Studies on bile duct Injury and the protective role of oxygenated machine perfusion in liver transplantation
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 CHAPTER 5

CCR5 Deficiency Results in Decreased Hepatic Recruitment of Regulatory T Cells after Obstructive Biliary Injury in Mice


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Chapter 5

ABSTRACT

CCR5Δ32 polymorphism, resulting in a nonfunctional chemokine receptor 5, is a known risk factor for the development of non-anastomotic biliary strictures (NAS) after liver transplantation. The underlying mechanism, however, is unclear. In the current study, we investigated the role of a functional CCR5 deficiency in mice on the influx of regulatory T cells (T\(_{\text{Regs}}\)) and the balance of effector Th cell subsets after bile duct ligation (BDL), an established model of biliary injury. Mononuclear cells were isolated from liver tissue at 12hr, 24hr, 36hr and 5 days after BDL in CCR5 KO or wild-type mice and stimulated \textit{in vitro}. The frequency of Th cell subsets (T\(_{\text{Regs}}\), Th1, Th2 and Th17) in the mononuclear cell population was analyzed by flow cytometric detection of their intracellular markers (FoxP3, IFN\(\gamma\), IL-4\(^+\), and IL-17\(^+\), respectively). The frequency of T\(_{\text{Regs}}\) after BDL in CCR5 KO mice was significantly less than in wild-type mice at 5 days after BDL (\(P=0.008\)). While the Th17 response decreased in wild-type mice, it stayed stable in CCR5 KO animals, resulting in significant change in Th17 / T\(_{\text{Regs}}\) ratio towards more Th17 response in CCR5 KO animals at 5 days after BDL (\(P=0.026\)). This study suggests that loss of CCR5 function results in less recruitment of immune-modulating T\(_{\text{Regs}}\) to the liver and an enhanced immune-stimulating Th17 response after biliary injury. This mechanism may explain the higher incidence of NAS in liver recipients carrying the CCR5Δ32 polymorphism.
INTRODUCTION

The occurrence of biliary strictures, particularly, non-anastomotic biliary strictures (NAS) remains a major burden in orthotopic liver transplantation (OLT). NAS after OLT are characterized by multiple intra- and extrahepatic biliary strictures and secondary dilatations of the larger donor bile ducts leading to cholestasis and ultimately progressive liver fibrosis. Immune mediated injury has been suggested as one of the underlying etiologies for development of NAS. However, the exact role of the immune system in the pathophysiology of NAS is not completely understood. Clinical evidence for a role of the immune system in the development of NAS includes an increased risk for NAS in case of ABO incompatibility between the donor and recipient, pre-existing autoimmune diseases such as primary sclerosing cholangitis (PSC), and carriership of the loss of function mutation in the CC-chemokine receptor 5 (CCR532 polymorphism) in the recipient (1,2).

The CCR532 polymorphism is caused by a 32-basepair deletion in the reading frame located on chromosome 3p21. This mutation leads to a lack of CCR5 membrane expression and thus a functional deficiency of the receptor (3,4). CCR5 is one of the chemokine receptors that is expressed on CD4 T helper cells (Th) and mediates their migration. Therefore, dysfunction in CCR5 may have an impact on the recruitment of Th cells to the site of injury or infection (5). Localization of effector Th (Th1/Th2/Th17) and suppressor regulatory T cells (TReg) to the inflammatory site is instrumental for the effective control of immune responses. Disturbed migration of T cells may result in an altered ratio of effector and regulatory T cells, and may causes tissue damage (6). CCR532 has been associated with many immunological diseases. In patients suffering from end-stage renal disease (ESRD), CCR532 had protective effects against inflammation-associated mortality (7). In addition, presence of CCR532 has been associated with better outcomes in populations with high cardiovascular risk (8) and reduced inflammation post myocardial infarction (9). CCR532 has been also associated with better survival in type 2 diabetes patients (10). However, in the liver the effects of the CCR532 polymorphism appear deleterious (1).

