LIVER REGENERATION: POTENTIAL ROLES OF MESENCHYMAL STEM CELLS AND TOLL LIKE RECEPTORS

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ABSTRACT

LIVER REGENERATION: POTENTIAL ROLES OF MESENCHYMAL STEM CELLS AND TOLL LIKE RECEPTORS

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Liver has unique capacity to regenerate in response to loss of hepatocytes depending on viral infections, toxic reactions and cancer formation. Although liver regeneration have been extensively studied, factors participate in the process are still under extensive investigation. Differentiation and immunosuppressive potential, as well as homing capacity to the site of injury of mesenchymal stem cells (MSCs) can be harnessed in liver regeneration. Moreover, toll like receptors (TLRs) which are known to involve immuneregulatory mechanisms of MSCs may also be a player in this process. Therefore, in this study, our aim was to understand the possible roles of MSCs and TLRs during liver regeneration. First, TLR message distribution upon partial hepatectomy induction was assessed. Then, homing capacity of MSCs in liver regeneration was examined by injecting labeled MSC generated from normal or already PH initiated rats back into partially hepatectomized (PH) rats. After investigating MSC homing, mRNA expression profiles of several key TLRs in the course of liver regeneration with or without MSC administration was examined. Finally, the role of TLRs in immunoregulatory properties of MSCs was explored. Our data showed that MSCs from normal rats and at day 1 post PH are localized in liver of PH generated animals. We have also determined that injected MSCs increased TLR2, 3 and 9 expressions in livers in comparison to that of hepatectomized liver that did not receive any MSC injection. Therefore, our data suggests that during liver regeneration at post PH day 3, homing of MSCs to the site of injury is at maximum and TLRs do not play any roles in recruiting these cells to liver, but rather localized MSCs either *cis* or at *trans* manner promote TLR expression. Despite the fact that MSCs are known to be non-immunogenic, in the present study isolated MSCs from BM are found to be expressing a panel of TLR mRNAs and our findings strongly implicate that these TLRs are functional in terms of cytokine secretion upon triggered by their proper ligands. Finally, we have identified that mouse MSC possesses different levels of critical surface markers such as CD11b, CD45, CD90 and CD117 at different passages and led us to think that either sub-populations or contaminating cell fractions may exist within the studied MSC population and furthermore may contribute to stimulatory potential of MSCs.

Keywords: Mesenchymal stem cells, liver regeneration, toll like receptors

ÖZET

KARACİĞER REJENERASYONU: MEZENKİMAL KÖK HÜCRE VE TOLL BENZERİ RESEPTÖRLERİN POTANSİYEL ROLLERİ

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Karaciğer, viral enfeksiyonlara toksik reaksiyonlara ve kanser oluşumuna bağlı olarak oluşan hepatosit kaybına cevap olarak kendini yenileyebilme kapasitesine sahiptir. Şimdiye kadar karaciğer rejenerasyonu geniş çapta çalışılmış olmasına rağmen, bu işleme katılan faktörler hala yoğun olarak çalışılmaktadır. Mezenkimal kök hücrelerin (MKH) farklılaşması ve immün baskılayıcı potansiyelleri kadar hasarlı bölgeye yönelme ve yerleşme kapasiteleri de onları karaciğer rejenerasyonunda önemli kılar. Ayrıca MKH'lerin bağışıklılığı düzenleme mekanizmalarına katılan toll benzeri reseptörler (TLR) de bu işlemde rol alıyor olabilirler. Bu çalışmadaki ana amacımız karaciğer rejenerasyonu esnasında MKH ve TLR'lerin iyileşme sürecine olası katkılarını araştırmaktır. Öncelikle, kısmi hepatektomi sonrasında TLR mesaj dağılımı belirlendi. Sonra, MKH'ların karaciğer rejenerasyonu esnasında hasarlı bölgeye yerleşme kapasiteleri kısmi hepatektomi (PH) yapılmış veya normal sıçanlardan alınan MKH'lerin işaretlenmesi sonrasında yine PH'lı sıçanlara geri verilmesiyle araştırıldı. Çalışmanın ileri evresinde, karaciğer rejenerasyonunda önemli olan bazı TLR'lerin mRNA ifade temelli profilleri ya sadece PH yapılmış ya da PH yapıldıktan sonra işaretli MKH enjekte edilmiş sıçanlarda araştırılmıştır. Son olarak, TLR'lerin, MKH'lerin bağışıklılığı düzenleyici özelliklerindeki rolleri araştırılmıştır. Sonuçlarımız normal ve bir günlük PH geçirmiş sıçanlardan alınan MKH'lerin, PH geçirmiş hayvanlara verildikten sonra o bölgeye yerleştiğini göstermektedir. Ayrıca PCR çalışmalarımız MKH enjeksiyonunun, MKH almamış PH geçirmiş karaciğerdekine göre TLR2, 3 ve 9 gen mesaj ifadelerini arttırdığını saptadık. Bu nedenle, verilerimiz gösteriyor ki PH 3. gün sonrası karaciğer rejenerasyonu süresince, MKH'lerin yaralanma bölgesine yerleşmesi en yüksek seviyededir ve TLR'ler bu hücrelerin karaciğere çağırılmalarında rol oynamamaktadırlar. Bunun aksine enjekte edilmiş MKH'ler TLR ifadesini cis veya trans şekilde yönlendirmektedirler. MKH'lerin non-immünogenik olarak bilinmelerine rağmen, bu çalışmada kemik iliğinden izole edilen MKH'lerin birçok TLRı ifade ettikleri saptanmıştır ve bulgularımız bu TLR'lerin uygun ligandlarla uyarıldıklarında sitokin salgılanmasında fonksiyonel olduklarını göstermiştir. Son olarak, bu hücrelerin CD11b, CD45, CD90 ve CD117 gibi çok önemli yüzey belirteçlerine pasaj sayısına bağlı olarak farklı derecelerde değiştiğini saptadık ve bu bulgular kullandığımız MKH popülasyonunda ya alt grupların, ya da kontamine hücre gruplarının bulunduğunu düşündürmektedir. Belki de bu hücre grupları MKH'lerin uyarıcı özelliklerini yönlendirmektedirler.

Anahtar Kelimeler: Mezenkimal kök hücre, karaciğer rejenerasyonu, toll benzeri reseptör

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ABBREVIATIONS

APC	Antigen Presenting Cell
bp	Base Pairs
BFB	Bromophenol Blue
BSA	Bovine Serum Albumin
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFU-F	Colony-forming Units-fibroblastic
CYC	Cyclophilin
ddH_2O	Double Distilled Water
DC	Dendritic Cell
DEPC	Diethylpyrocarbonate
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dsRNA	Double-stranded RNA
EAE	Experimental Autoimmune Encephalomyelitis
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked-Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
FLT3	Fms-related Tyrosine Kinase
g	Grams
GVHD	Graft-versus-host Disease
HGF	Hepatocyte Growth Factor
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
ICAM	Intercellular Adhesion Molecule
IF	Immunofluorescent
IFN	Interferon
IKK	IκB Kinase
IκB	Inhibitory of NFκB

Ig	Immunoglobulin
IL	Interleukin
IRAK	IL1R Associated Kinase
IRF	IFN- Regulatory Factor
μg	Microgram
μl	Microliter
lt	Liter
LCT	Liver Cell Transplantation
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
LTA	Lipoteichoic Acid
Mac-1	Macrophage-1 antigen
M	Molar
MetOH	Methyl Alcohol
mg	Milli Gram
mL	Milli Liter
mM	milliMolar
MOPS	4-Morpholinopropanesulfonic Acid
MS	Multiple Sclerosis
MSC	Mesenchymal Stem Cell
MyD88	Myeloid Differentiation Primary Response Gene 88
NF-κB	Nuclear Factor-kappa B
NaCl	Sodium Chloride
NK	Natural Killer
NLR	NOD-like Receptors
NOD	Nucleotide Binding Oligomerization Domain
OD	Optical Density
ODN	Oligodeoxynucleotide
OLT	Orthotopic Liver Transplantation
PAMP	Pathogen associated molecular pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cells

PFA	Paraformaldehyde
PGN	Peptidoglycan
PH	Partial Hepatectomy
PI3K	Phosphotidyl Inositol 3-Kinase
pI:C	Polyriboinosinic Polyribocytidylic Acid
PNPP	Para-nitrophenyl Pyro Phosphate
PRR	Pattern Recognition Receptor
RIG-I	Retinoic Acid-inducible Gene I
RLH	RIG-I-like helicase
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase
SA-AKP	Streptavidin Alkaline-phosphatase
SH	Sham
ssRNA	Single Stranded RNA
TAE	Tris Acetate EDTA
TGF	Transforming Growth Factor
TIR	Toll/IL1 Receptor
TIRAP	Toll/IL1 receptor-associated Protein
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF-associated Factor
TRAM	TRIF-related adaptor molecules
TRIF	TIR-domain containing adaptor inducing IFN-β
XC	Xylene Cyanol

1. INTRODUCTION

Since the first development of the technique of orthotopic liver transplantation (OLT) in the early 1960s (Starzl *et al*, 1963), several improvements have been made in this field. Although OLT is considered as a gold standard treatment for liver failure, organ shortage problem leads researchers to study on alternative solutions. Despite having limiting factors as cell viability, modest engraftment and limited tissue viability (Philippe *et al*, 2008), liver cell transplantation (LCT) is one of the developing alternative with increasing success (Najimi and Sokal, 2005; Stephenne *et al*, 2006). Stem cells are very potent candidates for LCT due to their plasticity (Verfaillie, Pera and Landsdrop, 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 potential (Philippe *et al*, 2008).

In addition to their hepatic potential, MSCs have two other important properties that make them critical for LCT. One of them is their preferential migration into injured site, which results in targeting of the cells to the site of liver failure to a certain extent. The other one is their immunoregulatory properties. They are not only non-immunogenic but also immunosuppressive. Due to these features MSCs shine as a very promising tool for cell-based therapies including liver diseases. Being non-immunogenic is extremely important in every kind of transplantation providing a wide size of donor population.

In recent decades, immunoregulatory properties of MSCs have been extensively studied. Although mechanism is unclear yet, there seems to be several factors playing role in immunoregulation. Pevsner-Fisher *et al.* (2007) has recently shown that murine MSCs express several Toll-like receptors (TLRs) and in particular TLR2 was found to be important in differentiation potential of MSCs. This leads us

to think whether there is a connection between TLRs and immunoregulatory property of MSC.

In this context, firstly liver regeneration will be explained. Then, next part will be about general characteristics and immunoregulatory properties of MSCs. In the last part, general characteristics and individual TLRs will be defined.

1.1. Liver Regeneration

Liver is an interesting organ with high regenerative capacity and complex functions (Michalopoulos and DeFrances, 1997; Taub, 2004). Liver regeneration has been an object of curiosity since the ancient Greeks recognized it in the myth of Prometheus. It is likely to be evolved to protect animals from the catastrophic results of liver loss caused by food toxins in nature (Fausto *et al*, 2006). It has also been shown that animals can survive surgical removal up to 75% of the total liver mass in experimental conditions (Bucher and Swaffield, 1964).

