

**Basic studies on autologous bone marrow cell infusion  
therapy for the dog with chronic liver disease**

(イヌの慢性肝疾患に対する自家骨髄細胞投与療法に関する基礎的研究)

Laboratory of Veterinary Internal Medicine  
Graduate School of Veterinary Science, Azabu University

dv0962 Takashi Kato

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# **General Introduction**

Canine liver failure is a common disease seen in veterinary small animal practice, and hepatitis, hepatic portal systemic shunts and hepatic fibrosis often occur in dogs [Allen *et al.*, 1999; Rutgers *et al.*, 1993; Watson, 2004; Poldervaart *et al.*, 2009]. As treatment of these illnesses is limited, an effective therapeutic regime would be very useful. Although, the liver has marked regenerative capacity, in severe disease, its ability to regenerate is decreased. Liver transplantation is often the only therapeutic option for dogs with severe hepatic failure. However, transplantation has many problems, including the lack of donors, the stress of surgery, and tissue rejection. In particular, a lack of knowledge regarding canine leukocyte antigens prevents successful liver transplantation from a donor. Thus, an effective therapeutic regime has been highly desirable, but alternative medicine of liver transplant is not established.

Recently, regenerative medicine using stem cells has been concerned as an attractive therapy for treating patients with severe liver diseases. Numerous reports have shown that bone marrow cells (BMCs) contain stem cell population and has the capacity to differentiate into a variety of non-hematopoietic cell lineages, including liver, neuron, intestinal, skin, and muscle tissues [Ferrari *et al.*, 1998; Alison *et al.*, 2000; Brazelton *et al.*, 2000; Dawn *et al.*, 2005; Okamoto *et al.*, 2002]. These results indicate that the BMC is one of the potent cell for source of regenerative medicine. The capacity of the BMC to differentiate into hepatocytes and intestinal cells has been shown by Y-chromosome detection in autopsy analysis of human female recipients of BMCs from male donors [Alison *et al.*, 2000; Okamoto *et al.* 2002; Theise *et al.*, 2000]. We also previous demonstrated that the capacity of canine BMC differentiation to hepatocyte-like cell in vitro using Hepatocyte Growth Medium and human placenta extract [Neo *et al.*, 2009]. However, clinical application for liver diseases using regeneration therapy is still difficult reason for precision and tissue construction of differentiated hepatocyte like cells. Especially, marked number of differentiated cell is required, since the liver is large organ. Therefor the transplantation of hepatic differentiated cell is difficult to use for clinical

application.

In human medicine, clinical trial to examine autologous bone marrow cell infusion (ABMi) therapy for cirrhotic patients have been initiated from 2003, and reported the clinical effect of the therapy [Terai *et al.*, 2006; Iwamoto *et al.*, 2011; Kim *et al.*, 2010]. Furthermore, many studies of rodents model demonstrated that BMC infusion reduced liver fibrosis [Hardjo *et al.*, 2009], corrected liver dysfunction [Allen *et al.*, 2004], and improved survival rate [Sakaida *et al.*, 2004]. Terai and colleagues have reported that BMCs infused via tail vein efficiently repopulate cirrhotic liver under the conditions of persistent liver damage induced by CCl<sub>4</sub> in mouse model [Terai *et al.*, 2000]. They also have indicated that infused BMCs expressed matrix metalloproteinase (MMP) in fibrotic liver and contributed to improvement of fibrosis [Terai *et al.*, 2003; Yamamoto *et al.*, 2004]. On the other hand, researchers who have a negative opinion on the plasticity of BMCs insist that little evidence exists for the use of bone marrow-derived hepatocytes in the replacement of injured liver, and that no data have demonstrated transdifferentiation of infused BMCs to hepatocytes or a major role in regeneration [Menthen *et al.* 2004; Wagers *et al.*, 2002]. Moreover, BMCs have been reported to be a possible source of functional hepatic stellate cells and myofibroblasts contributing to fibrosis [Baertschiger *et al.*, 2009; Forbes *et al.*, 2004; Russo *et al.*, 2006]. BMC transplantation has been performed to treat hematological diseases, and several clinical studies have applied BMC injection to induce regeneration of myocardium and blood vessels [Tateishi-Yuyama *et al.*, 2002]. Taken together, these findings suggest that BMCs will be effective sources for regenerative liver therapy. Although numerous studies have been done, a role of BMC infusion on liver function is still unknown. In dogs, causes of chronic hepatitis include microorganisms [Bishop *et al.*, 1979; Boomkens *et al.*, 2004; Fox *et al.*, 1996], toxins and drugs [Dayrell-Hart *et al.*, 1991; Newman *et al.* 2007], immune-mediated reactions [Poitout *et al.*, 1997; Thornburg *et al.* 1998; Weiss *et al.*, 1995], and breed-associated metabolic errors

[Smedley *et al.*, 2009; Webb *et al.*, 2002]. In spite of significant research efforts, the causes remain elusive [Watson., 2004]. However, the process of fibrotic change developed in chronic liver injury in dogs is similar to that of human [Kanemoto *et al.*, 2011]. Thus, ABMi may be an effective therapy for canine chronic hepatopathies in the future.

A series of the present studies were carried out to evaluate ability of canine BMC in the liver injured condition, and verify the feasibility, safety and efficacy of ABMi for primary chronic hepatitis in dogs. This study is composed of 3 chapters as follows. In chapter 1, I investigated the accumulation potential of canine BMCs in the liver by xenotransplant into immunodeficiency mice with CCl<sub>4</sub> acute hepatitis. In chapter 2, I evaluated the influence of canine BMC infusion on hepatic fibrosis by xenotransplant into immunodeficiency mice. In chapter 3, feasibility, safety and efficacy of ABMi therapy was assessed for primary chronic hepatitis in dogs.

# Chapter 1

**Accumulation of xenotransplanted canine bone marrow cells in the liver of NOD/SCID/ $\gamma_c^{\text{null}}$  mice with acute hepatitis induced by CCl<sub>4</sub>**

## **Abstract**

Bone marrow cell infusion (BMCi) has recently been suggested as an effective therapy for refractory liver diseases; however, the efficiency of BMCi using canine bone marrow cells (cBMCs) has not been reported. We evaluated the accumulation potential of cBMCs in a mouse model of acute liver failure. Acute hepatitis was induced by carbon tetrachloride (CCl<sub>4</sub>) treatment in NOD/SCID/ $\gamma_c^{null}$  (NOG) mice and wild-type (WT) C57BL mice, and the characteristics of liver dysfunction and the degree of hepatic injury and regeneration were compared between the two mouse models. Next, female CCl<sub>4</sub>-treated NOG mice were xenotransplanted with male PKH26-labeled cBMCs, and the potential of cBMCs to accumulate in the injured liver tissue compartments was examined. Fluorescence microscopy was performed to histologically detect the infused cBMCs, and DNA polymerase chain reaction was performed for detection of the male Y chromosome (SRY gene) in the recipient female NOG mice. The number of PKH26-positive cBMCs transplanted in the liver tissue gradually increased in the NOG mice. The infused cBMCs were located in the necrotic areas of the liver at an early stage after transplantation, and the most had accumulated a week after transplantation. However, the therapeutic efficacy of the xenotransplantation remained unclear because no significant differences were observed concerning the extent liver injury and regeneration between the cBMC-transplanted and saline control mice. These results suggest that cBMCs will specifically accumulate in injured liver tissue and that BMC transplantation may have the potential to repair liver deficiency.



## **Introduction**

Chronic hepatitis (CH) and cirrhosis have long been recognized in many dog breeds by both primary and referral veterinary practices [Poldervaart *et al.*, 2009; Rutgers *et al.*, 1988]. These disorders are often perceived as extremely difficult to treat, and there are no cures. Therefore, more effective therapies are needed. In human medicine, liver transplantation is the only effective cure for CH and cirrhosis, but the limitations of transplantation, such as a lack of donors, surgical complications, rejection and high cost, have led to proposals for less invasive regenerative therapy procedures.

Recent reports demonstrated that murine bone marrow cells (BMCs) transplanted via peripheral vein populated and differentiated into albumin-producing hepatocytes via hepatoblast intermediates [Almeida-Porada *et al.*, 2010; Terai *et al.*, 2003; Yamamoto *et al.*, 2004]. Interestingly, BMC infusion improves hepatic function, including elevation of serum albumin levels, and in mice or rats, it reduces liver fibrosis, corrects liver dysfunction and improves survival rate in human cirrhosis patients [Ishikawa *et al.*, 2006; Iwamoto *et al.*, 2012]. On the basis of these results, a clinical trial of autologous BMC infusion was conducted, wherein it was shown that BMCs administered through a peripheral vein improved liver function in patients with liver cirrhosis [Kim *et al.*, 2010; Saito *et al.*, 2011]. Therefore, BMC infusion may prove to be a curative therapy for liver cirrhosis in the future [Terai *et al.*, 2012].

Recent studies have shown that the effects of BMC therapy on liver failure depend on many factors such as specific accumulation ability, immunomodulation, trophic support, differentiation (plasticity), revascularization and tissue regeneration [Parekkadan *et al.*, 2007; Parekkadan *et al.*, 2007]. In particular, specific accumulation ability is one of the most important factors in cell transplant therapy [Cho *et al.*, 2011; Thomas *et al.*, 2003]. In murine models, accumulation ability has been established for specific disorders of the liver and pancreas [Takeshita *et al.*, 2006]. In another study of liver cirrhosis patients with

splenomegaly, splenectomy enhanced the repopulation of BMCs into the cirrhotic liver microenvironment and tended to result in greater improvement of liver function [Iwamoto *et al.*, 2012]. These studies suggest that the specific accumulation of BMCs in the disordered liver is important for effective results.

We previously demonstrated the capacity of canine BMCs (cBMCs) for differentiation into albumin-producing hepatocyte-like cells [Neo *et al.*, 2009]. It has also been shown that bone marrow stromal cells (BMSCs) may play a role matrix metalloproteinases (MMPs) production which resulting in improvement of liver fibrosis [Haraguchi *et al.*, 2012]. Furthermore, xenotransplantation of canine BMSCs may be effective in resolving inflammatory fibrotic liver in CCl<sub>4</sub>-treated immunodeficient mice [Haraguchi *et al.*, 2012]. Although autologous BMCs transplantation in liver injured models, studies demonstrating the ability of BMCs to accumulate in the disordered liver have been limited. In addition, the systemic effects of infused BMC populations are not well known in both dogs and rodents. Haraguchi *et al.* reported that the donor genomic DNAs of xenotransplanted canine BMSCs were detectable in the liver of recipient nude mice administered cyclosporin A (CSA) [Haraguchi *et al.*, 2012]. Cho *et al.* described that infused mice BMCs have a specific homing capacity to the liver in CCl<sub>4</sub>-injected recipient mice [Cho *et al.*, 2011]. However, these studies have not elucidated the localization of transplanted cells in the injured liver tissue. To analyze the accumulation of BMCs in the liver, we used NOD/SCID/ $\gamma_c^{\text{null}}$  (NOG) mice in this study because this model accepts heterologous cells much more readily compared with any other immunodeficient models [Ito *et al.*, 2002].

Therefore, the present study used an NOG mouse model with acute hepatitis induced by CCl<sub>4</sub> treatment to investigate the ability of xenotransplanted cBMCs to accumulate in injured murine liver tissue and estimate the specificity of this accumulation in the liver.

## **Materials and Methods**

### ***Animals:***

Six-week-old NOG mice were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). Same age C57BL6J Jcl mice were purchased from CLEA Japan, Inc. (Kanagawa, Japan) to serve as the wild-type (WT) murine control. All mice were shipped to the Research Institute of Biosciences of Azabu University and handled with humane care under pathogen-free conditions. The mice were housed in a room under controlled temperature (25°C), humidity and lighting (12-hr light/dark cycle). Access to food and tap water was ad libitum throughout the study period. All of male NOG and C57BL mice were used in research for an experimental model of liver injury, and female NOG mice were used for cBMC transplant experimentation. A 2-year-old male beagle dog that was clinically healthy was used as a donor of cBMCs for transplantation into NOG mice. All experiments in this study were performed in accordance with the Animal Protection Guidelines of Azabu University (authorization no. 10–79).

### ***Experimental model of liver injury:***

Forty male NOG mice and 20 male WT mice were divided into three groups: NOG + CCl<sub>4</sub> (n = 20), NOG + oil (n = 20) and WT + CCl<sub>4</sub> (n = 20). CCl<sub>4</sub> (Wako Pure Chemical Industries Ltd., Tokyo, Japan) dissolved in olive oil (1ml/kg) was administered via intraperitoneal injection in the NOG + CCl<sub>4</sub> and WT + CCl<sub>4</sub> groups, and the extent of liver injury and regeneration was investigated in these mice. The mice were sacrificed at 0 hr, 24 hr, 48 hr, 72 hr and 1 week after CCl<sub>4</sub> injection, and the livers were sectioned and fixed in phosphate-buffered 10% formaldehyde for histological examination.

### ***Preparation of donor BMCs:***

cBMCs were harvested from the humerus and femur of the dog under general anesthesia with intravenous injection of medetomidine (20 µg/kg) and pentobarbital (10 mg/kg). The cells were collected in a heparin-containing syringe. After fat removal, cBMCs were extracted, and single-cell suspensions were prepared by passing the cells through a 100-µm mesh filter (BD Falcon, Tokyo, Japan) into new tubes. Mononuclear cells (MNCs) were isolated with Ficoll solution (Lymphoprep; Axis-Shield, Oslo, Norway) and washed and concentrated three times. The isolated MNCs were labeled with red fluorescent dye using a PKH26 Red Fluorescent Cell Linker Kit (Sigma–Aldrich Japan, Tokyo, Japan) according to the manufacturer’s protocol. Incubation was performed for 5 min at room temperature with  $1 \times 10^7$  MNCs/ml, followed by washing in phosphate-buffered saline (PBS) to remove proteins from the culture medium for optimal staining. The staining reaction was stopped with addition of 1% bovine serum albumin, followed by washing with PBS. Labeling and viability were verified by cultivation in hepatocyte growth medium as previously described [Neo *et al.*, 2009].

