

ADMINISTRATION OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS MODULATE TLR EXPRESSION DURING LIVER REGENERATION

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Abstract: Liver cell transplantation is a powerful alternative to orthotopic cell transplantation in the treatment of liver failures. Recently, considerable effort is being channeled to understand the nature and kinetics of directing stem cells to effectively accumulate at the regenerating liver site. Mesenchymal stem cells are one of the promising cell sources modulating liver regeneration process. The present was designed to study how mesenchymal stem cells might modulate liver immune behaviors by changing Toll-like receptor (TLR) expression and increase regenerative potential during liver regeneration in rats.

Normal and partially hepatectomized rats were treated with mesenchymal stem cells isolated and expanded from rat bone marrows. Accumulation of mesenchymal stem cells was confirmed by Real Time-Polymerase Chain Reaction (RT-PCR), Fluorescence-Activated Cell Sorting (FACS), and Immunofluorescence Staining (IFS). Student's t-test analysis was used to evaluate the significance of differences between sham and partially hepatectomized rat groups.

Our results showed that mesenchymal stem cells expressed several TLRs, and their accumulation during regeneration was depended on the timing of injury. Mesenchymal stem cells isolated from bone marrow of normal rats were observed at the injured liver 3 days after the injection. There were no labeled mesenchymal stem cells in the liver sections of the uninjured animals. Mesenchymal stem cell administration significantly altered the expression of TLR2, 3 and 9 while retaining their migration potential to regenerating liver.

Our findings implicated that mesenchymal stem cell administration during liver regeneration modulate the immune response through changing the expression of the TLRs in the remaining liver parts into which the cells are recruited or infused. This alteration may contribute to the regeneration process following partial hepatectomy.

Key words: Mesenchymal stem cell, TLR, homing, liver regeneration, rat.

Özet: Karaciğer hücresi nakli, karaciğer yetmezliğinde ortotopik hücre nakline güçlü bir alternatiftir. Son yıllarda, kök hücrelerin doğalarının, kinetiklerinin ve yenilenen karaciğer bölgesinde etkili bir şekilde toplanmalarının sağlanmasının anlaşılması için hatırı sayılır gayretler sarf edilmektedir. Mesenkimal kök hücreler karaciğer yenilenme sürecini modüle eden ümit verici hücre kaynaklarından bir tanesidir. Bu çalışma, mezenkimal kök hücrelerin sıçanlarda toll benzeri reseptör (TLR) ifadesini değiştirmek suretiyle karaciğer immün yanıtını nasıl etkileyebildiklerini ve karaciğer yenilenmesi esnasında yenilenme potansiyelini arttırabildiklerini belirlemek için gerçekleştirilmiştir.

Normal ve karaciğerleri kısmen çıkarılmış sıçanlar, sıçan kemik iliğinden elde edilip çoğaltılan mesenkimal kök hücreler ile muamele edilmişlerdir. Mesenkimal kök hücrelerin toplanması Eş Zamanlı Polimeraz Zincir Reaksiyonu (RT-PCR), Floresan Aktivite Hücre Ayırma (FACS), ve Immunfloresan Boyama (IFS) ile doğrulanmıştır. Sham ve karaciğeri kısmen alınmış sıçan grupları arasındaki farklılığın istatistiki analizinde Student's t-testi kullanılmıştır.

Elde edilen sonuçlar mezenkimal kök hücrelerinde çeşitli TLR'lerin ifade edildiklerini ve bu hücrelerin yenilenme esnasında toplanmalarının meydana gelen hasarın zamanlamasına bağlı olduğunu göstermiştir. Normal sıçanların kemik iliğinden izole edilen mezenkimal kök hücreler hasarlı karaciğerde enjeksiyon sonrası 3. günde görülmüşlerdir. Hasarsız hayvanların karaciğer kesitlerinde işaretli bir mezenkimal kök hücre görülmemiştir. Mezenkimal kök hücre uygulaması TLR2, 3 ve 9'un ifadesinin anlamlı bir şekilde değiştirirken yenilenen karaciğere göç etme yeteneklerini devam ettirmişlerdir.

Sonuçlar, karaciğer yenilenmesi esnasında mezenkimal kök hücre uygulamasının, hücrelerin uygulandığı hasarsız karaciğer parçalarında TLR'lerin ifadelerini değiştirme yoluyla immün yanıtı modüle ettiğini ortaya koymaktadır. TLR ifadesindeki bu değişim kısmı hepatoktemi sonrası yenilenme sürecine katkı sağlayabilir niteliktedir.



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Introduction

Since its first development in the early 1960s (Starzl *et al.* 1963), orthotopic liver transplantation (OLT) has been considered as a gold standard in treatment of liver failure. However, other therapeutic strategies are also required due to the donor shortage. Despite its limiting factors such as cell viability, modest engraftment and limited tissue viability (Phillippe *et al.* 2008), liver cell transplantation (LCT) is one of the developing alternative solution with increasing success (Najimi & Sokal 2005, Stéphenne *et al.* 2006). Stem cells are very potent candidates for LCT due to their plasticity (Verfaillie *et al.* 2002). Besides hematopoietic stem cells (HSCs) and adult liver stem/progenitor cells, mesenchymal stem cells (MSCs) are also used in liver cell therapy because of their hepatic differentiation potential (Phillippe *et al.* 2008).