In the liver, ligands of CCR5 (CCL3, CCL4 or CCL5) are expressed by biliary epithelial cells (11). The CCR532 polymorphism has been reported to exacerbate the severity of hepatic inflammation and T cell-mediated liver diseases (11,12). In addition, the presence of a CCR532 allele has been associated with an increased incidence of NAS after liver transplantation (1,2,13). However, the mechanistic role of the CCR532 polymorphism in the development of NAS after transplantation remains unclear. We hypothesized that biliary injury in the presence of a nonfunctional expression of CCR5 leads to increased inflammation and subsequent fibrosis due to a reduced attraction of immune-modulating TRegs. The present study was designed to investigate the effects of a functional CCR5 deficiency on the recruitment of Th cell subsets in the liver after biliary injury in the well-established model of bile duct ligation in mice.
MATERIALS AND METHODS

Animals
In this study, we used male CCR5 KO mice (B6; 129P2-Ccr5tm1Kuz/J) and wild-type B6129PF2/J with median body weight of 25.3 gr (IQR: 23.8 - 26.4 gr), purchased from Jackson Laboratories (Charles River Inc., Boston, MA, USA). Animals were kept under standard laboratory conditions with free access to standard laboratory chow and water. The Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RuG) approved the experiments of this study and all the experiments were performed in accordance with the guidelines of this Committee.

Experimental Design
Forty-five KO mice and fifty-four WT mice were included in this study. In each arm of the study, the experimental mice underwent bile duct ligation (BDL) procedure and the control animals underwent sham operation. At 12 hr, 24 hr, 36 hr and 5 days after BDL or sham operation, the animals in both KO and WT arm of the study were sacrificed and livers were either perfused in situ for cell isolation (n=6 in each group) in order to perform further flow cytometry as described below or were used for liver tissue and blood sample collection (n=3 in each group).

Bile Duct Ligation
Mice were anesthetized by inhalation of a mixture of isoflurane and oxygen (5% isoflurane to induce anesthesia and 2-3% isoflurane to maintain deep anesthesia during the BDL). After a mid-line laparotomy, the common bile duct (CBD) was exposed and dissected above the pancreas. Two 8-0 nylon sutures were placed around the proximal end of the CBD, close to the hilum of the liver, and one 8-0 nylon suture was placed at the distal end of the CBD, close to the pancreatic entrance of CBD. The CBD was transected between the ligatures. Then, the abdominal muscle layer and skin were sutured separately to close the incision. The animals were examined every 12 hours during the first 24 hours and on a daily basis afterwards with respect to body weight, activity level, and jaundice.

Evaluation of Hepatobiliary Injury Markers
At the time of sacrifice, all mice were weighed and anesthetized. Blood samples (n= 3 per group, WT and KO mice) were obtained from the inferior vena cava after the injection of an anticoagulant solution containing heparin (500 IU heparin in 1 mL 0.9% NaCl). Plasma was collected after 5 min centrifugation at 2700 rpm, at 4°C, and was then stored at -80°C for further measurement of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and total bilirubin using standard biochemical methods.

Histological Evaluation
At the indicated time points, the liver was removed, weighed and processed for biochemical or histological analyses (n=3 per group). Samples were fixed in 10% neutral buffered formalin and were paraaffin embedded for further preparation for hematoxylin and eosin (H&E) staining.
Two independent investigators performed the histological assessment in a blinded fashion (i.e. the investigators were not aware of the assigned study group). Hepatocellular injury as defined by “biliary infarct” (BI) was quantified in ten random high-power fields (100X magnification) and the findings were expressed as percentage of BI relative to the total liver tissue surface area (14). Biliary infarcts were defined as coalescent injured hepatocytes in immediate proximity to a ruptured bile ductule (15).