### ***Transplantation:***

Fifteen female NOG mice were grouped as follows: CCl<sub>4</sub> + cBMC (n = 5); oil + cBMC (n = 5) and CCl<sub>4</sub> + saline (n = 5). At 1.5–2 hr after BMC harvesting, prepared MNCs ( $1 \times 10^6$ ) or saline were administered to the tail veins of CCl<sub>4</sub>- or oil-treated NOG mice. The mice were sacrificed after 1 week, and the livers, lungs, and spleens were removed to evaluate the systemic distribution of the administered cBMCs (Fig. 1A). To investigate the time course of BMC accumulation in the injured liver, 32 female NOG mice were grouped into CCl<sub>4</sub> + cBMC (n = 16) and CCl<sub>4</sub> + saline (n = 16) groups and injected as above. These mice were sacrificed, and the livers were removed at 0 hr, 24 hr, 48 hr and 1 week after BMC transplantation (Fig. 1B).

### ***Laboratory analysis:***

The mice were anesthetized with pentobarbital (Somnopenyl; Kyoritu Seiyaku Corp., Tokyo, Japan) and their blood was collected from the heart and centrifuged at 1500G for 15 min at 4°C. Albumin, alanine aminotransferase (ALT) and aspartate amino transferase (AST) levels were analyzed with a Hitachi 9000 series automatic analyzer.

***Histological examination:***

Liver samples were fixed in a 10% neutral-buffered formaldehyde solution, embedded in paraffin and sectioned. The 3- $\mu$ m-thick sections were stained with hematoxylin and eosin (HE). The relative necrotic area, expressed as a percentage of the total liver area, was determined in the HE-stained liver sections. Each field was acquired as 5 nonoverlapping random fields ( $\times 200$  magnification) and analyzed using Image-J software version 1.41 (<http://rsb.info.nih.gov/ij>).

***Immunohistochemistry:***

Tissue sections were subjected to immunohistochemistry using rat monoclonal antibody to the Ki-67 antigen (1:200; Dako, Tokyo, Japan), which detects proliferating cells. Microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 10–20 min. The sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min. For the primary antibody reaction, slides were incubated in a wet chamber overnight at 4°C. The immunoreactive materials were visualized using a peroxidase staining kit (Histofine Simple Stain MAX PO; Nichirei, Tokyo, Japan) and diaminobenzidine (Histofine Simple Stain DAB; Nichirei). The sections were counterstained with hematoxylin. The number of cells and the number of Ki-67-positive cells per field in each mouse liver were measured at  $\times 400$  magnification, and the ratio of Ki-67-positive cells was calculated.

### ***Double fluorescence analysis:***

For fluorescence analysis, freshly isolated lungs, spleens and livers were embedded in optimal cutting temperature medium and frozen. Frozen sections (5 µm) were obtained using a cryostat (model HM505; Microm) equipped with a tungsten carbide knife. After fixation with 3.7% paraformaldehyde, polymerized actin was stained with phalloidin (Alexa Fluor 488 phalloidin, Invitrogen, Carlsbad, CA, USA). Vectashield mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA) was used for nuclear-staining. The lung and spleen sections were nuclear stained with DAPI alone. Images were observed and captured by fluorescence microscopy (FSX100; Olympus, Tokyo, Japan). Computer-assisted image analysis was performed using ImageJ software version 1.41.

### ***Canine Y chromosome (SRY)-specific PCR:***

Genomic DNA was isolated from NOG mouse liver tissue homogenates using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The presence or absence of the sex-determination region of the male Y chromosome (SRY gene) in the recipient female NOG mice was assessed by PCR as previously described [Tateishi-Yuyama *et al.*, 2002]. Primer sequences for the SRY gene were obtained from published sequences (forward primer, 5'-CAAGATGGCTCTAGAGAATCCC-3'; reverse primer, 5'-AGCTGTCCGTGTAGG TGA-3'), which amplified a 284-bp product. The PCR conditions were as follows: incubation at 94°C for 2 min; 40 cycles of incubation at 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. PCR products were separated using 2% agarose gel electrophoresis and stained with ethidium bromide. Positive (male canine genomic DNA) and negative (female canine genomic DNA) controls were included in each assay.

### ***Statistical analysis:***

Data are expressed as means  $\pm$  standard error of the mean (SEM). The Mann–Whitney U test was used to compare the different groups as appropriate. P values of  $<0.05$  were considered statistically significant. Analyses were performed using GraphPad Prism 5.

## Results

### *Characterization of CCl<sub>4</sub>-induced acute liver dysfunction in NOG mice:*

The degree of liver injury was assessed by measuring serum liver enzymes (Fig. 2A). Serum albumin levels were significantly decreased at 48 hr after CCl<sub>4</sub> injection, after which the levels improved to within the normal range in both NOG and WT mice. Both serum ALT and AST increased markedly 24 hr after CCl<sub>4</sub> injection; however, they decreased to within the normal range 48 hr after CCl<sub>4</sub> injection, with no differences between the NOG and WT mice. To evaluate liver damage and regeneration, the necrotic areas of the liver were measured in NOG and WT mice injected with CCl<sub>4</sub>. There were no significant differences in the extent of liver necrosis between the NOG and WT mice (Fig 2B). Immunohistochemistry using Ki-67, a marker for cell proliferation, was performed to assess hepatocyte regeneration (Fig. 2C). Ki-67-positive hepatocytes were limited 24 hr after CCl<sub>4</sub> injection; however, there were many positive cells 48 hr after CCl<sub>4</sub> injection, with no differences between the NOG and WT mice.

### *Migration of PKH26-positive cBMCs into injured liver lobules:*

PKH26-positive cBMCs ( $1 \times 10^6$ ) were injected into liver-damaged and control (undamaged) female NOG mice. Infused cBMCs could be visualized by fluorescence microscopy a week after transplantation (Fig. 3). A few PKH26-positive cells were detected in sections of the liver from undamaged controls (Fig. 3A), and there were no PKH26-positive cells in CCl<sub>4</sub>-damaged mice without cBMC transplantation. In contrast, in the transplanted CCl<sub>4</sub>-treated mice, the number of PKH26-positive cells gradually increased, spreading into the liver lobules (Figs. 3A, B). Some PKH26-positive cells were observed in the lung tissue of both liver-damaged and control mice (Figs. 3A, B), while no PKH26-positive cells were detected in the spleen tissue from any of the groups (Fig. 3A).



***Detection of the SRY gene in transplanted NOG mice:***

PCR amplification of the canine SRY gene, located on the Y chromosome, demonstrated that transplanted cBMCs were present in the livers of the transplanted female mice, indicating that male cBMCs accumulated in the liver tissue of the female mice (Fig. 3C).

***Localization of infused cBMCs in the injured Liver:***

Phalloidin staining of frozen liver sections was performed to further elucidate the localization of cBMCs in the mouse liver. The actin cytoskeleton was disordered in necrotic tissue, and PKH26-positive cells were slightly accumulated in the necrotic areas 24 and 48 hr after transplantation (Fig. 4A). One week after transplantation, the number of accumulated cBMCs (PKH26-positive cells) gradually increased and spread into the liver lobules (Figs. 4A, B).

***Effects of cBMC transplantation on CCl<sub>4</sub>-induced acute liver dysfunction in the NOG mice:***

To clarify the therapeutic efficacy of cBMC transplantation in an acute liver hepatitis model, the degrees of hepatic injury and regeneration were compared between the cBMC-transplantation and saline (control) groups (Fig. 5). As parameters of hepatic injury, serum albumin and hepatic enzyme levels (Fig. 5A) and necrotic areas (Fig. 5B) were measured. As a measure of hepatocyte regeneration, the Ki-67-positive ratios were measured in each group (Fig. 4C). However, none of these parameters were significantly different between the control and transplantation groups ( $P = 0.872$ ).

## Discussion

In the present study, the infused cBMCs not only accumulated in the injured mouse liver at an early stage after transplantation but also accumulated specifically in injured tissue. Although several investigators have reported the ability of BMCs to engraft in experimental models, including rodent models [Brulport *et al.*, 2007; Doshi *et al.*, 2006; Meyerrose *et al.*, 2007], to our knowledge, this is the first study to investigate whether canine BMCs can accumulate in the injured liver tissue of an immunodeficient mouse model.

Transplantation of cBMCs into recipient mice was successful, as indicated by the detection of both PKH26-labeled cBMCs and the canine SRY gene in the liver tissue of female NOG mice. In particular, infused cBMCs were located in the necrotic area of the liver at an early stage after transplantation, with the largest accumulation at 1 week after transplantation. CCl<sub>4</sub>-induced acute liver injury in NOG mice peaked at 24 hr, and most liver regeneration occurred at 48 hr after CCl<sub>4</sub> injection. However, the time course of cBMC migration to the injured liver tissue did not correspond to the time course of liver injury or regeneration because most cBMC accumulation occurred later 48 hr after transplantation. Previously, Thomas *et al.* demonstrated that macrophage therapy was effective for improving liver fibrosis in a murine hepatic fibrosis model. Their migration of infused macrophages was detected in liver tissue from 1 to 7 days after transplantation, but the number of cells was not increased [Thomas *et al.*, 2011]. Chronic hepatitis and developed hepatic fibrosis models were used for their study, which they were established by a long-term protocol of CCl<sub>4</sub> injection on two consecutive days per week for at least 6 weeks. In the present study, NOG mice were singly injected with CCl<sub>4</sub> and used as an acute hepatitis model, and so its pathogenesis might be the cause of the different time course of cell accumulation in chronic hepatic injury. Therefore, we suggested that transplanted cell accumulation in the injured liver might be influenced by the difference in the course of hepatic injury, or transplanted cell fraction.

Several immunodeficiency models such as NOG mice have been developed for heterograft experimentation [Bosma *et al.*, 1983]. NOG mice can accept heterologous cells much more readily than any other immunodeficient rodent model [Hasegawa *et al.*, 2011; Iwamoto *et al.*, 2012]. Although the immune system plays an important role in liver regeneration [Diehl *et al.*, 2000; Racanelli *et al.*, 2006], it is not known whether NOG mice are an appropriate model for spontaneous acute hepatitis. In the present study, we further confirmed liver regeneration by comparing CCl<sub>4</sub>-induced hepatitis and liver regeneration between NOG and WT mouse models of acute hepatitis, which suggested that immune systems may not influence CCl<sub>4</sub>-hepatocyte injury and regeneration. Thus, NOG mice can be used as an acute liver injury model similar to WT mice. Both serum hepatic enzyme levels and necrotic areas showed maximum increases 24 hr after CCl<sub>4</sub> injection, and Ki-67-positive hepatocytes appeared 48 hr after CCl<sub>4</sub> injection (Fig. 2). These findings were consistent with those of a previous study [Recknagel *et al.*, 1967]. It is well known that the hepatocyte growth factor (HGF) is the most important factor for hepatic regeneration [Strain *et al.*, 1991]. We expect that liver regeneration is not influenced by the immunodeficient states of NOG mice because HGF may be secreted normally in these mice.

Infused cBMCs also accumulated in the liver and lung but not in the spleen. Thomas *et al.* and Cho *et al.* have demonstrated the efficient trafficking of rodent BMSCs to various target organs following transplantation, with a major portion of the input cells retained in the lung [Thomas *et al.*, 2011; Cho *et al.*, 2011]. In the green fluorescent protein (GFP)/CCl<sub>4</sub> mouse model, Iwamoto *et al.* demonstrated that splenectomy enhanced the migration of GFP-positive BMCs to the damaged liver [Iwamoto *et al.*, 2012]. With the exception of migration to the lung, our findings agree with these data, although it is difficult to precisely evaluate the accumulation in the spleen in NOG mice because they have natural splenic atrophy.

In this study, cBMC transplantation did not improve hepatic function and regeneration.

Serum ALT increased extremely (approximately 15000IU/L) 24 hr after CCl<sub>4</sub> injection in both the saline and cBMC groups. In this study, the hepatic necrotic area 24 hr after CCl<sub>4</sub> injection accounted 50% of the liver, indicating that change was the greatest value in this acute hepatitis model. We suggested that CCl<sub>4</sub>-induced acute liver injury may have been so severe that it became impossible to evaluate the therapeutic effects of cBMC transplantation. It might be necessary to evaluate other regenerative factor such as serum HGF and IL-6. In the hepatic fibrosis model, some studies indicated that bone marrow-derived cells could decrease collagen fibers and reduce hepatic fibrosis through expression of matrix metalloproteinases (MMPs) [Cho *et al.*, 2011; Haraguchi *et al.*, 2012; Haraguchi *et al.*, 2012]. Haraguchi et al. reported that transplanted cBMSCs migrated into liver regions exhibiting severe inflammation and fibrosis, and fibrosis was significantly reduced in CCl<sub>4</sub>/CSA mice transplanted with canine bone marrow stromal cells (cBMSCs) [Haraguchi *et al.*, 2012]. Therefore, the therapeutic effects of cBMC transplantation should be evaluated in clinical canine cases or in a hepatic fibrosis model.

We demonstrated that cBMCs accumulated after xenotransplantation in NOG mice with chemically induced liver injury. These results suggest that cBMCs may have potential in relation to liver regeneration, although the precise mechanisms for regulating cBMC stimulation in the damaged liver remain uncertain. Further study of these mechanisms is required to develop cell therapies that utilize cBMCs for repair of the damaged liver.

