chronic HCV patients. At present, the cell types responsible for the relatively high serum IL-10 levels in these patients are not known. However, some studies have shown that HCV encoded proteins, such as HCV core, NS3 and NS4 proteins have the ability to induce IL-10 production by monocytes isolated from both patients and healthy individuals [19, 28-33]. The importance of monocyte-derived IL-10 was further highlighted in a study that demonstrated that patients with self-limiting HCV infections produced significantly less IL-10 than chronic HCV patients [28]. Moreover, in the chronic LCMV model in mice, the role for IL-10 in preventing viral clearance was demonstrated in which therapeutic administration of an antibody that blocks the IL-10R restored T-cell function and eliminated LCMV infection [34].

In contrast to activation via TLR4, we demonstrated that monocytes from chronic HCV patients are more responsive to TLR8 ligation than monocytes from healthy individuals by producing cytokines. Similar to healthy individuals, also monocytes from chronic HCV patients were unresponsive to pure TLR7 ligands (data not shown). The enhanced response to TLR8 ligation could not be fully explained by elevated TLR8 mRNA expression in

monocytes from chronic HCV patients in our patient group, as has been reported before [35-36]. Interestingly IL-10 has less effect on TLR8 signalling in suppressing TNF and IL-12p40 production, since the TLR8 pathway is only weakly sensitive to the suppressive effects of IL-10, even at high concentrations. Also, serum from chronic HCV patients has no effect in increasing IL-10 production by monocytes after triggering of TLR8 signalling pathway. At present, it is unknown why IL-10 is able to inhibit the TLR4, but not the TLR8-induced responses. One possibility is that IL-10 signalling events may differentially affect the MyD88 and TRIF signalling pathways, since the MyD88-independent TRIF pathway, is activated upon TLR4 ligation, but not TLR8 ligation [37]. To further add to the complexity, we observed that upon combined triggering of TLR4 and TLR8, TLR8 ligation was able to overcome the inhibitory effect of IL-10 on TLR4 stimulation (Supplementary Figure S1B). Detailed signalling studies need to be conducted in order to delineate the underlying mechanisms. However, the specific inhibition by IL-10 of responses induced by LPS, but not R848 *in vitro*, is reflected by the selective inhibition of the TLR4 pathway as observed in chronic HCV patients.

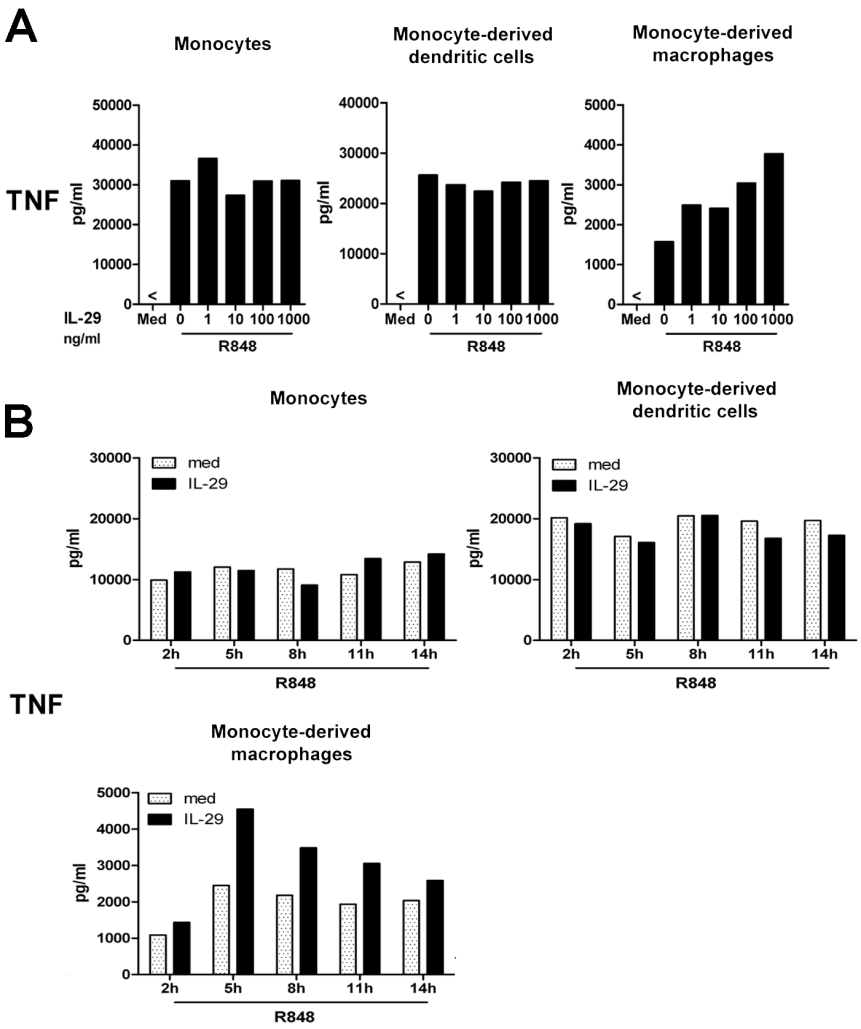
In contrast to our findings, enhanced LPS-induced TNF production by monocytes from chronic HCV patients was observed by some groups [14, 18, 20], whereas others - similar to our findings - did not [17, 24]. Besides the method of purification, the choice of medium and serum, and the read-out assay, also the specific LPS preparation used to stimulate monocytes is important as we demonstrate in this study. Great differences in TNF and IL-12p40 production by healthy monocytes stimulated with ultrapure LPS and the commonly used LPS preparations suggest that contaminants present in some LPS preparations activate monocytes, which may explain, at least in part, the opposing findings in literature. Although it is well-known that many preparations of LPS contain low amounts of TLR2 ligands [38], we observed that stimulation with Pam3CSK4 resulted in similar levels of TNF produced by monocytes from patients and healthy controls (data not shown). However, triggering by a different TLR2 ligand present in the LPS preparations, or synergistic triggering of the TLR2 and TLR4 pathways may be important in this.

Together, our results indicate that, by differentially affecting TLR4 and TLR8 pathways, IL-10 may mediate highly selective modulation of the function of monocytes observed in chronic HCV patients. This suggests that there is no overall increased susceptibility to pathogens, but a specific inhibition of the functionality of TLR4 signaling pathway in monocytes, which is likely mediated by IL-10.