Accumulating evidences has shown that there are at least three distinct liver regeneration mechanisms (Grompe and Finegold, 2001):

1. liver regeneration during normal tissue turnover
2. hepatocyte-driven regeneration after liver injury
3. progenitor-dependent regeneration after liver injury

1.1.1. Liver Regeneration During Normal Tissue Turnover

The average life span of adult mammalian hepatocyte has been estimated to be around 200–300 days. One of the models regarding the mechanism of normal liver turnover is termed as “streaming liver”. Ponder (1996) suggested that the normal turnover in adult animals was proceeded primarily by *in situ* cell division of hepatocyte and not by stem cells. According to this model, young hepatocytes

emerge in portal zone and migrate toward central vein. In parallel to this model, differential gene expression patterns were seen in different zones of liver and this was explained by aging during the migration representing typical lineage progression. Moreover, ploidy and size of hepatocytes depend on the location within the lobule (Zajicek *et al*, 1985; Arber *et al*, 1988). However, there were other studies opposing "streaming liver" hypothesis. In 1985, Thurman and Kauffman showed that the difference in gene expression was due to the direction of blood flow. Thus, lobular zonation seems to depend on metabolite-induced gene regulation rather than lineage progression. Besides, hepatocyte migration during normal hepatocyte turnover was also opposed by retroviral marking studies (Bralet *et al*, 1994; Kennedy *et al*, 1995). These results were later confirmed in studies of hepatocyte growth pattern analysis in mosaic pattern of X inactivation in female mice (Shiojiri *et al*, 1997, 2000).

1.1.2. Hepatocyte-Driven Regeneration After Partial Hepatectomy

Higgins and Anderson (1931) introduced the best experimental model with a precisely defined initiation of the regenerative stimulus, partial hepatectomy (PH), for the study of liver regeneration in response to liver injury. In partial hepatectomy, two-thirds of the liver is removed. Specific liver lobes are removed intact, without damage to the lobes left behind. Although the resected lobes never grow back, the residual lobes enlarge to compensate for the mass of the removed lobes in one week (Fausto *et al*, 2006).

The difference of liver regeneration from other regenerating tissues (bone marrow, skin) is that regeneration is not dependent on a small group of progenitor or stem cells except the case of large number of hepatocyte loss (see section 1.1.3). In order to rebuild the lost hepatic tissue, all the existing mature cellular populations in intact organ proceed liver regeneration by proliferating after PH. These include hepatocytes (the main functional cells of the organ), biliary epithelial cells (lining biliary ducts), fenestrated endothelial cells [a unique type of endothelial cells with large cytoplasmic gaps (fenestrae) that allow maximal contact between circulating blood and hepatocytes], Kupffer cells (macrophages in hepatic sinusoids) and cells of

Ito (satellite cells unique to the liver and located under the sinusoids; which surround hepatocytes with long processes, store vitamin A, synthesize connective tissue proteins, and secrete several growth factors) (Michalopolus and DeFrances, 1997).

Hepatocytes are the first to proliferate with a peak of DNA synthesis at around 24 hours (Figure 1.1.). The other cells of the liver enter into DNA synthesis about 24 hours after the hepatocytes and peak at 48 hours or later. After two to three days during PH, all cellular elements of the liver proliferate. Liver histology at day three to four after PH is characterized by clumps of small hepatocytes surrounding capillaries (Martinez-Hernandez and Amenta, 1995). By day 7, original number of cells is restored and hepatic histology consists of lobules that are larger in size prior to regeneration (Ogawa *et al*, 1979; Michalopolus and DeFrances, 1997).

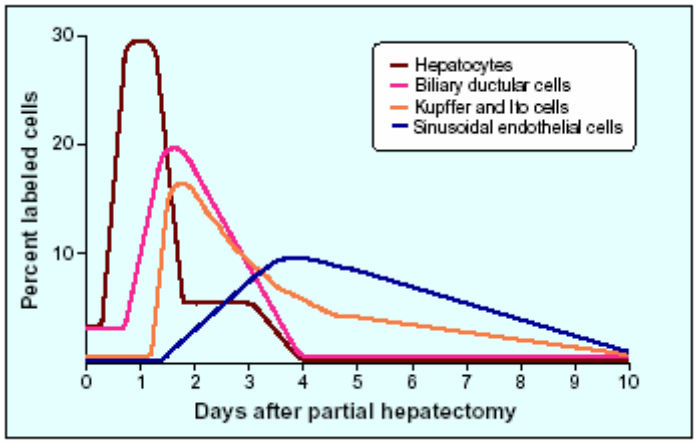


Figure 1.1. Time kinetics of DNA synthesis in different liver cell types during liver regeneration after partial hepatectomy.

1.1.2.1. Mitogenic Signals Associated With Initiation of Liver Regeneration

There are several factors affecting the induction of hepatocyte cell division. As indicated above, hepatocytes are the first cells of the liver to enter into the cell cycle and undergo proliferation, and they produce mitogenic signals for other hepatic cell types. Several studies have shown that mitogenic signals are present in the blood during liver regeneration. Initiation of liver regeneration may be attributed to that of mitogenic signals in a variety of mechanisms.

Amongst these factors, Hepatocyte growth factor (HGF) and its receptor Met-1 are key factors in liver growth and function (Naldini *et al*, 1991). HGF is responsible for the early events after PH. Several studies in humans have shown that plasma concentrations of HGF rise substantially when hepatic mass is decreased and plasma concentrations of HGF rise more than 20-fold within one hour after PH in rats (Michalopoulos *et a.*, 1984; Tomiya *et al*, 1992, Lindroos *et al*, 1991). It is thought that PH confers stored HGF to be released by remodeling extracellular matrix and releases HGF which triggers hepatocytes to re-enter cell cycle through a signal transduction pathway (Bottaro *et al*, 1991).

Besides HGF, there are a variety of factors associated with liver regeneration both directly and indirectly. Most important ones are TNFα, IL6, EGF and TGFα. In deficiency of IL6 and TNFα receptor 1 (TNFR1), there seems slow and incomplete response following PH (Yamada *et al*, 1997, 1998; Yamada and Fausto, 1998). Although TNFα is not a direct mitogen for hepatocytes, its level increases after PH. TNFα was found to enhance the mitogenic effects of direct mitogens such as HGF, both *in vitro* and *in vivo* (Webber *et al*, 1998). IL6 also is not a direct mitogen for hepatocytes and does not enhance the mitogenic effect of other growth factors. However, it is a direct mitogen for biliary cells (Liu *et al*, 1998) and has important effects on integrity of the intrahepatic biliary tree by regulating production of small proline-rich proteins by cholangiocytes (Nozaki *et al*, 2005; Demetris *et al*, 2006). IL6 increases in the plasma following PH.

Although plasma EGF concentrations rise only slightly after PH, EGF may play a mitogenic role in liver regeneration by quickly becoming more available to hepatocytes after PH.

TGFα mRNA is induced in hepatocytes within 2 - 3 hours after PH, with a peak between 12 and 24 hours, and remains elevated for at least 48 hours after PH, and its over expression is shown to drive hepatocyte replication *in vivo*.

Termination of liver regeneration is also as important as initiation. However, compared to initiation mechanism, termination is less understood. It is found that a small wave of apoptosis in hepatocytes occurs at the end of regeneration (Sakamoto *et al*, 1999). Some studies suggest TGFβ as a potent terminating signal for liver regeneration (Jirtle *et al*, 1991). No other specific candidate for termination is known at this point, though potential ones can be predicted depending on their function in physiological conditions such as tumor suppressor genes.

1.1.3. Progenitor Dependent Regeneration After Liver Injury

1.1.3.1. Hepatic Oval Cells

When there is a severe liver damage because of losing large number of hepatocyte and/or lack of their proliferation due to hepatoxins or carcinogens, liver utilizes a new source of cells to help repairing the liver. These small cells with high nucleus to cytoplasm ratio and ovoid shape are called hepatic "oval" cells. Oval cell proliferation is the result of progenitor-dependent regeneration since they are not derived from hepatocyte; rather they are associated with canals of Hering. However, the origins of oval cells are still debatable. Although most researchers tend to believe that the cells reside in the canals of Hering, it has been clearly indicated that at least a portion of the oval cells can be derived from bone marrow cells. In response to severe damage, these cells emerge in the portal zone, proliferate extensively and migrate into the lobule and eventually differentiate into hepatocytes (Farber, 1956; Shinozuka *et al*, 1978). These cells have the ability to proliferate-clonogenically and their bipotential capacity provides to differentiate into both hepatocytes and bile ductular cells (Oh *et al*, 2002). When hepatocytes do not respond to growth signals, oval cells are activated and proliferate rapidly as "facultative stem cells". They initially appear near bile ductules and then migrate into the hepatic parenchyma. Oval cells were shown to change first into basophilic small hepatocytes and then differentiate into mature hepatocytes. There are several surface markers expressed on oval cells including hepatic markers and hematopoietic stem cell markers. Most commonly used markers are AFP, Thy-1, C-KIT, FLT3, CH18, CK19, GGT and OC2.

In order to induce progenitor-dependent regeneration, in rat liver, the combination of 2-AAF treatment with either two-thirds partial hepatectomy (PH) or LD50 dose of carbon tetrachloride can be used. The continuous administration of low dose 2-AAF suppress proliferation of hepatocytes. Thus, hepatocytes of rats can not proliferate for regeneration after PH resulting in rapid proliferation of oval cells.

1.2. Mesenchymal Stem Cells

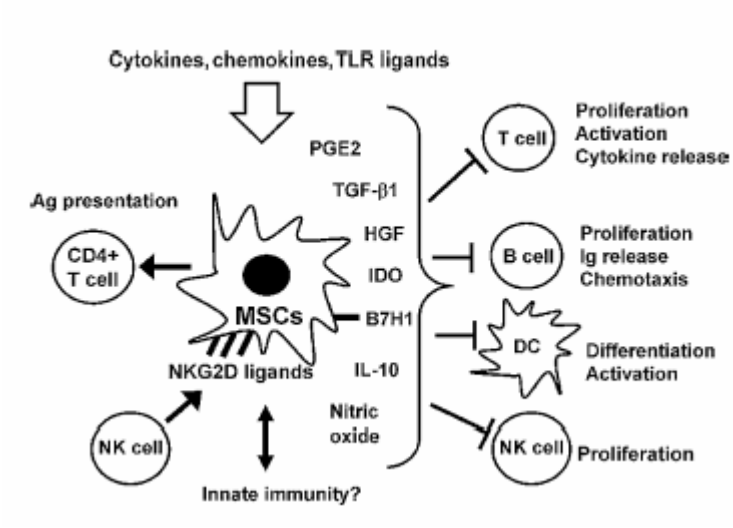
Mesenchymal stem cells (MSCs) are adult stem cells constituting a rare population of non hematopoietic origin (0.01-0.001 % of total nucleated cells) in the adult bone marrow (Pitteger *et al*, 1999). They were identified during isolation of bone-forming progenitor cells from rat marrow by Friedenstein *et al* in 1966. They have the capacity to proliferate extensively and form colonies of fibroblastic cells which are defined as colony-forming units-fibroblastic; CFU-F (Friedenstein *et al*, 1970). They differentiate into multiple mesenchymal lineage including adipocytes, osteoblasts, chondrocytes and myocytes (Pitteger *et al*, 1999) and cardiomyoctes *in vitro* (Makino *et al*, 1999). Besides mesenchymal lineage, it has been shown that they can differentiate along a number of endodermal and ectodermal tissues such as neurons (Woodbury, 2000), epithelial cells in skin, lung, intestine, kidney, spleen and liver (Chapel *et al*, 2003). To be able to differentiate into non-mesenchymal cell lineages demonstrate their plasticity and their potential in cellular therapies in several tissue repair and regeneration processes (Baksh *et al*, 2004).

