

Regulatory T cells suppress sickness behaviour development without altering liver injury in cholestatic mice

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Background & Aims: Cholestatic liver diseases are commonly accompanied by debilitating symptoms, collectively termed sickness behaviours. Regulatory T cells (T_{regs}) can suppress inflammation; however, a role for T_{regs} in modulating sickness behaviours has not been evaluated.

Methods: A mouse model of cholestatic liver injury due to bile duct ligation (BDL) was used to study the role of T_{regs} in sickness behaviour development.

Results: BDL mice developed reproducible sickness behaviours, as assessed in a social investigation paradigm, characterized by decreased social investigative behaviour and increased immobility. Depletion of peripheral T_{regs} in BDL mice worsened BDL-associated sickness behaviours, whereas infusion of T_{regs} improved these behaviours; however, liver injury severity was not altered by T_{reg} manipulation. Hepatic *IL-6* mRNA and circulating IL-6 levels were elevated in BDL vs. control mice, and were elevated further in T_{reg}-depleted BDL mice, but were decreased after infusion of T_{regs} in BDL mice. IL-6 knock out (KO) BDL mice exhibited a marked reduction in sickness behaviours, compared to wildtype BDL mice. Furthermore, IL-6 KO BDL mice injected with rIL-6 displayed sickness behaviours similar to wildtype BDL mice, whereas saline injection did not alter behaviour in IL-6 KO BDL mice. BDL was associated with increased hippocampal cerebral endothelial cell p-STAT3 expression, which was significantly reduced in IL-6 KO BDL mice.

Conclusions: T_{regs} modulate sickness behaviour development in the setting of cholestatic liver injury, driven mainly through T_{reg} inhibition of circulating monocyte and hepatic IL-6 production, and subsequent signalling via circulating IL-6 acting at the level of the cerebral endothelium.

Keywords: Fatigue; Cytokine; Liver–brain axis; Symptom; Endothelium; IL-6.
Received 17 April 2011; received in revised form 6 September 2011; accepted 15 September 2011; available online 23 October 2011

* DOI of original article: [10.1016/j.jhep.2011.11.003](https://doi.org/10.1016/j.jhep.2011.11.003).

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Abbreviations: BDL, bile duct ligation; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; T_{reg}, regulatory T cell.

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Introduction

Patients with cholestatic liver diseases commonly exhibit a number of associated symptoms including fatigue, malaise, and a loss of interest in engaging in social activity; collectively termed sickness behaviours [1,5,11,30,34,42]. Patients with cholestasis (e.g. PBC, PSC, obstructive cholestasis) often describe these symptoms as having the greatest detrimental impact upon their quality of life [5,11,30,40]. These symptoms are major causes of disability and work absenteeism, not to mention the high associated social and economic costs [8,17,24]. Moreover, currently available therapies for most cholestatic liver diseases have minimal, if any, impact on most of these sickness behaviours [2,21].

It is widely accepted that the brain can orchestrate changes in behaviour upon receiving signals which originate outside of the brain, within an inflamed tissue [26,42]. However, how the periphery and brain communicate in this fashion, and how this process is regulated, are poorly understood. Cytokines, released into the circulation during peripheral immune responses signal the brain to elicit sickness behaviours [15,16,42]. In particular, three cytokines have most commonly been implicated in the induction of sickness behaviours; namely, tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 [15,23]. In the setting of cholestatic liver injury, TNF- α , and IL-6 are most commonly measurable in the peripheral circulation of patients, as well as in animal models of cholestasis [4,14,22,50]. The activation of various immune cells is a tightly regulated process that is orchestrated in part by regulatory T lymphocytes (T_{regs}) [38,47]. T_{regs} are able to control the production of pro-inflammatory cytokines by activated immune cells during peripheral inflammation, and are being investigated clinically as potential therapeutic agents for the treatment of numerous immune-mediated diseases [35]. Therefore, we speculated that T_{regs} may also be able to modulate periphery-to-CNS immune signalling in the setting of cholestatic liver injury, to alter sickness behaviour development. Through a



series of loss and gain of function experiments, we identify an important role for T_{reg} in the development of sickness behaviours in mice with cholestatic liver injury due to bile duct ligation (BDL), in part, via the regulation of IL-6 production in the liver and by peripheral blood monocytes and subsequent IL-6-driven signalling to the brain.