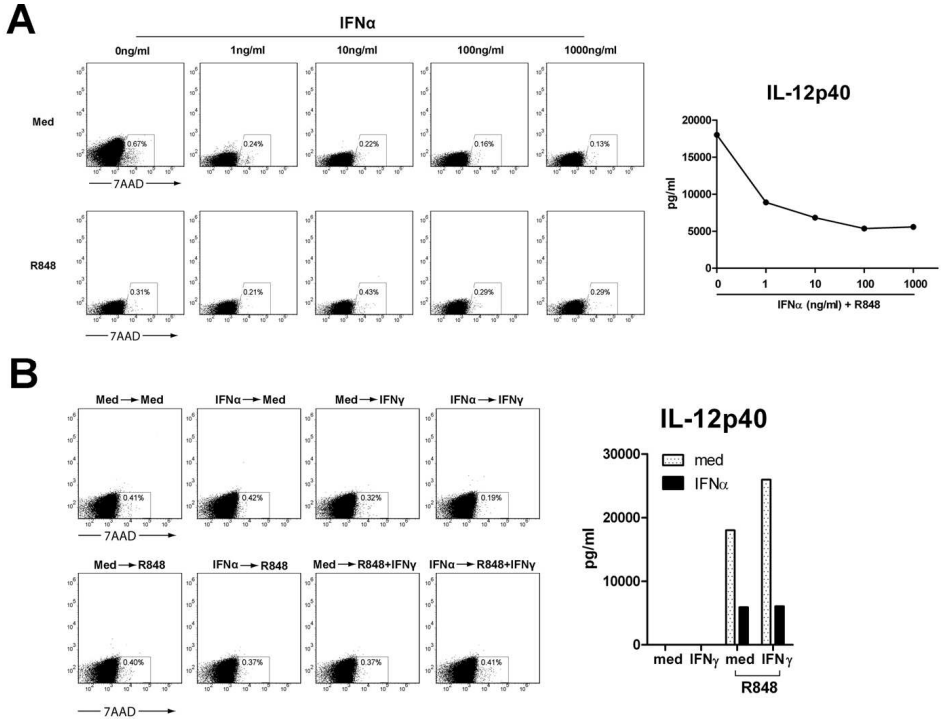
Acknowledgment

We would like to thank Robert de Knegt, Robert Roomer and Daphne Hotho for their help with collecting blood samples from chronic HCV patients visiting our outpatient clinic, and Andrea Woltman and Mark Claassen for critically reading the manuscript. We also want to thank Cheng Peng for performing the experiments on intracellular TNF staining on PBMC.

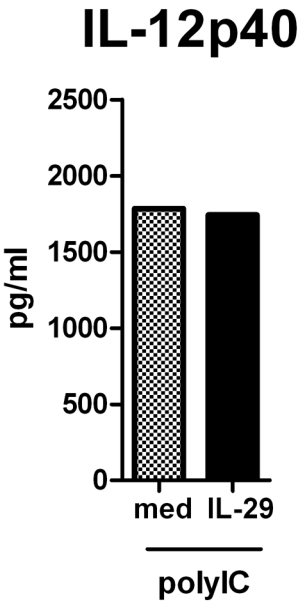
Supplementary Figures



Supplemntary Figure 1. Both monocytes and monocyte-derived dendritic cells did not respond to IL-29 with different pretreatment periods as well as different doses of IL-29 were evaluated. **(A)**. Monocytes, monocyte-derived DC and monocyte-derived macrophages were pretreated with IL-29 (100ng/ml) for the indicated periods and then further stimulated with R848 for 24h. TNF production was measured by ELISA. IL-29 alone did not induce TNF production by all three types of cells. The values depicted show representative data from 2 independent experiments. **(B)**. Monocytes, monocyte-derived DC and monocyte-derived macrophages were pretreated with IL-29 at indicated concentrations for 5h and then further stimulated with R848 for 24h. TNF production was measured by ELISA. IL-29 alone did not induce TNF production by all three types of cells. The values depicted show representative data from 2 independent experiments.



Supplementary Figure S2. The inhibitory effect of IFNα on IL12p40 production by monocyte-derived macrophages was not due to IFNα-induced cytotoxicty. **(A)** Monocyte-derived macrophages were pretreated with IFNα for indicated concentrations and further stimulated with R848 for 5h. Cells were then harvested and checked for viability with 7-AAD. The level of IL-12p40 was measured in the supernatants from cells that were stimulated for 24h. **(B)** Monocyte-derived macrophages were pretreated with IFNα for indicated concentrations and further stimulated with R848 plus IFNγ for 5h. Cells were then harvested and checked for viability with 7-AAD. The level of IL-12p40 was measured in the supernatants from cells that were stimulated for 24h.



Supplementary Figure S3. Blood myeloid DC did not respond to IL-29. $CD14^+$ and $CD19^+$ cells were depleted from PBMC.Blood myeloid DC were then purified using DBCA-1-PE and anti-PE-microbeads (Miltenyi). The purity of $BDCA1^+$ myeloid DC was higher than 95%. Myeloid DC were pretreated with IL-29 for 5h, stimulated with polyIC for 24, and IL-12p40 levels were measured in supernatant by ELISA. The data depicted show representative data from 3 independent experiments.

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Chapter 8

General Discussion and Summary

Worldwide, chronic HCV infection causes major health problems. Currently, the underlying mechanisms for the chronicity of HCV infection are still not fully understood. Besides mutational escape, HCV is generally believed to be able to induce immune suppressive mechanisms to evade from host immunity, such as the induction of IL-10 production, the functional impairment of APC (reviewed in Chapter 2) and the increase of the number of regulatory T cells. Human monocytes, containing at least two subpopulations: $CD14^+CD16^-$ and $CD16^+CD14^-$ monocytes [1-4], are important players in the first-line of defense against numerous pathogens, as well as in initiating and controlling adaptive immunity [1]. Currently, the immune status of human monocytes in chronic HCV patients is still not clear.

Due its potent antiviral effects, IFNα forms the backbone of current therapy for chronic HCV patients, and to a lesser extent for chronic HBV infection. However, only about 50% of chronic HCV and HBV patients respond to IFN-based therapy. It has been suggested that the activity of type I IFN on APC is weakened via modulation of the immunosuppressive cytokine IL-10. However, the effect of type I IFN on IL-10 production by immune cells is still under debate, and how IL-10 signaling is affected by type I IFN is not clear. IFNα was shown to be able to enhance IL-10 production by human monocyte-derived DC in response to LPS stimulation [5], and DC derived from human monocytes in the presence of IFNα or IFNβ produced high levels of IL-10 [6-9]. Furthermore, human monocyte-derived macrophages when primed by IFNα produced a higher level of IL-10 in response to LPS or R848 stimulation [10]. However, conflicting data was reported about the effect of IFNα on IL-10 by human monocytes. IFNα was reported to enhance IL-10 production by monocytes in response to TLR stimulation [11-12], whereas other group showed that IFNα inhibited IL-10 production by monocytes in response to TLR stimulation [13-14]. Moreover, how IFNα regulates IL-10 signal events is not well studied either.

Studies to find alternative therapy for chronic HCV patients are currently active, due to many side-effects and the relatively low response rates of the standard IFN-based therapy in some patients. In addition to the standard IFNα-based therapy, different TLR7/8 agonists, which are potent inducers of endogenous IFNα, for the treatment of chronic HCV patients are under investigation. Furthermore, polymorphisms close to the *IL-28B* gene that are associated with disease progression and the response to IFNα-based therapy have sparked interest in type III IFN [15-18]. Clinical studies are being conducted to examine the benefits of treatment with pegylated IL-29 [19]. However, the immunoregulatory effect of type III IFN is currently poorly understood.