MSCs are multipotent cells capable of self-renewal and differentiating into multiple lineages such as osteocytes, adipocytes, chondrocytes, myoblasts, hepatocytes and cardiomyocytes (Friedenstein *et al.* 1970, da Silva Meirelles *et al.* 2008, Tokcaer-Keskin *et al.* 2009). In addition to their hepatic potential, MSCs have two other important properties that make them critical for LCT. The first one is their preferential migration into the injured site. Migration of MSCs and their engraftment after the injury have been shown in several tissues including myocardium (Orlic *et al.* 2001) spinal cord (Hofstetter *et al.* 2002), brain (Kopen *et al.* 1999), kidney (Bussolati *et al.* 2008) and liver (Zhao *et al.* 2008). Secondly, MSCs can evade immune recognition and were implicated to suppress immune responses (Rasmusson 2006). Through multiple pathways, *ex-vivo* expanded MSCs were reported to down regulate a broad range of innate and adaptive immune cells including T cells, B cells, NK cells and antigen presenting cells (Stagg 2006) demonstrating their versatile immunoregulatory properties (Bartholomew *et al.* 2002, Aggrawal & Pittenger 2005, Inoue *et al.* 2006). Although the mechanism is unclear yet, there seems to be several factors playing role in this immunoregulation. Pevsner-Fisher *et al.* (2007) showed that murine MSCs express several Toll-like receptors (TLRs) and in particular TLR2 was found to be important in differentiation potential of MSCs. It was also recently shown that human MSCs express several TLRs (Pevsner-Fischer *et al.* 2007, Tomchuck *et al.* 2008).

Cell surface or endosome-associated TLRs recognize a wide range of pathogen-associated molecular patterns (PAMPs) including carbohydrates, lipids, proteins and nucleic acids (Akira *et al.* 2006). Several immune cells such as macrophages, dendritic cells (DCs), B cells, specific types of T cells but also non-immune cells including fibroblasts and epithelial cells (Kumagai *et al.* 2008) signaling through TLRs were reported (Takeshita *et al.* 2004). In all cell types, expression of TLRs is modulated in response to pathogens, cytokines and environmental stress. While TLR1, 2, 4, 5 and 6 are expressed on the cell surface, TLR3, 7, 8 and 9 are found in intracellular compartments such as endosomes

(Arancibia *et al.* 2007). Ligands of the latter ones, mainly nucleic acids, are required to be internalized to endosomes for recognition.

The collaborative role of TLRs on MSCs' homing during liver regeneration has not been evaluated so far. By using a well established liver regeneration model of partially hepatectomized (PH) rats, the present study was undertaken to investigate the interplay between MSCs and TLRs. Our findings implicated that MSC administration causes an increase in the expression of TLR2, 3 and 9 in the remaining liver and may facilitate MSC homing during liver regeneration.

Materials and Methods

Animals and the experimental design

Nine-week-old, 280–300 g, male Sprague–Dawley rats were obtained from the Experimental Animal Center of Bilkent University. The rats were housed in wire mesh cages under optimum laboratory conditions (temperature 22 °C±2 °C, humidity 50–55 %, light-dark period: 12h/12h) and daily fed with tap water and pellet foods, including % 21 pure proteins. All experimental protocols were approved by the Local Animal Ethics Committee of Bilkent University. Our protocol complied with Bilkent University's guidelines on the humane care and use of laboratory animals.

Animals were divided randomly in 3 groups as isolation group of MSCs, normal group (sham; SH) and partially hepatectomized (PH) group (Fig. 1). In the PH group, 70 % of the liver mass was resected (Higgins *et al.* 1931), and in the normal group, identical surgical procedures were carried out without resection after injecting 30 mg/kg ketamine (Ketalar, Pfizer, Istanbul, Turkey). Three animals per group were used in the experiments. Operations were performed between 08:00 and 12:00 hours to minimize diurnal effects. For mRNA expression experiments, animals from both groups were killed by cervical dislocation at 2 h, 4 h, 12 h, 18 h, and 24 h and after the operation. The remnant liver lobes were excised and immediately frozen in liquid nitrogen.

Isolation and culture labeling of MSCs and administration to PH rats

MSCs were also obtained from male, nine-week-old, 280–300 g Sprague–Dawley rat bone marrow (BM) according to the methods described elsewhere (Tokcaer-Keskin *et al.* 2009). 2×10^6 of MSCs were treated with Chloromethylbenzamido-1, 1–dioctadecyl- 3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI) (Molecular Probes, USA) at a concentration of 5 µg/mL in 1X PBS. Four days after CM-DiI labeling, cells were removed and 1×10^6 cells/mL labeled MSCs were injected to the normal rats to obtain SH and partially hepatectomized PH groups (Fig. 1). Animals were sacrificed by cervical dislocation at 1, 3, and 5 days following partial hepatectomy. The remnant liver lobes were excised and immediately frozen in liquid nitrogen.

