Antiviral Research 98 (2013) 469–475

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Erythropoietin administration suppresses human monocyte function *in vitro* and during therapy-induced anemia in HCV patients

Michelle Spaan, Zwier M.A. Groothuismink, Ludi Koning, Robert Roomer, Harry L.A. Janssen, Robert J. De Knegt, André Boonstra *

Department of Gastroenterology and Hepatology, Erasmus MC – University Medical Center Rotterdam, The Netherlands

ARTICLE INFO

Article history:
Received 9 December 2012
Revised 26 March 2013
Accepted 3 April 2013
Available online 11 April 2013

Keywords:
Erythropoietin
Monocytes
Cytokines
Viral hepatitis
Innate immunity

ABSTRACT

Erythropoietin (EPO) is a hormone that controls red blood cell production. Binding of EPO to the EPO-receptor results in increased numbers of red blood cells in the circulation, which makes EPO a potent molecule to treat anemia in various groups of patients. Although numerous studies have examined the clinical effects of EPO, its immunological effects have received less attention.

In this study, we examined the immunological effects of EPO on human monocytes. We show that human monocytes express EPO receptor mRNA, and are responsive to EPO in cell culture. *In vitro* exposure of PBMC from individuals to EPO and the TLR4 ligand LPS showed a significant reduction of monocytes producing IL-6 and TNF, while the frequencies of IL-12p40, IL-10, MIP-1β and IL-8-producing cells did not change upon incubation with EPO. In addition, EPO did increase the phagocytic activity but did not affect the ability to produce ROS by monocytes. Moreover, we studied eight chronic HCV patients undergoing treatment with peg-IFN and ribavirin, who were administered EPO for treatment-induced anemia. Blood was collected before and 7 days after EPO injection. In 7 patients, we observed a significant decline at day 7 after EPO administration of the frequency of monocytes producing various pro-inflammatory cytokines following stimulation with the TLR4 ligand LPS and the TLR7/8 ligand R848, which is in line with our *in vitro* findings. Our findings demonstrate an inhibitory effect of EPO on the secretion of effector molecules by monocytes and a stimulatory effect on the phagocytic activity by monocytes.

1. Introduction

Erythropoietin (EPO) is a renally secreted hormone that promotes red blood cell production in bone marrow by binding to the EPO-receptor (EPO-R). This interaction results in an increased number of red blood cells in the circulation, which makes EPO a potent molecule to treat anemia in various groups of patients (Alavian et al., 2012). Although numerous studies have examined the clinical effects of EPO, its immunological effects have received less attention. Immune cells have been shown to bear the EPO-R, making them probable targets (Brines and Cerami, 2005; Jelkmann, 2007). Indeed, in polyclonally stimulated whole blood cell cultures from hemodialysis patients, EPO increased IL-2, IL-10 and IL-12 production, while IL-6 and TNF production was reduced (Bryl et al., 1998, 1999; Trzonkowski et al., 2002). Also granulocytes and neutrophils have been shown to be activated by EPO (Costa et al., 2008) and incubation of B cells with EPO led to increased IgM production (Kimata et al., 1991). Recently, it was shown that administration of EPO to mice reduced the production of IL-6 and TNF, as well as nitric oxide. Furthermore, systemic bacterial infection and impaired pathogen clearance was observed in these mice, which resulted in reduced survival (Nairz et al., 2011).

Eighty percent of patients infected with the hepatitis C virus (HCV) are unable to resolve the infection by their own immune system. It has been shown that natural killer (NK) cells as well as dendritic cells (DCs) are functionally impaired in chronic HCV patients compared to healthy individuals (Jinushi et al., 2004; Oliviero et al., 2009; Woltman et al., 2010). Besides innate immunity, adaptive immunity is affected as well. The continuous presence of high levels of viral antigens leads to a weaker effector function of HCV-specific T cells, which is a characteristic feature of immunity in chronic HCV patients (Shoukry et al., 2003; Spaan et al., 2012). Antiviral therapy consisting of pegylated interferon-alpha

Abbreviations: EPO, erythropoietin; EPO-R, EPO receptor; HCV, hepatitis C virus; NK, natural killer; DC, dendritic cell; PEG-IFN, pegylated interferon-alpha; Hb, hemoglobin; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxidative species; PHA, phytohemagglutimin.

* Corresponding author. Address: Department of Gastroenterology and Hepatology, Erasmus MC – University Medical Center Rotterdam, 's-Gravendijkwal 230, Room Na-1011, 3015 CE Rotterdam, The Netherlands. Tel.: +31 10 7035944; fax: +31 10 7032793.

E-mail address: p.a.boonstra@erasmusmc.nl (A. Boonstra).