Differential responsiveness of $CD14^+CD16^-$ and $CD16^+CD14^-$ monocytes to TLR4 and TLR8 agonists

Both $CD16^+CD14^-$ and $CD14^+CD16^-$ monocytes express pathogen-recognition receptors, such as TLR, which recognize microorganisms, resulting in the production of cytokines and chemokines [1]. Monocytes are important in the defense of bacterial pathogens [1, 20], as partially evidenced by the factors that monocytes are highly responsive to TLR4 and TLR2 agonists (**Chapter 3**). However, how monocytes respond to viral-derived pathogens is still not clear. In **Chapter 3 and 4**, we studied in detail the responsiveness of human $CD14^+CD16^-$ and $CD16^+CD14^-$ monocytes to LPS, a TLR4 agonist and R848, a TLR8

agonist. While TLR4 recognizes bacterial derived LPS, TLR8 recognizes the single-stranded RNA (ssRNA), which is present in many viruses, including HCV.

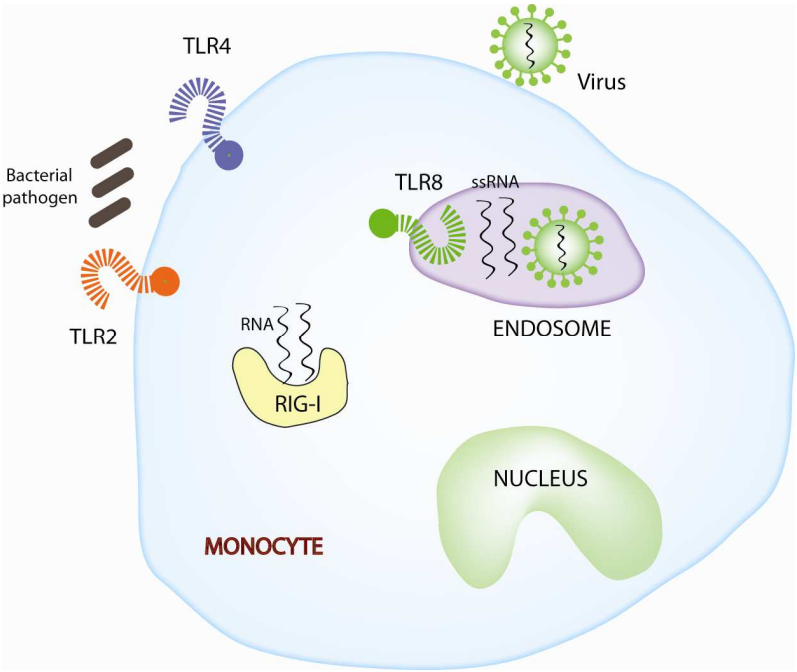

Figure 1. Recognition of bacterial- and viral-derived pathogens by human monocytes. While human monocytes are highly activated by bacterial derived pathogens via TLR2 and 4, they can also recognize viral-derived RNA via intracellular receptors, such as TLR8 and RIG-I.

We found that $CD14^+CD16^-$ monocytes were highly activated by both LPS and R848, as demonstrated with high level of TNF, IL-12p40 production (**Chapter 3**). Interestingly, $CD16^+CD14^-$ monocytes were more responsive to TLR8 ligation by their production of TNF and IL-12p40 as compared to $CD14^+CD16^-$ monocytes, while $CD16^+CD14^-$ monocytes were less responsive to TLR4 ligation than $CD14^+CD16^-$ monocytes (**Chapter 4**). These findings suggest that in addition to their well-known function in defense to bacterial invasions, monocytes may also play a role in some viral infections (**Figure 1**). Indeed, it is reported that human $CD16^+CD14^-$ monocytes patrol and sense nucleic acids and viruses via TLR7/8 receptors [21]. In addition to TLR8, RIG-I expressed in human monocytes, can also recognize RNA oligonucleotides to produce IFNα [22]. Interestingly, compared with $CD14^+CD16^-$ monocytes, $CD16^+CD14^-$ monocytes may be specialized to recognize RNA viruses, since they highly respond to TLR8 agonists, but not TLR4 agonists.

Role for IL-10 in modulating the function of monocytes isolated from chronic HCV patients

IL-10 has been suggested as one of the mechanisms that favor the development of chronic HCV infection. Indeed, a higher systemic level of IL-10 was found in our chronic HCV patients (**Chapter 3**), which is supported by previous reports [23-24]. Interestingly, we found that $CD14^+CD16^-$ monocytes isolated from chronic HCV patients had higher levels of spontaneous IL-10 production and also accordingly higher levels of IL-10 mRNA expression, as compared with $CD14^+CD16^-$ monocytes isolated from healthy individuals. This is important, since monocytes are the main producers of IL-10 in peripherial blood in response

to LPS or R848 stimulation (**Chapter 3 and 5**). Furthermore, serum isolated from chronic HCV patients had the ability to enhance IL-10 production by $CD14^+CD16^-$ monocytes upon LPS stimulation (**Chapter 3**). HCV mainly infects hepatocytes, but not monocytes in the periphery [25]; we thus hypnotized that HCV might have an indirect effect on the function on monocytes via IL-10. As expected, we found that LPS-induced TNF and IL-12p40 production by monocytes isolated from chronic HCV was suppressed, since we observed that the TLR4 pathway in $CD14^+CD16^-$ monocytes was very sensitive to IL-10. However, TLR8-derived TNF and IL-12p40 was not inhibited which was explained by our finding that the TLR8 pathway in $CD14^+CD16^-$ monocytes was less sensitive to IL-10 as compared with the TLR4 pathway (**Chapter 3**). Our results indicate that, by differentially affecting the TLR4 and TLR8 pathways, IL-10 may mediate highly selective modulation of the function of monocytes observed in chronic HCV patients. This suggests that there is no overall increased susceptibility to pathogens, but a specific suppression of the functionality of the TLR4 signaling pathway in monocytes, which is, at least partly, mediated via IL-10. Although the function of $CD14^+CD16^-$ monocytes isolated from chronic HCV patients is selectively impaired in TLR pathways, the response of human $CD16^+CD14^-$ monocytes to TLR ligation was weakly modulated as a consequence of persistent infection with HCV (**Chapter 4**). Therefore, in monocytes from chronic HCV patients, the TLR4 pathway, which recognizes bacterial pathogens, is impaired in $CD14^+CD16^-$ monocytes, but the TLR8 pathway, which recognizes viral-derived ssRNA, is not affected in both $CD14^+CD16^-$ and $CD16^+CD14^-$ monocytes (**Figure 2**).