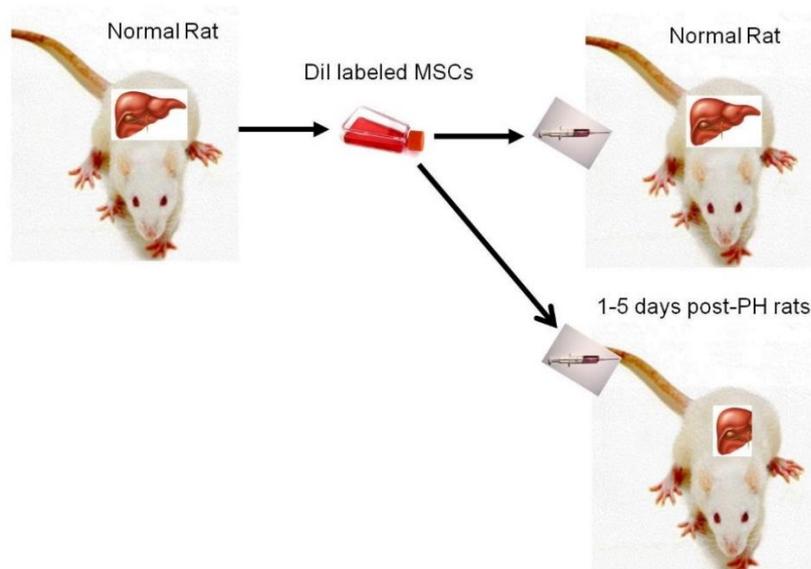


Fig. 1. MSCs were isolated and expanded from BM. CM-DiI labeled MSCs were injected into the normal and hepatectomized animals.

Total RNA Isolation from Rat MSCs and Liver Tissues

Total RNA from MSCs was isolated from the cell precipitate by using RNeasy Mini Kit (Qiagen, Hilden, Germany) and from liver tissues by using TriPure solution (Roche, Indiana, USA) according to the manufacturer’s protocol. The cDNAs were synthesized with the DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer’s protocol.

RT-PCR

RT-PCR conditions for CD11b, CD29, CD34, CD45, CD71, CD73, CD90, CD105, CD166 and GAPDH as the house keeping gene including corresponding primer sets are listed in Table 1. RT-PCR was done with DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes) according to the manufacturer’s protocol.

Table 1. Primer sequences for RT-PCR, conditions and product sizes.

Gene Acronyms		Primer Sequences	Product Size (bp)	Cycle #
CD11b ^{*a}	Forward Reverse	GCTGGGAGATGTGAATGGAG TGATGCTGGCTACTGATGCT	113	30
CD29 ^{**a}	Forward Reverse	ACTTCAGACTTCCGCATTGG GCTGCTGACCAACAAGTTCA	190	26
CD34 ^{**b}	Forward Reverse	TGTCGCTCCTTGAATCT CCTGTGGGACTCCAAC	281	30
CD45 ^{**a}	Forward Reverse	ATGTTATTGGGAGGGTGCAA AAAATGTAACGCGCTTCAGG	175	26
CD71 [§]	Forward Reverse	ATGGTTCGTACAGCAGCAGA CGAGCAGAATACAGCCATTG	182	35
CD73 ^{*a}	Forward Reverse	GAACTTGGGAGGGAGGAGAG CATTGGCAGGAAGAGAGGAG	282	30
CD90 ^{**b}	Forward Reverse	CCAGTCATCAGCATCACTCT AGCTTGCTCTGATCACATT	374	30
CD105 ^{*a}	Forward Reverse	CGGGAGGTGTTTCTGGTCT GTGCTGGGTTTCGTGGTTG	331	30
CD166 ^{*a}	Forward Reverse	CTTTGTTCTGGGAGTGGCTG GGTGTGCGGTATGTGTTTG	303	30
GAPDH ^{*a}	Forward Reverse	AGACAGCCGCATCTTCTTGT CTTGCCGTGGGTAGATCAT	207	30

^{*} Initial Denaturation 95°C 10', Denaturation 94°C 40'', Extension 72°C 40'', Final Extension 72°C 5'.

^{**} Initial Denaturation 95°C 5', Denaturation 94°C 30'', Extension 72°C 30'', Final Extension 72°C 10'.

^a Annealing 60°C 30'', ^b Annealing 55°C 30''.

[§] Initial Denaturation 95°C 5', Denaturation 94°C 45'', Annealing 66°C 60'', Extension 72°C 45'', Final Extension 72°C 10'.

Real-Time RT-PCR Studies

Rat TLR primer sequences were adopted from Hubert *et al.* (2006) and *CYC* was used as the house keeping gene. Efficiency of all primers were tested, standard curves were derived and E values were calculated (Table 2). The *Ct* values of the *i*) normal liver and PH groups (post PH at 2, 4, 12, 18 and 24 hours) for *TLR* genes and *CYC* and *ii*) PH groups (normal liver vs. 1 and 3 days post-PH) and MSC-administered PH groups (normal liver vs. 1 and 3 days post-PH) for *TLR2*, *3*, and *9* genes and *CYC* were calculated. By using normalized *Ct* values, ΔCt ($\Delta Ct_{TLR} / \Delta Ct_{CYC}$) for each *TLR* at indicated time was calculated. Fold changes in the expression of the tested *TLR* genes were calculated by using the $2^{-\Delta Ct}$ formula. Real-time RT-PCR reactions followed by a melting curve analysis were carried out in iCycler™ (Bio-Rad, Hercules, CA, USA). Q-RT-PCR conditions for all investigated genes have an initial denaturation 95°C, 10 minutes followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing for 60 seconds at 60°C, followed by extension of 60 seconds at 60°C. Final extension was set at 72°C for 5 minutes. Samples from PH and SH groups obtained at different time points were detected in duplicates, and readings from each sample and its internal control were used to calculate gene expression level.

Cell Surface Marker Staining and Analyses by FACS

Staining protocol was slightly modified from previously published protocol (Gursel *et al.* 2006). Rat specific antibodies against CD90-FITC (Santa Cruz

Biotechnology, CA, USA), and CD45-PE/Cy5 (Abcam, Cambridge, UK) with isotype controls were used to characterize MSCs. The cells were incubated in dark for 30 minutes and were washed twice, resuspended in 500 μ l PBS-BSA-Na azide and analyzed in FACSCalibur (BD, USA). FACS dot plots were assessed by Cell Quest Pro software.