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http://dx.doi.org/10.1016/j.antiviral.2013.04.003

(peg-IFN) and ribavirin has been the standard of care for chronic HCV patients for many years, with the recent addition of protease inhibitors to this treatment further improving the efficacy (Pawlotsky, 2011b). A major side effect of treatment with ribavirin and peg-IFN is anemia, which is even more pronounced by the addition of protease inhibitors (Pawlotsky, 2011a). Normalization of hemoglobin (Hb) levels can be achieved by ribavirin dose reductions, but this may lower the treatment efficacy. As an alternative to manage anemia, EPO can be administered to stimulate the generation of erythrocytes (Dieterich et al., 2003; Gergely et al., 2002; Pockros et al., 2004; Shiffman et al., 2007), while reducing the necessity of ribavirin dose adjustments, which may benefit the efficacy of therapy (Shiffman et al., 2007; Stickel et al., 2012). However, recent data showed no beneficial effect of EPO compared to ribavirin dose reductions on sustained viral response rates in HCV patients treated with boceprevir, peg-IFN and ribavirin (Lawitz et al., 2012; Poordad et al., 2012). To get more insight into the immunological effects of EPO in humans, we defined which human leukocyte subpopulations are potential targets for EPO, and explored the functional effects of EPO on these cells. We observed that EPO affected monocytes *in vitro*, which was in line with detectable EPO-R mRNA expression by monocytes. Moreover, similar to the *in vitro* effects, administration of EPO during antiviral therapy of chronic HCV patients resulted in reduced frequencies of monocytes producing cytokines.

2. Material and methods

2.1. Patients

Patients were treated at the Erasmus MC according to a study protocol and were seen at our outpatient clinic (EudraCT 2007-005344-25). Patients were treatment-naive, infected with HCV genotype 1 and were treated for 12–48 weeks with peg-IFN (Pegasys, 180 μg once weekly, Roche) and ribavirin (Copegus, 1200–2400 mg daily, Roche). Hb levels were monitored throughout therapy. Per protocol, at Hb levels below 6.8 mmol/l, EPO (NeoRecormon, Roche) was administered at a dose of 30,000 IU once weekly. When Hb levels increased above 7.5 mmol/l, EPO administration was discontinued. Eight patients were treated with EPO, heparinized blood was collected during antiviral therapy before administration of EPO and 7 days after the first injection of EPO. Furthermore, we included seven healthy volunteers outside the study protocol who were used as control(s). The institutional review board of the Erasmus MC approved the protocols, and informed consent was obtained from all individuals.

2.2. Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque™ plus, Amersham). For all *in vitro* experiments, PBMC were suspended in serum-free X-VIVO15 medium (BioWhittaker) supplemented with L-glutamin (Cambrex), Pen-Strep (Invitrogen/Gibco) and HEPES (Cambrex) and used for the various assays.

2.3. EPO-R mRNA expression

For determination of the expression of EPO-R mRNA, PBMC from healthy volunteers were separated by cell sorting into $CD19^+$ B cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD56^+$ NK cells, $CD14^+$ monocytes and granulocytes (FACS Aria SORP, BD). Granulocytes were sorted from full blood on the basis of the FSC-SSC profile. Cells were stored in RNAlater. Total RNA was extracted using the RNeasy kit (Qiagen) and cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR reactions were performed using a MyIQ5 detection system (Bio-Rad). Primers-probes for GAPDH (Hs00959427_m1) and EPO-R (Hs00959427) were obtained from Applied Biosystems. The expression of target genes was normalized to GAPDH using the formula: $2^{-\Delta Ct}$, $\Delta Ct = Ct_{EPOr} - Ct_{GAPDH}$.

2.4. ROS production and phagocytosis by monocytes

PBMC were rested for 1 h at room temperature, and DHR123 (0.1 μg/ml; Sigma) was added for 10 min, followed by pretreatment for 15 min with EPO (125 IU/ml, NeoRecormon). Next, cells were primed with fMLP (1 mM; F3506, Sigma) and incubated at 37 °C for 0, 5, 15 and 30 min. ROS was detected by flow cytometry (FACS Calibur 4, BD). EPO-pretreated PBMC were also used for the detection of phagocytosis. *Escherichia coli* FITC (2 μg/ml; Invitrogen) was added and incubated at 37 °C for 15 min. Cells were washed with trypan blue to remove unbound *E. coli* FITC. Phagocytosis was measured by flowcytometry (FACS Calibur 4, BD).