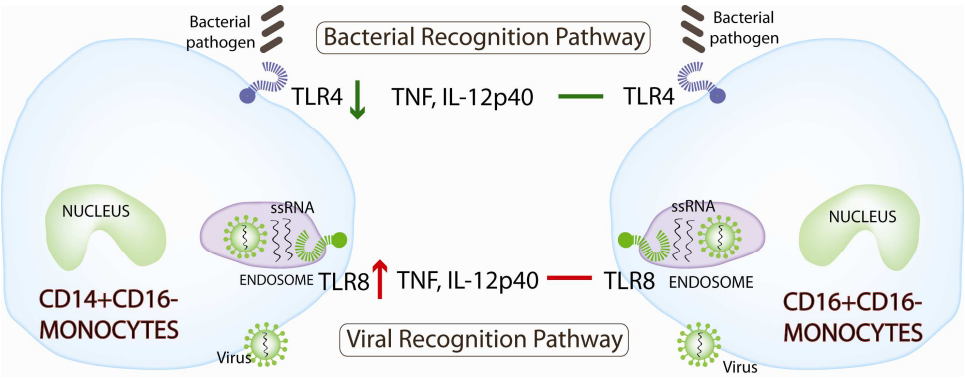

Figure 2. Monocytes from chronic HCV patients are impaired in bacterial recognition the TLR4 pathway, but not in the TLR8 pathway, which is able to recognize viral-derived ssRNA. In chronic HCV patients, the TLR4 pathway, which sense bacterial pathogen, is impaired in $CD14^+CD16^-$ monocytes possibly via IL-10, but the TLR8 pathway, which is able to recognize ssRNA, is not impaired. The response to TLR ligation of human $CD16^+CD14^-$ monocytes is weakly modulated as a consequence of persistent infection with HCV.

Indirect modulatory effects of HCV on human APC

The functional modulation of human APC by HCV has been observed (**Chapter 2**) despite the fact that HCV mainly infects liver hepatocytes, but not human monocytes nor DC. This indicates that HCV infection may induce indirect mechanisms to affect the function of peripheral APC (**Figure 3**). Indeed, we have shown in **Chapter 4** that chronic HCV infection elevates systemic IL-10 levels as well as increases TLR-induced IL-10 production by monocytes, and subsequently affects the function of monocytes via IL-10, and possibly other

immune cells. Furthermore, it is reported that other cytokines, such as IFNγ, MIP-1β, are also induced by chronic HCV infections and affect the function of human APC [26-27]. In addition to cytokines, HCV encoded proteins, such as core, NS3 etc., have reported to have immune modulatory effects on human APC [28-31]. In the liver, similar indirect effects via cytokines or HCV proteins could be speculated during chronic HCV infection. Interestingly, it has been suggested by *in vitro* studies [32] that the function of liver APC could be impaired by the debris from HCV-infected apoptotic hepatocytes, despite liver APC are not directly infected by HCV.

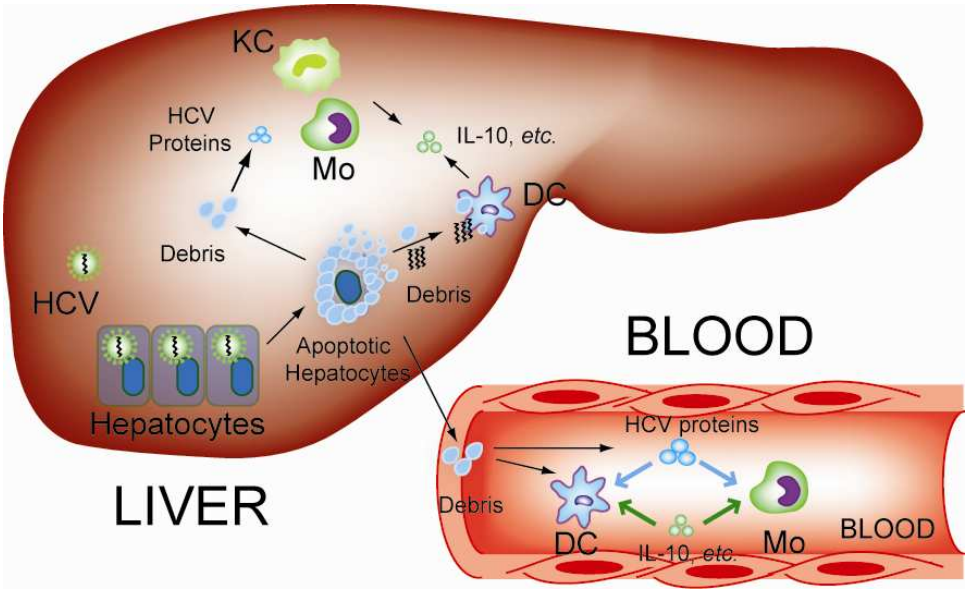

Figure 3. Hypothesized indirect modulatory effects of HCV on liver and peripheral APC. During chronic HCV infection, HCV induces apoptosis in liver hepatocytes and the apoptotic debris and the viral encode proteins could impair the function of liver APC. The debris of apoptotic and the viral components may circulate into the periphery and affect the function of immune cells in the blood.

Therefore, during chronic HCV infection, HCV-infected liver hepatocytes may slowly undergo apoptosis, as indicated by the higher ALT levels in chronic HCV patients. Liver APC could be exposed to the apoptotic debris as well as the viral components and then be functionally impaired. It could be speculated that the debris of apoptotic and the viral components may circulate into the periphery and affect the function of immune cells in the blood (**Figure 3**).

Type I and III IFN priming increase the sensitivity of human monocytes and macrophages to IL-10

IFNα is well known for its antiviral and immunomodulatory effects. In this, IFNα has been shown to increase TLR-induced IL-10 production by human monocyte-derived DC and macrophages [5-10] and mouse bone marrow derived macrophages [33]. Previously, conflicting data was reported about the effect of IFNα on IL-10 by human monocytes, which are important IL-10 producers in human leukocytes. IFNα was reported to enhance IL-10 production by monocytes in response to TLR stimulation [11-12]; whereas other groups

showed that IFNα inhibited IL-10 production by monocytes in response to TLR stimulation [13-14]. In **Chapter 5**, we aimed to settle these controversies about the effect of IFNα on monocyte-derived IL-10 production by using several different techniques. We showed that IFNα decreased the percentages of TLR-induced IL-10-producing monocytes in human PBMC and also inhibited IL-10 production by purified monocytes in response to TLR ligation. Interestingly, despite IFNα-mediated inhibition of IL-10 production by human monocytes, TLR-induced IL-12p70 secretion by IFNα-primed cells was strongly controlled by IL-10. We observed that priming of monocytes with IFNα or IFNβ up-regulated membrane IL-10 receptor 1 (IL-10R1) expression, which may –at least partly- be responsible for enhanced IL-10 induced phosphorylation of STAT3 (**Figure 4**). Moreover, type I and III IFN potentiated IL-10 signalling in a comparable manner in macrophages, indicating a more general effect of IFN on modulating the activity of IL-10 in APC. In summary, in this study, we demonstrated that one of the consequences of priming of human APC with type I and III IFN was to promote the cells' sensitivity to IL-10 rather than to promote IL-10 production.