**Isolation of mononuclear cells from the livers**

Mononuclear cells were isolated from the livers of WT (n=6 per group) and KO (n=6 per group) mice in two phases:

1. The liver cells were isolated by a two-step perfusion method using collagenase as described before (16). Briefly, after anesthesia and heparinization, the abdominal inferior vena cava was cannulated below the liver. The portal vein was transected to be used for outflow during perfusion and the abdominal aorta and inferior vena cava below the diaphragm were cross clamped. The liver was perfused through the cannulated abdominal inferior vena cava with Ca\(^{2+}\)-free Krebs Ringer Hepes buffer (pH 7.4) at 37\( ^\circ \)C for 5-8 min until the portal vein effluent was clear (flow rate 10 ml/min; controlled by a rotatory pump that was used for perfusion). This was followed by perfusion with Mg\(^{2+}\)-free Krebs Ringer Hepes buffer containing Ca\(^{2+}\) (5.7 mmol/L) and Collagenase type IV (0.120-0.16 U/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands) for 10 min at a flow rate of 8 ml/min. The liver was then removed and placed in a petri dish containing RPMI 1640 medium (Gibco, Life Technologies Inc.), 10% fetal calf serum (FCS; Sigma-Aldrich) and gentamicin (Gibco, Life Technologies Inc., Darmstadt Germany). The cells were released by gentle teasing of the liver capsule and the suspension was filtered through 60-mesh sterile nylon gauze. All buffers were oxygenated prior to perfusion. The cell suspension was washed three times with RPMI 1640 medium at 50 g for 5 min and the supernatant was discarded and the cell pellet was re-suspended in fresh medium.

2. Mononuclear cells were isolated from the prepared cell suspension by density gradient centrifugation on Lymphoprep (Stem Cell Technologies Inc., Grenoble, France). Cells recovered from the gradient interface were washed twice in phosphate buffered saline (PBS), and adjusted to 1 x 10\(^7\) cells/ml in RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 5% FCS and 50 g/ml of gentamycin.

**Ex-vivo Stimulation**

Isolated mononuclear cells were incubated in presence or absence of 4 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 0.2 nM Calcium ionophore (Sigma-Aldrich). Brefeldin A (Sigma-Aldrich) with a final concentration of 10 L/ml was added to each tube in order to disrupt protein transport to allow for intracellular accumulation of cytokines. Next, culture tubes were incubated for a total of 16 hours.
Flow Cytometry
After stimulation, the cells were washed and incubated with eFluor605-conjugated anti-CD8 and eFluor450-conjugated anti-CD3 for 30 minutes in the dark on ice. Cells were then washed with cold PBS, followed by fixation and permeabilization in Fix/Perm buffer (FoxP3 staining kit, eBioscience, Uithoorn, The Netherlands). Subsequently, cells were stained with Peridinin Chlorophyll Protein Complex (PerCP)-Cyanine5.5-conjugated anti-IFNγ, Alexa Fluor488-conjugated anti-IL17A, Phycoerythrin(PE)-conjugated anti-IL4 and Allophycocyanin(APC)-conjugated anti-FoxP3. After incubation for 30min at room temperature, the cell suspension was washed twice with cold permeabilization buffer, and immediately analyzed on a LSRII flow cytometer (BD).

For all flow cytometry analyses, data were collected for at least 2 x 10^6 cells, and plotted using the Kaluza software package (Beckman Coulter, Woerden, Netherlands). Because stimulation reduces surface expression of CD4 on T-cells, CD4-positive T-cells were identified indirectly by gating on CD3-positive cells and CD8-negative lymphocytes. Next, cells were analyzed for the expression of intracellular cytokine production of IFNγ, IL-4, and IL-17. Unstimulated samples were used for setting the linear gates to delineate positive and negative populations as well as defining the expression of FoxP3. Results were expressed as frequencies, reflecting the percentage within the total CD4+ T-cell population.

Statistical Analysis
Continuous data are presented as medians and interquartile range (IQR). Mann-Whitney U test was used to determine the significance of differences between groups. A P-value <0.05 was considered statistically significant. All the statistical analyses were performed using GraphPad Prism software version 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA).

