JOURNAL OF VIRAL HEPATITIS
JVH

Journal of Viral Hepatitis, 2011, **18,** e284–e291 doi:10.1111/j.1365-2893.2010.01408.x

Persistent pro-inflammatory cytokines following the initiation of pegylated IFN therapy in hepatitis C infection is associated with treatment-induced depression

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Received July 2010; accepted for publication October 2010

SUMMARY. Pegylated interferon (IFN), the basis for chronic hepatitis C virus (HCV) treatment, causes depression in 30–40% of patients. The potential for cytokine mRNA patterns from baseline into early treatment to associate with the onset of treatment-induced depression (TID) was examined. Depression was measured by the Beck Depression Inventory at baseline and weeks 2, 4, 8 and 12 of treatment ($n = 38$). At baseline and weeks 2 and 4, peripheral blood mononuclear cell (PMBC, $n = 28$), isolated *ex vivo*, were examined for tumour neurosis factor (TNF)-alpha, interleukin (IL)-1beta and IL-10 mRNA expression. In patients that developed treatment-induced depression, pro-inflammatory TNF-alpha mRNA levels from baseline into week 4 of therapy remained constant (1.1-fold increase); whereas IL-1beta transcripts decreased 3.5 fold. However, corresponding TNF-alpha (3-fold, $P < 0.05$) and IL-1beta (7.5-fold) transcript expression diminished to a greater extent in the absence of TID. Changes in TNF-alpha mRNA values correlated to the average change in BDI scores over the 12 weeks ($r = 0.56$, $P < 0.05$). Concomitantly, anti-inflammatory IL-10 transcript levels decreased in (TID), relative to increased expression in the absence of TID ($P < 0.05$). The potential influence of IL-10 was observed upon calculation of individual pro- verses anti-inflammatory mRNA ratios. Stable in the presence of depression, TNF-alpha/IL-10 and IL-1beta/IL-10 mRNA ratios declined significantly over time in its absence ($P < 0.05$). This study suggests that in chronic HCV infection, upon pegylated IFN administration persistent pro-inflammatory cytokine MRNA expression associates with TID. In contrast, therapeutic activation of mechanisms that decrease pro-inflammatory immunity may protect against depression during therapy.

Keywords: cytokines, depression, hepatitis C virus, pegylated IFN.

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is currently treated with pegylated interferon (IFN) in combination with ribavirin. This therapy has an efficacy rate of ~45–80% and is subject to serious side effects including depression [1–4]. Depression and other neuropsychiatric side-effects occur in up to 40% of individuals, primarily within the first 12 weeks of treatment. They are the main indication for treatment discontinuation, compromising therapeutic outcomes [5,6].

The accumulative evidence suggests that pro-inflammatory cytokines play an important role in the pathogenesis of depression. Studies on major depression disorders have found elevations of one or more serum/plasma pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and/or IL-8 levels compared to control subjects [7–10]. In HCV infected individuals, an association between circulating TNF-α concentrations and Beck Depression Inventory (BDI) scores was observed [11]. Cerebrospinal fluid concentrations of IL-1β and IL-6, but not TNF-α, also appeared enhanced in clinical depression [12].

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BDI, Beck Depression Inventory; CRH, corticotropin releasing hormone; EVR, early viral responses; HPA, hypothalamic pituitary axis; HCV, hepatitis C virus; IDO, indoleamine-2,3-dioxygenase; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cells; RT, reverse transcription; SVR, sustained viral responses; TID, treatment-induced depression; TNF, tumour neurosis factor.

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In addition, administration of pro-inflammatory cytokines in animals elicits sickness behaviors connected with clinical depression in humans [13–16].

Although the mechanism whereby pro-inflammatory cytokines contribute to depression remains to be identified, aberrant cytokine has been reported to alter serotonin (5-HT) bioavailability [3,17] and hypothalamic pituitary axis (HPA) activation [16,18]. As a result, it is theorized that IFN-induced depression occurs upon activation of cytokine cascades that subsequently derail the 5-HT and HPA pathways [19–21].