Materials and methods

Model of cholestatic liver injury

C57BL/6 male wildtype mice or B6.129S6-*Il6^{tm1Kopf}/J* (IL-6 KO mice) were used (6–8 weeks; Jackson Laboratory, Bar Harbour, ME). Bile duct ligation (BDL) or sham surgery were performed as previously described [4,14] and all experiments were performed at day 5 post-surgery. Although, like all animal models, this model has potential limitations with regards to translation of results to patients, the BDL model has been extremely useful for generating and testing clinically relevant hypotheses which can, and have been, taken to the clinic [3,31,45]. Experimental protocols were in accordance with local guidelines for animal experimentation.

Sickness behaviour measurement

Sickness behaviour was assessed as previously described using a social investigation paradigm [7,14,20]. BDL surgery results in the reproducible development of sickness behaviours which parallel in many ways symptoms experienced by many patients with liver disease; including decreased activity and immobility, social withdrawal, and anhedonia [10,14,30,42–44]. A 3–4 week old juvenile male C57BL/6 mouse was introduced into the home cage of the test mouse and behaviour was assessed (total time of social investigation, total time of immobility, and number of social interactions) for 10 min [14].

Detailed materials and methods used for assessment of liver injury and inflammation, peripheral blood and liver mononuclear cell isolation, and flow cytometry, T_{reg} depletion and infusion, real time-PCR of hepatic cytokine mRNA, and serum cytokine levels, Western blotting, immunohistochemistry for phosphorylated signal transducer and activator of transcription 3 (p-STAT3) expression in cerebral blood vessels, and recombinant murine IL-6 (rmIL-6) administration for sickness behaviour assessment and hippocampal p-STAT3 expression in IL-6 KO BDL mice can be found online in [Supplementary Materials and methods](#).

Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM). For comparisons between two means a Student's unpaired *t*-test was performed and for more than two groups a one-way analysis of variance (ANOVA), followed by Student–Newman-Keuls *post hoc* test was performed (GraphPad Prism Version 4.0).

Results

Characterization of mouse model of cholestatic liver injury

BDL mice had increased serum ALT (sham 16.5 ± 1.7 U/L vs. BDL 419.8 ± 58.1 U/L; $n = 4/\text{group}$, $p < 0.001$) and total bilirubin (sham 1.8 ± 0.5 $\mu\text{mol/L}$ vs. BDL: 230.3 ± 14.7 $\mu\text{mol/L}$; $n = 4$ mice/group, $p < 0.001$) levels compared to shams. Hematoxylin and eosin (H&E) stained liver sections demonstrated portal based inflammatory cell infiltrates in BDL mice (absent in sham mice; [Supplementary Fig. 1A](#)).

BDL mice exhibit reproducible sickness behaviours

BDL mice displayed overt sickness behaviours as reflected by a significant reduction in time spent in social interaction ([Fig. 1A](#)); however, total number of social interaction attempts were similar in both groups ([Fig. 1B](#)). In addition, BDL mice were more immobile than sham mice ([Fig. 1C](#)).

Sickness behaviours in BDL mice are enhanced by T_{reg} depletion, and improved with T_{reg} infusion

T_{reg} depletion in peripheral blood was confirmed by FACS (IgG-treated BDL: $1.2 \pm 0.09\%$ or $1.4 \times 10^3 \pm 0.1 \times 10^3$ cells/ml of blood vs. anti-CD25-treated BDL: $0.6 \pm 0.04\%$ or $0.6 \times 10^3 \pm 0.07 \times 10^3$ cells/ml of blood; $n = 4\text{--}5$ mice/group, $p < 0.01$). T_{reg} -depleted BDL mice exhibited increased sickness behaviours; decrease in the total time spent in social interactive behaviours, fewer social interactions, and increased immobility ([Fig. 1](#)). T_{reg} depletion did not alter the severity of BDL-induced cholestatic liver injury ([Supplementary Fig. 1B and E](#)).

In contrast, T_{reg} -infused BDL mice demonstrated less sickness behaviours; increase in the total time spent in social exploration behaviours, and a decrease in immobility time, compared to control cell infused BDL mice ([Fig. 1A and C](#)). In contrast, no significant difference was observed in the total number of social interactions ([Fig. 1B](#)). The severity of cholestatic liver injury was similar in T_{reg} and control cell infused BDL mice ([Supplementary Fig. 1C and E](#)).