RESULTS
Hepatocellular Injury Induced by Bile Duct Ligation
The course of hepatocellular injury following BDL was investigated by measuring serum levels of ALT and by histological quantification of biliary infarcts. In both WT and CCR5 KO mice, serum levels of ALT rapidly increased after BDL. ALT levels increased from a baseline median of 6 IU/L in sham operated animals, peaking at 36 hr after BDL (median of 1505 IU/L in WT and 574 IU/L in KO mice), and remained elevated at 5 days after BDL (median of 62 IU/L in WT and 205 IU/L in KO mice; p=0.034), compared to the sham mice of both the WT and CCR5 KO group (Figure-1A). We observed no significant differences in ALT levels between the CCR5 KO and WT mice at all the time points after BDL.

Serum markers of cholestasis such as ALP and total bilirubin were evaluated after BDL. In both WT and CCR5 KO mice, ALP increased from median baseline of 40 IU/L in WT and 65 IU/L in CCR5 KO sham operated mice to 150 IU/L in WT and 294 IU/L in CCR5 KO mice at 5 days after BDL (Figure-1B). Along with this, total bilirubin increased steadily from a median baseline of 3 mol/L in sham operated mice to a median of 124 mol/L in WT mice and 150 mol/L in KO mice at 5 days after BDL (Figure-1C). There were no significant differences between the WT and KO mice regarding the increase in the serum markers of cholestasis.
Biliary infarcts were detected as soon as 12 hr after BDL in both WT and CCR5 KO mice and infarcts increased in size and number (indicated as percentage of biliary infarct relative to the total liver surface area) (Figure-1B) during the observation period. However, there were no significant differences in the number and size of biliary infarcts between the CCR5 KO and WT mice. Histological examination of liver tissue showed increasing dilatation and proliferation of bile ductules during the course of injury after BDL (Figure-1E). Altogether, these data suggest that CCR5 loss of function does not affect the extent of hepatocellular injury or severity of bile duct injury up until 5 days after BDL.

**Frequency of Hepatic T\textsubscript{Regs} after Bile Duct Ligation**

The frequency of hepatic T\textsubscript{Regs} among the CD4\textsuperscript{+} T cells was evaluated in both WT and CCR5 KO mice by measuring the frequency of total FoxP3\textsuperscript{+} CD4\textsuperscript{+} T cells in isolated mononuclear cell preparations. The percentage of total FoxP3\textsuperscript{+} CD4\textsuperscript{+} T cells among the unstimulated samples decreased in both WT and CCR5 KO mice after BDL. In WT mice, total of FoxP3\textsuperscript{+} cells decreased from median of 7.5% in sham operated animals to 6.7% at 5 days after BDL and in KO mice it decreased from a median of 5.8% in sham operated animals to 2.3% at 5 days after BDL, which was significantly less compared to WT animals at 5 days after BDL (p=0.008) (Figure-2A). A representative flow cytometric characterization of FoxP3\textsuperscript{+} CD4\textsuperscript{+} T cells is demonstrated in Figure-2B.

It is known that a part of the FoxP3\textsuperscript{+} T\textsubscript{Reg} cells has the ability to lose their suppressive function in response to the inflammatory environment and convert towards IL-17 producing effector cells. Therefore the suppressive function of T\textsubscript{Regs} is attributed to FoxP3\textsuperscript{+} IL-17\textsuperscript{−} CD4\textsuperscript{+} cells, or so called true T\textsubscript{Regs} (6). At 5 days after BDL, the frequency of true T\textsubscript{Regs} was significantly lower in CCR5 KO mice (median of 2.0%), compared to sham operated animals (median of 5.6% of CD4\textsuperscript{+} cells; p=0.015; Figure-2C), while in WT mice the frequency of true T\textsubscript{Regs} was significantly higher in sham operated animals (median of 5.3% of CD4\textsuperscript{+} cell) , compared to the group at 36 hours after BDL (median of 10.1%; p=0.019; Figure-2C). These data suggest that CCR5 loss of function leads to decreased recruitment of T\textsubscript{Regs} to the liver after bile duct ligation.