2.5. Expression of intracellular and cell surface molecules by flow cytometry

The frequencies of cytokine producing $CD14^+$ monocytes were determined by flow cytometry (Liu et al., 2011; Peng et al., 2011). PBMC from healthy individuals were first pretreated with or without EPO variant alpha (125 IU/ml, EPREX) or EPO (125 IU/ml, NeoRecormon) for 30 min. PBMC from HCV patients on therapy were not pretreated *in vitro* with EPO. For the expression of activation markers, cells were stained with CD80-FITC (MAB104, Beckman) CD86-APC (IT2.2, Biolegend) and HLA-DR-FITC (L243, BD Bioscience). For determining cytokine expression, cells were stimulated overnight with an optimal concentration of LPS (0.8 ng/ml, InvivoGen) or R848 (1 μg/ml, Alexis). Brefeldin A (10 μg/ml; Sigma) was added 2 h after the addition of TLR agonists. Samples were fixed, permeabilized and stained with MIP-1β-PE (D21-1351, BD Pharmingen), IL-6-FITC (MQ2-13A5, BD Pharmingen), TNF-PE-Cy7 (MAb11, eBioscience), MCP-1-APC (5D3-F7, eBioscience), IL-8-FITC (6217, R&D), IL-12p40-PE (C11.5, BD Pharmingen), IL-10-APC (JES3-19F7, Biolegend) and CD14-eFluor450 (61D3, eBioscience). Cytokine producing cells were detected by flowcytometry (Canto-II, BD).

2.6. Statistics

The Wilcoxon signed-rank test was used for paired non-parametric analyses. The level of significance for all tests was $P \leqslant 0.05$.

3. Results

3.1. The EPO-R is expressed by human monocytes

To determine if human monocytes are responsive to EPO, we first assessed the expression levels of EPO-R mRNA. As shown in Fig. 1, using highly purified monocytes, EPO-R mRNA was detected by real-time PCR. The EPO-R mRNA expression levels in monocytes were lower than observed in B cells and granulocytes, but higher than by other lymphocyte subtypes, like T cells and NK cells. These findings demonstrate that monocytes, next to B cells and granulocytes, are putative targets for EPO.

3.2. EPO increases phagocytosis ability of monocytes in vitro

To study the influence of EPO on monocyte function, we first investigated the ability of EPO-treated monocytes to perform phagocytosis of *E. coli* and to produce reactive oxidative species

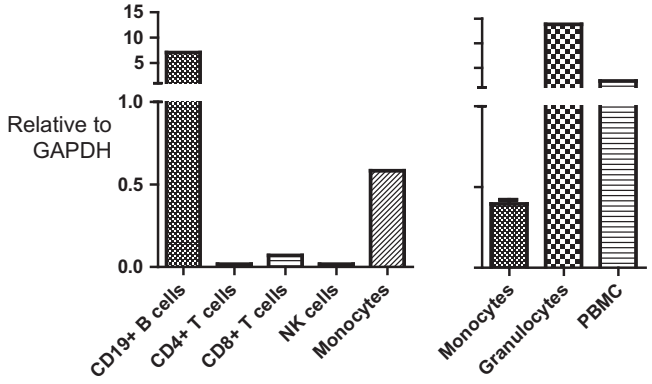

Fig. 1. Monocytes express EPO-R mRNA. CD19$^+$ B cells, CD4$^+$ and CD8$^+$ T cells, CD56$^+$ NK cells, CD14$^+$ monocytes and granulocytes were purified by flow cytometry. EPO-R mRNA was measured by real time PCR, and expression was determined relative to GAPDH mRNA.

(ROS) upon stimulation. As shown in Fig. 2, upon incubation of PBMC from healthy individuals, phagocytosis of *E. coli* by monocytes was observed. Pretreatment of PBMC with EPO for 15 min resulted in an increased uptake of *E. coli* in 5 out of 8 cultures from healthy volunteers, while the other 3 cultures showed no modulation of phagocytic ability of monocytes ($p = 0.05$).

To study the effect of EPO on the function of monocytes in more detail, we examined their ROS production. As shown in Fig. 3, unstimulated PBMC induced ROS production by monocytes, which was further enhanced by pretreatment with EPO (Fig. 3A). On average, the mean fluorescence intensity, representing the ROS levels, increased from 25.0 without EPO to 30.2 with EPO, although this result was not statistically significant ($p = 0.06$; Fig. 3B). Importantly, the kinetics of ROS production by fMLP-stimulated monocytes in the presence of EPO were comparable as ROS production in the absence of EPO, and again no significant differences were observed ($p = 0.12$; Fig. 3C).