One of the most challenging aspects of working with MSCs is the lack of specific cell surface markers for identification and characterization. They have common characteristics with other types of cells including endothelial and epithelial cells rather than displaying non-unique antigen profile. They express a number of different adhesion-related antigens, such as integrin subunits α4, α5, β1, integrins αvβ3 and αvβ5, ICAM-1, and CD44H (Conget and Minguell, 1999). Besides these nonspecific markers, there are some widely used cell surface antigens for *in vitro* characterization of MSCs, such as STRO-1 in human (Gronthos *et al*, 1994) and SH-

2, SH-3, SH-4 (Baksh *et al.*, 2004), CD71, CD90, CD106 and CD117 (Mangi *et al*, 2003) in rodents. Despite this controversy about surface markers defining "mesenchymal stem cell", there is a general agreement that the absence of CD34, CD45, CD31, Mac1 (CD11b), CD19 and glycophorin A expression distinguishes MSCs from hematopoietic cells, endothelial cells and endothelial progenitors (Deans and Moseley, 2000).

In recent decades, MSCs received increased interest due to their intrinsic self-renewal capacity and ability to differentiate functional cell types in specific tissues. These properties provide MSC as a promising candidate for cell and gene therapies. There are several heartening therapeutical applications of MSCs including osteogenesis imperfecta (Horwitz *et al*, 1999), hematopoietic recovery (Koç *et al.*, 2000), bone tissue regeneration (Pettite *et al*, 2000), cardiovascular repair, spinal cord injury, coronary artery disease (Minguell, 2001) and in also several organ failures such as lung fibrosis in animal models (Matty, 2008).

Besides their involvement in tissue repair and regeneration, one of the most crucial characteristics of MSCs is their immunoregulatory property (Bartholomew *et al*, 2002; Inoue S. *et al*, 2006; Aggarwal and Pitteger, 2005). They can evade from immunerecognition (Rasmusson, 2006), therefore they are ideal carriers for gene delivery. In particular, generating stable clones with high efficiency with adenovirus-mediated gene transfer make them popular in gene therapy (Baksh *et al*, 2004). Moreover, MSCs suppress immune responses (Rasmusson, 2006). Through multiple pathways, *ex-vivo* expanded MSCs have been shown to suppress a broad range of immune cells including T cells, B cells, NK cells and antigen presenting cells as shown in Figure 1.2. On the other hand, recent studies showed that MSCs have the dual ability to suppress and/or activate immune responses due to exposed stimulus (Stagg, 2007).



(Stagg, 2007)

Figure 1.2. Immunemodulating effects of *ex-vivo* expanded MSCs.

MSCs also suppress immune responses *in vivo*. Acute graft-versus-host disease (GVHD) is a common complication of allogeneic hematopoietic stem cell transplantation (HSCT) in which the immunocompetent cells in the graft reacts against host-derived antigens. After transplantation, mature T cells in the graft attack recipient tissue resulting in activation of host with secretion of proinflammatory cytokines including TNF-α and IL-1. In acute GVHD, donor T cells react to host APCs by activating donor T cells in a sequential manner. Since human MSC suppress the formation of cytotoxic T cells and alter the cytokine profile and maturation of antigen presenting cells, they may be used in potential cellular therapy in GVHD (Rasmusson, 2006). However, conflicting results have been reported regarding benefits of using MSC on GVHD. Among several studies, one of the most striking one showed that intravenous injection of MSC was found to be effective therapeutically in humans (Le Blanc *et al*, 2004). On the other hand, MSCs were found to be suppressing lymphocytes *in vitro* but have no effect on GVHD in mice (Sudres *et al*, 2006). MSCs are also used in autoimmune diseases. Experimental autoimmune encephalomyelitis (EAE), which is the "paradigmatic" model of multiple sclerosis (MS), is an autoimmune inflammatory disease of the central nervous system mediated by T cells and macrophages. Zappia E. *et al* (2005)

reported that intravenous injection of murine MSCs attenuated EAE by inducing tolerance of peripheral T cell against the pathogenic antigen.

1.3. Immune System Responses

Immune system can be categorized into two distinct branches known as the "innate immunity" and the "adaptive immunity". Adaptive immunity detects non-self through recognition of peptide antigens using antigen receptors expressed on the surface of B and T cells (Takeda *et al*, 2003) which is instructed by the innate immune arm. The initial immune response is mediated by the cells of the innate immunity that can recognize pathogens as well as altered self proteins expressed on host cells. One of the major and well studied receptor families of innate immunity is the toll like receptor family, which is a part of a broader group known as the pathogen recognition receptors.

1.3.1. Pathogen Recognition Receptors

Although the innate immune system lacks the fine specificity of adaptive immune system that is crucial for immunological memory, it can discriminate self from non-self. The basic machinery of innate immune recognition is highly conserved among species, from plants and fruit flies to higher mammals. Microbial pathogens possess specific molecular patterns called pathogen-associated molecular patterns (PAMPs). The host innate immune system recognizes these patterns with germ-line encoded pattern recognition receptors (PRRs) and elicits immune responses for eliminating pathogen and also activating adaptive immunity (Akira *et al*, 2001).

Besides recognizing PAMPs which are crucial for survival of microorganism, there are several common characteristics of PRRs. First, they are expressed constitutively in the host. Second, PRRs are germ line encoded, nonclonal, expressed on all cells of a given type. Their expression is independent of immunologic memory. Different PRRs react with specific PAMPs and they can detect pathogens in

all stages of pathogen's lifecycle. They show distinct expression patterns, activate specific signaling pathways, and lead to distinct anti-pathogen responses (Akira *et al.*, 2006).

Toll-like receptors (TLRs), which recognize a wide range of PAMPs including carbohydrates, lipids, proteins and nucleic acids, are well-known PRRs (Akira *et al*, 2006). Besides TLRs, there are two other important PPRs that play important roles in cytosolic recognition of invading pathogens: nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) which have role in TLR-independent activation of several signaling pathways and retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) which have role in antiviral responses (Kawai and Akira, 2006). I would like to focus only on TLRs for the scope of my thesis work.

1.3.2. TLRs as Pattern-Recognition Receptors

TLRs are type I integral membrane glycoproteins that are characterized by the extracellular domains containing varying numbers of leucine-rich-repeat (LRR) motifs and a cytosolic signaling domain called the Toll/IL1R (interleukin 1 receptor) homology (TIR) domain which is homologous to the cytoplasmic signaling domain of IL-1 (Bowie and O'Neil, 2000). Toll, a receptor playing critical role in development of embryonic dorsoventral polarity and also antifungal response in *Drosophila*, is the first identified member of TLR family (Lemaitre *et al*, 1996). TLRs are also characterized by their evolutionary conservation from *Caenorhabditis elegans* to mammals (Roach *et al*, 2005; Akira *et al*, 2006).To date, 13 murine TLRs and 10 human TLRs have been identified (Kumagai *et al*, 2008). They have different ligand specificities and they presumably induce different target genes by same or different signaling pathways.

TLRs are expressed not only on various immune cells such as macrophages, dendritic cells (DCs), B cells, specific types of T cells but also on nonimmune cells including fibroblasts and epithelial cells (Kumagai *et al*, 2008). In addition, murine

MSCs have been shown recently to express several TLR molecules (Pervsner-Fischer *et al*, 2007). 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 (Figure 1.3. and Figure 1.4.). Ligands of latter ones, mainly nucleic acids, are required to be internalized to endosomes for recognition.

1.3.2.1. TLR1, 2 and 6

One of the most extensively studied TLRs is TLR2. It recognizes a wide range of microbial components including lipoproteins/lipopeptides from various pathogens, peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, lipoarabinomannan from mycobacteria, glycosylphosphatidylinositol anchors from *Trypanosoma cruzi*, a phenol-soluble modulin from *Staphylococcus epidermis*, zymosan from fungi and glycolipids from *Treponema maltophilum* (Takeda *et al.*, 2003), LPS (lipoploysaccharide) preparation from non-enterobacteria such as *Helicobacter pylori* (Smith *et al*, 2003).

Although there is no certainty about mechanism of the wide spectrum of TLR2 recognition, it is thought that functional cooperation of TLR2 with several proteins accomplishes this task. For recognition of lipopeptide, TLR2 functions by forming heterodimers with structurally related TLRs such as TLR1 or TLR6. Forming heterodimers with either TLR6 or TLR1 may provide the recognition of diacyl or triacyl lipopeptides, respectively. This discrimination may be attributed to LRR domain topology, which is a unique property of TLR2 (Takeuchi *et al*, 2001; Takeuchi *et al*, 2002; Omueti *et al*, 2005). For recognition of fungal-derived components, TLR2 functions with members of the lectin receptor family, which may facilitate its activation by β-Glucan ligands (Gantner *et al.*, 2003). In particular, C-type lectin, Dectin-1, with ITAM motif in its intracellular domain, was found to interact with TLR2 to recognize yeast pathogens (Brown *et al*, 2003).

The expression and activity of TLR2 was shown to be cooperatively regulated by pro-inflammatory molecules such as TNFα and anti-inflammatory molecules such as glucocorticoids (Hermoso *et al*, 2004).

1.3.2.2. TLR3

TLR3 is also an important PRR in the host defense against viral infection. In life cycle of most viruses (in replication), double-stranded RNA (dsRNA) is produced and triggers antiviral and immunostimulatory activities by inducing synthesis of type I interferon (IFNα/β). It was found that expression of human TLR3 in the dsRNA-non-responsive cell line 293 elicits activation of NF-κB in response to dsRNA. Moreover, TLR3 deficient mouse was found to lack antiviral activity in response to dsRNA (Alexopoulou *et al*, 2001). These showed that TLR3 has a major role in the recognition of dsRNA (Takeda *et al*, 2005). TLR3 is known to be expressed by dendritic cells (DCs), macrophages, and epithelial cells. Moreover NK cells were found to respond to polyriboinosinic polyribocytidylic acid (polyI: C; synthetic dsRNA) through production of proinflammatory cytokines, including IL6 and IL-8, as well as the antiviral cytokine IFN-γ (Schmidt, 2004).

1.3.2.3. TLR4

In last decade, importance of TLR4 in LPS (major component of the outer membrane of Gram-negative bacteria) recognition was demonstrated with the studies showing that TLR4 point mutation causes LPS hyposensitivity in mice (Poltorak *et al*, 1998; Hoshino *et al*, 1999). Similar to TLR2, TLR4 respond not only to LPS but also other molecules such as taxol derived from *Taxus brevifolia* (Kawasaki *et al*, 2000) and respiratory syncytial virus fusion protein (Kurt-Jones *et al*, 2000). In addition, TLR4 has been shown to be activated by very high concentrations of endogenous ligands, such as heat shock proteins (HSP60 and HSP70), extra domain A of fibronectin, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen (Takeda and Akira, 2005).

LPS recognition by TLR4 requires several accessory molecules. LPS-binding protein (LBP) is a serum protein to which LPS is bound. It transfers LPS monomers to CD14 (Wright, 1989). CD14 which can be either secreted to serum or expressed as membrane-associated glycosylphosphatidyl inositol-linked protein at the surface of cells (especially in peripheral blood monocytes and macrophages) is a co-receptor of TLR4. After binding to CD14, LPS comes in close proximity with TLR4. Besides, MD-2 is another component of receptor complex (Shimazu, 1999).

1.3.2.4. TLR5

TLR5 recognizes flagellin subunits of flagellum, which is a complex structure required for bacterial motility (Smith *et al*, 2003). These flagellin subunits induce inflammatory mediators, such as TNFα and IL-8 in epithelial cells (Eaves-pyles *et al*, 2001). Moreover, it was found that flagellin signaling is abrogated by the TLR5 allele polymorphism 392STOP and it has been associated with *Legionella pneumophila* pneumonia (Hawn *et al*, 2003).