Nonetheless, in HCV infected individuals pre-treatment peripheral cytokine levels do not appear to predict the onset of depression with therapy [22–24]. Upon the initiation of treatment, however, changes in circulating serum levels from baseline have suggested an association with treatment-induced depression (TID) [21,25,26], though not always [27].

As an alternative approach, TNF-α, IL-1β and IL-10 mRNA profiles of peripheral blood mononuclear cells (PBMC) isolated *ex vivo* from HCV infected patients were examined to determine whether treatment modulation of baseline pro-inflammatory immunity associated with the subsequent onset of TID.

MATERIALS AND METHODS

Study participants

This study was approved by the University of Manitoba Research Ethics Board. Participants were recruited at the Viral Hepatitis Investigation Unit, Winnipeg, MB. Adults with chronic HCV infection preparing to undergo Peg-IFN and ribavirin, were invited to participate in the study. In addition to standard exclusion criteria for Peg-IFN and ribavirin treatment, participants were also negative for HIV and HBV infection. Two participants were stable on low dose antidepressant or anxiety medications. The remaining subjects were not receiving an anti-psychotic medications, including antidepressants. Study participants were treated with 1.5 μg/kg/day of Peg-IFN-α2b (67%, Schering-Plough Canada, Kirkland, QC, Canada) or 180 μg/day of Peg-IFN-α2a (33%, Roche, Mississauga, ON, Canada) plus ribavirin (800–1200 mg/day) according to standard of care. Hepatic inflammation was monitored by serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Early viral responses (EVR, defined as undetectable virus in the serum at 3 months of treatment) and sustained viral responses (SVR, defined as undetectable virus in the serum at 6 months after the end of treatment) were recorded.

Depression symptom assessment

All participants were evaluated at baseline for depression symptoms using the BDI, a widely used and standardized instrument [11]. The shorter 13 item questionnaire version was chosen because the reduced somatic symptom content is considered less influenced by concurrent medical illness [28]. Questionnaires were administered by a research nurse during regularly scheduled clinical appointments on weeks 2, 4, 8 and 12 of therapy. A BDI score of ≥10 indicated the presence of depression.

Blood sample collection and PBMC isolation

Blood samples (n = 28) were collected at baseline and at weeks 2 and 4 of treatment for *ex vivo* PBMC cytokine mRNA analysis. Sampling was performed on average at noon ± 30 min (SE). PBMC were isolated from whole blood with Ficoll (Sigma, St Louis, MO, USA) method. The buffy coat cells were washed with saline, counted and incubated with 10% (v/v) fetal calf serum containing medium (Invitrogen Life Technologies, Grand Island, NY, USA). Cells consistently exhibited >98% viability as determined by trypan blue exclusion [29]. PBMC were stored at −80 °C in Trizol® (Invitrogen, Carlsbad, CA, USA) until all samples were collected for an individual. *In vitro* cultures were established the day blood was collected.

Total RNA isolation and real time PCR

Total RNA from PBMC was isolated using Trizol® (Invitrogen) and RNeasy® MinElute® clean-up kit (Qiagen, Mississauga, ON, Canada) according to the manufacturers' instructions. Reverse transcription (RT) was used to produce cDNA from RNA (3 μg) using a first-strand cDNA synthesis kit (Invitrogen). Real-time PCR analysis was performed with SYBR green PCR master mix and Quanti-Tect® primer (Qiagen). Previously generated DNA standards (TOPO TA® cloning kit, Invitrogen) were run in duplicate or triplicate in a series of 10-fold dilutions spanning at least 7 points for each plate. Amplification was carried out (40 cycles) in an Applied Biosystems (ABI) 7500 instrument (Applied Biosystems, Foster City, CA, USA). Data was analysed using ABI software and cytokine mRNA expression was normalized to the housekeeping gene β-actin by dividing cytokine copy number by β-actin copy number.