3.3. EPO moderately down regulates cytokine production by monocytes in vitro

Another important function of monocytes is the production of pro-inflammatory as well as anti-inflammatory cytokines. After treatment of PBMC with EPO, the frequencies of IL-6 and TNF producing monocytes were decreased upon stimulation with the TLR4 ligand LPS, compared to PBMC not treated with EPO (Fig. 4A). Using PBMC from seven healthy volunteers, we showed a significant reduction of monocytes producing IL-6 (average from 23.2% to

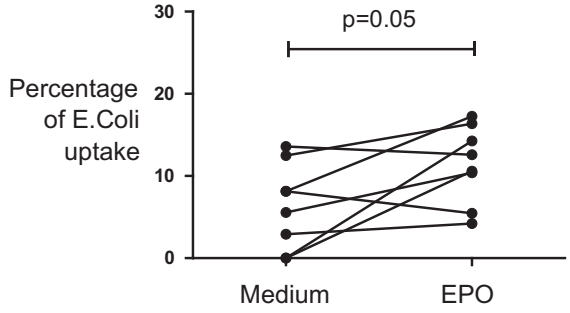

Fig. 2. Increased phagocytosis of *E. coli* by monocytes *in vitro*. PBMC from healthy individuals ($n = 8$) were treated with medium or EPO, and subsequently exposed to *E. coli*-FITC. *E. coli* uptake by CD14$^+$ monocytes was measured by flow cytometry as the difference between the frequency at 37 °C and at 0 °C.

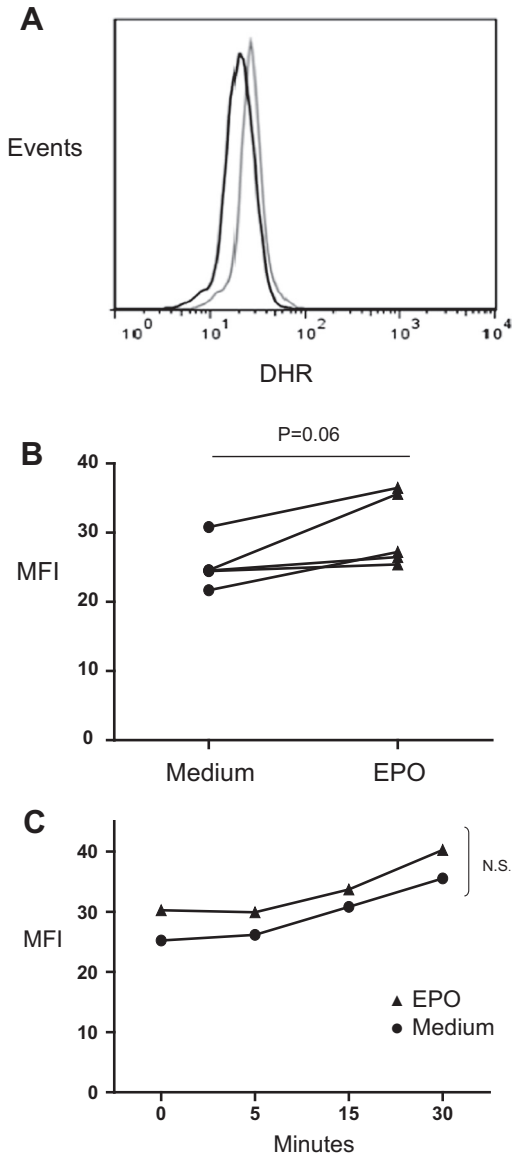

Fig. 3. The frequency of ROS producing monocytes is not altered by EPO *in vitro*. (A) A representative histogram showing the DHR expression of monocytes in the absence (black line) or presence of EPO (grey line). (B) PBMC from healthy individuals ($n = 5$) were treated with medium or EPO, and the direct effect of EPO on ROS-producing monocytes was determined by flow cytometry. (C) PBMC from healthy individuals ($n = 5$) were incubated with or without EPO and stimulated with fMLP. ROS was determined in CD14$^+$ monocytes at the indicated time points after stimulation.

17.9%; $p = 0.03$), and TNF (42.2–36.8%; $p = 0.02$) upon LPS stimulation (Fig. 4B). No significant effects of EPO were observed on the frequencies of monocytes producing IL-8, IL-12p40, IL-10, and MIP-1β upon LPS stimulation (Fig. 4B and results not shown). Pretreatment of R848-stimulated PBMC did not lead to differences in cytokine production by monocytes (results not shown).

Ribavirin-induced anemia in chronic HCV patients can be treated by EPO as well as EPO-alpha variant. To examine if these two different EPO variants have comparable effects on the functionality of monocytes, PBMC were also incubated with EPO-alpha variant *in vitro*. As presented in Fig. 4C, we showed that incubation of PBMC with EPO-alpha variant significantly reduced the frequency of monocytes that produced IL-6 ($p = 0.01$), TNF ($p = 0.01$) and IL-8 ($p = 0.03$) upon LPS stimulation. Thus, the inhibitory effect on cytokine production was observed for both EPO variants.