1.3.2.5. TLR7, TLR8

TLR7 and TLR8 are structurally highly conserved proteins, and recognize same ligands in some cases. Both TLRs are expressed in mice but mouse TLR8 appears to be non functional (Akira *et al*, 2006). TLR7 is highly expressed in plasmacytoid dendritic cells. These cells produce IFN type I (α/β IFN) in response to viral infection. TLR7 and TLR8 recognize viral and nonviral single stranded RNA (ssRNA) and activate cytokine production through interferon regulatory pathway; IRF3 and IRF7 (Kawai *et al*, 2004; Ito *et al*, 2005). TLR7 and human TLR8 was found to recognize guanidine- or uridine-rich ssRNA from viruses including human immunodeficiency virus (Heil *et al*, 2004), vesicular stomatitis and influenza virus (Lund *et al.*, 2004; Diebold *et al*, 2004). Moreover, imidazoquinoline (R848), which is crucial in treatment of genital warts, is also recognized by human TLR7 and TLR8 (Hemmi *et al*, 2002).

1.3.2.6. TLR9

CpG dinucleotides have recently gained attention because of their immunostimulatory properties. Although bacterial DNA contains several unmethylated CpG motifs, the frequency of CpG motifs decreases significantly in vertebrates. In addition to reduction in terms of quantity, cystein residues of CpG motifs are highly methylated in vertebrate. Methylation in vertebrates leads impairment of immunostimulatory effect of CpG (Krieg *et al*, 1995). TLR9 was found to be responsible for unmethylated CpG recognition in TLR9-deficient mice studies (Hemmi *et al*, 2000). It is primarily expressed on B cells, NK cells and DCs in the course of proliferation, maturation and cytokine, chemokine of immunoglobulin (Ig) secretion (Krieg, 2000). There are at least two types of CpG DNA which differentially activate plasmacytoid dendritic cells (pDC) versus B cells. First identified, conventional CpG is B/K- type CpG. It is made up of phosphorothioate backbone and it has more than one CpG motifs on a single backbone with no poly (G) tail. The other one, A/D- type CpG, is structurally different from conventional CpG in terms of having phosphodiester/phosphorothioate mixed backbone. It has a single palindromic sequence outside the CpG motif linked to a poly (G) tail at the 3' and 5' ends. B/K-type CpG can induce inflammatory cytokines including IL12 and TNFα and B cell proliferation. On the other hand, A/D-type CpG induce IFNα from pDC, but it has less ability to induce IL-12 and fails to stimulate B cells (Gursel *et al*, 2002; Verthelyi *et al*, 2001). Although TLR9 has found to be essential for recognition of both types of CpG (Hemmi, 2003), difference of these CpG-ODNs in terms of cellular specificity and functional effects are still under question. However, CXCL16, a co-receptor expressed on human pDC but not B cells, can be possible explanation for these differences since it selectively recognizes and mediates the subcellular localization of D ODN (Gursel *et al*, 2006). Besides bacterial CpG, virus-derived CpG, such as murine cytomegalovirus in PDC has been found to be recognized by TLR9 (Krug *et al*, 2004). Moreover, TLR9 appears to be involved in the pathogenesis of autoimmune disorders, such as systemic lupus erythematosus through chromatin structure recognition (Takeda and Akira, 2005).

1.3.2.7. TLR Signaling

Expressions of several genes that are involved in immune response are triggered by stimulation of TLRs by microbial components. Molecular mechanisms underlying induction of these genes through TLR signaling pathways are now being rapidly elucidated (Akira and Takeda, 2004). Microbial recognition of TLRs facilitates dimerization of TLRs either homo-or heterodimers similar to TLR2-TLR1 or TLR2-TLR6 heterodimers (Saito, 2004). TLR dimerization triggers activation of signaling pathway through their cytoplasmic TIR domain. A TIR domain-containing adaptor protein, particularly, MyD88, associates with TIR domain of receptor and provide induction of several pro-inflammatory cytokines such as TNFα and IL12 (Hemmi *et al*, 2002; Takeuchi *et al*, 2000). Activation of different TLRs leads to different pattern of gene expression through individual TLR signaling cascades, although MyD88 is common to all. However, there are also MyD88 independent pathways as well as MyD88 dependent pathways.

1.3.2.7.1. MyD88 Dependent Pathways

In almost all TLR signaling pathways, there is an adaptor molecule forming a complex with TIR domain to initiate signaling events. MyD88 functions as such for innate response activation. It has been shown that MyD88-deficient mice do not produce inflammatory cytokines including TNF-α and IL12p40 in response to all TLR ligands (Takeuchi *et al*, 2000; Kawai *et al*, 1999; Schnare *et al*, 2000). Therefore, MyD88 is essential for inflammatory cytokine production through all TLRs.

MyD88 has a TIR domain, through which it associates with TLRs, on its C-terminal portion between residues 155-296 (Hultmark, 1994). Besides, it has a death domain (DD) in its N-terminal that confers association with other DD-containing proteins (Boldin *et al*, 1995). This domain is found to be important for TLR-induced cell death (Aliprantis *et al*, 1999). MyD88 has also an intermediate domain (ID) that

is essential for TLR signaling in terms of interacting with IL1R associated kinases (IRAKs) (Arancibia *et al*, 2007).

Upon stimulation (Figure 1.3. and Figure 1.4.), MyD88 recruits IRAK4 to TLRs through interaction between their DD and this facilitates IRAK-4 mediated phosphorylation of IRAK-1. Activated IRAK1 associates with TRAF6 leading to activation of two distinct signaling pathways. In the first one, AP-1 transcription factors are activated through activation of MAP kinases. In the second one, TAK/TAB complex, which enhances the activity of IκB kinase (IKK) complex, is activated. Then the IKK complex induces phosphorylation and subsequent degradation of IκB leading to nuclear localization of transcription factor NF-κB (Takeda and Akira, 2005)

1.3.2.7.2. MyD88 Independent Pathways

In MyD88-deficient macrophages, despite the lack of TLR-4 induced production of proinflammatory cytokines, NF-κB activation is seen with delayed kinetics (Kawai, 1999). This indicates that although proinflammatory cytokine production is completely MyD-88 dependent, but yet there is a MyD88 independent component leading to the activation of NF-κB in TLR4 signaling. Furthermore later it has been shown that transcription factor IRF-3 is also activated in response to TLR4 stimulation in a MyD88-independent manner (Kawai, 2001). TLR4-induced activation of IRF3 leads IFN-β production which activates Stat1 and results in the induction of several IFN-inducible genes (Toshchakov *et al*, 2002; Hoshino *et al.*, 2002; Doyle *et al*, 2002). Viral infection or dsRNA was also found to activate IRF-3 (Yoneyama *et al*, 1998). Thus, TLR3 and TLR4 utilize the MyD88-independent pathways to induce IFN- β (Figure 1.3. and Figure 1.4.). TLR3 and TLR4 expressed on APCs require the TIR-domain containing adaptor inducing IFN- β (TRIF) protein for the induction of NF-κB activation (Brint *et al*, 2002).

TLR4 activation by LPS may induce the expression of the TRIF-related adaptor molecule (TRAM/TICAM-2) and TRAM binds to both TLR4 and TRIF.

TLR4 utilizes TRIF and TRAM independently of other adaptor molecules, such as MyD88, to initiate late phase of NF-κB activation. It also induces the expression of IFN-β and other IFN-inducible genes via IRF-3 (Brint *et al*, 2004; Oshiuma *et al*, 2003, Figure 1.3.). TRAM is indispensably required for bridging TRIF and TLR4 in TLR4 mediated responses. $TRAM^{-/-}$ mice are completely impaired in their response to LPS, while response was seen in My88 deficient mice with delayed kinetics (Horng *et al*, 2002; Yamamoto *et al*, 2002).

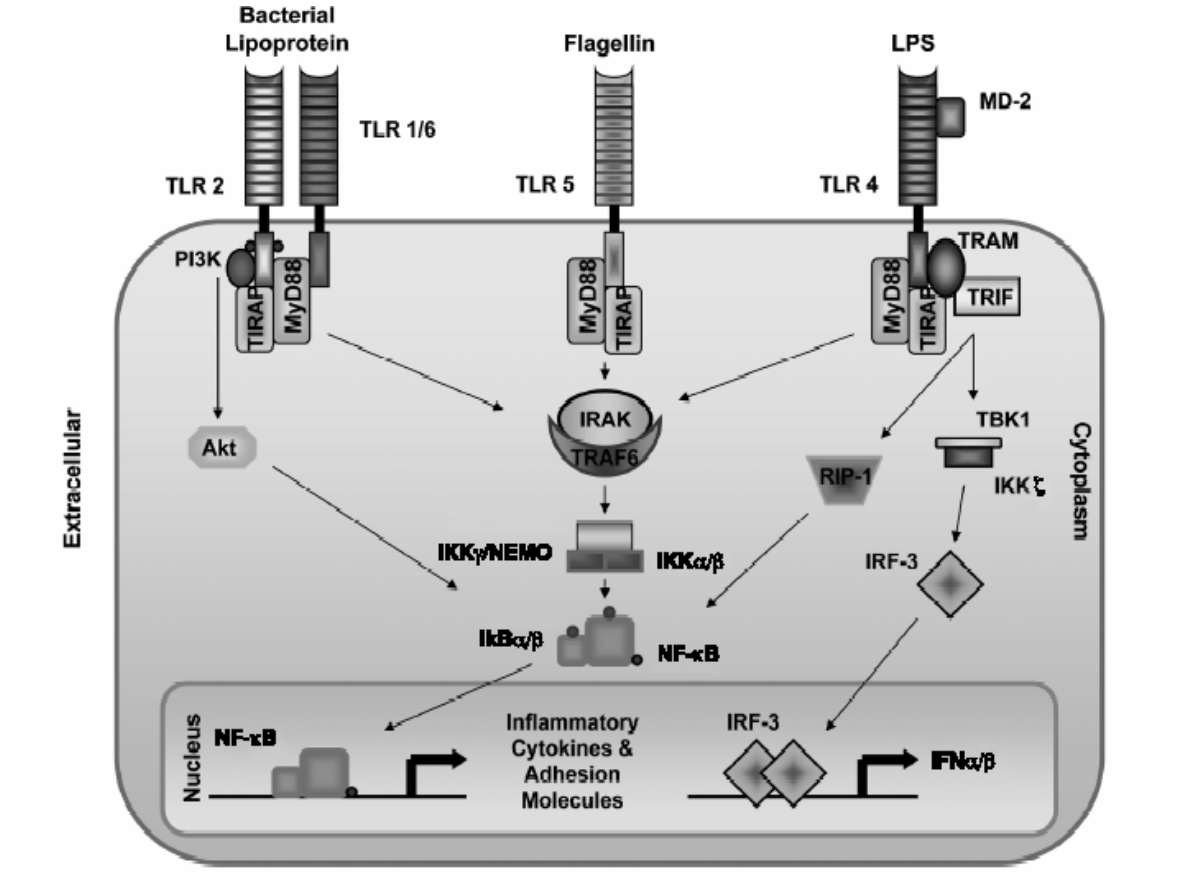


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Figure 1. 3. TLR signaling activation by bacterial ligands

In response to TLR3 signaling activation by viral dsRNA, TRIF is recruited to the receptor and induces IFN type I through TRIF-IKK-ζ (Figure 1.4.). In addition, agonist activation of TLR3 may promote tyrosine phosphorylation leading phosphotidyl inositol 3-kinase (PI3K) recruitment, Akt activation and eventually

IRF-3 phosphorylation (Sarkar *et al*, 2004). TLR3 can also stimulate pro-inflammatory cytokine expression through connecting the TRIF downstream signaling molecule to activation of NF-κB (Meylan *et al*, 2004).