## **Chapter 2**

**Canine bone marrow cell xenotransplantation into NOD/Shi-scid  
*IL2Rg<sup>null</sup>* mice promotes carbon tetrachloride-induced hepatic fibrosis.**

## Abstract

Clinical studies on bone marrow-derived cell (BMC) transplantation for liver cirrhosis are under way, but the safety of this procedure has been unknown. NOD/Shi-scid *IL2Rg<sup>null</sup>* (NOG) mice lacking a lymphoid immune system are new model of severe immunodeficiency, and these mice can be used for xenogeneic transplantation experiments. Here we tested the influence of canine BMCs on NOG mice with carbon tetrachloride (CCl<sub>4</sub>)-induced hepatic fibrosis. Hepatic fibrosis was induced in NOG and wild-type (C57BL/6) mice by continuous injection of CCl<sub>4</sub> (1 ml/kg). After disease induction, we performed canine bone marrow cell (cBMC) xenotransplantation into CCl<sub>4</sub>-treated NOG mice and assessed the extent of hepatic fibrosis. Engrafted cBMCs were detected in the liver by fluorescent in situ hybridization (FISH). Picrosirius red staining and immunohistochemistry for alpha smooth muscle actin ( $\alpha$ -SMA), CD3, CD20 and type 1 collagen were performed to assess the extent of fibrosis and to analyse disease pathogenesis in xenotransplanted NOG mice. Western blots for  $\alpha$ -SMA,  $\beta$ -actin and TGF- $\beta$ 1 were also performed. At 4 weeks, hepatic fibrosis and hepatic stellate cell (HSC) activation was significantly lower in CCl<sub>4</sub>-treated NOG mice than in wild-type mice. As a result of cBMC xenotransplantation, more severe hepatic fibrosis was induced in CCl<sub>4</sub>-treated NOG mice compared with non-xenotransplanted and non-CCl<sub>4</sub>-treated NOG mice. This hepatic fibrosis was characterized by a number of CD3<sup>+</sup> and CD20<sup>+</sup> lymphocytes infiltrating around the portal vein,  $\alpha$ -SMA-positive activated HSCs and excess accumulation of type 1 collagen. In addition, the infiltrating lymphocytes were positive by FISH analysis, indicating that transplanted cells proliferated and affected the development of fibrosis. CCl<sub>4</sub>-induced hepatic fibrosis was suppressed in NOG mice due to severe immunodeficiency. cBMC xenotransplantation into NOG mice promoted CCl<sub>4</sub>-induced hepatic fibrosis. These results suggest that lymphocytes included in BMCs may adversely affect liver fibrosis in cases of BMC therapy.

## Introduction

Bone marrow-derived cell (BMC) populations influence the progression and recovery phases of liver fibrosis [Sakaida *et al.*, 2004; Russo *et al.*, 2006; Higashiyama *et al.*, 2007; Cho *et al.*, 2011]. Clinical studies on BMC therapy for liver cirrhosis are under way, but the therapeutic mechanisms remain undefined [Terai *et al.*, 2006; Lyra *et al.*, 2007; Kim *et al.*, 2010; Iwamoto *et al.*, 2012.]. Identification of defined cell types with beneficial effects will enable a more rational and predictable course of therapy to be administered. Numerous studies have suggested that BMC therapy for liver cirrhosis will have minimal side effects. However, use of mixed cell populations has limited our understanding of the action mechanisms. Researchers who do not agree with the plasticity of BMCs insist that little evidence exists for the use of BM-derived hepatocytes in the replacement of injured liver tissue [Kanazawa *et al.*, 2003; Nakamura *et al.*, 2012] and argue that no data has demonstrated transdifferentiation of BMCs into hepatocytes or a major role in regeneration [Wagers *et al.*, 2002; Wagers *et al.*, 2004; Menthenas *et al.*, 2004]. In contrast, BMCs have been reported to be a possible source of functional hepatic stellate cells and myofibroblasts, contributing to fibrosis [Russo *et al.*, 2006]. However, safety of BMC therapy for liver cirrhosis has not been discussed in the report.

Establishment of an immunodeficient model is necessary to evaluate cell transplantation therapies for liver cirrhosis in humans and animals [16-18]. NOD/Shi-scid IL2Rg<sup>null</sup> (NOG) mice are a strain among the new generation of severe immunodeficient mice. NOG mice can accept heterologous cells much more easily than any other type of immunodeficient rodent model [Ito *et al.*, 2007]. Thus, the NOG mouse demonstrates markedly better engraftment of human cells, enabling engraftment of human cancer cells, hepatocytes and other cell types at high rates [Watanabe, *et al.*, 2007; Suemizu *et al.*, 2008; Watanabe *et al.*, 2009; Hasegawa *et al.*, 2011]. This excellent acceptability results from the absence of functional T and B lymphocytes due to Rag-deficiency and the absence of NK cells due to IL2Rg-deficiency.

Hepatic fibrosis is caused by an excessive deposition of extracellular matrix (ECM), which leads to the loss of organ structure and impairment of organ function [Gutiérrez-Ruiz *et al.*, 2007]. Liver cirrhosis caused by progressive fibrosis occurs due to a variety of reasons, including viral hepatitis, metabolic or autoimmune diseases, toxic injury, congenital abnormalities and chemical agents. Carbon tetrachloride (CCl<sub>4</sub>) is one of the most useful chemical agents for medical research and can be used to induce liver fibrosis in rodent models [Weber *et al.*, 2003].

Here we continuously treated NOG mice with CCl<sub>4</sub> to compare the extent of hepatic fibrosis with wild-type (C57BL/6) mice and to determine the influence of canine bone marrow cell (cBMC) xenotransplantation on CCl<sub>4</sub>-induced hepatic fibrosis in NOG mice.



## **Materials and Methods**

### ***Animals***

Male 6-week-old NOG mice were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). Age-matched C57BL/6J Jcl mice were purchased from CLEA Japan, Inc. (Kanagawa, Japan) to serve as wild-type (WT) murine controls. All mice were shipped to the Research Institute of Biosciences at Azabu University and were handled with care under pathogen-free conditions. Mice were housed in a room under controlled temperature (25°C), humidity and lighting (12 h light/dark cycle). Access to food and tap water was ad libitum throughout the study period. All male NOG and C57BL/6 mice were used for cBMC transplantation experiments. A clinically healthy 2-year-old male beagle dog was used as a cBMC donor for transplantation into NOG mice. All experiments in this study were performed in accordance with the Animal Protection Guidelines of Azabu University (authorization No. 10-79).

### ***Induction of hepatic fibrosis***

A total of 24 male NOG mice and male C57BL/6 (WT) mice were randomly divided into three groups: NOG/CCl<sub>4</sub>, NOG/Oil and WT/CCl<sub>4</sub> (each group, n = 12). The mice were intraperitoneally injected with CCl<sub>4</sub> (1 ml/kg, diluted 1:10 in olive oil, Wako, Osaka, Japan) weekly twice for 4 weeks. CCl<sub>4</sub> administration induces necroinflammatory liver injury that reproducibly causes hepatic fibrosis. Mice were sacrificed at 0, 2 and 4 weeks. NOG mice that were continuously treated for 10 weeks with CCl<sub>4</sub> received cBMCs ( $1 \times 10^6$  cells in 200  $\mu$ l saline) via the tail vein midway through the CCl<sub>4</sub> treatment. Control groups consisted of cBMCs, saline and oil/cBMCs. Each cohort consisted of five mice. Animals were sacrificed at 2 weeks after commencing the experiment, and blood and liver tissue were collected. Spleen, heart, lungs and kidney were also collected from mice that received cBMC infusion.

### ***Preparation of donor BMCs and transplantation***

Bone marrow was harvested from humeri and femora of a male dog under general anaesthesia and was collected in a syringe containing heparin. After fat was removed, BMCs were extracted and a single-cell suspension was prepared by passing the cells through a 100- $\mu$ m mesh filter (BD Falcon, Tokyo, Japan) and into a new tube. Mononuclear cells (MNCs) were isolated with Ficoll solution (Lymphoprep, Axis-Shield, Oslo, Norway), washed and then concentrated three times. At 1.5–2 h after BM harvest, NOG mice were administered with a preparation of  $1 \times 10^6$  MNCs via the tail vein.

### ***Histology and Immunohistochemistry***

For histopathological analysis, liver and spleen samples were quickly obtained and fixed in a 10% neutral buffered formaldehyde solution, embedded in paraffin and sectioned. The 4- $\mu$ m thick sections were stained with hematoxylin and eosin (HE). For hepatic fibrosis evaluation, picrosirius red staining was performed using 0.1% picrosirius red solution. The picrosirius red-stained area, considered to be the fibrotic area, was assessed by image analysis using the Image J software, version 1.41. Mean value of three randomly selected areas per sample was used as the expressed percentage area of fibrosis. For immunohistochemistry, a monoclonal antibody against  $\alpha$ -SMA (1:100, Dako, Kyoto, Japan) and polyclonal antibodies against CD3 (1:100, Dako), CD20 (1:100, Dako) and type 1 collagen (1:200, Abcam, Cambridge, UK) were used. Microwave antigen retrieval was performed in pH 6.0 citrate buffer for 10–20 min. For antigen retrieval of CD3 samples, tissue sections were digested with pepsin for 20 min at 37°C and washed in phosphate-buffered saline (PBS). The sections were sequentially treated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min. For the primary antibody reaction, slides were incubated in a wet chamber overnight at 4°C. Immunoreactive materials were then visualized using a

peroxidase staining kit (Histofine simple stain MAX-PO kit, Nichirei Bioscience, Tokyo, Japan) and diaminobenzidine (Histofine simple stain DAB, Nichirei). Finally, sections were counterstained with hematoxylin. Five non-overlapping random fields (200× magnification) per slide were visualized and then analysed using Image J software, version 1.41.

### ***Western blotting***

Liver samples (approximately 20 mg) were homogenized in 1 ml of 1% SDS buffer (Bio Rad, CA, USA) and subjected to centrifugation in a mini-centrifuge. The supernatant represented the whole protein. A total of 10 µg of protein was separated by 10.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto a PVDF membrane (ATTO, Tokyo, Japan). The membrane was treated with 0.1% Tween-20 (Wako, Osaka, Japan) and 5% skim milk (Wako) in PBS for 1 h. Membranes were incubated overnight at 4°C with a monoclonal antibody against  $\alpha$ -SMA (1:1,000, Dako) and polyclonal antibodies against  $\beta$ -actin (1:1000, Santa Cruz) and TGF- $\beta$ 1 (1:500, Santa Cruz). After washing, the membranes were incubated with rabbit anti-mouse and goat anti-rabbit IgG-HRP-conjugated secondary antibodies (1: 1,000, Dako) in wash buffer containing 5% skim milk for 1 h. Reactive bands were identified using enhanced chemiluminescence (Luminata, Millipore, Billerica, MA, USA), and bands were captured with LAS4000 mini (GE healthcare, NJ, USA).

### ***Fluorescent in situ hybridization (FISH)***

Samples for FISH were paraffin-embedded sections of liver and spleen, which were also used for histological analysis. The sections were heated for 20 min at 90°C in citrate buffer, pH 6.0. After washing in 2× SSC, the tissue sections were digested with 0.4% pepsin for 30 min at 37°C and washed in PBS. Sections were then dehydrated in an ethanol series, and a canine-specific FISH probe labelled with fluorescein isothiocyanate (FITC) (Chromosome Science Laboratory, Hokkaido, Japan) was applied. After plating the slides on an 80° C hot

plate, sections were incubated overnight at 40° C. After washing in 50% formamide/2× SSC at 37° C for 20 min, slides were incubated in 1× SSC for 15 min. Finally, Vectashield mounting medium was used that included 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc.) for nuclear staining.

### *Statistical Analysis*

Data are presented as the mean ± standard error of the mean (SEM). A Mann–Witney U-test was used to compare the different groups as appropriate. P value < 0.05 was considered to represent a statistically significant difference. All data were analysed using GraphPad Prism 5.

## Results

The degree of liver injury was assessed by measuring serum liver enzymes (Fig. 6). In the NOG/CCl<sub>4</sub> and WT/CCl<sub>4</sub> groups, serum ALT and AST levels increased significantly compared with NOG/Oil group. Serum albumin levels did not change during the course of CCl<sub>4</sub> administration in any group.

Sirius red staining was performed to assess the extent of hepatic fibrosis at each time point, and this data indicated that CCl<sub>4</sub>-induced hepatic fibrosis in NOG mice did not develop to a high degree (Fig. 7a–c). At each time point the NOG/Oil group demonstrated a normal distribution of collagen, whereas extensive collagen deposition area evident in the liver tissue from the continuous CCl<sub>4</sub>-treated groups. The level of hepatic fibrosis in the NOG/CCl<sub>4</sub> group was lower than that in the WT/CCl<sub>4</sub> group at 4 weeks. In the liver of the NOG/Oil group,  $\alpha$ -SMA immunopositive cells were restricted to the smooth musculature of the arterial tunica media, whereas liver cells area observed to be negative. Continuous CCl<sub>4</sub> administration induced perisinuoidal  $\alpha$ -SMA expression, which was recognized as activation of hepatic stellate cells (aHSCs) in WT mice (Fig. 7e). However, at 4 weeks in the NOG/CCl<sub>4</sub> group, only a small population of  $\alpha$ -SMA-positive cells was observed in the fibrotic area (Fig. 7d). We then investigated the expression levels of  $\alpha$ -SMA and TGF- $\beta$ , since TGF- $\beta$  is a key cytokine to develop hepatic fibrosis. In comparison with the WT/CCl<sub>4</sub> group, the expression levels of these proteins were decreased in the NOG/CCl<sub>4</sub> group (Fig. 7g).