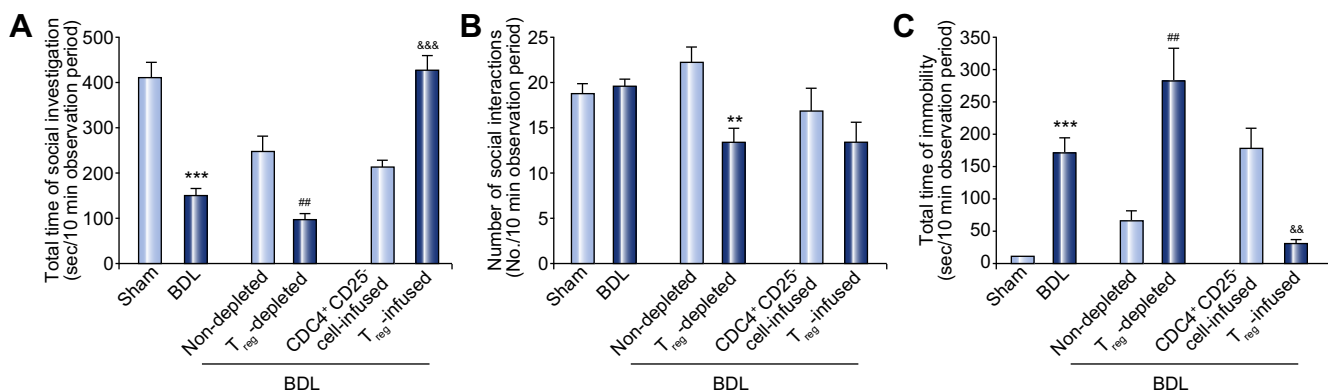


Fig. 1. T_{reg} depletion in mice with cholestatic liver injury exacerbates sickness behaviours while T_{reg} infusion decreases sickness behaviour development. Sickness behaviour was assessed using a social exploration study, a widely accepted method of quantifying these behaviours in mice. (A) Total time of social investigation (***) $p < 0.001$ as compared to sham, (***) $p < 0.01$ as compared to non-depleted BDL, (***) $p < 0.001$ as compared to CD4⁺CD25⁺ cell-infused BDL; $n = 5\text{--}12/\text{group}$), (B) number of interactions between the test mouse and the juvenile mouse (***) $p < 0.01$ as compared to non-depleted BDL; $n = 5\text{--}12/\text{group}$) and (C) total time of immobility of the test mouse (***) $p < 0.001$ as compared to sham, (***) $p < 0.01$ as compared to non-depleted BDL, (***) $p < 0.01$ as compared to CD4⁺CD25⁺ cell-infused BDL; $n = 5\text{--}12/\text{group}$) were significantly different between non- T_{reg} -depleted BDL mice compared to T_{reg} -depleted BDL mice and between control cell-infused BDL mice compared to T_{reg} -infused BDL mice. Bars represent mean values \pm SEM.

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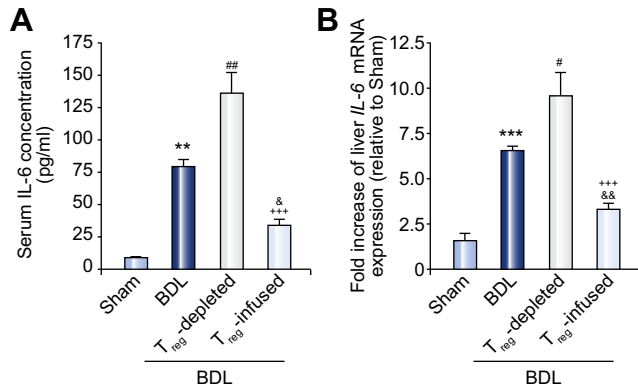


Fig. 2. Serum protein and hepatic mRNA IL-6 levels change with T_{reg} cell population manipulation. (A) Serum IL-6 levels in sham, BDL, T_{reg}-depleted, and T_{reg}-infused BDL mice (***p* < 0.01 as compared to sham controls, ###*p* < 0.01 as compared to BDL, #*p* < 0.05 as compared to BDL, ****p* < 0.001 as compared to T_{reg}-depleted BDL; *n* = 5–15/group). (B) Hepatic IL-6 mRNA levels in sham, BDL, T_{reg}-depleted, and T_{reg}-infused BDL mice (****p* < 0.001 as compared to sham controls, #*p* < 0.05 as compared to BDL, ##*p* < 0.01 as compared to BDL, ****p* < 0.001 as compared to T_{reg}-depleted BDL; *n* = 4–6/group). Bars represent mean values ± SEM.