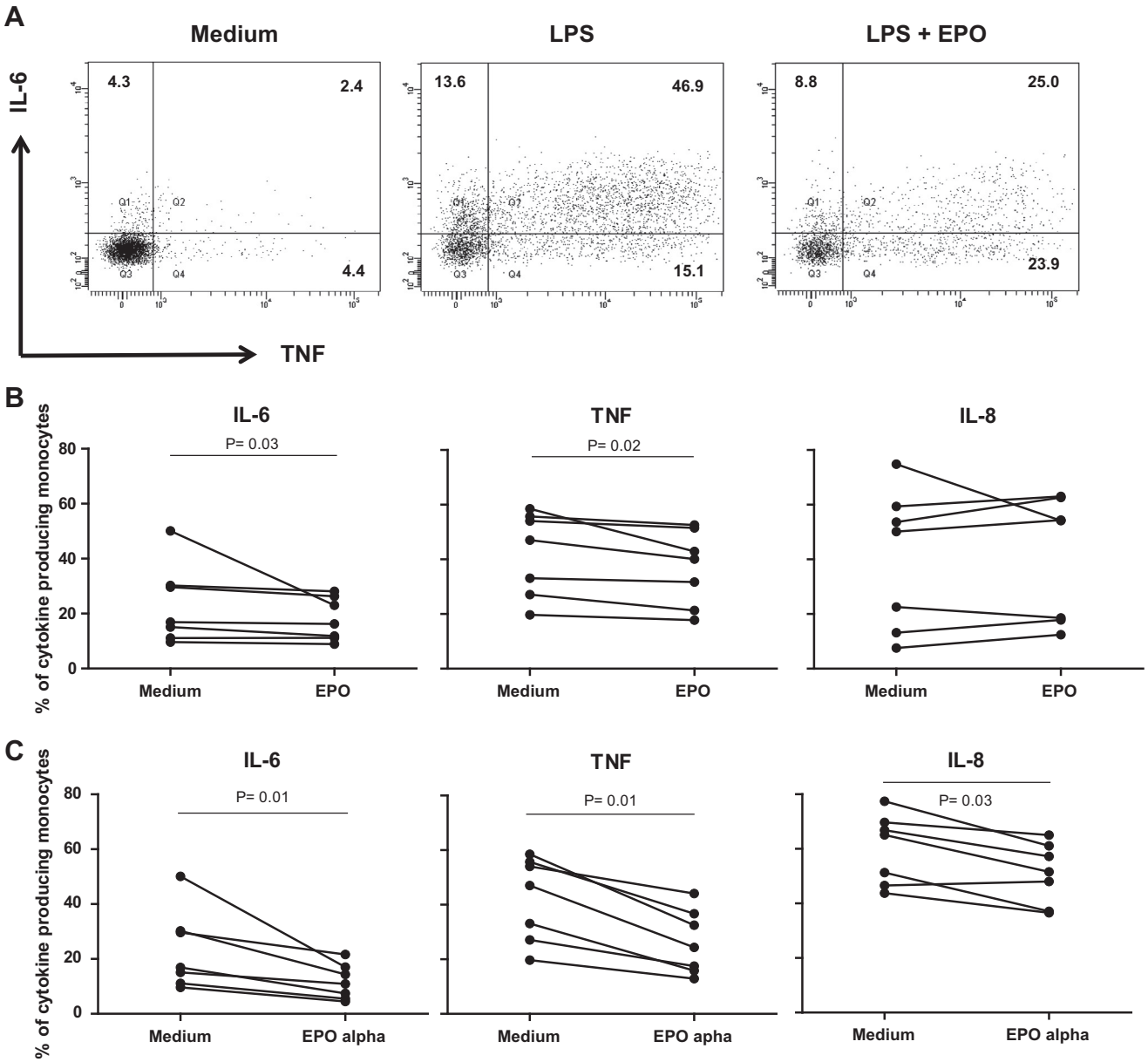

Fig. 4. Pretreatment with EPO of LPS stimulated PBMC shows decreased frequencies of IL-6 and TNF producing monocytes *in vitro*. (A). Representative dot-plots showing IL-6 and TNF producing $CD14^+$ monocytes upon LPS stimulation of PBMC after incubation with medium or EPO. (B) PBMC from healthy individuals ($n = 7$) were pretreated with EPO and stimulated with LPS. The frequency of cytokine producing monocytes was determined by flow cytometry. (C) Similar as in *B*, except that PBMC were pretreated with EPO-alpha variant.