Immunofluorescence Staining (IFS)

5 μ m sections of frozen liver tissues were fixed in 4 % paraformaldehyde for 30 minutes and were immersed in 3 % H₂O₂ (in methanol v/v) for 30 minutes. After washing with phosphate buffered saline pH: 7.2-7.4 (PBS), the specimen was blocked with 2 % bovine serum albumin (BSA) for 1 hour at room temperature (RT) in a humid chamber. Sections were then incubated with anti-FLT3 (Santa Cruz Biotechnology) and anti-CD90 (Chemicon, Temecula, Canada) antibodies at RT for one hour at a dilution of 1/50 and 1/500 in 1 % BSA, respectively. The tissues were incubated with FITC-labeled anti-mouse IgG (Sigma, St. Louis, MO, USA) and FITC-labeled anti-rabbit IgG (Sigma) for CD90 and FLT3, respectively. After incubating in dark for 1 hour at RT, the specimens were mounted using UltraCruz™ (Santa Cruz) medium with DAPI and examined under fluorescence microscope.

Statistical Analysis

Statistical significance between PH and SH groups was determined using the Student's *t*-test analysis. *P*-value <0.05 was considered to be statistically significant.

Table 2. The sequences of different rat TLR primers (adopted from Hubert *et al.* 2006) and efficiency values of the primer sets (E value).

Gene Acronyms		Primer Sequences	Product Size (bp)	E value
TLR1	Forward	CAGCAGCCTCAAGCATGTCT	82	1.94
	Reverse	CAGCCCTAAGACAACAATACAATAGAAGA		
TLR2	Forward	CTCCTGTGAACCTCCTGTCTT	74	1.94
	Reverse	AGCTGTCTGGCCAGTCAAC		
TLR3	Forward	GCACTGTGAGATACAACGTAGCT	66	1.98
	Reverse	GAAGGTCATCAGGTATGTGTGTCA		
TLR4	Forward	TGCTACAGTTCATCTGGGTTTCTG	78	1.88
	Reverse	CTGTGAGGTCGTTGAGGTTAGAAG		
TLR5	Forward	GGGCAGCAGAAAGACGGTAT	61	1.86
	Reverse	CAGGCACCAGCCATCCTTAA		
TLR6	Forward	AGAACCTTACTCATGTCCCAAAAGAC	79	1.99
	Reverse	AGATCAGATATGGAGTTTTGAGACAGACT		
TLR7	Forward	GTTTTACGTCTACACAGTAACTCTTCA	75	2.00
	Reverse	TTCCTGGAGGTTGCTCATGTTTT		
TLR8	Forward	GGCTTCGGCAGAGGATCT	75	2.00
	Reverse	GCCAAAACAAGTTTTCCGCTTTG		
TLR9	Forward	CCGAAGACCTAGCCAACCT	70	2.00
	Reverse	TGATCACAGCGACGGCAATT		
TLR10	Forward	CTCCAACATGGCTTTAAGGAAGGT	90	1.90
	Reverse	TGGAATTGATAGAGGAGGTTGTAGGA		
CYC	Forward	GGGAGGGTGAAAGAAGGCAT	211	1.90
	Reverse	GAGAGCAGAGATTACAGGGT		

Results

Characterization of MSC by PCR and by FACS

We first isolated MSCs from rat bone marrow. Consistent with previous observations by us and others (Tokcaer-Keskin *et al.* 2010, Mangi *et al.* 2003, Pittenger *et al.* 1999), the cells were positive for MCS markers such as CD90, CD71, CD73, CD29, CD105 and CD166 and negative for hematopoietic cell lineage markers such as CD11b, CD34 and CD45 at the transcript level (Fig. 2A). We further confirmed that these MSCs were positive for CD90 (84.5±7.2 % of total population, MFI: 334.5±150.3) and negative for CD45 (96.6±1.2 % of total population, MFI: 10.4±0.5) by FACS (Fig. 2B). Then we investigated the expression of TLRs in MSCs (Fig. 2C). Our results showed that rat MSCs express TLR1, 2, 3, 4, 6, and 9 mRNA (Fig. 2C).

Homing of MSCs in Partially Hepatectomized Rats

We partially hepatectomized rats and then injected CM-DiI labeled MSCs isolated from normal animal's bone marrow (Fig. 3). No injection of MSCs was our negative group (Fig. 3A-B). CM-DiI labeled MSCs were administered to the animal that did not undergo PH (Fig. 3C-D), to 1 day post-PH group (Fig. 3E-F), to 3 days post-PH group (Fig. 3G-H) and to 5 days post-PH group (Fig. 3I-J) through their tail vein. Three days after MSC administration, animals were sacrificed. Fluorescence microscopy studies revealed that a thin layer of CM-DiI positive MSCs were localized at the outermost border of the PH liver sections only in 3 days post-PH animals (Fig. 3H). Untreated, 1 day and 5 days post-PH animals gave no detectable CM-DiI specific signal (Fig. 3D, 3F and 3J respectively). As expected, no staining in the liver sections was observed when MSCs were not injected (Fig. 3B). This data implicated that MSCs were accumulating in the liver upon liver injury in a specific timing (Fig. 3H).