Data analyses

Differences in categorical data were determined by χ^2 Fisher's exact test. Differences in the medians of a parameter (BDI or mRNA levels) between cohorts with or without TID at a specific time point were evaluated using the non-parametric Mann–Whitney U test. When considering the influence of multiple time points (e.g. baseline, week 2 and week 4) within a given cohort, median differences were evaluated with the Kruskal–Wallis test (non-parametric one way ANOVA) and subsequent Dunn's multiple comparisons *post hoc* analysis. Correlations between baseline cytokine mRNA

levels and BDI scores were determined with the Spearman's rank test. For all tests, $P < 0.05$ (two-tailed) was considered significant. Data from early terminations were included in the analysis.

RESULTS

Cohort demographics

At total of 38 individuals agreed to participate in the study. Four individuals exhibited baseline depression as defined by BDI scores of ≥10. They continued with treatment under observation, but were not enrolled in study. The remaining 34 participants were generally male (70.6%), Caucasian (67.6%), with genotype 1 infections (64.7%) and a median age of 52.5 (range 35–62) years old.

Treatment-induced depression

During the first 12 weeks of IFN therapy, 38% of participants developed TID in keeping with previous reports [5,30,31]. Between TID and non-TID cohorts, there were limited differences in demographics (age, sex or race), biochemical evidence of hepatic inflammation, as indicated by ALT or AST values, and viral characteristics of genotype infection or baseline loads (Table 1). In contrast, baseline BDI scores were higher (but not at levels required for a definition of depression) in individuals that subsequently progressed to TID than those that did not ($P < 0.001$, Table 1).

All 13 individuals who experienced TID (BDI ≥ 10) were referred to a counsellor. Seven declined the referral and completed treatment. Six patients accepted referral with three completing treatment, including one that was also referred to a psychiatrist and provided anti-depressant medication (week 3). Treatment for the remaining three individuals was terminated at weeks 5 (acute confusion), 12 (pneumonia) and 20 (no relief of symptoms despite psychiatric care and anti-depressant medication at baseline). In the cohort that did not experience TID, treatment was terminated early for three individuals due to anxiety (week 7), changes in vision (week 6) and travel out of the country (week 6).

Pro- and anti-inflammatory cytokine changes and depression

To examine the involvement of endogenous cytokine activity in the development of TID with treatment, changes in pro-inflammatory mRNA expression from baseline to weeks 2 and 4 of treatment were investigated. In the TID cohort, TNF-α cytokine transcript levels tended to remain stable over time. In the absence of TID, a 3-fold decrease in the TNF-α mRNA levels into week 4 was observed ($P < 0.05$, Fig. 1). Over the same timeframe IL-1β mRNA levels declined at comparable rates in the presence (3.5-fold) or absence (7.7-fold) of TID (Fig. 1).

Table 1 Demographics for depression cohorts

Parameters	TID	No TID*
Number	13	21
Age (median)	53.1	49.9
Male (%)	61.5	76.2
Caucasian (%)	76.9	61.9
Viral load (IU/mL, median)	3.5×10^6	4.7×10^6
Genotype 1 (%)	61.9	69.2
ALT/AST (IU/L, median)†‡		
Baseline	63/54	94/53
Week 2	42/50	49/41
Week 4	46/40	37/40
EVR (%)‡	61.5	66.7
SVR (%)‡	50.0	66.7
Early termination (%)	23.1	14.3

*There were no significant differences between the cohorts for displayed parameters. Categorical differences were determined by χ^2 Fisher's exact test and quantitative data was assessed by Mann–Whitney. †Markers of liver inflammation serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were documented. Normal levels of ALT are 2–40 IU/L and AST are 2–45 IU/L. ‡Serum viral loads of participants were determined at 3 and 6 months after the end of treatment. If viral loads were negative at these time points, they are designated as early viral responses (EVR) and sustained viral responses (SVR) respectively (EVR and SVR data does not include individuals who were subject to early termination).

The impact of IFN treatment on endogenous IL-10 levels was also evaluated. From baseline into week 4 there were marginal changes in median IL-10 mRNA levels in the TID and non-TID cohorts (Fig. 2a). However, when the fold difference in IL-10 mRNA expression from baseline to week 2 or 4 for each individual was examined, a 3-fold difference was apparent between the presence and absence of TID ($P < 0.05$, Fig. 2b). As such, in the TID cohort median transcript levels at baseline fell 1.7- and 1.9-fold by weeks 2 and 4 respectively; whereas, in the absence of TID mRNA values rose 1.6 and 2.1 fold from baseline to weeks 2 and 4.