Manipulations of T_{reg} numbers are paralleled by changes in serum and hepatic IL-6 levels in BDL mice

Serum levels of TNF- α , IL-1 β , and IL-6 were assessed in sham, BDL, T_{reg}-depleted, and T_{reg}-infused BDL mice. Serum TNF- α levels were detectable at the lower limit of assay detectability and were slightly increased in BDL vs. sham mice; however, serum TNF- α levels were unchanged in T_{reg}-depleted and non-depleted BDL mice (data not shown). In contrast, serum IL-1 β was undetectable in all groups of mice. However, serum IL-6 levels were ~20-fold higher in BDL compared to sham mice (*p* < 0.01) (Fig. 2A). T_{reg}-depleted BDL mice demonstrated a ~2-fold increase in serum IL-6 levels compared to non-T_{reg} depleted BDL mice, and infusion of T_{regs} into BDL mice resulted in a significant decrease in serum IL-6 levels, to levels below those documented in BDL mice and in BDL mice depleted of T_{regs} (Fig. 2A).

The liver is a main source of circulating IL-6, and hepatic IL-6 expression is increased in BDL mice [6]. Moreover, hepatic IL-6

mRNA and circulating IL-6 protein levels are increased in patients with the cholestatic liver disease PBC [25,29]. BDL mice demonstrated a significant ~4-fold increase in hepatic IL-6 mRNA expression compared to sham mice (*p* < 0.001). Changes in hepatic IL-6 mRNA levels paralleled changes in serum IL-6 concentrations in response to decreasing or increasing relative numbers of peripheral T_{regs} in BDL mice (Fig. 2B). In addition to increased hepatic IL-6 mRNA expression, depletion of peripheral T_{regs} in BDL mice also resulted in an increase peripheral blood mononuclear cell expression of IL-6 when compared to non-T_{reg} depleted BDL mice (Supplementary Results).

IL-6 knockout BDL mice exhibit a striking reduction in the development of sickness behaviours

The potential role of IL-6 in sickness behaviour development in BDL mice was examined through social exploration studies involving IL-6 KO mice, compared to wildtype BDL mice. Baseline behavioural assessments were similar in un-operated IL-6 KO and wildtype mice. IL-6-deficient BDL mice spent more time in social investigation than wildtype BDL mice (Fig. 3A); however, the total number of social interactions in these two groups were similar (Fig. 3B). In addition, IL-6 KO BDL mice exhibited less immobility than wildtype BDL mice (Fig. 3C). The severity of cholestatic liver injury was similar in IL-6 KO and wildtype BDL mice (Supplementary Fig. 1D and E).

rmlL-6 Injection induces sickness behaviours in IL-6 KO BDL mice

IL-6 KO BDL mice injected with rmlL-6 developed significant sickness behaviours at 2 h post-injection, as reflected by decreased total time of social investigation (Fig. 3A) and number of social interactions (Fig. 3B), as well as by an increase in total time of immobility (Fig. 3C) (similar to observations in wildtype BDL mice), in comparison to IL-6 KO BDL mice that were injected with saline (exhibited sickness behaviours similar to non-injected IL-6 KO BDL mice).