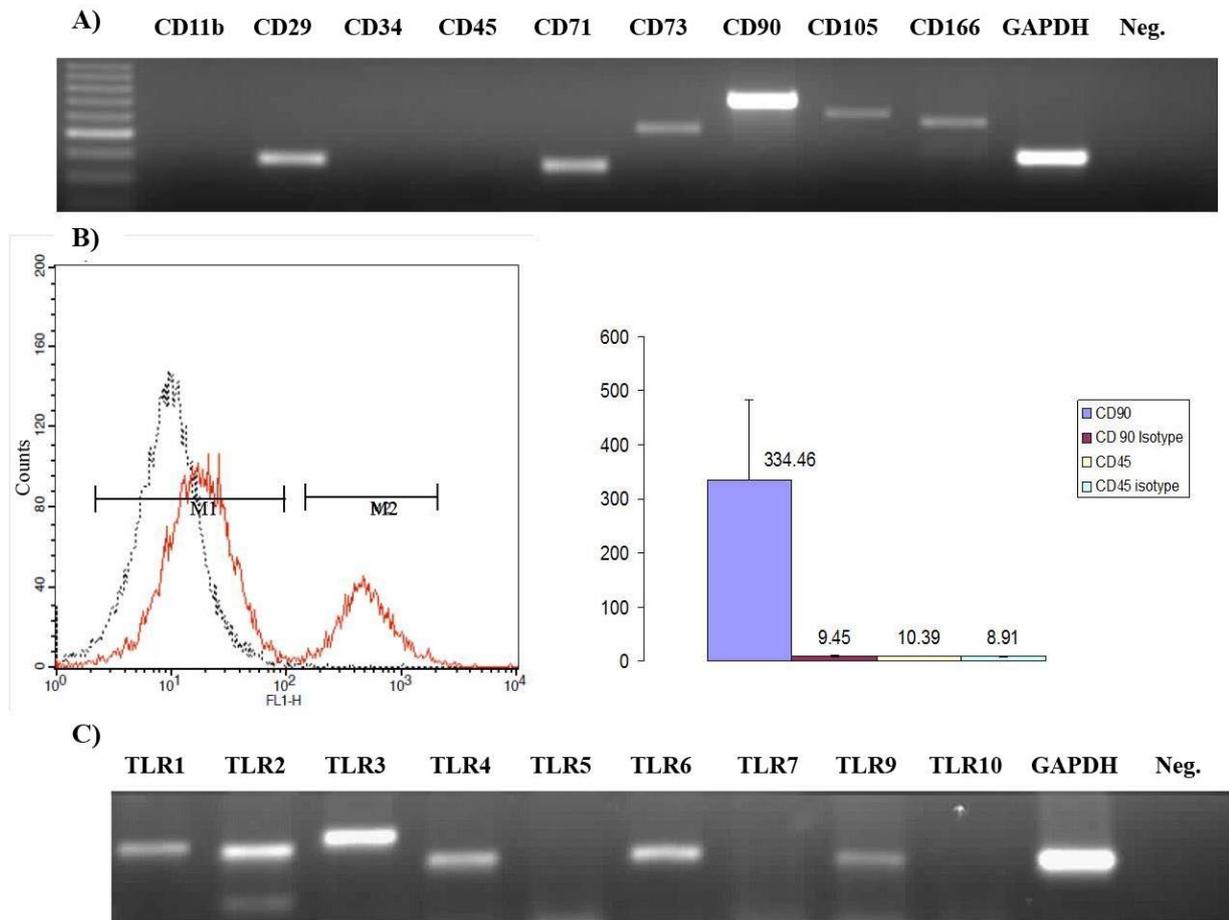


Fig. 2. Characterization and TLR expression profile of MSCs. A) Gel picture showing MSC-specific positive (CD 29, CD71, CD73, CD90, CD105 and CD 106) and negative (CD 11b, CD34 and CD45) marker transcripts, B) Quantification of CD90 and CD45 proteins expressed by MSCs (MSCs were positive for CD90 (84.5±7.2 % of total population, MFI: 334.5±150.3) and negative for CD45 (96.6±1.2 % of total population, MFI: 10.4±0.5)) by FACS (at day14), C) PCR gel image showing the MSC expression levels of rat (TLR1, 2, 3, 4, 6, and 9 mRNA) amongst TLR1-10.

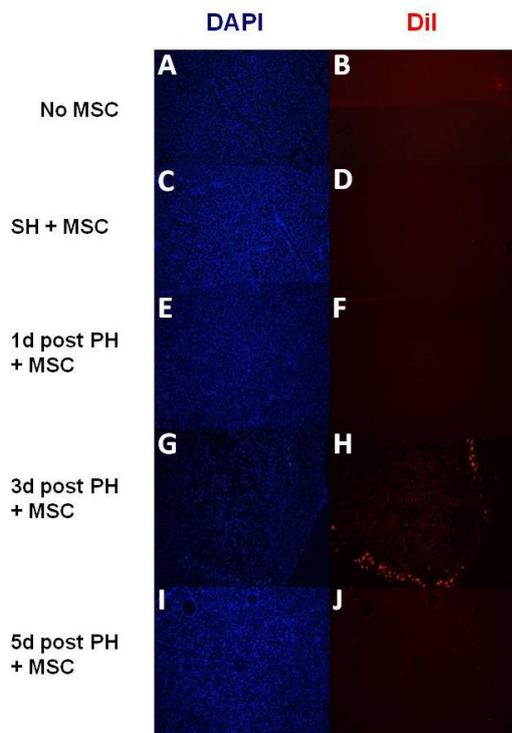


Fig. 3. Fluorescence photomicrographs of the liver sections injected with CM-DiI labeled MSCs. CM-DiI labeled MSCs were injected into SH (C, D), 1 day post-PH (E, F) 3 days post-PH (G, H) and 5 days post-PH (I, J) rats. Background staining from untreated and no MSC received liver sections (A, B). Thin layer of CM-DiI positive MSCs were localized at the outermost border of the PH liver sections only in 3 days post-PH animals (H). Arrows: CM-DiI labeled MSCs. Magnification: 20X.