To estimate modifications to the pro-/anti-inflammatory microenvironment for each patient, individual pro-inflammatory/IL-10 mRNA ratios were calculated (Fig. 3). Within the TID cohort, TNF-α/IL-10 ratios were consistent over time. In the absence of TID, the TNF-α/IL-10 mRNA ratio declined into week 4. A similar pattern was observed with the IL-1β/IL-10 ratios were median mRNA values remained stable in the TID cohort, but diminished from baseline into week 2, 2-fold ($P < 0.05$) and into week 4, 6-fold ($P < 0.05$) in the non-TID cohort (Fig. 3b). This suggests that a stable

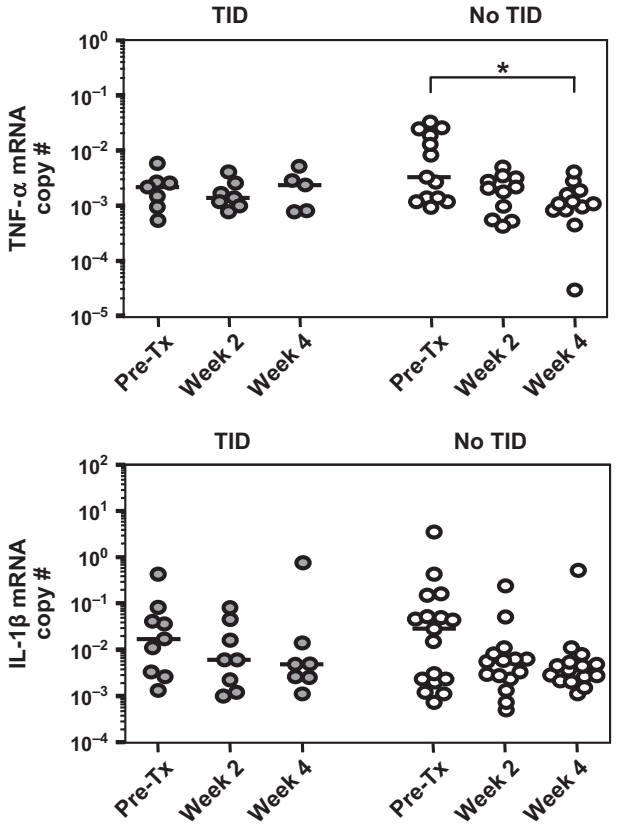

Fig. 1 A decrease in TNF-α mRNA values from baseline into week 4 occurs in the absence of TID. Whole blood was collected from study participants at baseline and 2 and 4 weeks of treatment. PBMC *ex vivo* TNF-α and IL-1β mRNA expression was determined and actin normalized. At end of treatment, values were analysed as to whether individuals subsequently experienced TID (n = 7) or not (n = 12). Medians are indicated by horizontal bar. Statistical differences *between* cohorts were not found. Statistical differences *within* the no TID cohort occurred from baseline to week 4 (*P < 0.05, Kruskal–Wallis test).