IL-6 mediates activation of STAT3 in brains of BDL mice

We addressed the possibility that circulating IL-6 signalled the brain via the cerebral endothelium, to bring about changes in

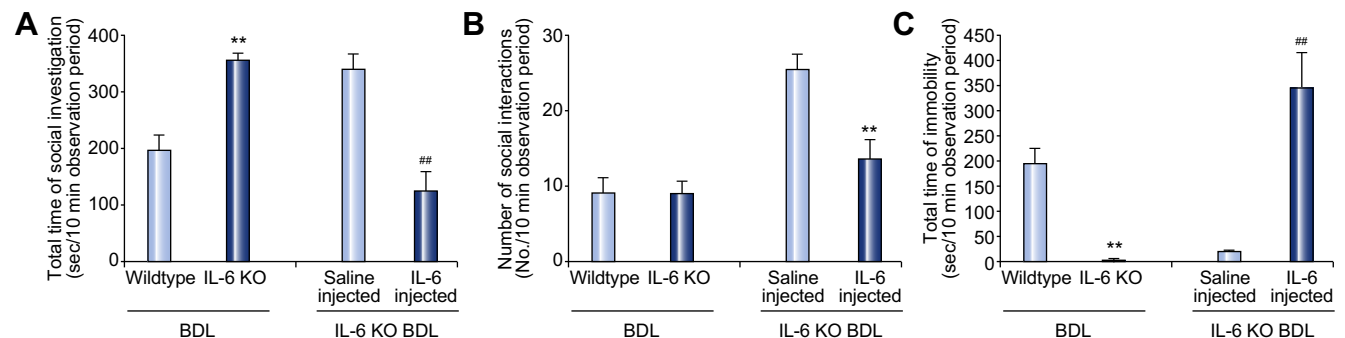


Fig. 3. IL-6 KO cholestatic mice demonstrate a marked reduction in liver injury-associated sickness behaviours compared to wildtype cholestatic mice, an effect that is reversed in IL-6 KO BDL mice injected with IL-6. (A) Total time of social investigation (***p* < 0.01 as compared to wildtype BDL, ###*p* < 0.01 as compared to saline injected IL-6 KO BDL; *n* = 5–7/group), (B) number of interactions between the test mouse and the juvenile mouse (***p* < 0.01 as compared to saline injected IL-6 KO BDL; *n* = 5–7/group) and (C) total time of immobility of the test mouse (***p* < 0.01 as compared to wildtype BDL, ##*p* < 0.01 as compared to saline injected IL-6 KO BDL; *n* = 5–7/group) were significantly different between wildtype BDL mice compared to IL-6 KO BDL mice and between saline injected IL-6 KO BDL mice compared to IL-6 injected (1 μ g/mouse i.p.) IL-6 KO BDL. Bars represent mean values ± SEM.

behaviour. IL-6 activates endothelium through stimulation of STAT3, generating p-STAT3 [36,37,49]. Therefore, we examined p-STAT3 expression within the hippocampus in general (by Western blot), and within cerebral endothelium of hippocampal blood vessels (by immunohistochemistry) of BDL vs. sham mice. The hippocampus is an area of the brain commonly implicated in sickness behaviour regulation [12,18,27]. Western blotting of total hippocampal protein homogenates revealed a significant increase in the p-STAT3/STAT3 ratio in BDL vs. sham mice (Supplementary Fig. 3B). Hippocampal STAT3/actin (loading control) ratios were similar in sham and BDL mice (Supplementary Fig. 3A).

In addition, we determined endothelial p-STAT3 expression (by immunohistochemistry) in hippocampal brain sections of BDL and sham mice. Numerous p-STAT3 positive staining endothelial cells were identified within hippocampal blood vessels in BDL mice, whereas none were evident in sham mice (Fig. 4A). The percentage of endothelial p-STAT3 positive staining hippocampal blood vessels was significantly reduced in IL-6 KO

BDL (and similarly in saline injected IL-6 KO BDL mice) compared to wildtype BDL mice, but the percentage of p-STAT3 positive staining hippocampal blood vessels was increased in rmlIL-6 injected IL-6 KO BDL mice to levels similar to those observed in wildtype BDL mice (Fig. 4B).

Discussion

In this study we have identified a novel role for T_{regs} in suppressing the development of sickness behaviours in BDL mice; independent of overt changes in the severity of liver injury. Our findings suggest a novel pathway whereby T_{regs} can regulate the development of sickness behaviours by inhibiting the production and release of IL-6 from the liver.