CD90 Expression in Partially Hepatectomized Livers

After observing the presence of CM-DiI positive MSCs in PH liver, contribution of resident MSCs mediating liver regeneration process in the injured niche was examined. We performed immune staining for CD90 to investigate the recruitment of resident MSCs adjacent to CM-DiI labeled allogeneic MSCs (Fig. 4). The presence of the cells was shown by DAPI staining (Fig. 4A). The accumulation of the injected MSCs was evident by CM-DiI staining (Fig. 4B). Several resident CD90 positive MSCs (Fig. 4C) were present and scattered around CM-DiI-labeled MSCs as evidenced by merging of CM-DiI and FITC-CD90 (Fig. 4D) in the liver section of the 3 days post-PH rats. Consistent with earlier observations, no specific CD90 staining was observed for normal, 1 day and 5 days post-PH rats (data not shown).

FLT3 Expression in Partially Hepatectomized Livers

Oval cells are known to play pivotal role during progenitor-dependent liver regeneration and they express FLT3 on their cell surface (Alison 1998). Immunofluorescent staining against FLT3 was performed to check the existence of hepatic oval cells in the hepatectomized liver sections after the administration of CM-DiI-labeled MSCs (Fig. 5). FLT3 positivity was observed in the vicinity of CM-DiI positive MSCs in the liver of 3 days post-PH rats (Fig. 5A-D).

TLR Expression Following MSC Infusion During Liver Regeneration

In order to understand the contribution of TLRs during homing process of MSCs in liver regeneration, the changes of the expression patterns of several TLRs from normal and hepatectomized livers over 24 h by real-time RT-PCR were examined. From *Ct* values, fold change in expression for each TLR was plotted (Fig. 6A-F). From these plots, it was apparent that specifically four genes (TLR2, TLR3, TLR5 and TLR 9) remained unaltered (i.e. expression levels ~ 1.0) over the course of first 24 h post-PH compared to normal liver (Fig. 6A, 6B, 6D and 6F). Before investigating further, we omitted the potential contribution of TLR 5 due to the fact that MSCs do not express this mRNA (Fig. 2C).

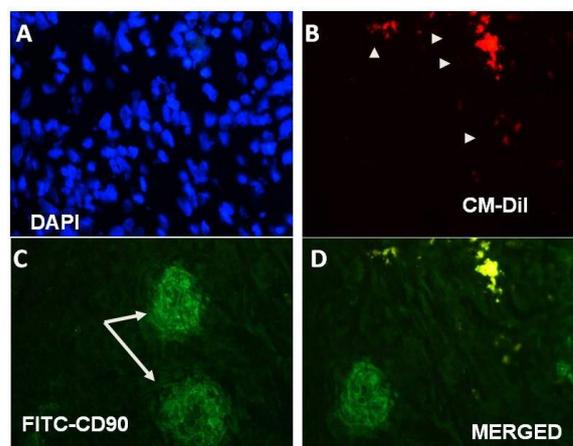


Fig. 4. Immunofluorescence photomicrographs showing CD90 expression. Sections were from 3 days post PH liver that had been injected with CM-DiI labeled MSCs revealing the contribution of resident MSCs mediating liver regeneration process in the injured niche. (A) DAPI, (B) CM-DiI (C) CD90-FITC (D) merged. Arrows: CD90+ areas. Arrow Heads: CM-DiI positive areas. Magnification: 20X.

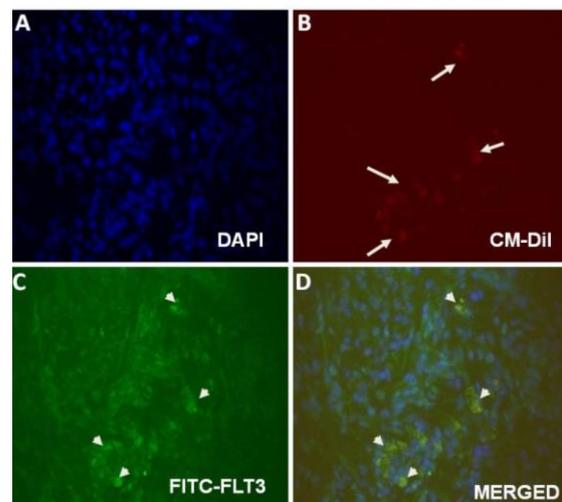


Fig. 5. Immunofluorescence photomicrograph showing FLT3 expression. Sections were from 3 days post PH liver that had been injected with CM-DiI labeled MSCs revealing the existence of hepatic oval cells in the hepatectomized liver sections. (A) DAPI (B) CM-DiI (C) FLT3-FITC and (D) Merged. Arrows: CM-DiI and arrow heads: FITC-FLT3 positive areas. Magnification: 20X.