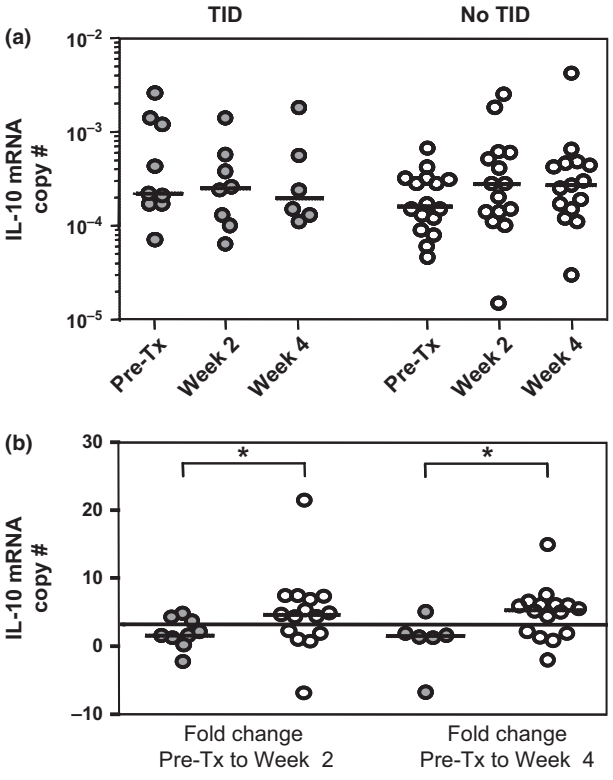

Fig. 2 IL-10 mRNA levels decrease in the TID cohort relative to patients that did not experience TID. Whole blood was collected from study participants at baseline and 2 and 4 weeks of treatment. IL-10 mRNA levels were evaluated by RT-PCR and actin normalized. Values were analysed against the subsequent onset of TID or not. (a) IL-10 mRNA values did not differ between depression cohorts. Horizontal bar indicate median value for panel horizontal. (b) IL-10 mRNA values decreased from baseline into treatment for the TID cohort (grey circles) compared to the no TID cohort (white circles). Median values are indicated by horizontal bars. Statistical differences in fold change of mRNA values between TID and no TID cohorts are shown as *P < 0.05 (Mann–Whitney).

pro-/anti-inflammatory microenvironment is consistent with the onset of TID.

Association of genotypes with liver enzymes

The possibility that the observed changes in cytokine mRNA levels could reflect liver inflammation was explored. No discrepancy in liver enzyme levels between the presence, or absence, of TID was found (Table 1). In addition, changes in cytokine values did not correlate with decreases in ALT or AST levels (Table 3). This suggests that cytokine mRNA associations with TID are independent of liver inflammation.

DISCUSSION

Peg-IFN and ribavirin is the current treatment for chronic HCV infection. Pharmaceutical doses of IFN induce depression in 30–40% of cases and are a primary reason for the early termination of therapy, thereby compromising viral clearance. Here, we explored the effect of pegylated IFN induction of pro- and anti-inflammatory cytokine MRNA expression to determine whether there was an association with TID.

As predicted by the literature, TID occurred in 38% of our patients (Table 1). The onset of depression did not appear to be subject to host and viral parameters (Table 1). Though a relationship between TID and SVR was not found (Table 1), discrepancies to this finding have been observed in evaluations of divergent populations. In one investigation [32], a positive association between TID and SVR occurred with a lower percentage of Caucasians (49%) than our study (77%); while the converse was found in a cohort with a higher

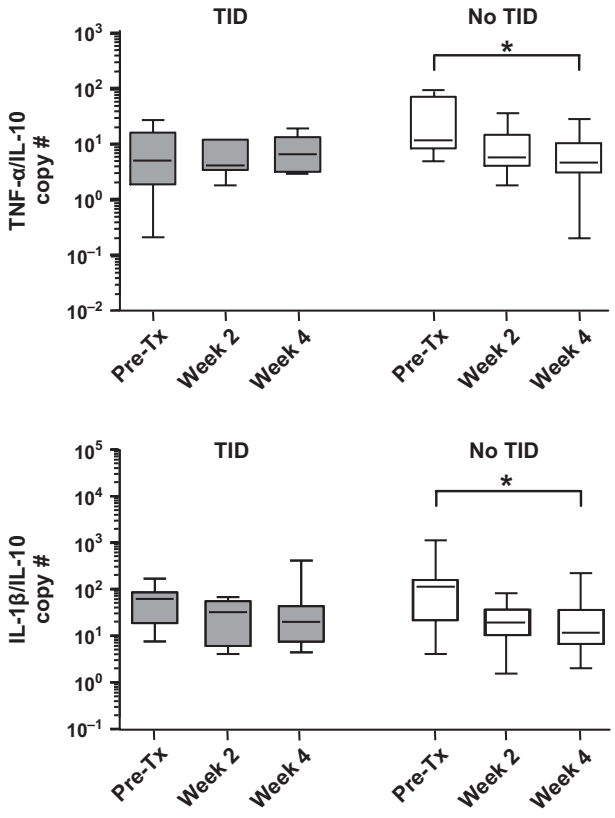

Fig. 3 In the absence of TID, pro-inflammatory verses anti-inflammatory mRNA values decline from baseline into week 4 of treatment. For each individual, the mRNA value of TNF-α and IL-1β was calculated against that of IL-10. Cohort medians and ranges (as indicated in Whisker plots) were determined. Statistical differences *within* the no TID cohort occurred from baseline to week 4 (*$P < 0.05$, Kruskal–Wallis test).