In NOG mice administered with cBMC infusion, the gross finding of the liver appeared to be fragile and discoloured. To investigate the influence of cBMC transplantation into NOG mice continuously treated with CCl<sub>4</sub>, a histological examination was performed (Fig. 8). Ten weeks after CCl<sub>4</sub> treatment, a number of inflammatory cells were presented around portal veins of the liver tissue in CCl<sub>4</sub>/cBMC group, whereas mononuclear infiltration was not seen in both of CCl<sub>4</sub>/saline and oil/cBMCs groups (Fig. 8a–c). Most of inflammatory cells were defined as T or

B lymphocytes, since they were positive for CD3 or CD20, respectively (Fig. 9b, 9d). In the CCl<sub>4</sub>/cBMC group, fibrotic facilitation was seen throughout the liver, marked hepatic fibrosis was developed around the portal in sirius red stained sections, and the fibrotic areas were expanded significantly compared with the saline/cBMC and oil/cBMC groups (Fig. 8d–f). Furthermore, semi-quantitative analysis indicated that the extent of fibrotic areas increased in the CCl<sub>4</sub>/cBMC group (positive area: saline/cBMC, 0.69%; oil/cBMC, 3.1%; CCl<sub>4</sub>/cBMC, 12.1%). Immunohistochemical analyses of type 1 collagen and  $\alpha$ -SMA were carried out to confirm the impact of cBMC transplantation and investigate the pathogenesis of this hepatic fibrosis, (Fig. 8h–m). In the liver of CCl<sub>4</sub>/cBMC groups, high collagen-1 expression was seen, and  $\alpha$ -SMA-positive cells also were increased more than CCl<sub>4</sub>/saline and oil/cBMC group. Indeed, there were no significant differences between CCl<sub>4</sub>/saline and oil/cBMC group. The protein expression levels of  $\alpha$ -SMA and TGF- $\beta$  were assessed by western blot analyses to investigate the process of fibrosis. These results indicated that the expressed of both  $\alpha$ -SMA and TGF- $\beta$  protein were enhanced in CCl<sub>4</sub>/cBMC group compared with CCl<sub>4</sub>/saline and oil/cBMC groups. (Fig. 8o).

The spleen was enlarged in mice from the CCl<sub>4</sub>/cBMC and oil/cBMC group, even though the spleen had atrophied due to lymphocyte deficiency (Fig. 9a, 4b). Hematopoietic cells, such as erythroblasts and megakaryocytes, were histologically observed in NOG mice infused with saline (Fig. 9d). In CCl<sub>4</sub>/cBMC group, numerous mononuclear cells including CD3<sup>+</sup> and CD20<sup>+</sup> cells emerged and accumulated in the spleen (Fig. 9c, 9e). Furthermore, FISH using canine-specific FITC-labelled probes was applied to detect the transplanted canine cells in NOG mouse tissues, and it indicated that canine-derived cells stained FITC were accumulated in the liver and spleen of CCl<sub>4</sub>/cBMC mice. (Fig. 10).

## Discussion

The safety of BMC therapy for liver cirrhosis has been still unknown. In the present study, cBMCs xenotransplantation into hepatic fibrosis model of NOG mice induced by CCl<sub>4</sub> was carried out to reveal the influence of cBMCs. From the results of cBMC xenotransplantation into NOG mice, severe hepatic fibrosis was developed by CCl<sub>4</sub> treating (CCl<sub>4</sub>/cBMC group), and the characteristic of this hepatic fibrosis is intense lymphocyte infiltration including migrated canine T-cell and B-cell around portal vein. BMCs contain various hematopoietic cells, and bone marrow is also a key component of the lymphoid system, producing the lymphocytes that support the immune system [Zhang *et al.*, 2009; Akiyama *et al.*, 2012]. These results suggested that lymphocytes infiltration is important process for hepatic fibrosis progression, because various immune systems contribute to develop the hepatic fibrosis. In canine chronic hepatitis, hepatic T lymphocytes may play a central role in disease pathogenesis [Boisclair *et al.* 2001]. The CD3<sup>+</sup> lymphocyte are notably the most dominant lymphoid cell type in the liver of dogs with chronic hepatitis [Sakai *et al.* 2006]. However, we did not isolate lymphocyte populations, so the influence of this cell population was not evaluated.

Concerning BMC therapy for liver disease, BMCs have been an attractive cell source in regenerative medicine, because obtaining BMC is easier than isolating other tissue-specific stem cells. However, the results of recent studies have been controversial such that some studies found that BMC rarely transdifferentiated, whereas others documented high rates of transdifferentiation in cell therapy settings [Wagers *et al.*, 2002; Wagers *et al.*, 2004; Menthenia *et al.*, 2004]. Successful transdifferentiation of infused stem cells involves various cellular and recipient factors, and these factors interact in a complex manner. It is therefore important to identify the effective cell lineage and to exclude adverse cell lineages from BMCs to improve therapies for hepatic fibrosis in the future. It has been documented that infused BMCs secrete

matrix metalloproteases and have an important role in the improvement of hepatic fibrosis [Higashiyama *et al.*, 2007; Hardjo *et al.*, 2009; Cho *et al.*, 2011; Iwamoto *et al.*, 2012]. Thomas *et al.* reported that cells of the monocyte–macrophage lineage have key roles in the development and resolution of the hepatic fibrosis [Thomas *et al.*, 2011]. However, concerning cell therapy using BMC, past studies did not discuss the roles of donor BMC-derived lymphocytes. The results of our study suggest that donor BMC-derived lymphocytes possibly affect liver fibrosis adversely. Therefore, a BMC fraction that excludes lymphocytes may be more beneficial for treating liver cirrhosis.

To date, a study on hepatic fibrosis using NOG mice has not been reported. In particular, various immune systems contribute to the progression of hepatic fibrosis [Friedman *et al.*, 2000; Svegliati-Baroni *et al.*, 2008], and it was unknown whether NOG mice could develop CCl<sub>4</sub>-induced fibrosis. The CCl<sub>4</sub> metabolite directly impairs hepatocytes by altering the permeability of the plasma, lysosomal and mitochondrial membranes [Recknagel *et al.*, 1967; Raucy *et al.*, 1993]. The mechanism of CCl<sub>4</sub>-induced hepatotoxicity, besides direct cytotoxicity, is considered to result from inflammatory cell infiltration and the activation of HSCs. The aHSC are key mediators of liver fibrosis, because of their transformation to myofibroblasts to secrete collagen by TGF- $\beta$  [Gressner *et al.*, 2002; Seki *et al.*, 2007]. Thus, the lack of an immune system may prevent the development of fibrosis.

In this study, we showed that hepatic fibrosis could be induced in NOG mice by injection of CCl<sub>4</sub>; however, the fibrosis level was lower than that in WT mice. There was little correlation between aHSCs in NOG mice and hepatic fibrosis, although numerous  $\alpha$ -SMA-positive aHSCs appeared in areas corresponding to the fibrotic area in WT mice. Hyon *et al.* previously reported that mice developed hepatic fibrosis in NOD/SCID mice treated with dimethylnitrosamine [Hyon *et al.*, 2011]. Although the toxic substance, dose interval and period were different in our study, the  $\alpha$ -SMA expression ratio in the liver of NOG mice appeared to be lower than that in



NOD/SCID mice. Experimental evidence suggests that TGF- $\beta$  greatly contributes to the profibrogenic role of HSC [Gressner *et al.*, 2002; Seki *et al.*, 2007]. TGF- $\beta$  is the most potent fibrogenic cytokine known to stimulate HSCs, and it is secreted by macrophages, T cells and aHSCs. In our study, TGF- $\beta$  protein expression in the liver did not increase in CCl<sub>4</sub>-treated NOG mice, although it did increase in WT mice. These results suggested that CCl<sub>4</sub>-induced hepatic fibrosis is suppressed in NOG mice due to lymphocyte defection.

In this study, we described fibrotic changes involved in the appearance of a number of  $\alpha$ -SMA-positive aHSCs and the excessive deposition of ECM, including type 1 collagen. This hepatic fibrosis observed in the CCl<sub>4</sub>/cBMC group was more severe than that in the CCl<sub>4</sub> or cBMC-only groups. These results suggest that transplanted cBMCs engraft into NOG mice and continuous CCl<sub>4</sub>-induced liver injury stimulate the infiltration of lymphocytes, including cBMCs. Subsequently, infiltrating canine lymphocytes secreted various proinflammatory cytokines such as TGF- $\beta$  and stimulated the HSCs. Thus, HSC activation might have resulted in severe hepatic fibrosis. The pathophysiology in this model of hepatic fibrosis might be a result of graft-versus-host disease (GVHD). After intravenous transplantation of human BMCs (hBMCs), it has been known that NOG mice showed an early onset of GVHD symptoms compared with BALB/cA-*Rag2*<sup>null</sup> *IL2 $\gamma$* <sup>null</sup> or NOD/SCID mice [Ito *et al.*, 2009]. Moreover, remarkably abundant invading human CD45<sup>+</sup> cells were observed around the veins in the liver [Ito *et al.*, 2009]. However, severe hepatic fibrosis was not shown in that report, in contrast with our results.

In conclusion, cBMC xenotransplantation into NOG mice promotes CCl<sub>4</sub>-induced hepatic fibrosis. Our results suggest that lymphocytes in BMCs may be associated with adverse effects on liver fibrosis, which should be considered when designing BMC therapy regimens for clinical use.

## **Chapter 3**

### **Autologous bone marrow cell transplantation in dogs**

## **Abstract**

Clinical trials of autologous bone marrow cell (BMC) therapy for liver cirrhosis have been performed in humans; however, the feasibility of autologous BMC therapy in dogs has not been assessed, and there is no study on BMC therapy for canine liver disease. We evaluated the influence of autologous BMC therapy in 4 clinically healthy dogs and two dogs with spontaneous chronic hepatitis. Mononuclear cell (MNC) preparation was administered via the peripheral vein, and characteristics of MNCs were analyzed by flow cytometry. After autologous BMC infusion, blood samples from each dog were evaluated by haematology and serum biochemistry. The viability of MNCs was more than 80 % in each dog, and no abnormal changes were observed in haematological and biochemical parameters after autologous BMC infusion in clinically healthy dogs during the study period. Two clinical cases with chronic hepatitis underwent autologous BMC therapy. MNCs included 1.26% and 1.01% of the CD45+ and CD34+ cell population, respectively. After autologous BMC therapy, serum albumin levels remained low in both dogs. Other serum parameters did not improve during the study period in both dogs. One dog died 45 days after autologous BMC therapy. These results suggested that BMC therapy was not effective in improving hepatic function. Although the feasibility of autologous BMC infusion was shown in clinically healthy dogs and dogs with spontaneous chronic hepatitis, further large-scale studies would be required to assess the efficiency and to develop a transplantation protocol in future.

## **Introduction**

Canine hepatitis, hepatic portal systemic shunts, hepatic fibrosis and hepatic failure are commonly observed in veterinary practice [Allen *et al.* 1999; Rutgers *et al.* 1993; Watson 2004]. Although liver tissue has regenerative ability, this ability is decreased in some severe diseases. Therefore, liver transplantation is often the only therapeutic option for dogs with severe hepatic failure. However, transplantation has many problems, including a lack of donors, the stress of surgery and tissue rejection. In particular, a lack of knowledge regarding canine leukocyte antigens can prevent successful liver transplantation. Moreover, alternatives to liver transplantation have not yet been established. Thus, treatments for canine liver disease are limited, and an effective therapeutic regimen is required.

Clinical trials of autologous bone marrow cell (BMC) therapy for liver cirrhosis in humans began in 2003, and a multicenter clinical trial was performed in 2005 [Terai *et al.* 2006]. In these trials, the patients who were treated with BMC therapy with decompensated hepatitis B virus (HBV)-related liver cirrhosis and elevated serum albumin (Alb) levels showed improvement of Child–Pugh scores. The efficiency of BMC therapy was attributed to the activation of hepatic progenitor cells [Kim *et al.* 2010]. Furthermore, Saito *et al.* (2011) reported the efficacy and safety of autologous BMC therapy in cases of alcoholic liver cirrhosis. Terai *et al.* (2006) evaluated the safety of cell therapy using autologous BMCs for liver diseases in 9 patients with terminal liver cirrhosis [Terai *et al.* 2006]. Subsequently, Kim *et al.* (2010) investigated the same in 10 patients with HBV liver cirrhosis. Lyra *et al.* (2007) advocated the safety of ABMi in 10 patients with alcoholic, HCV, cholestatic and cryptogenic liver cirrhosis [Lyra *et al.* 2007]. However, the feasibility and safety of ABMi has not been assessed in animals. Moreover, there is no evidence regarding ABMi treatment for canine liver diseases. In the present study, we performed autologous bone marrow transplantation in 4 clinically healthy dogs and 2 dogs with chronic liver disease.

## **Materials and Methods**

### ***Dogs***

We evaluated the safety of BMC therapy in 4 clinically healthy dogs (Beagle, 2 males, 2 females) with a mean age of 2.1 years (range, 1–6 years) and a mean body weight of 9.8 kg (8.0–11.0 kg). The experiments in this study were performed in accordance with the Animal Protection Guidelines of Azabu University (authorisation no. 10–79).

### ***BMC preparation and transplantation: clinically healthy dogs***

Dogs were fasted for 8 h before ABMi. Anaesthesia was induced through intravenous administration of medetomidine (20 µg/kg) and pentobarbital (10 mg/kg). Bone marrow (6 mL/kg) was then harvested from the humerus and femur using a heparin-coated syringe. After fat removal, BMCs were extracted and single-cell suspensions were prepared by passing BMCs through a 100-µm mesh filter (BD Falcon). Mononuclear cells (MNCs) were isolated using Ficoll solution (Lymphoprep: Axis-Shield, Oslo, Norway) and washed and concentrated 3 times. The supernatant was then removed, and the cells were re-suspended to prepare the BMC solution ( $5 \times 10^8$  cells/mL). The final concentrated and washed cell product was prepared to a final volume of 30 mL. Approximately 3 hrs after harvesting the bone marrow, the final MNC preparation was administered via the peripheral vein over a 20-min duration.

### ***Cases***

In addition to the 4 clinically healthy dogs, we examined 2 dogs with a clinical diagnosis of chronic hepatitis that was confirmed by histological examination. They had previously received general medical treatments that included hepatotonic, cholagogues and anti-fibrotic agents. The cases selected for autologous BMC therapy were based on the human criteria reported by Terai et al. (2006), which included chronic hepatitis with a total bilirubin level less than 7.0 mg/dL, a

platelet count greater than  $5 \times 10^4/\mu\text{L}$  and no viable malignant tumour. Dogs were excluded from the study if they showed evidence of disease or dysfunction in organs other than the liver.