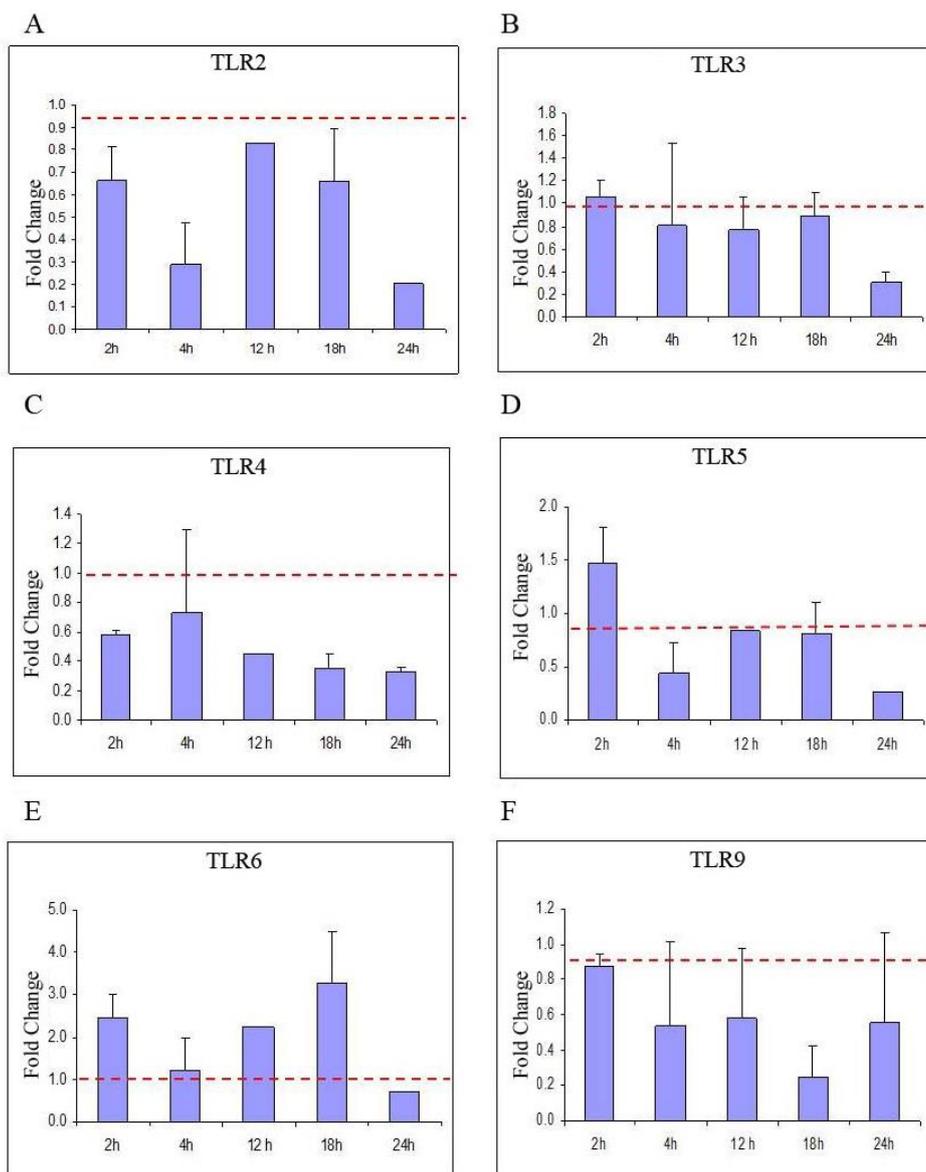


Fig. 6. qRT-PCR profiles showing time course fold change of (A) TLR2, (B) TLR3, (C) TLR4, (D) TLR5, (E) TLR6 and (F) TLR9 gene transcript levels following PH over 24 h. TLR2, TLR 3, TLR 5 and TLR 9 remained unaltered over the course of first 24 h post-PH compared to normal liver.

Next, we checked changes in TLR expression level upon MSC administration during liver regeneration. To our knowledge, no study has attempted to delineate the changes in TLR expression and MSC accumulation following injury. In order to differentiate whether TLR2, TLR3 and TLR9 are involved in the homing of administered MSCs to the injured site, the time-course fold change in their transcript levels was investigated. Our results revealed that TLR2, TLR3 and TLR9 mRNA expression levels increased in liver samples of partially hepatectomized rats that received labeled MSC in comparison to those animals that did not have any MSC treatment (Table 3). Following MSC injection, TLR2 and TLR9 mRNA levels were significantly higher than TLR3 in regenerating liver (ca. 7 fold for TLR2 and 9 vs ~2 fold for TLR3).

Table 3. Fold change in TLR2, TLR3 and TLR9 expression of PH animals before and after MSC administration from normal rats.

Rx groups	TLR2	TLR3	TLR9
Mock + MSC	1.9 ± 0.3	2.2 ± 0.5	2.8 ± 0.1
PH (1d)	1.3 ± 0.2	0.3 ± 0.1	1.6 ± 0.6
PH (1d) + MSC	5.7 ± 1.1	1.7 ± 0.4	3.9 ± 0.7
PH (3d)	1.3 ± 0.3	0.6 ± 0.2	1.2 ± 0.4
PH (3d) + MSC	7.7 ± 0.9	2.1 ± 0.1	4.3 ± 0.5

Discussion

In this study, we aimed to understand the immune response of liver upon BM derived MSCs infusion through changes in TLR expression of remaining liver and delineate their contribution to regeneration process.