T_{regs} regulate numerous inflammatory diseases, and have been considered as having potential therapeutic benefit for treating these diseases [38,47,53]. Importantly, T_{reg} manipulation may have a potential role in the treatment of liver diseases [53]. BDL surgery in mice results in the development of significant cholestatic liver injury and sickness behaviours, coupled with an innate immune cell driven inflammatory response [6,48]; associated with a significant reduction in circulating, but no change in liver recruited, T_{reg} numbers compared to sham controls. Somewhat surprisingly, neither depletion nor augmentation of T_{reg} numbers in BDL mice altered the degree of cholestatic liver injury. Therefore, T_{regs} appear to be relatively ineffective in regulating overt hepatic inflammatory injury in BDL mice, consistent with findings often indicating a failure of increased numbers of T_{regs} to suppress hepatic inflammation in the clinical setting [9,41]. In contrast, the development of sickness behaviours in BDL mice was significantly augmented by T_{reg} depletion, and suppressed by T_{reg} infusion. These findings strongly suggest that T_{regs} are capable of regulating the development of sickness behaviours in the setting of cholestatic liver injury, independent of overt changes in the degree of liver injury.

T_{regs} are capable of regulating the innate immune response, including the activation of macrophages and their production of cytokines, including IL-6 [46,52]. Cytokines have been implicated historically in sickness behaviour development, both in humans with inflammatory disease and in animal model disease correlates [15,16]. Moreover, anti-TNF, and more recently anti-IL-6, neutralizing antibodies have been used clinically to treat inflammatory diseases, and are typically associated with improvements in sickness behaviours (e.g. fatigue, malaise) in these patients; often well before changes in local inflammation severity have been noted [13,32,33]. Similarly, inhibition of these cytokines can also improve sickness behaviours in animal models of inflammatory disease [7,15]. We could not detect circulating IL-1 β in BDL mice, and only a minimal elevation in circulating TNF- α levels were noted in BDL mice which were unchanged with T_{reg} depletion or augmentation. In contrast, BDL mice exhibited a striking increase in hepatic IL-6 mRNA and circulating IL-6 levels compared to shams. Moreover, depletion of T_{regs} in BDL mice further increased IL-6 levels (both circulating protein, hepatic mRNA), and increasing T_{reg} numbers significantly reduced IL-6 levels; in parallel to changes observed in sickness behaviour development. Interestingly, the effect of T_{reg} manipulation did not appear to be strictly limited to the liver, as depletion of T_{regs} was also