#### ***Autologous BMC therapy for dogs with chronic hepatitis***

The general procedure for autologous BMC therapy for the dogs with chronic hepatitis was similar to that for the clinically healthy dogs. In brief, the bone marrow (6 mL/kg) was collected under general anaesthesia from the humerus, femur and ilium using a 10-mL heparin-coated syringe using standard procedures. After fat was removed, BMCs were extracted, and single-cell suspensions were prepared by passing the cells through a 100- $\mu\text{m}$  mesh filter (BD Falcon) into new tubes. BMCs were washed and concentrated 3 times. Finally, suspensions were prepared by passing the cells through a 100- $\mu\text{m}$  mesh filter. The final concentrated and washed cell product was prepared to a final volume of 100 mL. Approximately 3 h after harvesting the bone marrow, the final MNC preparation was administered via the peripheral vein over a 20-min duration.

#### ***Measurement of laboratory data***

For clinically healthy dogs, blood samples were obtained from the jugular vein at 1, 2, 7 and 14 days after BMC infusion. Complete blood count (CBC) analysis was performed using Celltac  $\alpha$  (Nihon koden, Tokyo, Japan). The remaining blood sample was centrifuged at 1500 g for 20 min to separate the serum. The blood levels for total protein (TP), Alb, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (T-Bil), creatinine (Cre) and urea nitrogen (BUN) were analysed using a Hitachi 9000 series automatic analyser (Hitachi, Tokyo, Japan). Values for the laboratory data are presented as the mean  $\pm$  standard error of the mean (SEM).

#### ***Flow cytometry***

The final cell product (5 mL) was subjected to a trypan blue dye exclusion test and fluorescence-activated cell sorting analysis. Analysis of cell surface expression of BMCs was performed with a flow cytometer (EC800, Sony, Tokyo, Japan) using anti-CD34 (R-PE-conjugated mouse anti-canine CD34, BD Pharmingen, Japan), anti-CD45 (FITC-conjugated mouse anti-canine CD45, AbD Serotec, UK) and anti-CD133 (PE-conjugated rat anti-mouse CD133, eBioscience, USA) monoclonal antibodies.

***Follow-up (laboratory data)***

After autologous BMC therapy for cases of chronic hepatitis, dogs were followed-up during 1 and 2 months, and laboratory data were monthly analyzed for 2 months. During the study period, medication was not changed before and after ABMi therapy. Primary outcomes were safety and feasibility of autologous BMC therapy. TP and Alb levels were measured as markers of nutrition, and serum ALT, ALP and T-Bil levels were assessed to determine the presence of hepatopathy.

## Results

We obtained  $8.9 \pm 1.2 \times 10^8$  MNCs from each of the 4 clinically healthy dogs and injected  $6.8 \pm 0.7 \times 10^8$  MNCs via the peripheral vein. Thus, MNC viability was more than 80% for these 4 dogs. Following autologous BMC infusion, all haematological and biochemical measures remained within normal ranges (Table 1). Thus, no adverse effects were observed in these 4 dogs following autologous BMC infusion.

The clinical information for the 2 dogs with chronic liver disease is presented in Table 2. Both dogs were followed-up 1 day after autologous BMC infusion. As shown in Figure. 11 and Table 3, we obtained  $9.2 \times 10^8$  (case 1) and  $6.8 \times 10^8$  (case 2) MNCs and injected  $8.2 \times 10^8$  (case 1) and  $6.8 \times 10^8$  (case 2) MNCs. Thus, MNC viability was greater than 90% for the 2 dogs with liver disease. In case 1, 1.26% of CD45+ and CD34+ cells were included in whole MNCs, indicating a haematopoietic stem cell. In case 2, 1.01% of CD45+ and CD34+ cells were included in whole MNCs. However, CD45- cells indicating a mesenchymal fraction in whole MNCs were rare.

### Case descriptions

Case 1 was a 10-year-old casted male American Cocker Spaniel that showed foreleg oedema during a physical examination. Severe ascites also were detected with subsequent ultrasonography. Liver biopsy was performed, which revealed micro-hepatica and diffuse nodular lesions (Fig. 12). Histologically, hepatocytes were markedly reduced and the surviving hepatocytes showed swelling and vacuolisation. In addition, numerous small bile ducts were increased in the stromal tissue; however, no lobular structures were observed. Therefore, based on the number of observed regenerative nodular, this case was diagnosed as liver cirrhosis. The dog was treated with branched-chain amino acid granules (Livact, 0.3 g/kg, PO, q 24 h,



Ajinomoto Co., Inc., Japan) to improve the nutritional condition. Spironolactone (Aldactone, 1 mg/kg, PO, q 12 h, Pfizer Co., Inc., Japan) was used to control the ascites. Porcine placental granules (Laennec, 10 mg/kg, PO, q 24 h, Japan Bio Products Co., Ltd.) were administered for liver regeneration but without subsequent improvement (Table. 2). The dog was evaluated at follow-up consultations 1 and 2 months after autologous BMC infusion at the Veterinary Teaching Hospital, Azabu University. Serum Alb levels remained low (1 month, 1.8 g/dL; 2 month, 2.1 g/dL), although serum ALT levels decreased, and serum ALP levels increased following autologous BMC infusion (Fig. 13). No adverse effects were observed during the 2 months that followed autologous BMC infusion.

Case 2 was an 8-year-old spayed female Miniature Schnauzer that showed severe jaundice on physical examination. Liver biopsy was performed, which revealed liver discolouration involving entire lobules (Fig. 12). Histologically, marked increases in small bile ducts and diffused fibrosis were observed (Fig. 12). No regenerative nodular lesions were seen, and the pathogenesis suggested chronic hepatitis. Liver protecting agents were administered, including S-adenosyl-methionine (Nutramax Pet Denosyl, 20 mg/kg, PO, q 24 h, Nutramax laboratories, Inc., USA). Ursodeoxycholic acid (Urso, 10 mg/kg, PO, q 24 h, Mitsubishi Tanabe Pharma Corp., Japan) was administered as a cholagogue, and porcine placental granules (Laennec, 10 mg/kg, PO, q 24 h) were administered (Table. 2). The dog was followed-up a month after autologous BMC infusion; however, the dog died 45 days after Autologous BMC infusion, and laboratory data could not be obtained. The owner reported no changes in the dog's general condition, and no changes were detected in physical examination. Serum TP, Alb and BUN levels remained low, whereas serum ALT and ALP levels that were not stable before treatment decreased after treatment. No change in serum T-Bil levels was observed (Figure. 13).

## Discussion

In the present study, improvement of hepatic function was not observed in the 2 clinical cases following autologous BMC therapy. Terai et al. (2006) infused autologous BMCs into 9 patients with decompensated liver cirrhosis, which resulted in significant improvement in serum TP and Alb levels, Child–Pugh scores and AFP and proliferating cell nuclear antigen expression 24 weeks after transplantation. Kim et al. (2010) reported that serum TP and Alb levels, Child–Pugh scores, liver volume and expression of hepatic progenitor cells (ductular reaction) and quality of life assessed by questionnaire improved in patients with liver cirrhosis following autologous BMC infusion therapy.

The present study has some limitations. First, because the clinical trials were performed in only 2 cases, we could not assess the effect of autologous BMC infusion therapy. Second, we evaluated the efficacy only by serum biochemistry, and we did not assess histological changes after autologous BMC infusion. In human medicine, there are many parameters for monitoring hepatic fibrosis [Giannini *et al.* 2001]. A number of non-invasive techniques, including serum biomarker assay and imaging techniques, have been developed [Castera 2011; Baranova 2011]. In the veterinary field, however, there are no useful biomarkers for liver fibrosis at present. Although Kanemoto et al. (2009) studied HA as a marker for canine hepatic fibrosis, it has not been widely used till date. Thus, further studies that establish new biomarkers for assessing liver functions for fibrosis are required.

A large number of MNCs were obtained from both the clinically healthy dogs and the clinical cases, and the viability of MNCs was greater than 80%. The results of flow cytometry indicated that a sufficient number of haematopoietic stem cells for transplantation were obtained from the dogs with chronic liver disease. We evaluated the CD34+ and CD133+ cell populations obtained from MNCs, which were detected as haematopoietic stem cells. Recently, a number of

studies have suggested that CD34+ or CD133+ haematopoietic stem cells may play an important role in improving hepatic function. Esch et al. (2005) reported that portal administration of autologous CD133+ BMCs accelerated liver regeneration, and it is a novel therapy for hepatic resection. Hosny et al. also attempted CD34+ autologous haematopoietic stem cell transplantation via the portal vein and hepatic artery in 48 patients with end-stage chronic liver disease, and concluded the treatment improved liver function [Samala *et al.* 2010]. Terai et al. (2006) obtained 2.39% of CD45+ and CD34+ cells from 9 human patients with liver cirrhosis. Samala et al. (2010) isolated CD34+ cells from peripheral blood from 48 patients with liver cirrhosis to activate granulocyte-colony stimulating factor (G-CSF). Compared with these findings in humans, we observed a smaller population of CD34+ haematopoietic stem cells in dogs. Thus, the amount of CD34+ cells may affect autologous BMC treatment of chronic liver diseases. Further studies are required to clarify this point in canine case.

In the present study, 1 dog (case 2) with chronic hepatitis died 45 days after autologous BMC infusion. However, no significant adverse effects of bone marrow isolation or autologous BMC infusion were observed. A post-mortem examination was not performed, and the cause of death was unclear. Severe complications due to autologous BMC infusion have not been reported in humans [Terai *et al.* 2006; Kim *et al.* 2010; Saito *et al.* 2011]. However, some studies have suggested that BMCs contributed to fibrotic changes in the kidney and liver [Baertschiger *et al.* 2009; Forbes *et al.* 2004; Russo *et al.* 2006; Li *et al.* 2007]. Nevertheless, the safety of BMC therapy in dogs should be evaluated using many cases in detail through future studies.

In the present study, the short-term safety of autologous BMC infusion therapy was demonstrated in clinically healthy dogs and dogs with chronic hepatitis. The administration route and cellular processing methods were chosen to minimise the risk of complications. Direct

injection into the liver or infusion via the hepatic artery, portal vein or splenic vein may provide other options for administration. Other modifications such as clonal selection, cellular tracing, expansion by culture or repeated injection are important issues for autologous BMC infusion therapy. The results of the present study provide a basis for further advances and modifications in this treatment therapy. On the other hand, there is no evidence that infused BMCs differentiated into hepatocytes or participated in regeneration. Therefore, further study is required to confirm the efficacy of infused autologous BMCs.

Taken together, our results suggest that autologous BMCs infused via the peripheral vein in dogs with chronic hepatitis or cirrhosis may be safe. Because of a small number of cases, we could not generalise these findings. However, if further studies reveal benefits, autologous BMC infusion therapy may be helpful in dogs with advanced hepatitis or cirrhosis as a novel therapy.

# Conclusion

Regenerative medicine using bone marrow derived cells is an attractive treatment for patients with severe liver disease in human medicine. Recent studies discussed earlier provide novel and interesting data regarding the effect and safety of autologous BMC therapy in the treatment of chronic liver disease. To reveal usability of ABMi in dogs, I programed two experimental systems that the xenotransplantation and autologous transplantation.

In chapter 1, I demonstrated accumulation potential of infused canine BMCs to the injured liver using NOG mice. This phenomenon may be one of the most important for successful treatment for patients with liver disease. However, population of accumulated BMCs and role of these cells should be studied in future. If the subjects would be resolved, more effective novel treatment would be developed. Considering the future clinical trials, this research to design more effective trials, potentially using different BMC population cell types to target specific complications of liver disease. More modifications on administration route and cellular processing are important issue for this attractive therapy.

In chapter 2, I demonstrated that canine BMC xenotransplantation into NOG mice promoted CCl<sub>4</sub>-induced hepatic fibrosis. NOG mice could sustain various liver injuries and are good recipients for xenogenic rodent BMC transplantation. We think that the use of an immunodeficient animal model could also be a bias in xenogenic BMC transplantation studies. In fact, immune cells could modulate liver regeneration and fibrotic change. Their absence in immunodeficient animals could modify the liver response to acute injury and to chronic injury, as well as engraftment of BMCs. From our data, it is clear that canine BMCs at least included cell fraction which associate progress of hepatic fibrosis, and the cell fraction may be associated with adverse effects on hepatic fibrosis. It should be considered more in detail in future when designing BMC therapy regimens for clinical use.

In chapter 3, I demonstrated the short time safety in clinically healthy dogs dogs with spontaneous liver fibrosis. Thus, ABMi for treat dogs with liver disease can be used in future

general clinical application, and there were no serious adverse events. To my knowledge, this is the first study that performed ABMi in dogs. Previous studies using autologous BMCs for chronic liver disease including cirrhosis suggested the efficacy and the safety therapy. Data in present study reinforce the need for long term follow up of the dogs. Novel studies may also elucidate if CD34-positive hematopoietic stem cell or other cells induce improvement of liver function.

Finally, the mechanism of action of BMC within the liver remains elusive. If further studies reveal benefits, ABMi may be helpful in dogs with advanced liver cirrhosis as a novel therapy. The issues that need to be investigated will include identification of cells showing treatment effects from BMC fractions, and clarification of the underlying mechanisms. When cells with liver regeneration and restorative activity can be isolated from small amounts of bone marrow fluid, cultured, and then infused via peripheral vein, and that genuine treatment methods using less-invasive bone marrow derived cultured cells could be developed.

Some of these studies were released to below.

Kato T, Hisasue M, Segawa K, Fujimoto A, Makiishi E, Neo S, Yasuno K, Kobayashi R, Tsuchiya R. Accumulation of xenotransplanted canine bone marrow cells in NOD/SCID<sub>γ</sub><sup>c(null)</sup> mice with acute hepatitis induced by CCl<sub>4</sub>. J Vet Med Sci. 2013. 31; 75(7): 847-55.