**Hepatic Th-1, Th-2 and Th-17 Type Response after Bile Duct Ligation**

The functional phenotype of CD4\textsuperscript{+} cells was evaluated by analyzing the intracellular expression of the cytokines IFN-\gamma, IL-4 and IL-17 after stimulation of the cells. The percentage of IFN-\gamma\textsuperscript{+} secreting CD4\textsuperscript{+} cells (indicating a Th-1 type directed response), the percentage of IL-4\textsuperscript{+} secreting CD4\textsuperscript{+} cells (indicating a Th-2 type directed response), and the percentage of IL-17\textsuperscript{+} secreting CD4\textsuperscript{+} cells (defining a Th-17 response) were not significantly different between WT and KO mice without BDL (Figure-3A, B, C). The percentage of Th-1 cells was lower at 5 days after BDL in CCR5 KO (median of 12.8%), compared to sham operated animals (median of 26.6%) or WT animals at 5 days after BDL (median of 12.0%) (Figure-3A). The same pattern was observed after BDL in CCR5 KO mice in the Th-2 type directed response when compared to its relatively stable response in WT mice (Figure-3B). However, the differences in the Th-1 and Th-2 responses were not statistically significant. The percentage of Th-17 cells at 5 days after BDL in WT animals (median of 10.9%) was lower than the percentage in sham
Figure 1. Markers of hepatocellular injury, cholestatic injury and histological evaluation of liver tissue after BDL

Both WT and KO mice had a peak increase in the serum levels of ALT, as a marker of hepatocellular injury, at 36hr after BDL, which decreased afterwards during the course of injury (A). Serum markers of cholestatic injury as indicated by alkaline phosphatase (B) and bilirubin (C) progressively increased over the course of the injury in both WT and KO mice. Histological examination of the liver tissue showed progressive hepatocellular injury (biliary infarcts) in both WT and KO mice after BDL as shown in panel (D) and hematoxylin and eosin staining of the liver tissue in panel (E) [biliary infarcts indicated by arrows]. Graphs are presented as median ± IQR.
operated animals (median of 19.1%), however, this change in Th-17 cells was not observed in CCR5 KO mice after BDL (Figure-3C), suggesting an absence of the suppression of the Th-17 response in CCR5 KO mice after BDL, compared to WT animals. A representative flow cytometric characterization of CD4⁺ T cell subsets in WT and CCR5 KO mice is provided in Figure-4.

**Ratio of Th-17/TRegs after Bile Duct Ligation**

The balance of Th-17/TRegs was evaluated by calculating the ratio of IL-17⁺ secreting CD4⁺ cells to true TRegs as defined by FoxP3⁺ IL-17⁻ cells (Figure-3D). The ratio of Th-17/TRegs was significantly lower in CCR5 KO sham operated mice compared to WT animals (median of 1.8 vs. 3.8, respectively; \( p=0.019 \)), indicating the difference in hepatic T cell distribution influenced by absence of CCR5 ligands. Upon injury, an initial increase in the ratio of Th-17/TRegs was observed in WT animals while the ratio stayed stable in CCR5 KO mice, resulting in a significant difference between the two groups at 12hr after BDL (median of 6.6 vs. 1.00, 55

![Figure 2. The frequency of hepatic TRegs after BDL.](image)