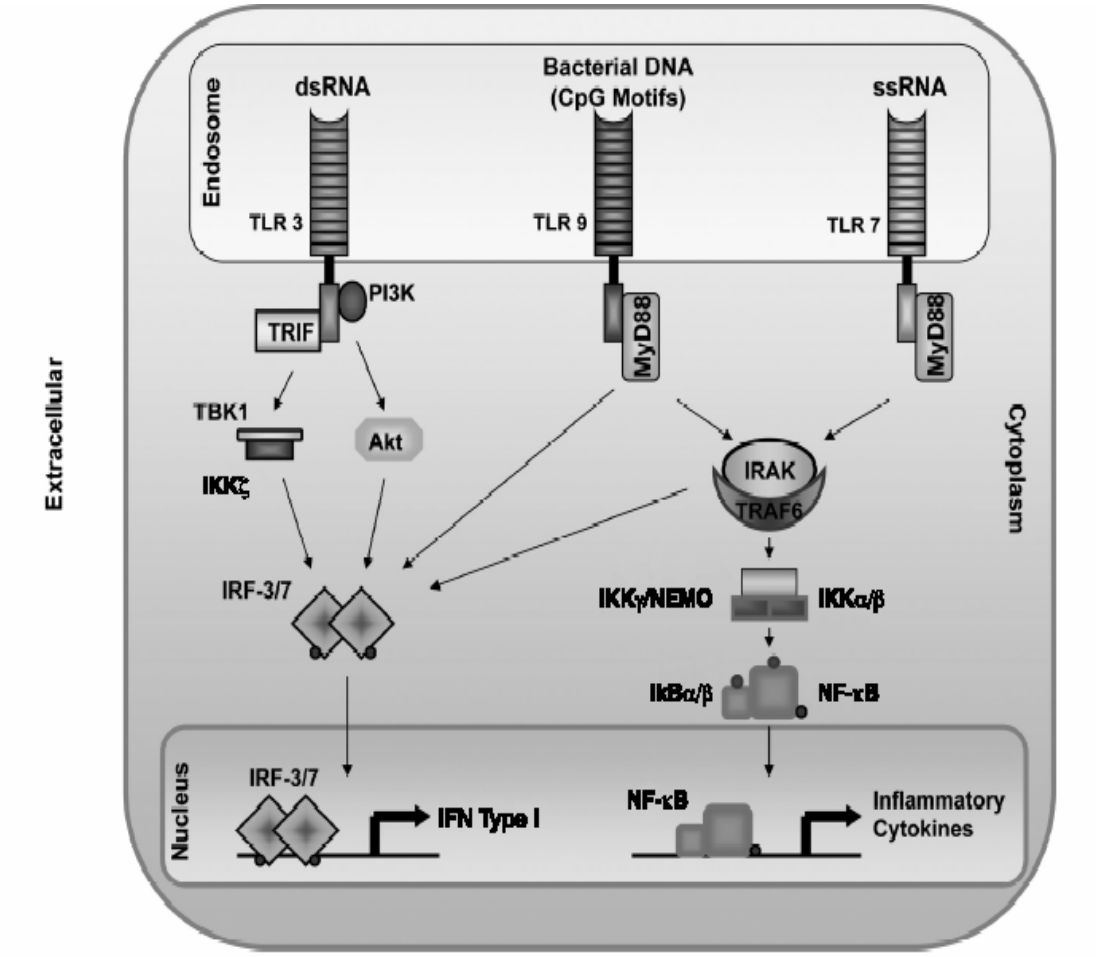

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Figure 1.4. TLR signaling activation by viral PAMPs

2. AIM OF STUDY

Liver transplantation is a well accepted treatment option for end-stage liver disease and acute liver failure. However, due to the donor shortage, new therapy strategies are becoming more important than ever. Amongst them liver cell transplantation (LCT) has been receiving great attention in recent years due to the improvement on the isolation and expansion of hepatocyte and stem/progenitor cells. In this context, MSCs are also important candidates as a source of cell due to their distinctive characteristics such as hepatic differentiation potential, homing to the site of injury and nonimmunogenicity.

By using a well established liver regeneration model of partial hepatectomy in rats, we aimed to investigate the followings in this thesis;

i) The effect of liver regeneration on the homing mechanisms of MSCs.

ii) Potential roles of MSCs and TLRs during liver regeneration.

iii) Role of TLRs expression on MSCs and their contribution to the immunoregulatory function of MSCs

3. MATERIALS AND METHODS

3.1. Animals

Adult male Spraque Dawley rats (7-9 months old) and adult male BALB/c mice (8-12 weeks old) were used for the entire study. The animals were kept in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled ambient conditions (22^{o} C ± 2) with 12 hour light and 12 hour dark cycles. They were provided with unlimited access of food and water. The experimental procedures have been approved by the animal ethical committee of Bilkent University (Protocol No: Bil-AEC 2005/2).

3.2 Standard Solutions and Buffers

For details of the solutions and buffers used for several assays, please refer to Appendix A section.

3.3. Liver Regeneration Model

3.3.1. Partial Hepatectomy and Sham Operations Alone

In this study, 7-9 months old male Sprague-Dawley rats were used. Partial hepatectomy procedure was standardized by Higgins and Anderson (1931).

3.3.2. Partial Hepatectomy Operation

The rats were pre-anesthetized firstly with ether chamber and then anesthetized with ketalar (2, 5-2, 8 mL/g) by intraperitonal injection. They were immobilized on the operation stand. Under aseptic conditions, the skin was cut and the abdomen was opened by cutting the peritoneal membrane through the midline (in Figure 3. 1.).

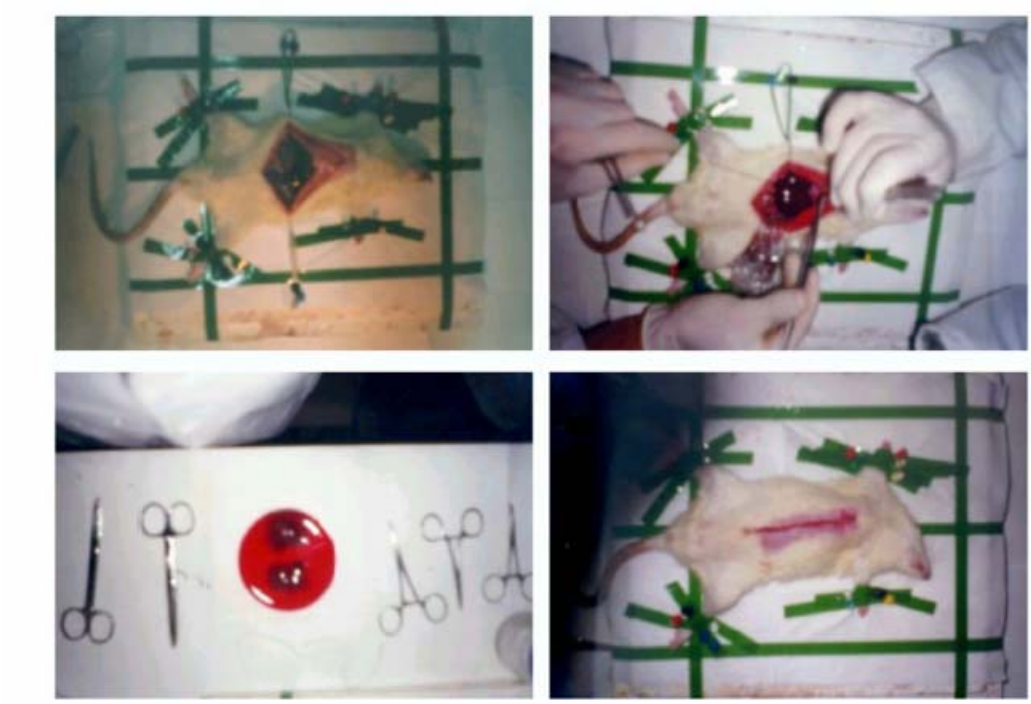


Figure 3.1. The steps of the liver resection surgery for partial hepatectomy (PH)

After opening of the abdomen, ligaments connecting liver to diaphragm and connecting the lobes of the liver was cut. The middle lobe (40% of total liver mass) and the front lobe (30% of total liver mass) were removed after the branches of vena cava inferior entering these lobes was tied with silk suture. The removed lobes were immediately washed with PBS and snap frozen in liquid nitrogen for further storage. The specimens (approximately 70% of the original liver) were stored in -80 ^{o}C until further use.

After removing the lobes, 10 mL of NaCl is injected into abdominal cavity to compensate dehydration. Then, the abdomen was sutured, and left in cages (one animal per cage); the cages were warmed and watched until the animals were conscious again.

For mRNA expression experiments, the animals (2 per time points) were sacrificed 2, 4, 12, 18, 24 hours after PH. Their livers were removed and washed

with PBS and were snap frozen in liquid nitrogen. The specimens were stored in -80 ^{o}C for further use.

3.3.3. Sham Operations

The surgical operations in sham group of rats were performed similar to PH operations (for details see Section 3.3.2.), but the exception in the procedure was that the livers of these animals (2 animals per time point as specified in previous section) were not removed.

3.4. Cell Culture

3.4.1. Cell Number Count With Thoma Cell Counter

After the heterogeneous bone marrow cells was washed and precipitated, they were suspended in 10 mL of MesenCult® (StemCell Technologies) media and the mixture was micropipetted into a hemocytometer.

The total number of cells in the chamber was determined by counting the cells under the light microscope from the four gridlines. The cell number was calculated according to the following formula:

$$\left[\frac{\text{Cell Number}}{4} \right] \left(10^{6} \right) = \text{Total cell number in 10 mL media}$$

3.4.2. Spleen Cell Preparation

Spleens were removed from the BALB/c male mice after cervical dislocation. In order to obtain single cell suspensions, the spleens were smashed with the back of a sterile syringe by circular movements and suspended in 2% FBS supplemented regular RPMI media. The cells were washed 3 times with media at 1500 rpm for 10 minutes. After removal of the cell debris, the cell pellet was gently dislodged with fresh media. The splenocyte suspension was counted as indicated in section 3.4.1.

3.4.3. Cell Distribution

For MSC and splenocyte interaction assays splenocytes were distributed into 96 well plates with a final cell number of 10^5 cells per well. Then MSCs were titrated on the splenocytes with a ratio of 1:1 to 1: 10^5 (MSC to splenocyte) in triplicate. Splenocyte alone (10^5cells per well) and MSC alone (10^5 cells per well) sets were also layered in triplicates as separate groups.

After magnetic bead separation cells, CD34- and CD34+ cells were distributed into 96 well plates with a final cell number of 10^5 cells per well. Stimulations were done in triple wells.

For TLR mediated morphological changes of mouse mesenchymal stem cells, the cell number was adjusted to 10^6 cells/well in 6 well plates. The ligands were replenished every three days for the duration of assay.