3.4. EPO administration to patients with HCV-treatment induced anemia mildly reduces cytokine production by monocytes

Next, we assessed whether the immunomodulatory effect of EPO on monocytes *in vitro* is also observed *in vivo*. Cytokine production by monocytes was measured at baseline and 7 days after EPO injection in eight patients with HCV treatment-induced anemia (Fig. 5A). The patient characteristics are shown in Table 1. Upon stimulation of PBMC with the TLR4 ligand LPS and the TLR7/8 ligand R848, a decreased frequency of cytokine producing monocytes at day 7 compared to baseline was observed in 7 out of 8 patients (Fig. 5B). Upon LPS stimulation, a decreased percentage of monocytes producing MIP-1β (average from 20.3% to 11.7%; $p = 0.02$, results not shown) and IL-8 (3.6–1.6%; $p = 0.03$) was found. Upon R848 stimulation, lower percentages of monocytes producing IL-6 (13.9–6.6%; $p = 0.05$), MCP-1 (13.0% till 8.5%; $p = 0.05$, results not shown) and IL-8 (25.2–14.6%; $p = 0.03$) were observed.

Despite the fact that we observed a trend towards lower cytokine levels, no significant differences were found for the frequency of TNF (Fig. 5B), IL-12p40 and IL-10 production (results not shown). Furthermore, the activation status of monocytes as reflected by their expression of CD80, CD86 and HLA-DR was determined at baseline and 7 days after administration of EPO. No differences were observed as a consequence of EPO administration in the frequency or mean fluorescence intensity (MFI) of monocytes expressing these markers (results not shown). Management of therapy-induced anemia in chronic HCV patients by administration of EPO resulted in reduced frequencies of cytokine producing monocytes, which may have consequences for inflammatory responses by monocytes upon challenge by pathogens.

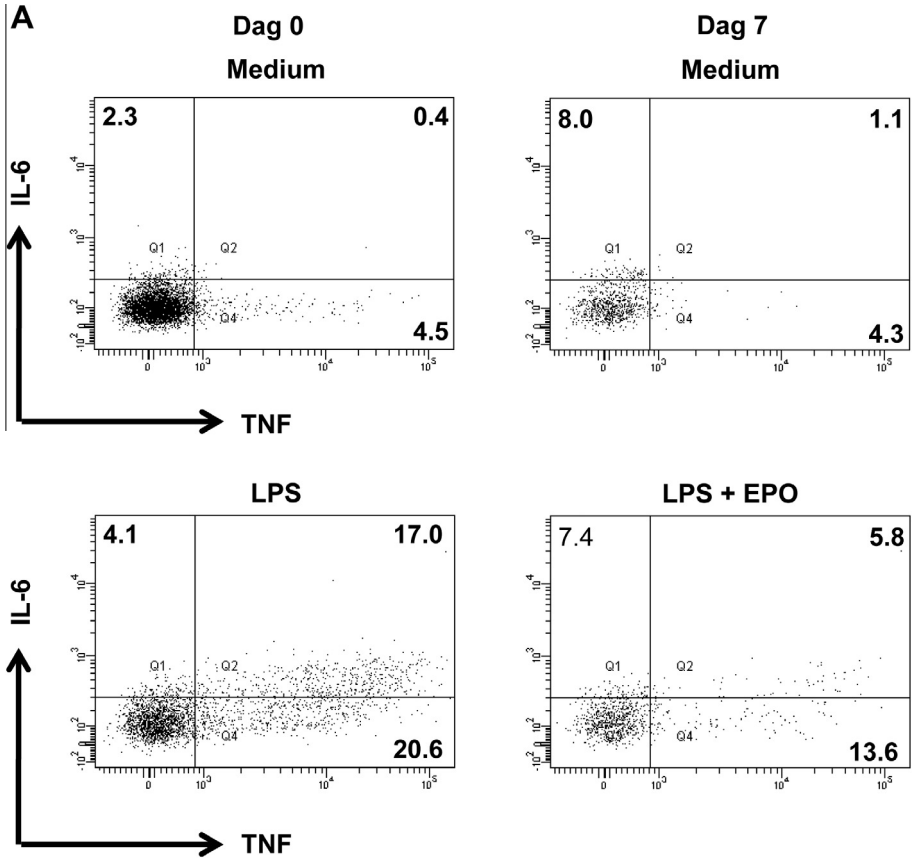

Fig. 5. EPO administration to chronic HCV patients with ribavirin-induced anemia mildly reduces cytokine production by monocytes. (A) Representative dot plots of IL-6 and TNF producing monocytes before and 7 days after injection of EPO from a chronic HCV patient on peg-IFN/ribavirin therapy who is being treated for anemia. (B) The frequency of IL-6, TNF, IL-8 producing monocytes upon LPS stimulation (left panels) or R848 stimulation (right panels) of PBMC collected from HCV patients ($n = 8$) before and 7 days after administration of EPO.