percentage of Caucasians (88%) [33]. The difference between these reports may be more complex than ethnicity. Further studies would be required to determine whether ethnicity or another factor influences the interaction between TID and SVR.

Table 2 TNF-α mRNA values correlate with changes in BDI scores

	r value†	
Cytokine (copy #)	Change week 4	Average change
TNF-α	0.44	0.56*
IL-1β	0.16	0.13
IL10	−0.46	−0.17

†Changes in cytokine mRNA expression from baseline into week 4 were examined against the average change in BDI scores over the 12 week evaluation. Spearman correlations were calculated (*$P < 0.05$).

Table 3 Cytokine mRNA expression does not correlate with liver enzyme levels

	r value†	
Cytokine (copy #)	ALT	AST
TNF-α	−0.005	0.005
IL-1β	0.147	−0.107
IL-10	0.220	−0.191

†Changes in cytokine mRNA expression from baseline into week 4 were examined against corresponding changes in liver enzyme values. Spearman correlations were calculated and did not prove to be significant.

Changes in cytokine mRNA expression from baseline into early treatment (week 4) were assessed against the development of TID. In contrary to our expectation that TID would associate with increased pro-inflammatory transcript levels, TNF-α mRNA expression remained stable in this cohort. In contrast, in the absence of TID TNF-α transcript levels declined over the course of the first 4 weeks of treatment ($P < 0.05$, Fig. 1). Moreover, this change in TNF-α mRNA expression positively correlated with the average change in BDI scores over the first 12 week of treatment (Table 2). These findings are supported by previous reports evaluating circulating TNF-α concentrations. Specifically, Wichers *et al.* described a positive correlation between serum TNF-α levels and total depression scores for 17 patients receiving treatment [25]. In addition, Raison *et al.* documented that changes in plasma TNF-α and sTNFR2, an indicator of previous TNF-α activity, from baseline to week 12 positively associated with corresponding variations in depression scores for 20 treated patients and 13 untreated healthy controls evaluated over the same timeframe [26].

The connection between reduced TNF-α activity with the absence of TID was echoed, albeit to a less extent, by IL-1β responses (Fig. 1). IL-1β transcript values fell slightly within the first 4 weeks for individuals that subsequently went on to develop TID (3.5-fold) compared to those that did not (7.7-fold). These decreases were not significant nor did they correlate with depression scores (Table 2). A lack of association was also found for soluble IL-1Ra concentrations, an inhibitor of IL-1 function [25].

Other investigators have investigated cytokines in an attempt to understand the involvement of pro-inflammatory mediators with TID. Bonaccorso and colleagues, evaluating 14 HCV infected individuals, noted that early changes in IL-6 and IL-8 concentrations significantly associated with depression scores at 4–6 months, but not within 3 months [21]. This finding with IL-6, but not IL-8, was subsequently replicated by Wichers *et al.* [25]. However, alterations in peripheral IL-6 with respect to

depression upon IFN treatment for HCV infected [26] or cancer ($n = 14$) patients (18, 27) have not always been found. Soluble IL-2 receptor levels have also been correlated with depression scores (25). Overall, our results describing an association between the maintenance of pro-inflammatory cytokine expression and depression extend to other reports that the induction of pro-inflammatory serum cytokine activity correlates with depression.