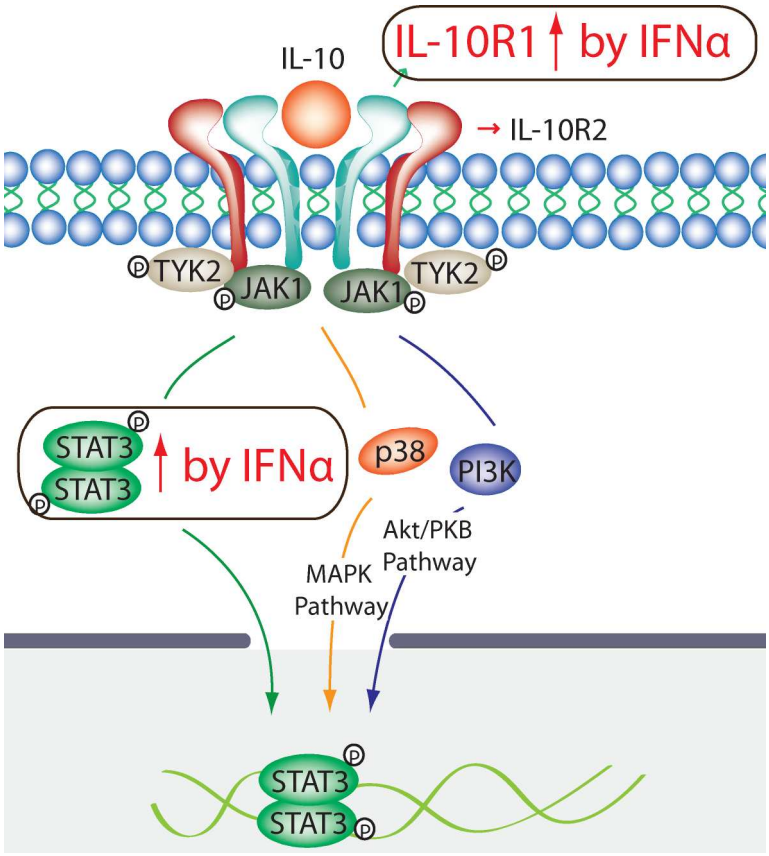

Figure 4. IFNα pretreatment enhances IL-10 signal events via up-regulating membrane IL-10R expression and enhancing IL-10-induced p-STAT3.

It is well known that IFNα can activate multiple signaling pathways and it is possible that the promotor of the gene that encodes IL-10R1 is regulated by one of the ISGs. This may be examined by future studies on the genetic structure of the IL-10R1 promotor. The up-regulation of IL-10R1 by IFNα may be sufficient to enhance IL-10 downstream signal events, as observed by enhanced IL-10-induced p-STAT3. However, it might also be possible that IFNα directly provides some molecules, such as p-JAK1, p-TYK2 or p-STAT3 for IL-10/IL-

10R complex, since these molecules are also involved in IL-10 signaling pathways. Interestingly, the IL-10/IL-10R1 complex is similar to the IFNγ/IFNγR complex [34]; and it has been shown that low concentrations of IFNα can provide p-STAT1 for the IFNγ/IFNγR complex [34]. Thus, it is possible that the downstream molecules in the IFNα pathway can also be recruited by the IL-10/IL-10R1 complex (**Figure 5**).

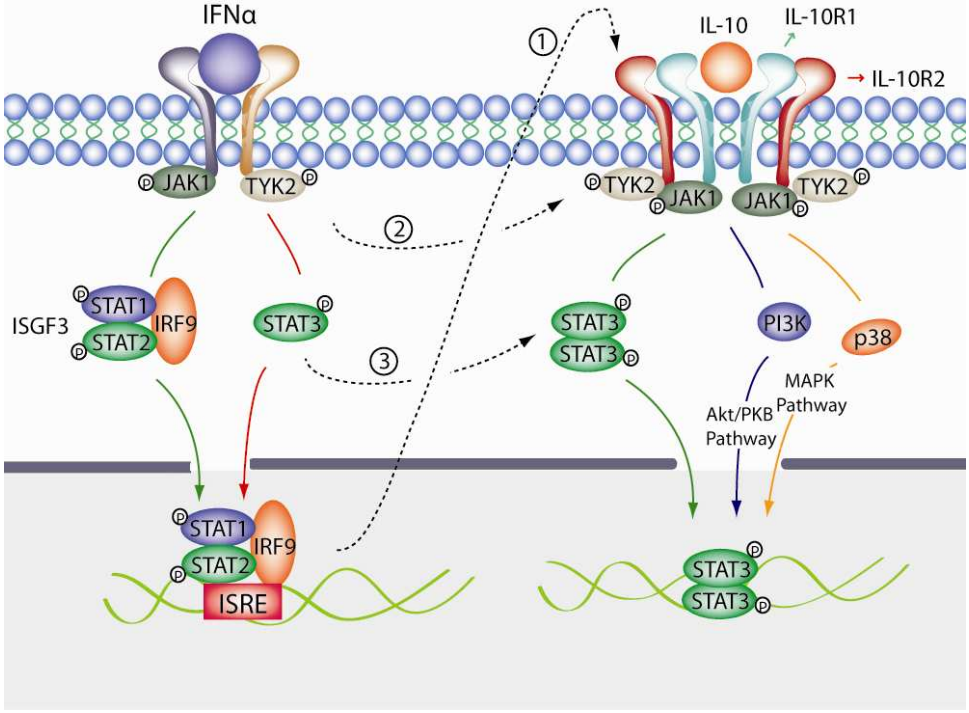

Figure 5. The possible explanations for the findings that IFNα pretreatment enhances the IL-10 signal events. (1) the IL-10R promoter might be one of the targets of IFNα signaling (1); IFNα-induced molecules, such as p-JAK1, p-TYK2 and p-STAT3 could be directly recruited by the IL-10/IL-10R complex (2, 3)

Due to the severe side-effects of IFNα-based therapy for chronic HCV patients, we were interested to study the effects of an oral TLR7 agonist, ANA773, which is a potent inducer of endogenous IFNα, in the treatment of chronic HCV patients in **Chapter 6**. Importantly, monocytes do not express TLR7, which means that monocytes will not be activated by ANA773. The administration of ANA773 resulted in transiently reduced numbers of mDC and pDC in blood. Interestingly, reduced pDC numbers as well as increased serum IFNα and IP-10 levels were observed only in virological responders (≥1 $\log_{10}$ IU/mL reduction of HCV RNA levels upon ANA773 treatment), but were absent in virological non-responders. *In vitro* stimulation of PBMC from virologic responders showed a high frequency of IFNα-producing pDC upon stimulation in vitro with ANA773, whereas no IFNα was induced in non-responders. These findings indicate that the efficacy of viral load decline in chronic HCV patients with the TLR7 agonist ANA773 is likely due to intrinsic differences in the induction of endogenous interferons and ISG products (IFNα and IP-10) upon TLR7 ligation.