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# Tables



**Table. 1.** Changes of hematological and serum biochemical parameters in healthy dogs after ABMi (n=4)

		Before	1day	2days	7days	14days
CBC (Mean±SE)	WBC ( $10^2/\mu\text{l}$ )	95 (28)	86.7 (22.61)	90.7 (20.8)	94 (12.67)	85.7 (15.7)
	RBC ( $10^4/\mu\text{l}$ )	764.7 (31.5)	791.7 (105.5)	771 (65.6)	731 (63.1)	746.7 (49.9)
	PCV (%)	53.6 (2.3)	54.6 (7.1)	54.4 (5.2)	51.2 (4.5)	52.2 (2.8)
	PLT ( $10^4/\mu\text{l}$ )	33 (7)	32.6 (3.8)	27.6 (2.2)	30.7 (2.8)	29.8 (2.1)
Biochemical (Mean±SE)	TP (g/dl)	5.7 (0.2)	5.35 (0.3)	5.45 (0.2)	5.75 (0.4)	5.9 (0.1)
	Alb (g/dl)	3.35 (0.2)	3.05 (0.1)	3 (0.2)	3.25 (0.2)	3.17 (0.2)
	AST (IU/L)	35.5 (2.4)	26 (6.4)	26 (6.6)	28.5 (5.7)	31.7 (8.1)
	ALT (IU/L)	38.5 (3.8)	38 (4.3)	37 (2.6)	37.5 (3.7)	37.7 (3.5)
	ALP (IU/L)	147 (48.9)	134.5 (73.1)	142.5 (55.5)	128 (45)	99 (49)
	T-Bil (mg/dl)	0.03 (0.01)	0.025 (0.01)	0.005 (0.005)	0.025 (0.02)	0.037 (0.01)
	Cre (mg/dl)	0.7 (0.03)	0.71 (0.07)	0.64 (0.04)	0.71 (0.06)	0.71 (0.05)
	BUN (mg/dl)	12.25 (2.2)	9.75 (4.5)	9.55 (4.1)	13.75 (4.5)	14.1 (2.1)

**Table. 2.** Clinical characteristics of two dogs with chronic hepatitis before ABMi therapy

	Case 1	Case 2
Breed	American Cocker Spaniel	Miniature Schnauzer
sex	Casted male	Spayed female
Age	10	8
Body weight	12.1 kg	4.8 kg
Ascites	+	-
Jaundice	-	+
Pathologic diagnosis	Liver cirrhosis	Liver fibrosis
Medical treatment	Livact (0.3 g/kg, PO, q 24h)	Denosyl (20 mg/kg, PO, q 24 h)
	Laennec (10 mg/kg, PO, q 24h)	Urso (10 mg/kg, PO, q 24h)
	Aldactone (1 mg/kg, PO, q 12h)	Laennec (10 mg/kg, PO, q 24h)

**Table 3.** Characteristics of processed bone marrow cells (BMCs) from two dogs with chronic hepatitis

	No. of isolated cells ( $\times 10^8$ )	No. of infused MNCs ( $\times 10^8$ )	CD45+ (%)	CD34+ (%)	CD45+, CD34+ (%)	CD45+, CD13 (%)	CD45- (%)
Case 1	9.2	8.2	91.67	1.46	1.26	1.69	7.07
Case 2	6.8	5.8	92.1	1.47	1.01	1.29	6.8

## **Legends for Figures**

**Figure. 1 :** Experimental design of cBMC transplantation into NOG mice.

**Figure. 2 :** CCl<sub>4</sub>-induced acute liver injury and regeneration in NOG and WT mice.

**Figure 3:** Engraftment of donor cBMCs in liver, lung, and spleen tissues was examined 1 week after transplantation.

**Figure. 4:** Combined analysis of actin phalloidin (green), PKH26 (red), and DAPI (blue).

**Figure 5:** Influence of cBMC transplantation on acute liver injury and regeneration.

**Figure. 6:** Changes of serum albumin, ALT and AST levels during continuous CCl<sub>4</sub> injection.

**Figure. 7:** Characteristics of hepatic fibrosis in NOG mice with continuous CCl<sub>4</sub> injection.

**Figure. 8:** Characteristics of hepatic fibrosis in each NOG mice group after 10 week after continuous CCl<sub>4</sub> injection.

**Figure. 9:** Gross appearance and histology of the spleen in each mice group.

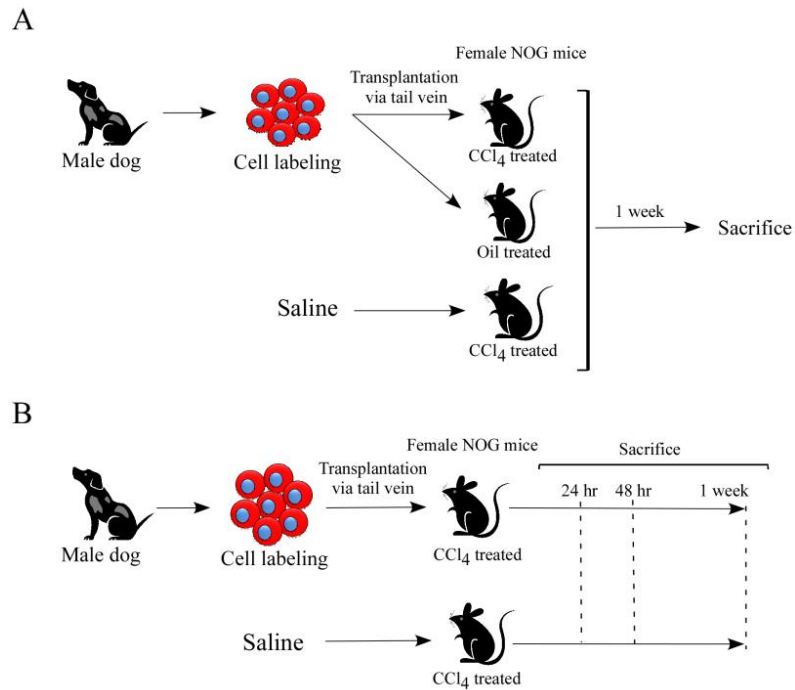
**Figure. 10:** Immunohistochemical analysis for CD3 and CD20, and FISH analysis using canine specific FITC-labeled probe to detect the xenotransplanted cBMC in NOG mice tissue.

**Figure. 11:** Cellular characterization of bone derived MNCs from 2 cases with by flowcytometric analysis.

**Figure. 12:** Gross appearance and histopathology of the liver in two dogs diagnosed chronic hepatitis (Case 2)/ cirrhosis (Case 1)

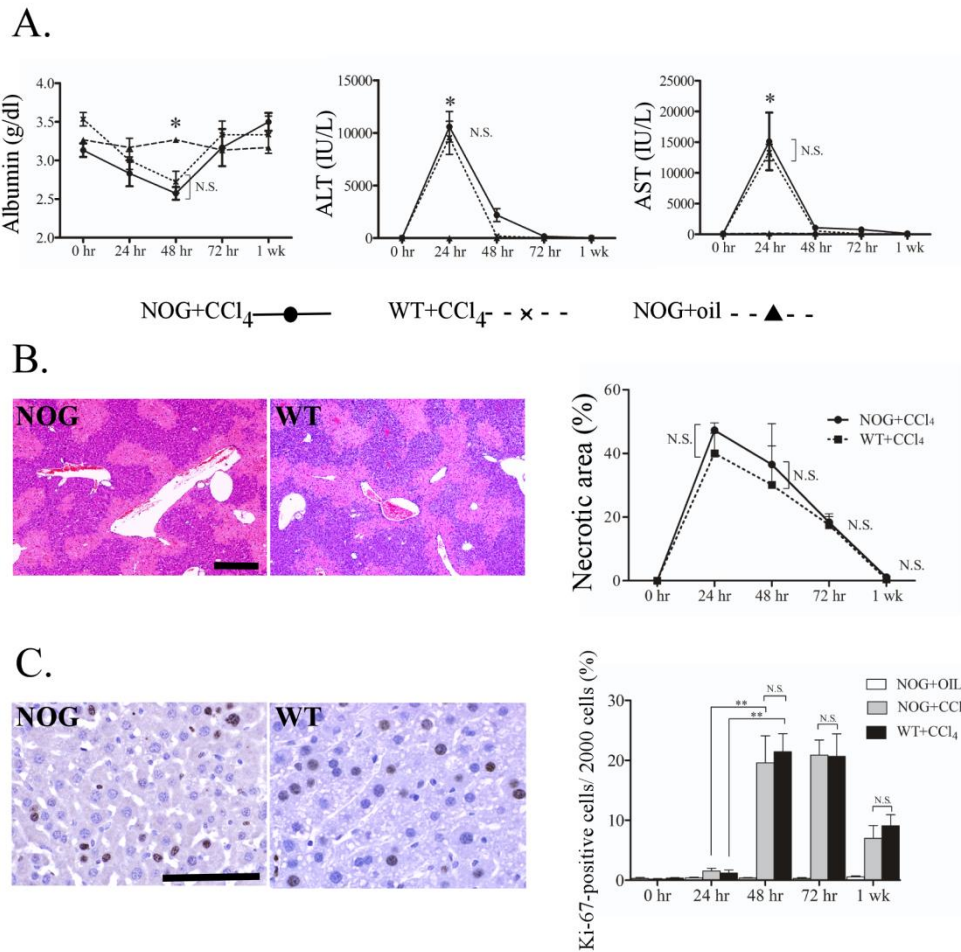
**Figure. 13:** Changes of the levels of serum biochemistry in two dogs with chronic hepatitis/ cirrhosis.

# Figures

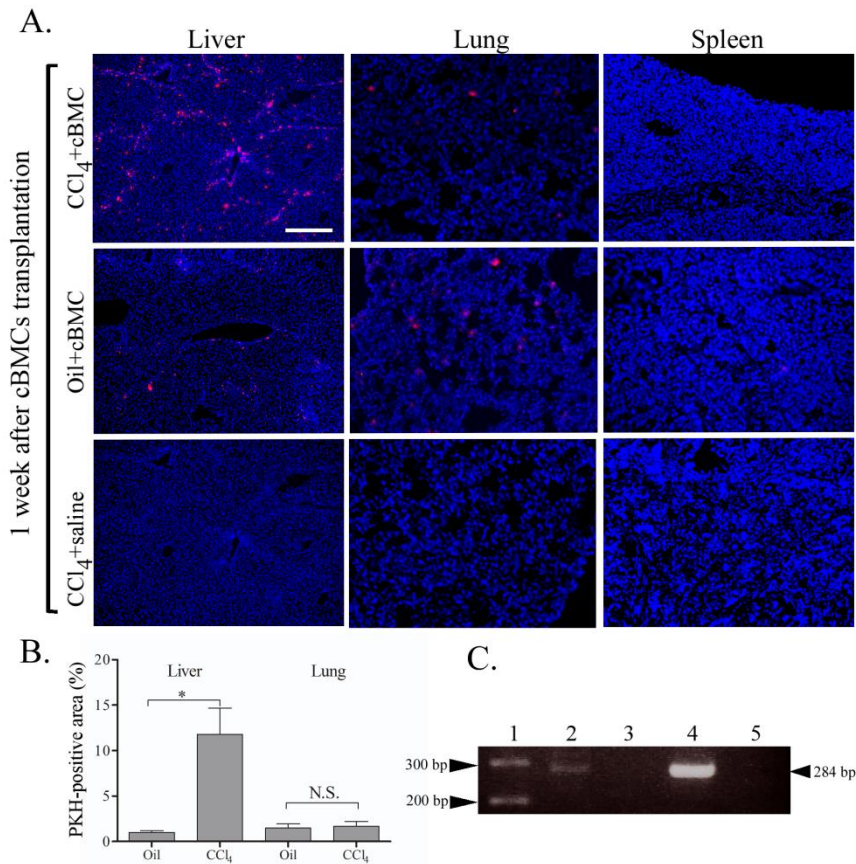


**Figure 1.** Experimental design of cBMC transplantation into NOG mice. (A) The cBMCs were collected from a male dog, red fluorescence-labeled a male dog, red fluorescence-labeled by CCl<sub>4</sub> treatment. The mice were grouped as CCl<sub>4</sub> + cBMC, oil + cBMC, and CCl<sub>4</sub> + saline groups (n = 5, respectively). The systemic distribution of the transplanted male cBMCs was evaluated 1 week after transplantation. (B) Sampling times after cBMC transplantation. The CCl<sub>4</sub> + cBMC (n = 12) and CCl<sub>4</sub> + saline (n = 12) groups were sacrificed, and their livers were removed at 0 hr, 24 hr, 48 hr and 1 week after BMC transplantation (n = 4, respectively).



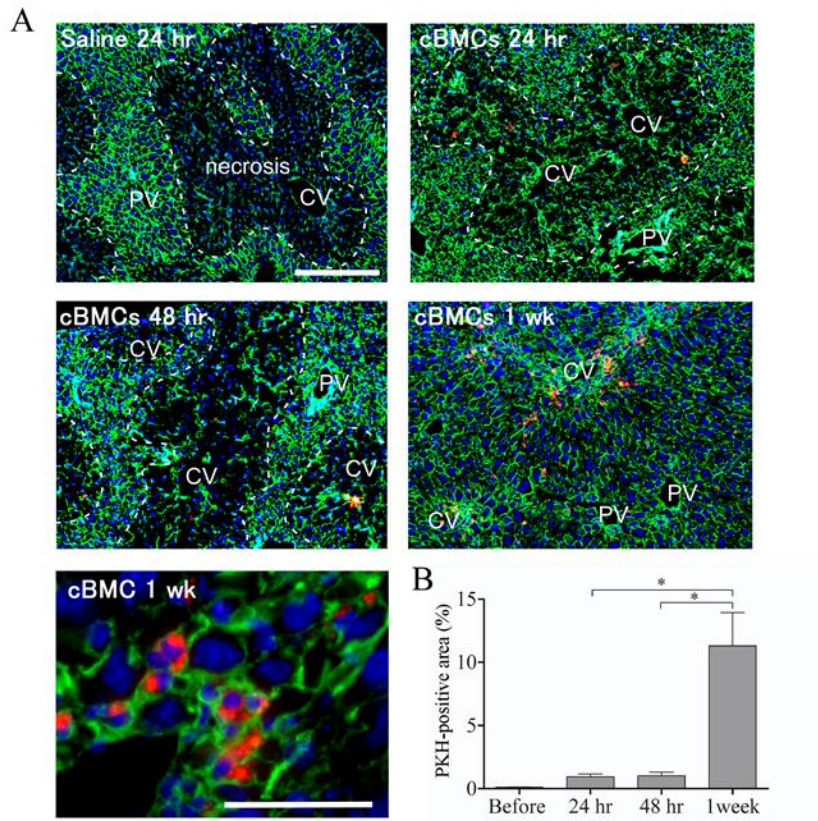


**Figure 2.** CCl<sub>4</sub>-induced acute liver injury and regeneration in NOG and WT mice. (A) Serum albumin, ALT, and AST levels before and 7 days after CCl<sub>4</sub> injection. (B) Histological findings of necrosis in the centrilobular and midzonal liver areas. The necrotic area of the liver was increased by more than 50% 24 hr after CCl<sub>4</sub> injection in both WT and NOG mice. Scale bar=200  $\mu$ m. (C) Regeneration of liver tissue was estimated by detection of Ki-67-positive cells. The ratios of Ki-67-positive cells were calculated in 2000 hepatocytes. Scale bar=100  $\mu$ m. Bars indicate means  $\pm$  SEM. N.S., not significant. \*Significant difference compared with the NOG + oil group (n = 4 per group; P < 0.05). \*\*Significant difference compared with that 24 hr after CCl<sub>4</sub> treatment of NOG and WT mice (n = 4 per group; P < 0.05).