4. Discussion

Although the stimulatory effect of EPO on the erythrocyte lineage in bone marrow is well examined, the immunomodulatory activity of EPO is not well understood. In this study, we investigated the *in vitro* and *in vivo* effects of EPO on human leukocytes, with a focus on monocytes, which express the EPO-R as we demonstrated in this study.

We show that *in vitro* exposure of PBMC to EPO resulted in decreased frequencies of IL-6 and IL-8 producing monocytes upon TLR ligation. Although differences were seen in potency, both EPO-alpha variant and EPO (EPO-beta, which was used throughout this study) showed comparable immunomodulatory effects on monocytes *in vitro*. In addition, we found EPO-induced modulation of phagocytic ability by monocytes *in vitro,* but no effect on ROS production. This demonstrates a shift in the function of EPO-exposed monocytes towards a more potent anti-microbial activity, and a weaker ability to produce effector cytokines. It is important to mention that we have no indications that the viability of monocytes was affected by short-term exposure to EPO as determined by annexin-V staining *in vitro*. Similar effects on inhibition of cytokine production were seen in the *ex vivo* model where monocytes were obtained from chronic HCV patients undergoing antiviral therapy. Decreased frequencies of cytokine producing monocytes upon TLR ligation were observed following administration of EPO to patients. This inhibitory effect was not restricted to TLR4 ligation, but also observed upon TLR8 ligation, leading to lower frequencies of monocytes producing pro-inflammatory cytokines. Although we observed significant modulation of monocyte function *in vivo* at day 7, we cannot exclude that more pronounced effects can be demonstrated at different time points following EPO administration, since we and others did not perform kinetics studies.

Our observations in humans are in line with a recently published study in mice that showed that EPO inhibited LPS-induced pro-inflammatory cytokines and nitric oxide production in peritoneal macrophages *in vitro*. *Salmonella typhimurium*-infected mice treated with EPO demonstrated a higher bacterial load and reduced expression of IL-6, TNF and Nos2 as compared to control mice treated with PBS (Nairz et al., 2011). Besides the studies in mice, the effect of EPO in hemodialysis patients has also been examined. In these patients, neutrophils were significantly more activated after hemodialysis and EPO therapy, which is in line with our results, where we show that neutrophils express the EPO-R and are likely responsive to EPO (Costa et al., 2008). In other studies on the direct effect of EPO, decreased TNF and increased IL-10 production were produced by phytohemagglutimin (PHA)-stimulated whole blood cell cultures of this patient population during therapy with EPO (Bryl et al., 1998, 1999). Lower TNF production as a result of EPO treatment is in line with our results, but we did not observe an increase of IL-10 producing monocytes. The discrepancy is likely the result of the use of different stimuli, with PHA also activating T cells, whereas TLR ligands do not. In this respect, it is important to note that we did not observe EPO-R mRNA expression by $CD4^+$ and $CD8^+$ T cells, and the activity of T cells upon stimulation with anti-CD3 antibodies was not affected by EPO, as reflected by their production of IL-2 or IFN-γ *in vitro* and *in vivo* (data not shown).

Chronic HCV patients undergoing antiviral therapy consisting of peg-IFN and ribavirin are more susceptible to develop bacterial infections (Antonini et al., 2008). The enhanced occurrence of