The regulatory effect of IL-29 on IL-12p40 production by human macrophages

Type III IFN including IL-28A (IFNλ2), IL-28B (IFNλ3) and IL-29 (IFNλ1), were first uncovered in 2003 [35-36]. Since then, the research was mainly focused on the antiviral activities of

these cytokines. Type III IFN have been shown to possess potent antiviral activities via mechanisms similar to IFNα despite triggering of a unique IL-28 receptor pair which is distinct from the IFNα receptor [37-39]. *In vitro*, IL-29 has been shown to be able to suppress the replication of HCV in human hepatocyte cell lines [40-42]. The current IFNα-based standard therapy for chronic HCV patients has many side-effects and a limited effectiveness in a group of patients. Due to the restricted expression of IL-28RA, type III IFN become a potential alternative medicine for chronic HCV patients. Clinical studies are being conducted to examine whether pegylated-IL-29 holds promise for future therapeutic use in the treatment of chronic HCV patients.[19]. The interests in type III IFN is further inspired by many recent findings that polymorphisms close to the *IL-28B* gene are associated with disease progression and response to IFNα-based therapy for chronic HCV patients. The antiviral activities of type III IFN have been extensively studied [42-46], however, little is known about the effect of type III IFN on innate immune cells and their immunoregulatory activity.

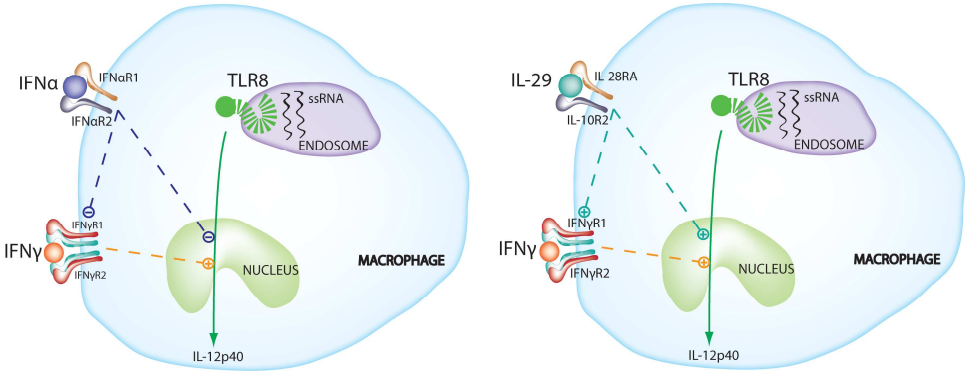

Figure 6. IL-29 and IFNα differentially regulate IL-12p40 production by human monocyte-derived macrophages. IFNα has a weak inhibitory effect on TLR8-inudeced IL-12p40 production by macrophages, whereas IFNα potently inhibits IFNγ-induced IL-12p40 by macrophages in response to TLR8 stimulation. In contrast, IL-29 enhances TLR8-induced IL-12p40 production as well as IFNγ-induced IL-12p40 by macrophages upon TLR8 stimulation.

We have shown in **Chapter 7** for the first time that IL-29 can increase TLR-induced IL-12p40 production by human monocyte-derived macrophages. Furthermore, IL-29-treated macrophages were more responsive to IFNγ, since IL-29 enhanced IFNγ-induced IL-12p40 and TNF production by macrophages upon R848 stimulation. However, IFNα suppressed IFNγ-induced IL-12p40 and TNF production by human macrophages (**Figure 6**). The differential effects of IL-29 and IFNα on the responsiveness of macrophages to IFNγ could not be explained by an effect on TLR7 or TLR8 mRNA expression, or by altered IL-10 signaling. However, we demonstrated that IL-29 up-regulated, whereas IFNα down-regulated, the surface expression of the IFNγR1 on macrophages, thereby resulting in differential responsiveness of TLR-challenged macrophages to IFNγ. Our findings on the differences between IFNα and IL-29 in modulating TLR-induced cytokine production by macrophages may further contribute to understand the role of interferons in regulating immunity to pathogens.

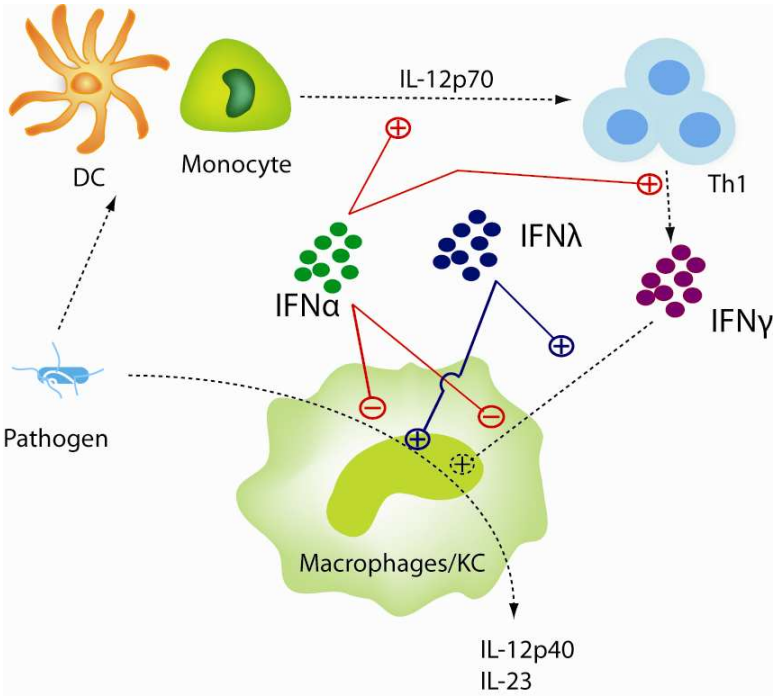

Figure 7. IL-29 and IFNα may co-operate to induce a Th1 response during bacterial or viral infections. IFNα favors the Th1 responses by enhancing IL-12p70 production by APC and amplifies the Th1 responses via increasing IFNγ production by Th1 cells in the presence of APC; IL-29 activates cytokine production by macrophages and also enhances the responses of macrophages to IFNγ. Also, type III IFN reduces IL-13 production by ConA-driven Th2 cells [51] and inhibits GATA3 expression in human naïve and memory T cells [52].

Despite the finding that IFNα inhibits IL-12p40 production by macrophages, IFNα is shown to potently enhance IL-12p70 production by both human and mouse monocytes and DC [47], which indicates that IFNα favors Th1 response upon bacterial and viral infections, since the level of IL-12p70, but not IL-12p40, is important for promoting a Th1 response. Also, IFNα can increase IFNγ production by Th1 cells indirectly via APC [48-50], showing that IFNα can further amplify Th1 responses. However, IL-29 is able to activate human macrophages and also enhances the responses of macrophages to IFNγ. Interestingly, type III IFN have no effect on DC and monocytes, and also they have no direct effect on Th1 responses, yet it has been reported that IL-29 is able to reduce IL-13 production by ConA-driven Th2 cells [51] and inhibits GATA3 expression in human naïve and memory T cells [52]. Therefore, IFNα and type III IFN, induced during bacterial and viral infections, might together favor the Th1 responses (**Figure 7**).