There are several heartening therapeutical applications of MSCs in osteogenesis imperfecta, hematopoietic recovery, bone tissue regeneration, cardiovascular repair, spinal cord injury, coronary artery disease and also in several organ failures such as lung fibrosis in animal models (Horwitz *et al.* 1999, Koc *et al.* 2000, Petite *et al.* 2000, Minguell *et al.* 2001, Matty 2008). Although Popp *et al.* (2007) reported that multipotent mesenchymal stromal cells do not differentiate into hepatocytes *in vivo* when transplanted in regenerative conditions, Lee *et al.* (2004) showed that bone marrow-derived MCS from human differentiate into functional hepatocyte-like cells under defined conditions pointing out the potential for clinical relevance. Moreover, recent findings suggested that these cells can effectively rescue experimental liver failure (upon CCl₄ administration) and contribute to liver regeneration. Collectively, accumulating data implicated that MSCs are suitable cell-based tools as an alternative therapy to organ transplantation for the treatment of liver diseases (Kuo *et al.* 2008). The signals driving MSCs to the site of injury during healing is not convincingly resolved and there are conflicting reports describing the physiologic roles of TLR expression on isolated/generated MSCs. These controversies prompted us to investigate in detail the relevance between migration of MSC to and contribution of TLRs to this process using hepatectomized rat as a model in experimental liver regeneration.

In order to chase homing of MSCs in liver after PH, CM-DiI labeled MSCs from normal rats were administrated into the 1, 3 and 5 post-PH rats. Our results revealed that only in 3 day post-PH rat, MSCs were localized in the liver (Fig. 3H).

Next, we asked the question of whether endogenous MSCs also show the similar homing pattern to allogeneic MSCs during liver regeneration in terms of timing and localization. Our *immunofluorescein* data for CD90 staining in the liver sections revealed that the time of the appearance of endogenous MSCs are synchronous to that of exogenously localized allogeneic MSCs. These cell types were co-localized at the liver regeneration site 3 days post-PH (Fig. 4C and 4D). This suggested that either localized administered MSCs induced further accumulation of the syngeneic MSCs, or the accumulation process of the resident MSCs reached to a plateau, and furthermore initiated injected MSCs to localize at the injury site. Further studies to clarify these alternatives are required.

Oval cells are playing pivotal role in progenitor-dependent liver regeneration and are known to express specific proteins at their surfaces (Allison 1998). Among these, FLT3 is one of the candidate proteins (Agnes *et al.* 1994). FLT3 is a receptor tyrosine kinase (RTK) and a well-known hematopoietic stem cell marker (Agnes *et al.* 1994). We previously showed the expression of FLT3 both at mRNA and protein level during progenitor cell-dependent liver regeneration (Aydin *et al.* 2007). In this study, FLT3 expression was observed only when MSCs

administrated into 3 days PH rats in the vicinity of labeled MSCs. Expression of FLT3 around this location suggests the onset of progenitor-dependent liver regeneration. However, we do not know the factors that regulate the specific pattern of MSC localization in the regenerated liver. Future studies to elucidate these factors (such as chemo attractant molecules) are expected to better understand liver regeneration process and provide very useful information for possible therapies.

Faust *et al.* (2006) proposed that three major pathways may regulate the circuitry required for liver regeneration. These are i) cytokine, ii) growth factor and iii) metabolic networks linking liver function to cell growth and proliferation. It is proposed that the innate immune system plays an important role in the initiation of liver regeneration after partial hepatectomy (Hritz *et al.* 2008). In particular, IL6 and TNF α production by Kupffer cells are found to be required for initiation of liver regeneration after PH, although the activation processes are still unknown. TLRs are important upstream elements of proinflammatory cytokine networks. Watanabe *et al.* (2007) showed that IL6 and TNF α production decrease significantly leading to defective liver regeneration in MyD88^{-/-} mice after PH. However, it was also shown that TLR 2, 4 and 9 are not essential for NF- κ B activation and IL6 secretion (Seki *et al.* 2005, Campbell *et al.* 2006). The latter observation is particularly surprising since enteric-derived LPS (ligand of TLR4) was shown as the stimulating agent for proinflammatory cytokine production at the start of liver regeneration (Cornell 1985, Cornell 1990, Shiratori *et al.* 1996). Therefore, we decided to investigate the mRNA expression of several TLR genes in 0 h, 2 h, 4 h, 12 h, 18 h and 24 h after PH (Fig. 6). Our results revealed that induction in the expression of TLR 2, 3, 5 and 9 remain nearly unchanged during this testing period compared to the expression levels of normal animals at 0h.

We hypothesized that the mechanism of MSC homing to injury site may be due to TLR expression of the organ in which injury occurs, and investigated the relationship between TLR expression profiles of hepatectomized liver upon MSC infusion. It was shown that TLR stimulation on human MSC drives their migration *in vitro* (Tomchuck *et al.* 2008). In the light of this data, one can hypothesize that certain TLR expression at the injured tissue/organ may be critical for the MSC migration leading to regenerative process. Our results strongly suggest the involvement of TLR 2, 3 and 9 due to following observations: i) their expressions are relatively unchanged over the period of 24 hours after PH of the host animals in the absence of MSC infusion (Fig. 6), ii) mRNA expression levels of TLR 2, 3 and 9 are increased in MSC-administrated 1 day PH and 3 days PH rat liver samples in comparison to that of PH animals that had no MSC injection (Table 1). Thus, the injected MSCs induced the alteration of TLR 2, 3 and 9 levels in the remaining liver during regeneration.

In conclusion, our data suggested that the driving force of MSC homing is not dependent on TLR expression of MSCs, rather TLR upregulation in the micro environment of the injured liver in part may dictate MSC homing. Thus, immune response mediated by TLR signaling during regeneration process is critical for MSC homing.

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