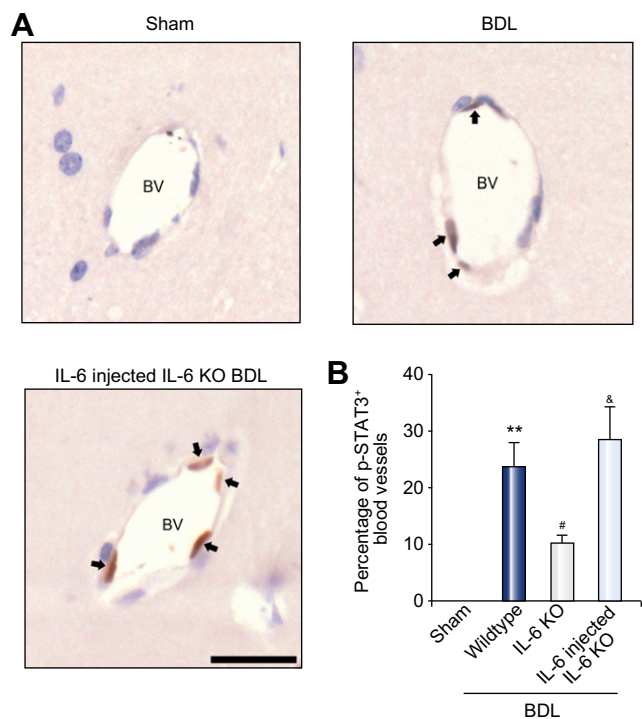


Fig. 4. IL-6 signalling induces endothelial cell p-STAT3 expression in the hippocampi of BDL mice. (A) Representative hippocampal blood vessels in sham and BDL mice, showing no p-STAT3 staining of endothelial cells in a sham hippocampal blood vessel compared to numerous p-STAT3 immunoreactive endothelial cells present in a hippocampal blood vessel of a BDL mouse. Endothelial p-STAT3 staining was reduced in IL-6 KO BDL mice injected with saline compared to wildtype BDL mice and increased in IL-6 injected IL-6 KO BDL mice compared to saline injected IL-6 KO BDL mice (magnification: 40 \times ; scale bar: 50 μ m; BV, blood vessel; black arrows, positive staining endothelial cells). (B) Total percentage of p-STAT3 positive staining hippocampal blood vessels in sham, BDL, IL-6 KO BDL, and IL-6 injected IL-6 KO BDL mouse hippocampi (** p < 0.01 as compared to sham, # p < 0.01 as compared to BDL, & p < 0.05 as compared to IL-6 KO BDL; n = 5/group). Since the total number of p-STAT3 positive blood vessels were similar in IL-6 injected IL-6 KO BDL and saline injected IL-6 KO BDL mice, data from these two groups were combined. Bars represent mean values \pm SEM.

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associated with increased circulating monocyte IL-6 production. These observations suggested that T_{reg}-mediated effects on BDL-associated sickness behaviour development are driven by alterations in circulating IL-6 levels. Therefore, we next examined how circulating IL-6 might drive alterations in behaviour in BDL mice.

To explore whether IL-6 is a mediator of sickness behaviour development in BDL mice, we employed IL-6 KO mice. The degree of liver injury was similar in IL-6 KO BDL and wildtype BDL mice, as reflected by serum ALT and bilirubin levels and histology; similar to reports by others [39]. This observation is consistent with our findings that changes in T_{reg} numbers in BDL mice are associated with changes in IL-6 protein and mRNA levels, but not in the degree of liver injury. Furthermore, IL-6 KO BDL mice demonstrated reduced sickness behaviours compared to wildtype BDL mice. These observations suggest that IL-6 is a critical signalling molecule from the liver to the brain in inducing sickness behaviours in BDL mice. IL-6 within the circulation would typically be excluded from the CNS by the blood–brain barrier; however, IL-6 can activate cerebral endothelial cells which could in turn generate secondary signals driving changes in behaviour [37,49]. Activation of cells by IL-6 leads to activation of STAT3, as reflected by the phosphorylation of STAT3 (i.e. p-STAT3) [28,37,49]. Therefore, endothelial cells activated by IL-6 may be quantified by their expression of p-STAT3 [28,37]. No p-STAT3 expressing endothelial cells were observed in blood vessels within the hippocampus (an area of the brain commonly implicated in the genesis of sickness behaviours) [12,18] of sham mice. In contrast, BDL mice demonstrated a marked increase in hippocampal endothelial cell p-STAT3 expression; an increase significantly blunted in IL-6 KO BDL mice. Importantly, IL-6 KO BDL mice injected with rIL-6 demonstrated a p-STAT3 expression profile in hippocampal cerebral endothelial cells similar to that observed in wildtype BDL mice at 4 h post-injection [19,51]. Furthermore, overt sickness behaviours developed in IL-6 KO BDL mice injected with rIL-6, whereas this did not occur in saline injected IL-6 KO BDL mice. These observations strongly suggest that IL-6 released into the circulation in BDL mice is a critical step in signalling the brain, at least in part, by activating cerebral endothelium, ultimately leading to the development of sickness behaviours.

In summary, the present study identifies T_{regs} as a novel modulator of sickness behaviour development during experimental cholestatic liver injury; an effect mediated mainly by T_{reg}-driven suppression of hepatic IL-6 production and release, and not through the attenuation of liver inflammation or injury. Moreover, our findings suggest that T_{reg} administration, or possibly inhibition of IL-6 signalling, could potentially be used for the treatment of severe or refractory sickness behaviours in patients with cholestatic liver disease.

Financial support

MGS holds the Cal Wenzel Family Foundation Chair in Hepatology. This work was funded through an operating grant awarded to MGS from the Canadian Institutes of Health Research (CIHR). KN and CD were funded through CIHR Frederick Banting and Charles Best Canada Graduate Scholarship Masters and Doctoral Awards, respectively.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgements

We would like to thank the University of Calgary Flow Cytometry Core (Laurie Robertson and Laurie Kennedy) and the Snyder Institute Live Cell Imaging Core Facilities and Dr. Quentin Pittman for his helpful comments.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jhep.2011.09.014](https://doi.org/10.1016/j.jhep.2011.09.014).

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