IFNα has been shown to have an effect on human liver Kupffer cells (KC) and play an important role in viral infection in the liver. We have found that IL-29 has a regulatory effect on human monocyte-derived macrophages, and we are interested to translate our *in vitro* findings on IL-29 to processes operational in the liver of chronic HCV patients. The future research will focus on liver KC. Since the SNPs close to *IL-28B* gene are associated with the IFNα-based therapy, it is also very interesting to study the cross-talk between IFNα and type III IFN in human liver.

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Summary

The consequences of chronic infection with the HCV on immunity to distinct pathogens are not fully appreciated despite the potent modulatory effects of HCV on the immune system. Our research shows that there is no overall increased susceptibility to pathogens, but a specific suppression of the functionality of TLR4 signaling pathway in $CD14^+CD16^-$ monocytes from patients chronically infection with HCV, which is, at least partly, mediated via IL-10 either present in the serum of the patients or produced by monocytes. Different from $CD14^+CD16^-$ monocytes, the function of $CD16^+CD14^-$ monocytes is only minimally altered as a consequence of the persistent HCV infection, indicating no roles for $CD16^+CD14^-$ monocytes in HCV pathogenesis.

Type I IFN form the backbone of current therapy for chronic HCV patients, and to a lesser extent for chronic HBV infection. However, only about 50% of chronic HCV and HBV patients respond to IFN-based therapy. We demonstrated that one of the effects of type I and III IFN on human APC is to promote the cells' sensitivity to IL-10. These findings suggest that IFNα also acts as a foe during infections or IFN-based therapy for chronic HCV patients due to its indirect immunosuppressive capacities on immune cells, and modulation of IL-10 signaling may improve the outcome of the standard IFN-based therapy for chronic HCV patients.

Type III and I IFN were previously shown to induce similar antiviral signaling pathways via different receptors. However, the effects of type III IFN on immune cells were not completely appreciated. Our research reports for the first time that type III and I IFN differ in the regulation of IL-12p40 and IL-23 production by TLR-activated human macrophages. These findings on the differences between type I and III IFN in modulating TLR-induced cytokine production by macrophages may further contribute to understand the role of IFNs in regulating immunity to pathogens as well as the therapy for patients with viral hepatitis.

Samenvatting

De gevolgen van chronische HCV infectie voor de immuniteit tegen verschillende ziekteverwekkers wordt niet volledig naar waarde geschat ondanks de krachtige modulerende effecten van HCV op het immuunsysteem. Ons onderzoek laat zien dat de vatbaarheid voor ziekteverwekkers in het algemeen niet verhoogd is, maar er een specifieke onderdrukking plaats vindt van de TLR4 signaaltransductie route in $CD14^+CD16^-$ monocyten van patiënten die chronisch geïnfecteerd zijn met HCV. Deze onderdrukking wordt, in ieder geval gedeeltelijk, tot stand gebracht door IL-10 dat aanwezig is in het serum van de patiënten of geproduceerd wordt door monocyten. In tegenstelling tot $CD14^+CD16^-$ monocyten is de functie van $CD16^+CD14^-$ monocyten slechts minimaal beïnvloed door de chronische HCV infectie, hetgeen er op duidt dat er geen rol is weggelegd voor $CD16^+$ $CD14^-$ monocyten in de HCV pathogenese.

IFNα vormt de basis van de huidige therapie voor chronische HCV patiënten, en in mindere mate van de therapie voor chronische HBV patiënten. Echter, slechts 50% van de chronische HCV en HBV patiënten reageren op deze op IFN gebaseerde therapie. Wij hebben aangetoond dat een van de effecten van type I en III IFN op humane antigeen-presenterende cellen is het bevorderen van de gevoeligheid van deze cellen voor IL-10. Deze bevindingen suggereren dat IFNα ook een nadelig effect kan hebben op de immuunrespons tijdens infecties of tijdens de op IFN gebaseerde therapie voor chronische HCV patiënten, als gevolg van de indirecte immuunsuppressieve werking op immuuncellen. Derhalve zou modulatie van de IL-10 signaaltransductie route mogelijk de uitkomst kunnen verbeteren van de op IFN gebaseerde standaard therapie voor chronische HCV-patiënten.

Van Type III en I IFN werd eerder aangetoond dat ze dezelfde antivirale signaaltransductie routes induceren via verschillende receptoren. De effecten van Type III IFN op immuuncellen zijn echter nog niet volledig onderkend. Ons onderzoek laat voor het eerst zien dat Type III en I IFN verschillen in de regulatie van IL-12p40 en IL-23 productie door TLR geactiveerde humane macrofagen. Dit verschil tussen Type I en III IFN in het moduleren van TLR-geïnduceerde cytokine productie door macrofagen kan verder bijdragen aan de kennis over de rol van IFN in zowel immuunregulatie tegen pathogenen als de therapie voor patiënten met virale hepatitis.

Acknowledgements

I have been much enjoyed with the time in the Netherlands. My life here started with the surprise from the first day that the Dutch people look all the same and ends up with the satisfaction that the Dutch do look different. To me, these four years time would not be so interesting and exciting without being together with the people in the lab and clinic.

I would like to first give my thanks to Prof. Harry HLA Janssen and Dr. Andre Boonstra, who supervised my research and also gave tremendous help for my life. **Prof. dr. Harry HLA Janssen** is a very nice person. I still remember he invited me and my wife to his home for dinner, which kept his wife busy for few hours. His kindness is more than I could tell. Even yesterday, I was still working on the desk he gave to me just after I moved to the Netherlands. In research, I was a bit afraid of him in the beginning, but I appreciated his trust and fully support. **Dr. Andre Boonstra** is a great mentor. The words, even my mind, would never, never be able to fully cover my gratitude to him. He has taught me the differences between Chinese English and real English, the skills to give presentation, and to be critical in research, thinking and writing. “Think it again, does that sentence make sense?” would be one of his most useful sentences he have given to me. Dr. Andre Boonstra is one of the most hard-working as well as most productive people I’ve ever met. He has improved my way of thinking and way of working in both life and research. Again, many thanks to both of you for the help and supervision!

To **Marion Hoogendoorn**, you have been giving me tremendous help in past 4 years. You are such a nice and careful person that you have never forgotten any thing of me, Again, many thanks for the great help to my PhD defence. You are always ready to help me with every small thing, making a call to pedel, helping to translate my certificates, arranging my date of defence, etc.

To **Dr. Andea Woltman**, thank you for giving useful comments and discussions on my presentation and writing. To **Prof. dr. Maikel Peppelenbosch**, thank you for giving an excellent recommendation for my new job.

To my roommates (**Arjan Boltjes**, **Colin de Haar**, **Gwenny Fuhler**, **and Nadine van Montfoort**), thank you for the nice discussion on the Dutch education, Dutch culture, Dutch food, Chinese one-child policy, etc., also for the comments on my presentations. I have been feeling thankful for you guys to be ready at any moment to help me with the trouble I encountered, Dutch mails, Dutch calls and so on. More special thanks for **Arjan Boltjes,** for without you, how could I fill in the forms from the Dutch tax office? I feel so lucky to have many of such nice people as you in the room.