3.4.4. Stimulation With Different Toll like Receptor Ligands

Co-cultured splenocytes and MSCs were stimulated with TLR ligands (i) PGN (TLR2L or TLR2/6L); 5µg/mL, (ii) pI:C (TLR3L); 20µg/mL, (iii) LPS (TLR4L); 5µg/mL, (iv) R848 (TLR7/8L); 5µg/mL, (v) CpG DNA (TLR9L) ODN1555 a 15 mer ODN or ODN K23 a 12 mer ODN) ; 1µM, and (vi) Control ODNs (ODN1612 or ODN K23CGflip ODN); 1µM. Supernatants were collected 36 to 42 hours after stimulation.

CD34- and CD34+ cells were also stimulated with above indicated TLR ligands with different concentrations ((i) PGN; 1µg/mL, (ii) pI: C; 10µg/mL, (iii) LPS; 10µg/mL, (iv) CpG ODN1555; 1µM, and (v) Control ODN1612; 1µM). Supernatants were collected 5 days after stimulation. For TLR mediated morphological changes, mesenchymal stem cells were stimulated with TLR ligands

for 12 days (in every 3rd day the ligands were replenished). Similar concentrations were used as for CD34 cells.

Cells were cultured with 5% oligo FBS supplemented RPMI 1640 or DMEM media when they were stimulated with ODNs or dsRNA and were cultured with 5% regular FBS supplemented RPMI-1640 or DMEM when they were stimulated with other ligands.

3.5. RNA Isolation and Quantification

3.5.1. Total RNA Isolation From The Liver Tissues

All solutions and materials were prepared/treated with diethylpyrocarbonate (DEPC, Sigma) in order to avoid RNase contamination. Throughout the isolation process the centrifugations were conducted at 4°C. The total RNAs were isolated by using TriPure solution (Roche/Boehringer Mannheim, Indiana, USA) according to the manufacturer's protocol. 100 mg of tissue sample was homogenized in 1 mL of TriPure solution by using the homogenizer. Then the homogenate was incubated for 5 minutes at room temperature (RT), and allowed complete dissociation of the nucleoprotein complexes. Afterwards, 0.2 mL of chloroform was added and the tube was shaken vigorously for 15 seconds. After incubation at RT for 15 minutes, the tube was centrifuged at 12000Xg for 15 minutes resulting in three distinct phases. The colorless upper part was transferred to a new eppendorf and 1mL isopropanol was added on the sample and immediately mixed gently by inverting the tube few times and left at RT for 10 minutes. After centrifugation at 12000Xg for 10 minutes the supernatant was discarded. The pellet was washed with 75% ethanol, and then centrifuged at 12000Xg for 5 minutes. The supernatant was discarded and pellet was air-dried on bench for about 10-15 minutes. The RNA pellets were resuspended in 100 µl of the DEPC-treated ddH_2O.

3.5.2. Total RNA Isolation From Murine Splenocytes and Mesenchymal Stem Cells

The total RNAs form murine mesenchymal stem cells (for details, please see Section 3.10.) and murine splenocytes were isolated using TriZol® reagent (Invitrogen, USA) according to the manufacturer's protocol. Adherent fraction of mouse MSCs were pooled (centrifuged at 1500 rpm for 5 minutes) following trypsinazation. Low Glucose DMEM (supplemented with 10 % FBS) was added to the trypsinized cells during centrifugation and the media was removed. The cell pellet was washed once with PBS and centrifuged again at the same setting. The cells were counted on hemocytometer. Afterwards, 1 mL of TriZol® was added onto per 10^7 cells and dislodged by 10-15 times successive mild pipetting. After incubating the cells in TriZol® for 15 minutes at RT, 0.2 mL of chloroform/mL of TriZol® was added to the cells and vigorously shaken for 15 seconds. The mixture was incubated for 5 minutes at RT and centrifuged at 1200Xg for 15 minutes at 4^{o}C. Following centrifugation, the upper aqueous transparent fraction which contains total RNA was transferred into a new tube and 0.5 mL of isopropanol per 1 mL of TriZol® reagent was added and the tube was incubated for another 10 minutes at RT, the samples were centrifuged at 12000Xg for 10 minutes. The supernatant removed and the pellet was washed with 1 mL of 75 % ethanol per 1 mL of TriZol® reagent used. The samples were gently vortexed and centrifuged at 7500Xg for 5 minutes at 4^{o}C. The supernatants were discarded and pellets were air-dried under laminar flow hood. The RNA pellets were resuspended in 20-30 µl of DEPC-treated water.

3.5.3. Total RNA Isolation From Rat Mesenchymal Stem Cells

On the 14th days of the cell culture, total RNAs from the MSCs were isolated. The cells were first washed with 1X PBS buffer in order to eliminate the non-adherent cells in the media. Then the cells were trypsinized and incubated at 37^{o}C for 5 minutes. MesenCult® (StemCell Technologies) media was added to the trypsinized cells and centrifuged at 1500 rpm for 5 minutes. Then the media was removed and the precipitated cells were washed with cold PBS buffer and centrifuged again at

1500 rpm for 5 minutes. Next, the buffer was removed and the total RNA was isolated from the precipitate by using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The RNA isolated from several sources was stored at -80^{o}C for further use.

3.5.4. Quantification of RNA

The concentrations of RNA samples were measured via NanoDrop® ND-1000 (Nano Drop Technologies, USA) and the quality of RNAs was determined by measuring the ratio of A_{260}/A_{280}. The OD_{260}/OD_{280} ratios were calculated in order to determine if there is a phenol, protein or DNA contamination in the RNA samples. The acceptable ratio was expected to be from 1.6 to 2.0. In order to check the integrity of the isolated RNAs denaturing agarose gel electrophoresis was performed.

3.5.4.1. Denaturing Gel Electrophoresis

In order to check the integrity of the isolated RNA denaturing agarose gel electrophoresis was performed for all samples. 1.2 % denaturing agarose gel was prepared with DEPC-treated water, including 10X MOPS, 3.6 % formaldehyde and 30 ng/mL ethidium bromide solution. The electrophoresis tank contained 1X MOPS during running the gels. The samples were prepared by addition of 2 µl agarose gel loading dye, 3 µl RNA and 3 µl DEPC-treated H_2O. The samples were incubated at 65ºC for 15 minutes in order to allow RNA denaturation. The gel was run at 90V, for 45 minutes and visualized under transilluminator (Gel-Doc BIO-RAD, USA or Vilber Lourmat, France). Chemicapture (Vilber Lourmat, France) software was used to take photographs of the gels.

3.6. The cDNA Synthesis

The cDNA was synthesized from the extracted total RNA sample using a cDNA synthesis kit (Finnzymes, Finland) according to the manufacturer's protocol. 2 µg RNA was mixed with 1.5 µl of Oligo (dT) primer and completed to a total volume of 14µl with DEPC-treated water. They were pre-denaturated at 65ºC for 5

minutes and incubated on ice for 3-5 minutes. 20 µl 2X RT buffer (includes dNTP mix and 10 mM $MgCl_2$). Then a 4 µl M-MuLV RNase H reverse transcriptase including RNase inhibitors were added to the mixture and incubated at 25ºC for 10 minutes, 40ºC for 45 minutes, 85ºC for 5 minutes, and on ice for 10 minutes, respectively. The cDNAs were stored at -20ºC for further use.

3.7. RT-PCR Studies

3.7.1. Conventional PCR

The reaction ingredients used in PCR reactions are shown in Table 3.1. and the condition of PCR reactions are shown in Table 3.2.

Table 3. 1. PCR reaction composition

Reaction Ingredients	Volume
cDNA	1.5 µl
2X DyNAyzme II Master Mix (Finnzymes)	12.5 µl
Forward Primer (from 10 pmol stock) (Alpha DNA)	1 µl
Reverse Primer (from 10 pmol stock) (Alpha DNA)	1 µl
DNase RNase free H_2O (HyClone)	9 µl
Total	25 µl

Table 3.2 PCR running conditions

30 sec @ 94°C
30 sec @ 55°C } **35 cycles**
1 min @ 72°C

Final Extension: 10 minutes @ 72°C

3.7.2. Agarose Gel For Visualization of PCR Products

2% agarose gel was prepared with 1X TAE buffer and 1 mg/mL ethidium bromide solution. Samples were prepared by addition of 5 µl Agarose Gel Loading dye to 10 µl of cDNA sample and loaded to the agarose gel. Agarose gel was run at

90V for 45 minutes and visualized under transilluminator (Gel-Doc BIO-RAD, USA and Vilber Lourmat, France). Chemicapture (Vilber Lourmat, France) software was used to take photographs of the gels. The low range DNA ladder (Jena Biosciences) was used as a marker and 5 µl was loaded to every gel.

3.8. The Real-Time RT-PCR Studies

The real time RT-PCR was done with DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Finland) according to the manufacturer's protocol. Rat TLR primer sequences were adopted from Hubert *et al* (2006) and the house keeping gene was selected to be cyclophillin (CYC) (Table 3.3). Before the real time RT-PCR, the efficiencies of the primers were tested using a standard dilution series. The reaction conditions of real-time PCR are shown in Table 3.4.

The real-time PCR reactions were carried out in iCycler™ (Bio-Rad, California, USA). After the amplification steps melting curve analysis was done.

Cyclophillin (CYC) gene was used as internal control. Samples from partial hepatectomized (PH) and sham (SH) animals obtained at different time points (please see Section 3.2) were detected in duplicates, and the readings from each sample and its internal control (CYC) were used to calculate gene expression level.

Table 3.3. The sequences and the sizes of the rat TLR primers used in this study (adopted from Hubert *et al*., 2006).

Gene Acronyms		**Primer Sequences**	**Product Size (bp)**
TLR1	Forward	CAGCAGCCTCAAGCATGTCT	82
	Reverse	CAGCCCTAAGACAACAATACAATAGAAGA	
TLR2	Forward	CTCCTGTGAACTCCTGTCCTT	74
	Reverse	AGCTGTCTGGCCAGTCAAC	
TLR3	Forward	GCACTGTGAGATACAACGTAGCT	66
	Reverse	GAAGGTCATCAGGTATGTGTGTCA	
TLR4	Forward	TGCTACAGTTCATCTGGGTTTCTG	78
	Reverse	CTGTGAGGTCGTTGAGGTTAGAAG	
TLR5	Forward	GGGCAGCAGAAAGACGGTAT	61
	Reverse	CAGGCACCAGCCATCCTTAA	
TLR6	Forward	AGAACCTTACTCATGTCCCAAAAGAC	79
	Reverse	AGATCAGATATGGAGTTTTGAGACAGACT	
TLR7	Forward	GTTTTACGTCTACACAGTAACTCTCTTCA	75
	Reverse	TTCCTGGAGGTTGCTCATGTTTT	
TLR8	Forward	GGCTTCGGCAGAGGATCT	75
	Reverse	GCCAAAACAAGTTTTCCGCTTTG	
TLR9	Forward	CCGAAGACCTAGCCAACCT	70
	Reverse	TGATCACAGCGACGGCAATT	
TLR10	Forward	CTCCAACATGGCTTTAAGGAAGGT	90
	Reverse	TGGAATTGATAGAGGAGGTTGTAGGA	
CYC	Forward	GGGAGGGTGAAAGAAGGCAT	211
	Reverse	GAGAGCAGAGATTACAGGGT	

Table 3.4**.** Reaction composition for real time PCR

Reaction Mixture	**Volume**
cDNA	1 µl
2X DyNAmo HS Master Mix	10 µl
10 pmol forward primer (Alpha DNA)	1 µl
10 pmol forward primer (Alpha DNA)	1 µl
ddH_2O	7 µl
Mineral oil	10 µl
Total	30 µl

3.9. Administration of CM-DiI Labeled Mesenchymal Stem Cells to Partially Hepatectomized Rats

3.9.1. Chloromethylbenzamido-1,1–dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (CM-DiI) Labeling

A stock solution of 2 mg/mL Chloromethylbenzamido-1, 1–dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI, Molecular Probes, USA) was prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. $2x10^6$ of cells isolated form normal, one day and three days after PH were stained with CM-DiI at a concentration of 5 µg/mL in 1X PBS. The flasks were first incubated for 5 minutes at 37°C and further for 15 minutes at 4°C. After washing twice with PBS, MesenCult was added to the flasks. The cells were left in the CO_2 incubator for further use.