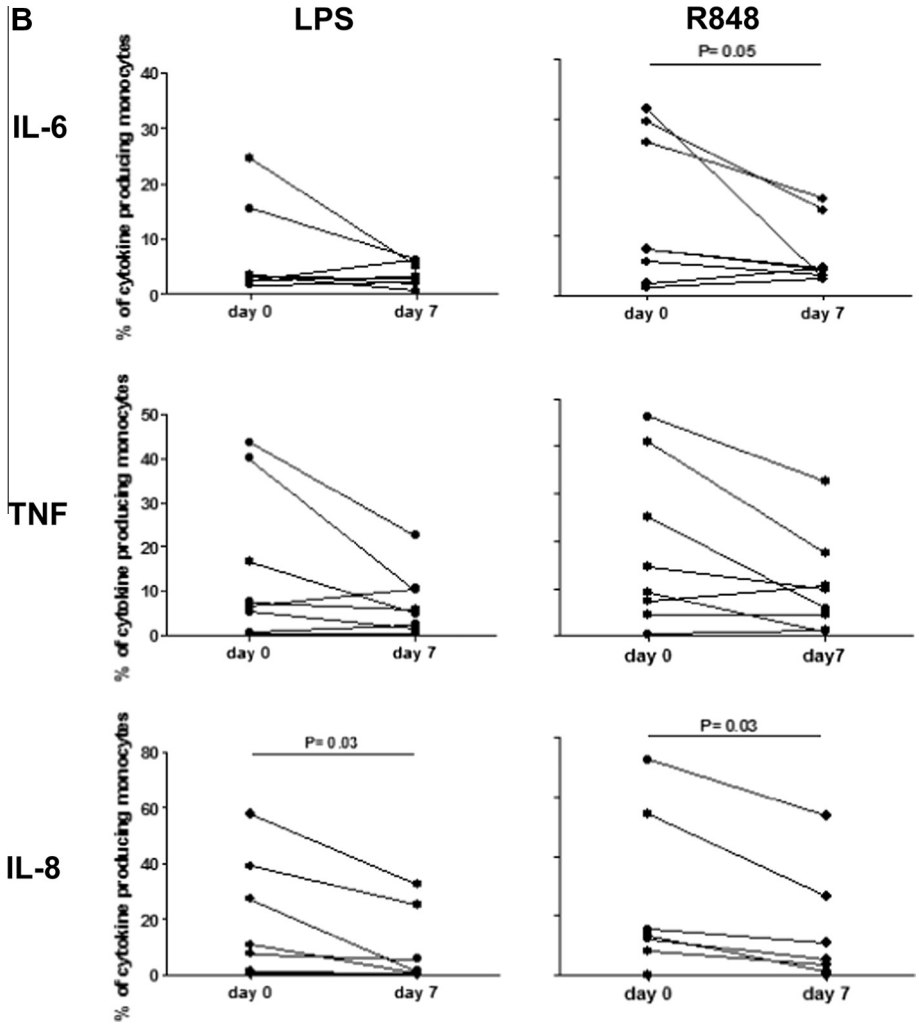

Fig. 5. (*continued*)

Table 1
Patient characteristics of eight HCV patients. Data presented are at start of therapy.

Patient ID	Gender	Genotype	Hb (mmol/l)	Bilirubin (μmol/l)	Albumin (g/l)	Platelet count (E9/l)	AST (U/l)	ALT (U/l)	Viral load (U/ml)
1	F	1	9.3	11	45	183	59	91	6.82E + 06
2	M	1	9.0	11	45	130	60	65	4.24E + 06
3	M	1	9.8	8	42	226	55	90	6.79E + 06
4	M	1	10.4	11	46	213	34	51	4.42E + 06
5	F	1	7.9	12	44	99	110	113	9.14E + 05
6	M	1	9.8	17	43	179	52	68	1.07E + 06
7	M	1	9.9	7	44	202	87	74	4.09E + 06
8	F	1	9.1	6	45	217	80	81	1.93E + 06

infection does not correlate with neutropenia (Antonini et al., 2008; Roomer et al., 2010), while the involvement of monocytes is unclear. Monocytes are important players in the first-line defense against numerous pathogens (Auffray et al., 2009), and are functionally modulated in chronic HCV patients as compared to control monocytes. We previously showed that TLR4 ligation of monocytes from chronic HCV patients induced lower TNF and IL-12p40 production as compared with healthy individuals (Liu et al., 2011). Little information is available on the effects of EPO administration on the clinical course of infections. In critically ill patients who are treated with antimicrobial therapy, it has been shown that administration of EPO is safe (Corwin et al., 2007). Our data suggest, however, that EPO reduces the pro-inflammatory function of monocytes, which might be relevant in patients not treated with antimicrobial therapy but who are at risk for these infections. Since our observed effects of EPO are influenced by the simultaneous presence and modulatory effect of IFN-alpha during the course of antiviral therapy, future studies that examine in more detail the effect of IFN-based therapy as well as of EPO on monocytes in relation to the increased infection risk in HCV patients are needed. It has been reported that with the introduction of protease inhibitors to antiviral therapy of chronic HCV patients, the beneficial effects of EPO in managing anemia during therapy are limited. The high costs of EPO and reported side effects argue against the use of EPO in these patients (Lawitz et al., 2012; Poordad et al., 2012).

In conclusion, although we were unable to demonstrate an effect of EPO on human NK cells and T cells, EPO inhibited TLR-induced cytokine production by monocytes *in vitro* as well as *in vivo*. The inhibitory effect of EPO on cytokine producing monocytes may hamper efficient immune responses by monocytes against bacterial infection in patients undergoing treatment for anemia. The clinical implications for patients using EPO to treat anemia needs to be further determined.

Acknowledgments

The study was supported by the Foundation for Liver Research (SLO), Rotterdam, and further supported by Roche and the Virgo consortium, funded by the Dutch government project number FES0908, and by the Netherlands Genomics Initiative (NGI) project number 050-060-452.

We also would like to thank Irene Brings, Heleen van Santen, Lucille Maarschalkerweerd, and Melek Polat for their assistance during various stages of the study.

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