The frequency of FoxP3⁺ CD4⁺ T cells (unstimulated TRegs) was significantly lower in KO mice at 5 days after BDL when compared to WT animals (A); as shown in a representative flow cytometric characterization of unstimulated FoxP3⁺ cells (B). Moreover, while the frequency of true TRegs was significantly higher in WT mice at 36hr after BDL (\(^*\)), compared to sham operated animals, it was significantly less in KO mice at 5 days after BDL (\(^**\)), when compared to sham operated animals (C). Graphs are presented as median± IQR.
respectively; \( p = 0.026 \)). Thereafter, WT animals showed a significantly lower ratio at 36hr after BDL (median of 0.96) compared to sham animals (median of 3.8; \( p = 0.019 \)). In contrast, the ratio of Th-17/T\(_{RegS}\) stayed stable during the acute phase of injury in CCR5 KO mice and increased to a median ratio of 3.5 at 5 days after BDL. This ratio was significantly higher than its ratio in sham operated animals (\( p = 0.026 \)), reflecting presence of less suppressor T cells and therefore less T cell suppression in CCR5 KO mice (Figure-3D). Altogether, these data suggest that CCR5 loss of function leads to altered Th-17/T\(_{RegS}\) ratio in the liver towards a higher Th-17 immune response.

![Figure 3](image.png)

**Figure 3.** An overview of Th-1, Th-2 and Th-17 immune response as well as the balance of Th-17/true T\(_{RegS}\) after BDL.

In the KO mice after BDL a decreasing trend in the Th-1 (A) and Th-2 (B) immune response was observed while in the WT animals these immune responses stayed stable. While the Th-17 response decreased after BDL in WT animals, it stayed stable in KO mice after BDL (C), resulting in an altered Th-17/true T\(_{RegS}\) ratio in the KO mice after BDL towards enhanced Th-17 immune response (D). Graphs are presented as median± IQR.
DISCUSSION

This is the first study to investigate the involvement of CCR5 loss of function on recruitment of Th cells to the liver following obstructive bile duct injury in a mouse model of bile duct ligation. Our data showed that CCR5 loss of function suppresses the recruitment of true T\textsubscript{regs} to the liver at 5 days after BDL when the chronic phase of the inflammation and fibrosis formation begins. These findings can explain the higher incidence of NAS after liver transplantation in recipients with a loss of function polymorphism of CCR5 (CCR5Δ32) (1,13).

NAS are biliary abnormalities that can occur at intrahepatic as well as extrahepatic bile ducts and usually have a multifocal pattern (18). The clinical presentation of NAS includes an obstructive cholestatic picture with progression to liver fibrosis in two-third of the patients (19). Clinical studies have suggested two different patterns in the occurrence of NAS: some patients develop biliary abnormalities early (<1 year) after transplantation, whereas others present with late (>1 year) occurrence of NAS (20). Based on differences in the association with clinical risk factors, it has been suggested that the early type of NAS is largely related to ischemia-reperfusion injury during the transplant procedure, while that late type of NAS is more often

**Figure 4.** Flow cytometric characteristics of Th-1, Th-2 and Th-17 in WT and CCR5 KO mice without injury (no BDL), and at 12hr, 24hr, 36hr and 5 days after BDL.
immune-mediated (21). To date, there is no established animal model for post-transplant NAS. In this study, using the established BDL model of biliary injury, we simulated the cholestatic picture of NAS as indicated by a steady increase in the serum levels of ALP and bilirubin after BDL in both WT and CCR5 KO mice. Our study showed that CCR5 loss of function was associated with a decreasing trend in the Th1 and Th2 immune responses after BDL. Th1 immune response has shown anti-fibrotic effects during liver injury by the induction IFN-\(\gamma\), resulting in less secretion of pro-fibrotic cytokines by Th2 cells (22,23). Therefore, the relative difference in the Th1 and Th2 immune response may influence fibrotic reactions upon liver injury (24,25). In addition, an imbalance of Th-1/Th-2 has been associated with progression of primary biliary cirrhosis (PBC) (26). Although the effect of CCR5 deletion on decreased level of Th-1 and Th-2 cells, shown in our study, was not statistically significant, it may suggest that the CCR5 loss of function has an effect on the distribution of T cell subsets after liver injury.