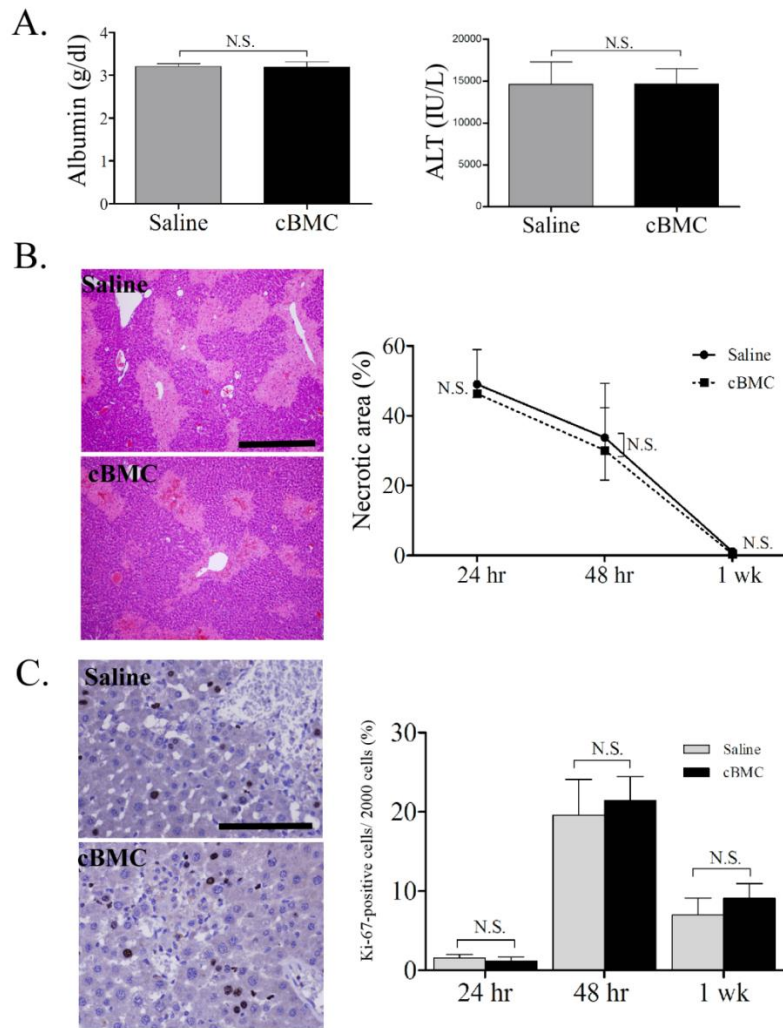


**Figure 3.** Engraftment of donor cBMCs in liver, lung, and spleen tissues was examined 1 week after transplantation. (A) Detection of PKH26-labeled infused cells in liver tissues by fluorescence microscopy. A number of PKH26-positive cBMCs were detected in livers of the CCl<sub>4</sub> + cBMC group, but only a few cBMCs were detected in livers of the oil + cBMC group. In both the CCl<sub>4</sub> + cBMC and oil + cBMC groups, some PKH26-positive cells were detected in the lung tissue but not in the spleen tissue. Large bar=50μm, small bar=200 μm (B) Quantitative comparison of PKH26-positive cBMCs in liver and lung tissues. PKH26-positive cells were significantly increased in the CCl<sub>4</sub> + cBMC group compared with the oil + cBMC group, but the number of PKH26-positive cells in the lung tissue did not differ between the groups. \*Significant difference compared with the Oil+cBMCs group (n = 4 per group; P < 0.05). (C) The canine male-specific SRY gene (284 bp) was amplified exclusively in the livers of the female xenotransplanted mice. Lane 1, molecular weight standard; lane 2, cBMC-infused liver

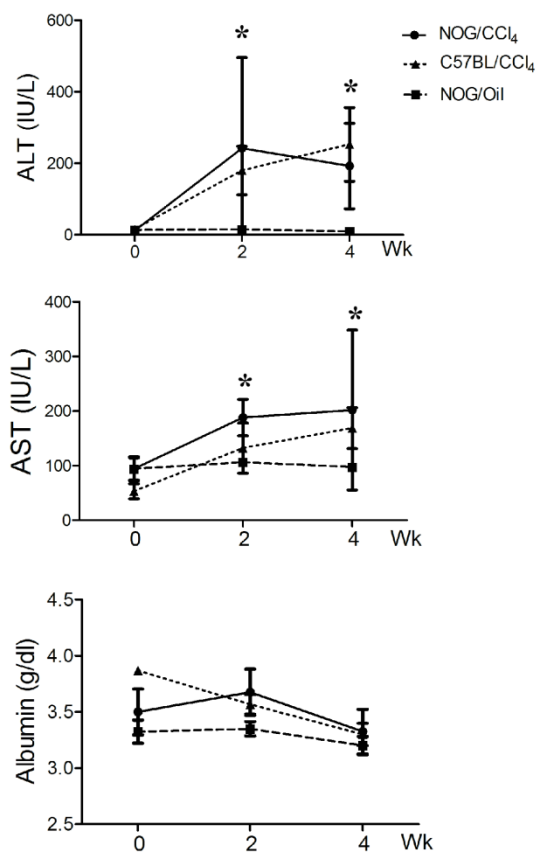
tissue; lane 3, saline-treated liver tissue; lane 4, male dog liver tissue (positive control); lane 5, female dog liver tissue (negative control).



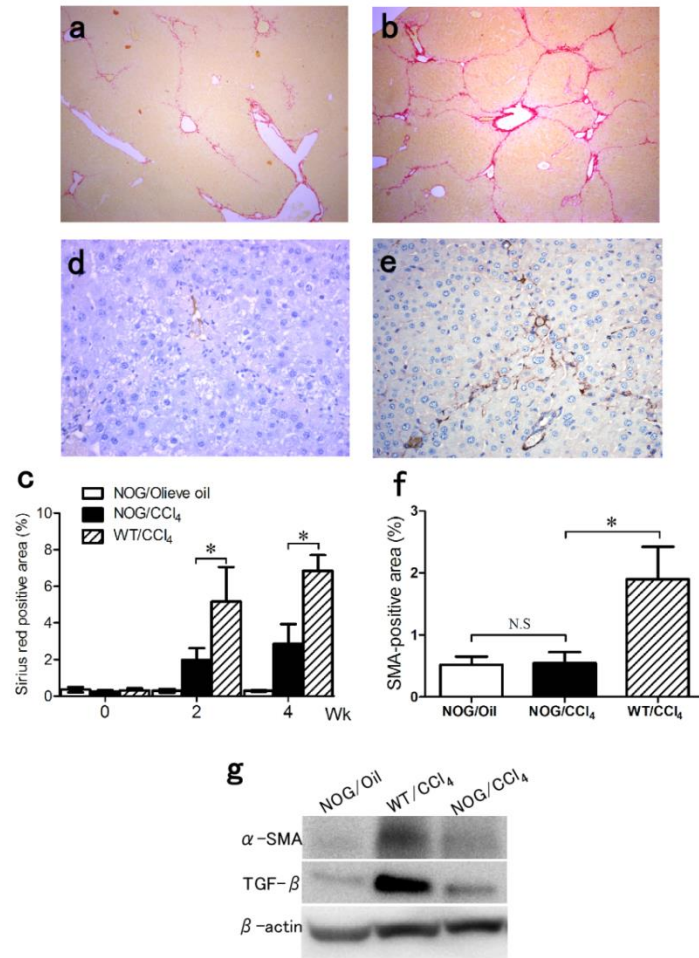
**Figure 4.** Combined analysis of actin phalloidin (green), PKH26 (red), and DAPI (blue). (A) The left liver lobes of NOG mice were analyzed 24 h, 48 h, and 1 week after cBMCs transplantation. The dotted lines indicated the necrotic area of the liver tissue. Red fluorescence indicates the position of the PKH26-labeled cBMCs. At 24 and 48 h after transplantation, cBMCs were sparsely distributed, corresponding to the necrotic areas. These cells had accumulated 1 week after transplantation. (B) Quantitative comparison of PKH26-positive cBMCs in liver tissues. Bars indicate means  $\pm$  SEM. C, Central vein; P, Portal vein. \*Significant difference compared with that in the oil + cBMC group ( $n = 4$  per group;  $P < 0.05$ ). Scale bar = 200  $\mu\text{m}$  (Fig. A. 1-4), 50 $\mu\text{m}$  (Fig. A. 5).



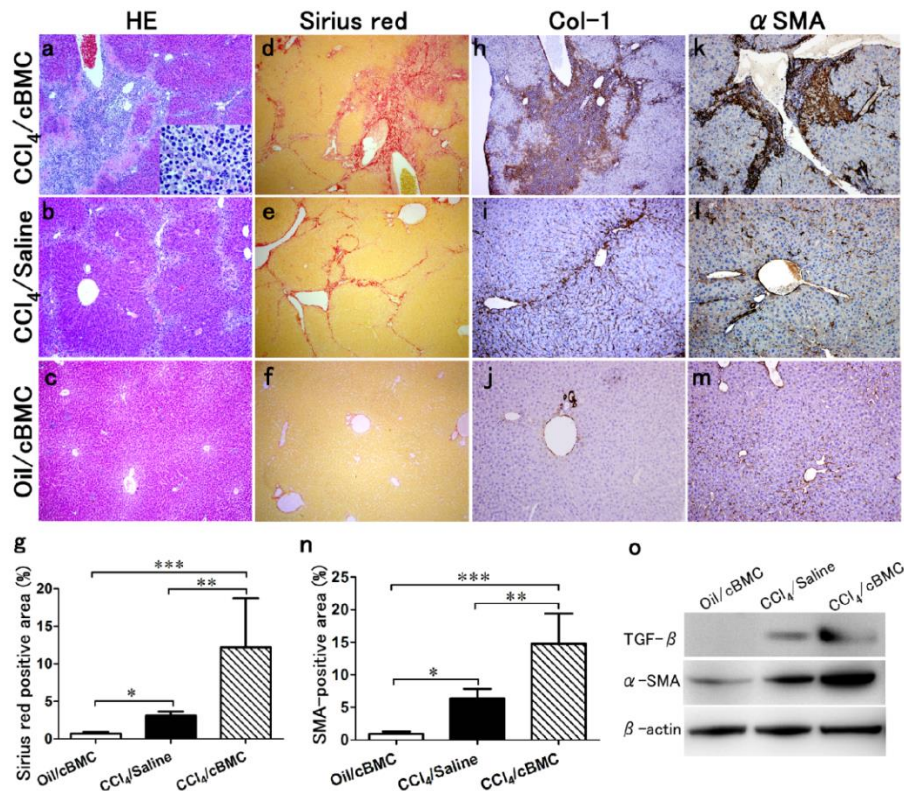
**Figure 5.** Influence of cBMC transplantation on acute liver injury and regeneration. (A) Serum albumin and ALT levels at 24 h after transplantation were not significantly different between the saline and cBMC groups. (B) Histology of the liver confirmed necrotic hepatocytes as eosinophilic cytoplasm in the centrilobular and midzonal areas. There were no significant differences at any time point between the saline and cBMC groups. Scale bar=200 $\mu$ m. (C) Hepatocyte regeneration was measured by immunohistochemistry of Ki-67 48 h after CCl<sub>4</sub> injection. Regeneration was unchanged at each time point in both the saline and cBMC groups. Scale bar=100 $\mu$ m. Bars indicate means  $\pm$  SEM. N.S., not significant.



**Figure 6.** Changes of serum albumin, ALT and AST levels during continuous CCl<sub>4</sub> injection. NOG/CCl<sub>4</sub> and C57BL/CCl<sub>4</sub> group mice were given intraperitoneal CCl<sub>4</sub> injections twice weekly for 4 weeks. Bars indicate means  $\pm$  SEM. \*Significant difference compared with NOG/oil group (n=4 per group; P<0.05).