The possibility that the reduction of pro-inflammatory responses in the absence of TID may result from negative feedback mechanisms such as triggering anti-inflammatory IL-10 activity was supported by the increasing IL-10 mRNA levels after the initiation of treatment in patients compared to patients with TID (Fig. 2b). Changes in IL-10 transcript expression also displayed a negative ($r = -0.46$), but not significant, trend with BDI scores (Table 2). Lastly, pro-inflammatory mRNA values for each individual were evaluated against corresponding IL-10 transcript levels. In TID, TNF-α/IL-10 and IL-1β/IL-10 mRNA ratios were not significantly altered with the initiation of treatment. However, in the absence of subsequent depression, diminishing pro- verses anti-inflammatory ratios were observed from baseline into 4 weeks following treatment (Fig. 3, $P < 0.05$). Degeneration of pro-/anti-inflammatory cytokine production may shield against depression [34], though early fluctuation in IL-10 observed in IL-2 therapy for cancer associated with matching depression scores [27]. The inability to detect modifications of serum IL-10 concentrations with respect to IFN-induced depression could also reflect timing or the inherit dilution of cytokine in the blood stream [25,27]. Nonetheless, our data suggests that in the treatment of HCV patients with pegylated IFN the production of IL-10 could constrain pro-inflammatory consequences including depression [35].

The mechanism whereby ongoing maintenance or increases in pro-inflammatory cytokines promote depression with pegylated IFN treatment remains to be determined. Cytokine amplification of indoleamine-2,3-dioxygenase (IDO) production, resulting in reduced 5-HT availability and enhanced levels of the neurotoxin metabolite quinolinic acid has been implicated in depression upon therapy [3,17,36]. TNF-α and IL-1β activation of the HPA, and may participate in the disruption of the HPA associated with the onset of IFN-induced depression [26, 37–39]. Conversely, IL-10 can support of 5-HT synthesis by inhibiting cytokine upregulation of IDO expression, as observed with neuronal cell lines [40]. IL-10 modulation of the HPA further results in the release of the immunosuppressive hormones including corticotropin releasing hormone (CRH) and corticotropin (ACTH) which theoretically would restrict the pro-inflammatory elements of depression [41]. Elevated ACTH and cortisol levels within hours of IFN administration, however, have been associated with the subsequent onset of TID [18]. Thus, deciphering the events initiating TID following pegylated IFN administration in HCV infection will be difficult [42]. This is further complicated by predilection for neuropsychiatric events in HCV infection [11,43–45], as illustrated by the presence of clinical depression in 10.5% ($n = 4/38$) of the patients initially recruited for this study.

There are a number of caveats to this study. First, the interpretation of the data is limited by sample size. Second, we chose to document mRNA levels rather than serum cytokines concentrations due to the inherent instability of serum cytokines because of proteases and dilution relative to their source [46]. Although changes in TNF-α mRNA expression did not reflect hepatic inflammation, as assessed by liver enzymes (Table 3), the involvement of other mechanism(s) was not investigated due to the nature of the study. In a disease phenotype as complex as TID in HCV infection, pathways beyond 5-HT and HPA may need to be addressed. Therefore, our finding must be consider associations and may not speak to causation.

In conclusion, the results of our study indicate that the maintenance of pro-inflammatory cytokine expression is associated with TID in patients undergoing IFN-based therapy for HCV infection. An alternative, but not mutually exclusive interpretation of this data is that a decrease in pro-inflammatory cytokine expression after the initiation of treatment is protective against TID. Whichever the case, monitoring pro-inflammatory cytokine expression early in the course of treatment may serve to identify subjects at risk of developing TID. Moreover, understanding the mechanism(s) by which pro-inflammatory events can be reduced upon IFN administration could lead to therapeutic protection against TID.

DISCLOSURES

A study initiation grant was provided to Drs. Rempel and Wong by Schering-Plough Canada. Dr. Wong also received support from the University Research Grants Program (UGRP). This primarily support was provided by The Thorlakson Foundation to Dr. Rempel. Carla Krueger received fellowships from Manitoba Health Research Council. Ms. Krueger, Ms. Hawkins, Dr. Enns and Dr. Minuk report no biomedical financial interests or potential conflicts of interest.

ACKNOWLEDGEMENTS

The authors would like to thank Claudia Watson and the other staff at the Viral Hepatitis Investigation Unit, Winnipeg, MB for their assistance with patient management. The diligence of Colleen McRae in assistance with compiling the manuscript was also appreciated.

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