To **Eric Twja** and **Mark Claassen** for your friendship and kindness, I really enjoyed the time with you. If it were not you, my time in the Netherlands would be much less perfect as it is now. I celebrated the first Christmas in my life with Eric and your family, and the Chinese

food you cooked is really tasteful! Also, from Mark and your family, I have known much Dutch life. The time the little kids played together will definitely be kept in my memory. To **Abdullah Pan (Qiuwei Pan) and your family**, for without you, both I and my family would feel lonely in the Netherlands. To **Dowty Movita** and **Viviana Moroso,** thank you for the nice time in the lab, the great encouragement in research. I firmly believe we will keep our friendship through the life time.

To **Duygu Turgut, Paula Biesta, Kim Kreefft** and **Anthonie Groothuismink**, to all the people from the clinic, HCV and HBV group and all the other people from the lab, thank you all for your great help in the research as well as the jokes. Without you, my time in the Netherland wouldn't be so much fun.

Finally, to my wife, **Jin Liu**. You have always been my side to support me, to encourage me, and to help me. For numbers and numbers of the weekends, you did the house work, prepared the food and took care of the child, without your fully support, I would not be able to go through these four years. I hope I have made you proud of my work.

PhD Portfolio

Name PhD Student	Bisheng Liu
Erasmus MC Department	Gastroenterology and Hepatology
PhD Period:	October 2007 – December 2011
Promotor:	Prof. dr. H.L.A. Janssen
Copromotor:	Dr. A. Boonstra

PhD Training

General academic and research skills (Erasmus MC)	Year
English biomedical writing and communication	2008
Course on radioactivity	2008
Basic course on 'R'	2010
Basic Introduction Course on SPSS	2010
Analysis of microarray gene expression data using R/BioC and web tools	2011
Browsing Genes and genomes with UCSC	2011

(Inter)national conferences	Year
Dutch Annual Virology Symposium, Amsterdam. The Netherlands	2008
EASL Monothematic Conferences: Immune Mediated Liver Injury. The European Association for the Study of the Liver, Hamburg, Germany	2008
Cellular and Cytokine Interactions in Health and Disease. Tri-Society Annual Conference, Lisbon, Portugal	2009
Dutch Society for Immunology, Lunteren, The Netherlands	2009
Symposium: New Frontiers in Pattern Recognition Receptors, Nijmegen, The Netherlands	2009
Dutch Society for Gastroenterology and Hepatology, Veldhoven, The Netherlands	2010
British Society for Immunology, Liverpool, UK	2010
Dutch Annual Virology Symposium, Amsterdam. The Netherlands	2011
Symposium on "Features and functions of T-cells in health and disease", Dutch Society for Immunology, Lunteren, The Netherlands	2011

Scientific Awards and Grants	*Year*
EASL (European Association for the Study of the Liver) Young Investigators' Bursary.	2008
Travel grant for oral presentation at the British Society for Immunology, Liverpool, UK	2010
The 4th prize winner for the annual publication (Erasmus MC, 2010)	2010

Invited Speakers	*Year*
Regulatory effect of IFNλ on IL-12p40 production by human macrophages. British Society for Immunology, Liverpool, UK.	2010
Distinct regulation of bacterial and viral recognition pathways in monocytes from patients with chronic HCV infection. Dutch Society for Gastroenterology and Hepatology, Veldhoven, The Netherlands.	2010

Curriculum vitae

Bisheng Liu was born on March 6 1982 in Binhai, northern Jangsu province, China, to parents Meng Liu and Mei Liu, brother to Bicheng Liu.

He obtained his Bachelor's degree in Bioengineering from Zhejiang Gongshang University, located in the capital city of Zhejiang province, where he studied the applied microbiology in food science from 2000 to 2004. While Bisheng's behaves in the university had resulted in several times of first-class scholarships, he found himself to be possessed by molecular biology from the second year. He then spent most of his time in the library and bookshops, where he learned molecular biology, genetics, and cellular biology.

From 2004-2007, Bisheng started his research work under the supervision of Prof. Xinyuan Liu and Prof. Chen Qian in Zhejiang Sci-Tech University, where he obtained his Master's degree. His research focused on constructing several tumour-targeting adenoviral delivery systems, followed by further in vitro and in vivo evaluating the antitumor activities of those oncolytic viruses. In the lab, he learned much knowledge and lab skills in molecular biology and virology, but more importantly, he met his wife, Jin Liu, a beautiful and smart lady.

At the end of 2007, Bisheng moved with his family to the Netherlands and started his PhD training in immunology of chronic hepatitis C viral infection, promoted by Prof.dr. H.L.A. Janssen and Dr. A Boonstra. During the four years of research, Bisheng have finished several interesting projects on examining the function antigen-presenting cells from chronic HCV patients, with the focuses on the effects of interferon and IL-10. While Bisheng have performed excellently in the lab, he also feels proud of the various opportunities to improve his scientific writing, presentation skills as well as to attend national and international conferences. Finally, Bisheng would defend this PhD thesis under the supervision of Prof.dr. H.L.A. Janssen and Dr. A Boonstra.

For his research career, Bisheng's next plan is to work as a post-doc. However, wherever Bisheng goes, he will always remember what his parents told him "Hard-working plus a good personality will lead you to your dreams!"

Publications

1. **Bi-Sheng Liu**, Andrea M. Woltman, Harry L.A. Janssen, André Boonstra. *Modulation of dendritic cell function by persistent viruses.* J Leukoc Biol. 2009;85(2):205-214.
2. **Bi-Sheng Liu**, Harry L.A. Janssen, André Boonstra. *IL-29 and IFNα differ in their ability to modulate IL-12 production by TLR-activated human macrophages, and exhibit differential regulation of the IFNγreceptor expression.* Blood. 2011;117(8):2385-2395.
3. **Bi-Sheng Liu**, Zwier M.A. Groothuismink, Harry L.A. Janssen, André Boonstra. *Role for IL-10 in inducing functional impairment of monocytes upon TLR4 ligation in patients with chronic HCV infections.* J Leukoc Biol. 2011 Jun;89(6):981-988.
4. **Bi-Sheng Liu,** Cheng Peng, Robert J. Knegt, Harry L.A. Janssen, André Boonstra. *The Response to TLR ligation of human CD16+CD14- monocytes is weakly modulated as a consequence of persistent Infection with the hepatitis C virus.* Mol Immunol. 2011 Jul;48(12-13):1505-1511.
5. André Boonstra, **Bi-Sheng Liu**, Z.M.A. Groothuismink, Harry L.A. Janssen. *Potent immune activation in chronic hepatitis C patients upon administration of an oral inducer of endogenous interferons that acts via TLR7.* Antivir Ther. in press.
6. **Bi-Sheng Liu**, Harry L.A. Janssen, André Boonstra. *Type I and III IFN enhance IL-10-induced STAT3 phosphorylation in human monocytes and macrophages.* Submitted.
7. **Bi-Sheng Liu**, Harry L.A. Janssen, André Boonstra. The immunosuppressive effects of *type I IFN on antigen-presenting cells.* In preparation.