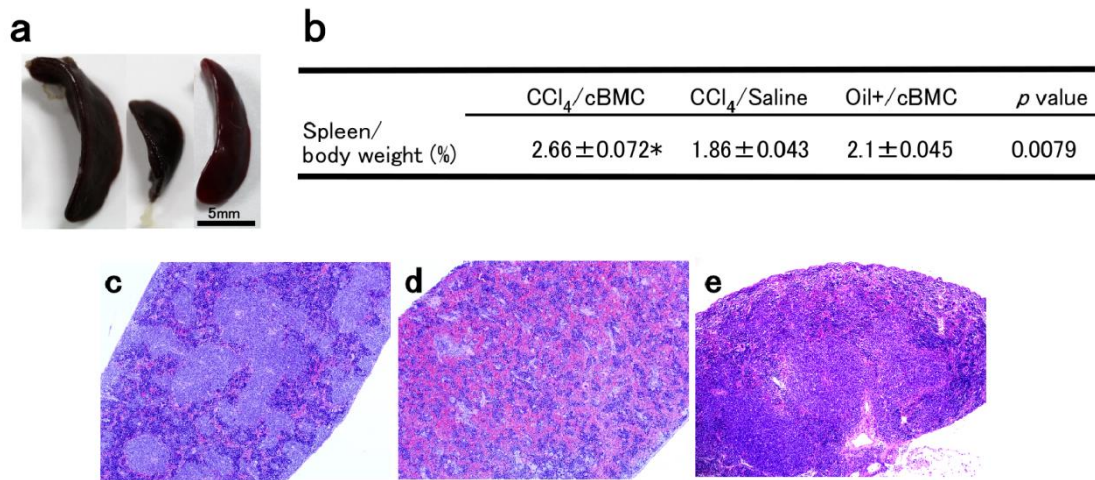


**Figure 7.** Characteristics of hepatic fibrosis in NOG mice with continuous CCl<sub>4</sub> injection. Ssirius red staining of liver tissue sections (a-c). The extensive collagen depositions were evident in liver tissue from the continuous CCl<sub>4</sub>-treated groups (c), but the level of hepatic fibrosis in the NOG/CCl<sub>4</sub> group (a) was lower than in the WT/CCl<sub>4</sub> group (b) at 4 weeks. Immunohistochemical analysis of  $\alpha$ -SMA expression in liver tissues (d-f).  $\alpha$ -SMA immune-positive fibrogenic cells which is recognized as aHSCs were appeared corresponded to fibrotic area in WT/CCl<sub>4</sub> group (e), while remained negative in NOG/CCl<sub>4</sub> group (d). Western blotting analysis of  $\alpha$ -SMA and TGF- $\beta$  expression in liver (g). Expression levels of each protein were decreased in NOG/CCl<sub>4</sub> group than WT/CCl<sub>4</sub> group. Bars indicate means  $\pm$  SEM. N.S., not significant. \*Significant difference compared with NOG/CCl<sub>4</sub> group (n=4 per group; P<0.05).

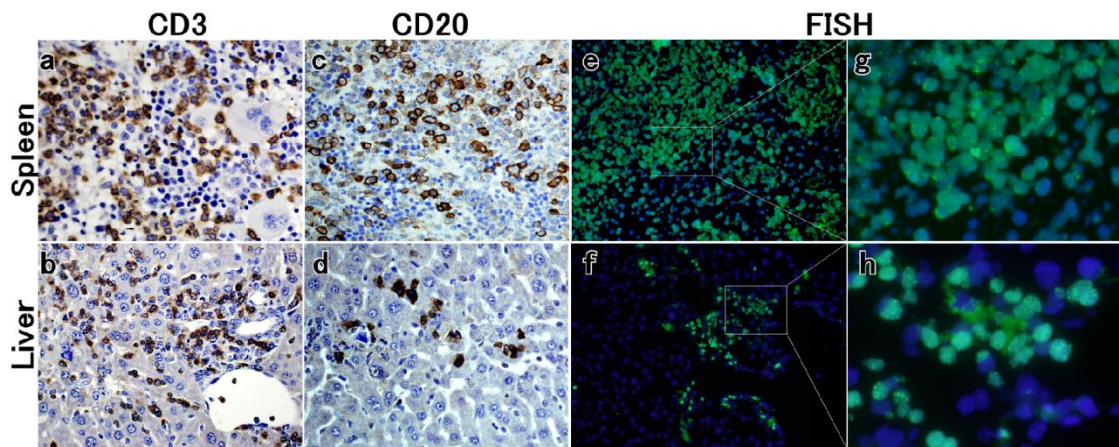


**Figure 8.** Characteristics of hepatic fibrosis in each NOG mice group 10 week after continuous CCl<sub>4</sub> injection. HE staining (a-c). Intense inflammation was observed around portal vein in CCl<sub>4</sub>/cBMC group (a) but, was not seen in CCl<sub>4</sub>/saline (b) and Oil/cBMCs group (c). Sirius red staining (d-g). Hepatic fibrosis was observed around portal vein, and fibrotic area increased significantly in CCl<sub>4</sub>/cBMC group (d) compared to saline/cBMC (e) and Oil/cBMC group (f). Immunohistochemical analysis for collagen-1 expression (h-j). Expression of collagen-1 was recognized extensively in liver tissue. There was higher collagen-1 expression in CCl<sub>4</sub>/cBMCs group (h) compared with CCl<sub>4</sub>/saline (i) and Oil/cBMC groups (j). Immunohistochemical analysis for  $\alpha$ -SMA expression (k-n). Extensive immune-positivity corresponded to fibrotic area in CCl<sub>4</sub>/cBMCs group (k), while remained negative in oil/cBMC group (m). A few number of immune-positive cells were seen in CCl<sub>4</sub>/cBMC group (l). Immune-positivity was significantly increased in CCl<sub>4</sub>/cBMCs group than oil/cBMC and CCl<sub>4</sub>/saline group (n). Western blotting for  $\alpha$ -SMA and TGF- $\beta$  expression (o). CCl<sub>4</sub>/cBMC group increased  $\alpha$ -SMA and TGF- $\beta$  expression in liver compared with other groups.

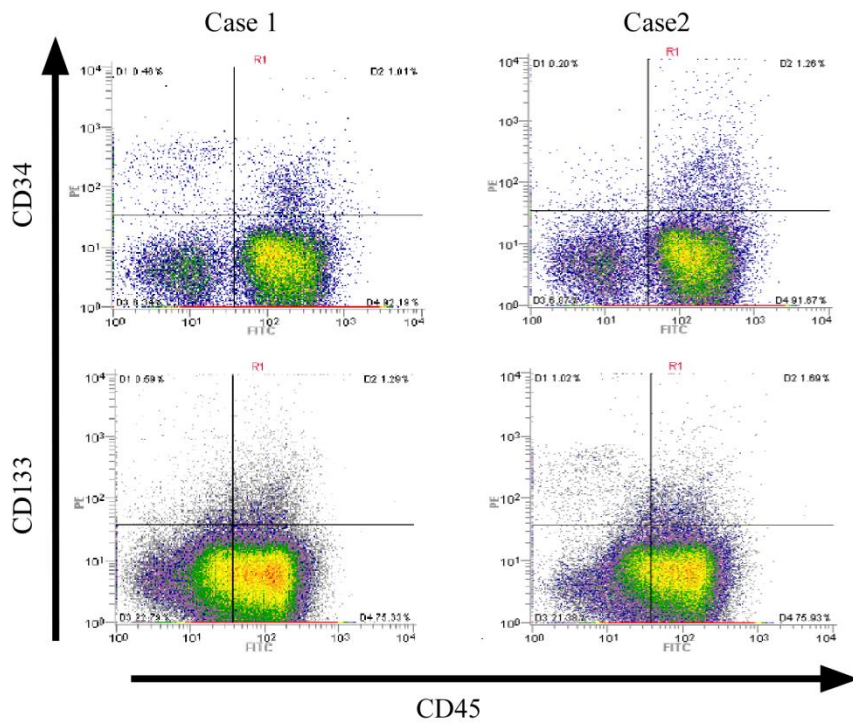




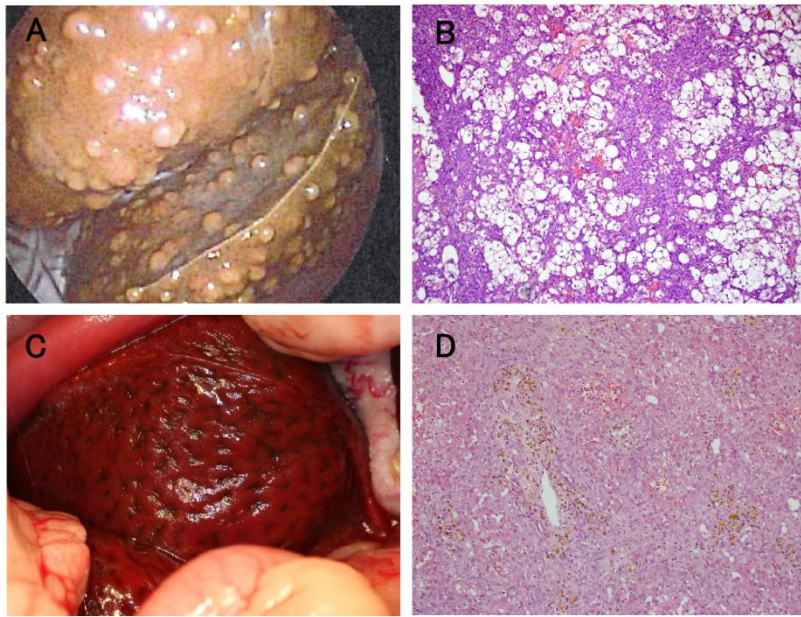
**Figure. 9.** Gross appearance and histology of the spleen in each mice group. Gross appearance of spleen (a) and spleen/body weight ratio (b) in cBMC infused group. The spleen in CCl<sub>4</sub>/Saline group was atrophic because of lymphocyte deficiency. Numerous mononuclear cells emerged and accumulated and these cells in cBMC xenotransplanted group (c, e). Hematopoietic cells were seen such as erythroblast and megakaryocyte in CCl<sub>4</sub>/Saline group (d).



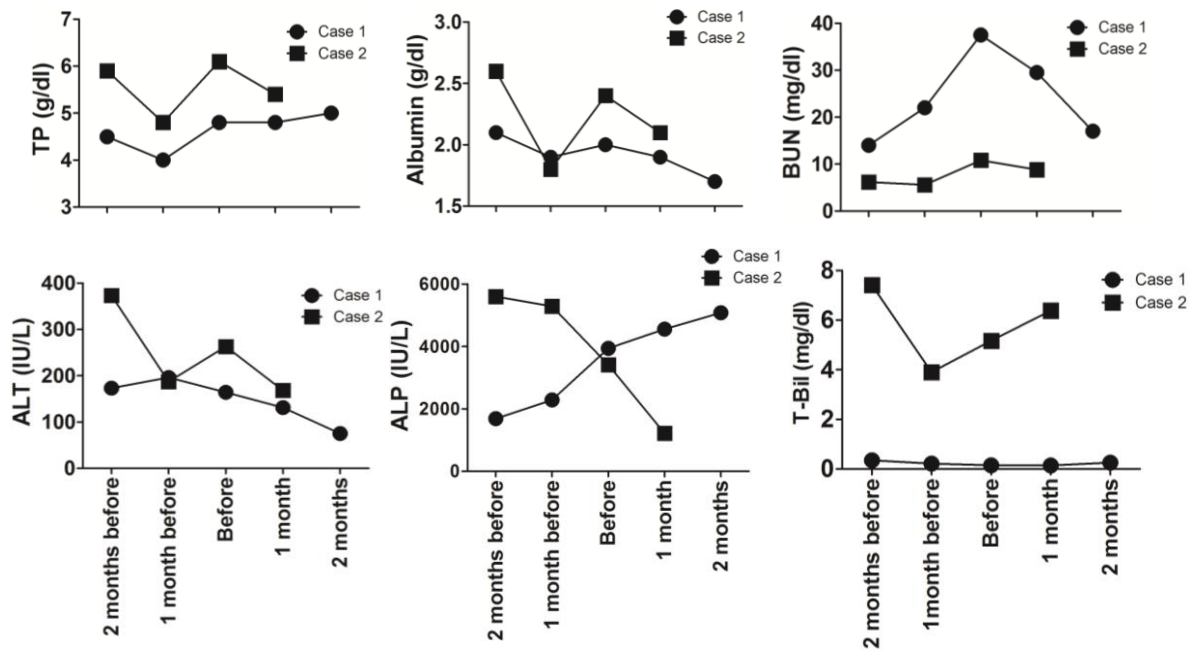
**Figure. 10.** Immunohistochemical analysis for CD3 and CD20, and FISH analysis using canine specific FITC-labeled probe to detect the xenotransplanted cBMCs in NOG mice tissue. Numerous mononuclear cells which emerged and accumulated in NOG mice liver and spleen tissues were included CD3 (a, b) and CD20 (c, d) positive lymphocytes. These accumulated cells were also positive canine specific FITC-labeled probe (e-h).



**Figure. 11.** Cellular characterization of bone marrow derived MNCs from two cases with chronic hepatitis by flow cytometric analysis. Representative flowcytometric analyses for CD34, CD45 and CD133 antigens in MNCs.



**Figure. 12.** Gross appearance and histopathology of the liver in two dogs diagnosed chronic hepatitis (Case 2)/ cirrhosis (Case 1). Case 1 was diagnosed as liver cirrhosis. At liver biopsy, micro-hepatica and diffuse nodular lesion were detected (A). HE staining of the liver of case 1 (B). There were no seen lobular structures, and hepatocytes reduced markedly. The swelling and vacuolization were seen in survive hepatocytes. The numerous small bile ducts were increased in stromal tissue in the liver, and numerous regenerative nodular was seen. In case 2, liver was discolored in whole lobular (C). Histologically, increased small bile duct and diffused fibrosis were seen markedly (D). Regenerative nodular lesion was not seen in this case, and pathogenesis might be chronic hepatitis.



**Figure. 13.** Changes of the levels of serum biochemistry in two dogs with chronic hepatitis/cirrhosis. Both dogs were followed-up a month after autologous BMC infusion. However, case 2 died 45 days after autologous BMC infusion, and laboratory data could not be obtained. All serum parameters did not improve after autologous BMC infusion treatment in both dogs. Total protein: TP, albumin: Alb, aspartate aminotransferase: AST, alanine aminotransferase: ALT, alkaline phosphatase: ALP, total bilirubin: T-Bil, creatinine: Cre and urea nitrogen: BUN.