3.9.2. Preparation of Labeled Mesenchymal Stem Cells For *in vivo* Injection

Four days after CM-DiI labeling, the cells were taken form flasks by trypsinization. The cells were first washed with PBS buffer in order to eliminate the non-adherent cells in the media. Then the cells were trypsinized and incubated at 37^oC for 5 minutes. Low Glucose DMEM (with 10% FBS) was added to the

trypsinized cells and centrifuged at 1500 rpm for 5 minutes. Then the media was removed and the precipitated cells were washed with PBS buffer and centrifuged again at 1500 rpm for 5 minutes. 10 mL of PBS was added to trypsinized cells and they were counted on hemocytometer. The cell concentration was adjusted to $1x10^6$ cells/mL for all injections (the cells were chilled on ice until administration).

3.9.3. Administration of Labeled Mesenchymal Stem Cells to PH Rats

Rats were hepatectomized as mentioned in section 3.3.2. CM-DiI labeled MSCs, which were isolated from normal rat, were injected to PH animals (1, 3, and 5 days following partial hepatectomy operation 1-2 animals/time point) as well as normal rats through tail vein in a volume of 1 mL. Other labeled MSCc that were isolated from both 1 day and 3 day PH rats, were injected only to 3 day PH and naive animals. 3 days after administration of labeled MSCs, all the animals were sacrificed and their organs (liver, heart, kidney, and spleen) were removed and stored for further studies.

In order to investigate the localization and existence of the CM-DiI-labeled cells from different tissues under fluorescence microscope, 5 μm tissue sections were taken and the specimens were mounted using UltraCruz^{TM} (Santa Cruz) mounting medium with DAPI and covered for further study.

3.10. Immunofluorescence Staining

For immunofluorescence staining, 5 μm sections were taken from frozen liver tissues. The tissues were fixed in 4% paraformaldehyde for 30 minutes and washed with dH_2O. Then the sections were immersed in 3% H_2O_2 for 30 minutes. After washing with PBS for 2 minutes, the specimen was blocked with 2% BSA for 1 hour at RT in humid chamber. Sections were then incubated with FLT3 (Santa Cruz biotechnology) and CD90 (Chemicon International) at RT for one hour in a dilution 1/50 and 1/500 in 1% BSA, respectively. Blocking peptide specific to FLT3 in the 3/50 dilution was incubated with FLT3 antibody. After washing the sections twice

with PBS, the specimen was incubated with FITC-labeled anti-mouse IgG (Sigma) with FITC-labeled anti-rabbit IgG (Sigma) for CD90 and Flt3, respectively. The sections were incubated in dark for 1 hour at RT in humid chamber. After washing with PBS, the specimens were mounted using UltraCruz^{TM} (Santa Cruz) mounting medium with DAPI and covered for further study.

3.11. Fluorescence Microscopy Studies

CM-DiI, CD90, FLT3, DAPI stainings were investigated under the fluorescence microscope (Leica TCS/SP5, JAPAN) equipped with Lecic software for image analysis at an excitation wavelength 359 nm for DAPI, 464 nm for FITC and 553 nm for CM-DiI. The images were taken by 10X, 25X, 40X and 63X objectives with or without water immersion.

3.12. Isolation of The Bone Marrow From The Animals

After the animals were sacrificed by cervical dislocation, the gastrocnemius region was shaved with a razor. Then, the femur and tibia were removed, and the ends of bones were cut and flushed with DMEM (Invitrogen) which contains 10% FBS (HyClone) and 1% penicillin/streptomycin solution (HyClone), by using a 5 mL syringe, with a 26 gauge needle (Figure 3.2.). The mixed media and bone marrow were centrifuged at 4000 rpm for 3 minutes. Then the media was removed and the cells were washed four times with PBS to prepare for tissue culturing.

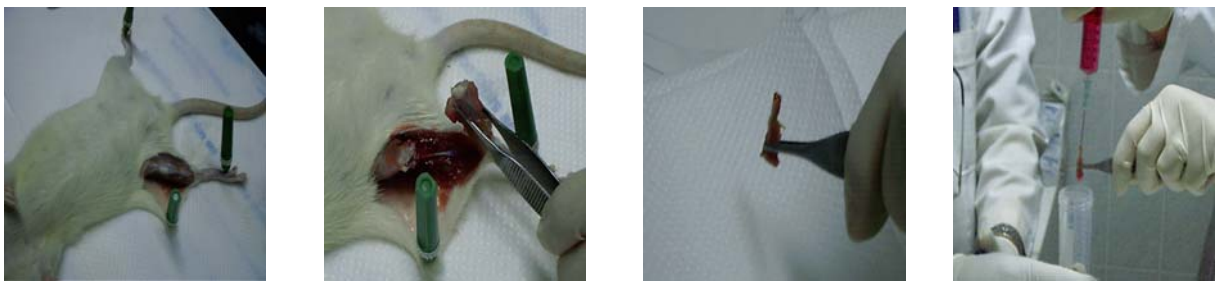


Figure 3.2. The isolation of the bone marrow of the rats

3.13. TLR Mediated Morphological Changes of MSC

In order to examine effect of individual TLR ligands on morphology and possibly differentiation potential of mesenchymal stem cells, the cells were stimulated with TLR ligands as indicated in section 3.4.4. Stimulation was repeated with ligand replenishment in every 3 days.

Light microscope images were taken at day 12 after 4^{th} stimulation using Nikon (Eclipse TS100) microscope.

3.14. Enzyme Linked-Immunoabsorbent Assay (ELISA)

3.14.1 Cytokine ELISA

Polysorp (F96 Nunc-Immunoplate, NUNC, Germany) plates were coated (50 µl/well) with anti-cytokine IL6, TNFα, IFNγ monoclonal antibodies (Pierce, Endogen USA) in PBS, final concentrations 10µl/mL, 5 µl/mL and 5 µl/mL respectively for 4-5 hours at RT (or overnight at 4^oC). The wells were blocked with 200 µl blocking buffer for 2 hours at RT and washed 5 times with ELISA wash buffer for 5 minutes. After washing, the wells were rinsed with ddH_2O and blot dried. Supernatants and serially diluted recombinant proteins of IL6 (starting concentration: 2000 ng/mL), TNFα (starting concentration 2000 ng/mL), IFNγ (starting concentration 2000 ng/mL) were added and incubated for 1-2 hours at RT (or overnight at 4^oC). Plates were washed in similar fashion as mentioned above. For detection of cytokine levels, biotinylated anti-cytokine antibodies (Pierce, Endogen) were prepared in a T-cell buffer, (at a 1:1000 dilution, 50 µl/well) added to the wells and incubated for 2 hours at RT (or overnight at 4^oC). After washing the wells as described above, 1:5000 diluted Streptavidin Alkaline-phosphatase (SA-AKP) was prepared in T-cell buffer and added to the plates (50 µl/well) for 1 hour at RT. After washing the plates; PNPP substrate (one tablet/5 mL PBS, 50 µl/well, Pierce, USA) was added and incubated. Continuous ELISA was performed to plates and after color

formation, multiple OD readings (at 405nm) were recorded on an ELISA plate reader (BioTek, μQuant, USA).

In order to determine concentrations of cytokines from cell supernatant, 4-parameter standard curves were constructed from recombinant proteins as mentioned above.

3.15. Magnetic Bead Cell Separation

MSCs are separated with Dynabeads®™ M-450 Epoxy magnetic beads (DYNAL®, Invitrogen™) coupled with CD34 antibody (Santa Cruz) according to the manufacturer's protocol. Briefly, 100 μl of Dynabeads were taken from stock and washed by placing the tube in a magnet (Dynal MPC®) for 60 seconds by discarding the supernatant. Then, the tube was taken from the magnet and the beads were resuspended in buffer A (0.1 M phosphate buffer) and mixed for 2 minutes. After repeated washing steps the beads were resuspended in a volume of buffer A equal to the original particle volume to obtain the recommended final coating concentration of 4-8x10^8 beads/mL. Afterwards, the beads were coated with CD34 antibody adding by dissolved antibody into bead solution with a final concentration of 20-40 μg of antibody/4-8x 10^8 beads/mL and incubating the mixture for 16-20 hours at 4-37^oC on a rotating/rocking rotor.

After isolation, washing and counting of MSCs, as mentioned in Section 3.11 and in Section 3.4.1., the cells were incubated with coated beads for 30 minutes at 2-8^oC on the same rotor. The tube was placed on the magnet and left to be separated for 2-3 minutes and the supernatant was transferred into a new tube as CD34- cells. Both CD34- supernatant and CD34+ cell pellets were plated in MesenCult media.

At day 14, CD34+ and CD34- cells were stimulated with TLR ligands as mentioned in section 3.4.4., and IL6 secretion were examined in response to indicated TLR ligand stimulation by sandwich ELISA.

3.16. Flow Cytometric Analysis of Cell Surface Markers

3.16.1. Fixation of Cells

Mesenchymal stem cells from different passages were first washed with PBS in order to eliminate the non-adherent cells in the culture and were removed from flasks by scraping. The cells were transferred into falcon tubes and centrifuged at 1500 rpm for 5 minutes. The cell number was adjusted to 1-4x10^6cells/tube and pelleted. After dislodging the cells, 100 µl of fixation medium A (Fix & Perm®, Caltag Lab. USA) per tube was added while vortexing for 10-20 seconds, the tubes were incubated at RT for 15 minutes. 2 mL of PBS-BSA-Na-Azide was added to the tubes. The tubes were centrifuged at 1500 rpm for 5 min and supernatant was discarded. The pellet was resuspended in 1 mL PBS-BSA-Na-Azide and stored at 4^{o}C at most for a week until for further use.

3.16.2. Surface Marker Staining

After dividing cells into four different falcon tubes, the tubes were centrifuged at 1500 rpm for 5 minutes. In order to prevent non-specific binding of antibodies, 0.5 µl of Fc block was added to each tube in 49.5 µl PBS-BSA-Na-Azide. After blocking, the cells were stained with CD90-FITC (Thy 1.2, Abcam, USA), CD45-PE/Cy5 (Abcam), CD11b-PE (ImmunoTools™, Germany), CD117-FITC (c-Kit, ImmunoTools™) for 30 minutes in dark at RT. Moreover, some cells were stained with FITC-labeled rat anti-mouse IgG to serve as isotype control. The cells were washed twice by adding 2 mL PBS-BSA-Na-Azide and centrifuging at 1500 rpm for 10 minutes. Finally the cells were resuspended in 500 µl of PBS-BSA-Na-Azide or PBS and transferred to FACS tubes, for analysis.

3.16.3. Flow Cytometric Analysis of Cells

Samples were analyzed (10,000–50,000 events) on a FACS Calibur instrument (Becton Dickinson, San Jose, CA) after gating on live cells with proper

electronic compensation. The data were analyzed using CELLQuestPro software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

3.17. Statistical Analysis

Statistical significance between animal groups was determined using Student's t–test analysis.