In addition, CCR5 loss of function was associated with less recruitment of T\(_{\text{Reg}}\)s and yet stable levels of Th-17 immune response after BDL. This resulted in an increased Th-17/T\(_{\text{Reg}}\)s ratio at 5 days after BDL. This finding suggests that less recruitment of T\(_{\text{Reg}}\)s to the liver results in less suppression of Th-17 response and therefore higher production of IL-17 cytokine. The IL-17 receptor is expressed on different cell types including cholangiocytes (25). It has been shown that depletion of hepatic FoxP3\(^+\) T\(_{\text{Reg}}\)s results in enhanced Th-17 response and therefore less suppression of a pro-fibrogenic inflammatory milieu upon liver injury, leading to more liver fibrosis (27). Moreover, increased Th-17/T\(_{\text{Reg}}\)s due to higher Th-17 response has been associated with bile duct damage in infants with biliary atresia (28), as well as chronic inflammation of bile ducts in patients with primary biliary cirrhosis (29,30). Increased hepatic levels of Th-17 cells have been reported in patients with acute and chronic cholestatic liver injury and IL-17 has been shown to play a key role in induction of liver damage after ischemia reperfusion by initiating neutrophil-induced inflammatory responses (31). Therefore, association of CCR5 loss of function with such an imbalance after cholestatic-induced liver injury in BDL mice may suggest a mechanism for higher incidence of late NAS in patients with CCR5\(\Delta32\) polymorphism.

The most prominent change in Th-17/T\(_{\text{Reg}}\)s ratio in our study was observed at 5 days after BDL; when the chronic phase of liver injury begins. At this phase, the T cells immune response becomes more prominent and plays an important role in the progression of liver injury (14). Clinical studies have reported that late-onset NAS (>1 year post-transplantation) is more frequently associated with immunological risk factors, including the CCR5\(\Delta32\) functional deletion (1,13,18,20). The prominent effect of CCR5 loss of function on T\(_{\text{Reg}}\)s recruitment to the liver during the later phases of injury after BDL in mice may be an explanation for the higher incidence of late-onset NAS in liver transplant recipients with the CCR5\(\Delta32\) polymorphism. To this end, it would have been interesting to have a longer follow up than the 5 days, which were used in the current study. However, this cannot be done in a BDL model, because the animals get too sick in the long term. In fact, in our study, CCR5 deletion showed no effect on the extent of BDL-induced hepatobiliary injury. BDL is a very severe model of acute cholestasis, leading to acute severe hepatobiliary injury and liver dysfunction with clinical characteristics that are similar to patients with acutely decompensated cirrhosis (32), whereas NAS is a chronic cholestatic disease that generally does not lead to liver failure (19,20). Therefore, BDL may
not be the best model to investigate the histological developments of a chronic mild cholestatic disease. Our interest was to study the immune response after the type of biliary injury that occurs after transplantation, i.e. NAS. However, there is no representative animal model for NAS while BDL is an established model of biliary injury. The difference between these two types of biliary injury is a limitation of our study. We suggest that the lack of effect of CCR5 deletion on BDL-induced hepatobiliary injury may not truly represent the histologic consequences of carriersonship of the CCR5Δ32 polymorphism in patients with NAS. However, in the current study we aimed to study the contributing immunologic factor that may explain the ongoing bile duct damage after transplantation due to reduced anti-inflammatory TRegs immune response in the liver, rather than the initiating cause of NAS. Moreover, performing adoptive transfer experiments such as BDL in CCR5Δ32 overexpressed mice or transplantation of livers from WT mice into CCR5Δ32 KO recipients in future studies would be interesting to investigate the role of CCR5 in pathogenesis of NAS. In conclusion, this study suggests that CCR5 loss of function results in less recruitment of immune-modulating TRegs to the liver after obstructive bile duct injury, resulting in enhanced response of immune stimulating and proinflammatory Th-17 cells. This mechanism may serve as the underlying reason for the higher incidence of NAS in liver transplantation recipients with CCR5Δ32 loss of function polymorphism.

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PART B

Oxygenated Machine Perfusion: A Potential Strategy to Improve Organ Quality prior to Transplantation