4. RESULTS

4. 1. MSC Homing In Partial Hepatectomized Rats

4.1.1 Administration of MSC From Normal Rat

Mesenchymal stem cells are multipotent progenitor adult cell population capable of differentiating into several lineages including adipocytes, osteoblasts and chondrocytes, and nerve cells (Pitteger *et al*, 1999; Woodbury, 2000). Moreover, MSC is able to migrate to the damaged tissue in (vivo Baksh *et al*, 2004). The mechanism of this phenomenon is still elusive. We have hypothesized that one mechanism triggering the MSCs to accumulate at these sites is the TLR expression ability of the host organ (or tissue). To demonstrate that this we have designed a set of experiments to investigate whether MSCs given systemically localize to the site of injury, we hepatectomized rats and then injected MSCs either prepared from already hepatectomized or normal animal bone marrows. In order to understand homing property of MSCs in partially hepatectomized rats, MSCs (from normal animals) were labeled with CM-DiI (see appendix B section) and administered into 1, 3, and 5 day post-PH and normal rats through their tail vein (Figure 4.1.). At the end of 3 days of post MSCs administration, animals were sacrificed, the liver and several other organs and fixed. The liver was sectioned and stained with DAPI.

As shown in Figure 4.1. our findings showed that a thin layer of CM-DiI positive MSCs localized at the outermost layer of the PH liver were detectable from the ultra thin sections of 3 day PH animals (please examine Figure 4.1. H). The livers studied for day 1 and 5 PH animals including normal and untreated livers gave no detectable CM-DiI specific signal.

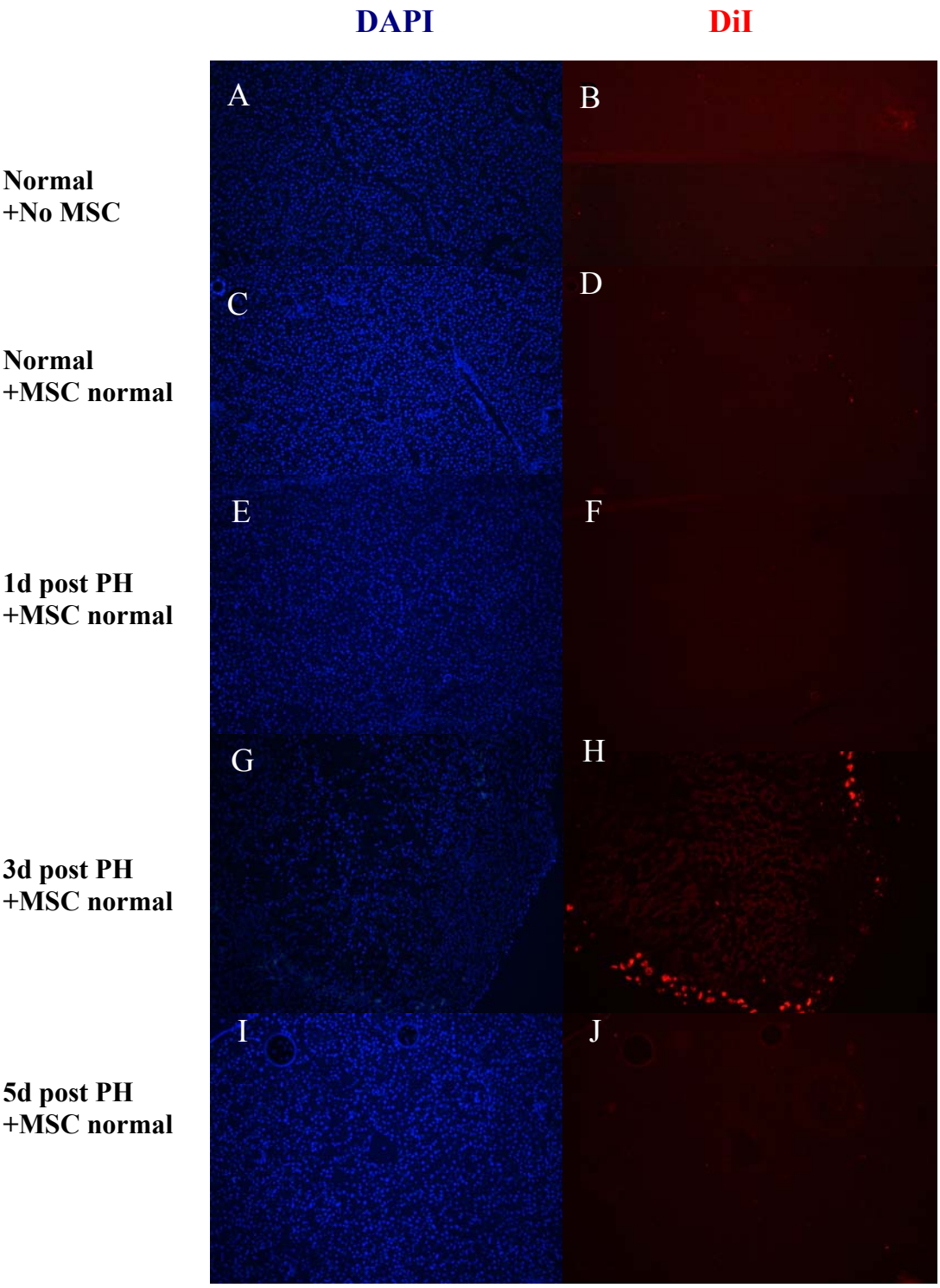


Figure 4.1. Photomicrographs of normal and PH liver sections that had labeled MSC generated from normal rat (Magnification: 20X). (A), (C), (E), (G), (I) =DAPI; (B), (D), (F), (H), (J) =DiI images

4.1.2 Administration of MSC From Hepatectomized Rats

It is also established in the literature that the critical time frame for the recovery of PH animals is the first 72 hours. Extending this observation we have postulated that the mobilization of MSCs in these hepatectomized animals may be different than normal rats. For this, we asked the question whether we can monitor any change in the localization yield of the MSCs isolated from day 1 or day 3 PH animals when they are administered into 3 day PH (or as a control group to normal rats) animals from their tail vein (Figure 4.2.).

Our results indicated that MSCs isolated from 1 day PH rat rather than 3 day PH animal is localized in the liver of 3 day PH liver in a similar fashion as seen in the case of MSC isolated from normal rat (section 4.1.1. and Figure 4.1.). However, no CM-DiI staining was observed in liver of rats that were injected with MSC from 3 day PH rat. This set of data implicate that, bone marrow cells isolated either from normal or day 1 PH rats can yield MSCs with a recruitment capacity to the site of injury (localize only to post injury day 3 only).

4.1.3. CD90 Expression in Hepatectomized Livers

Observing the CM-DiI positive MSC presence in post day 3 PH liver was encouraging. We further wanted to understand if there are additional MSCs from recipient animal in the niche of the injured organ that may play additional role mediating liver regeneration. One of the widely accepted surface markers for mesenchymal stem cells in rodents is CD90 (Thy1.2) (Mangi *et al*, 2003). Therefore, we performed immunofluorescent staining (IF) to investigate the presence of MSCs in DiI-labeled MSC administered hepatectomized liver sections (Figure 4.3.). We have stained the sections with anti-CD90- FITC labeled antibody for MSC presence and investigated under fluorescence microscope.

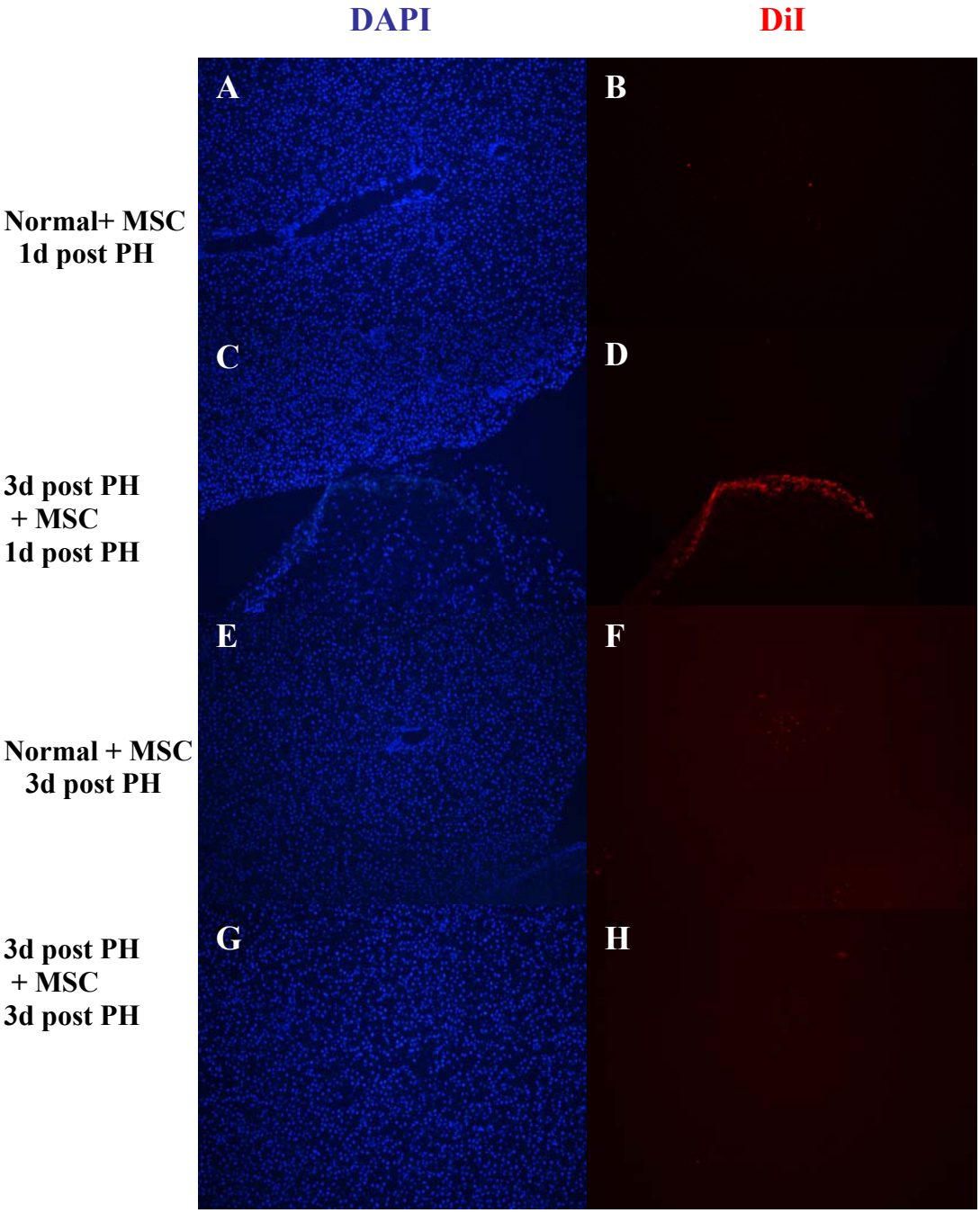


Figure 4.2. Photomicrograph of different liver sections that had labeled MSC generated from 1d and 3day post PH rats (Magnification: 20X). A, C, E, H = DAPI images; B, D, F, I = DiI images. A-B, Normal liver images that received MSC generated from 1d post PH rat BM. C-D, 3d post PH liver images that received MSC generated from 1d post PH BM. E-F, Normal liver images that received MSC generated from 1d post PH rat BM. G-H, post 3d PH liver images that received MSC generated from 3d post PH.

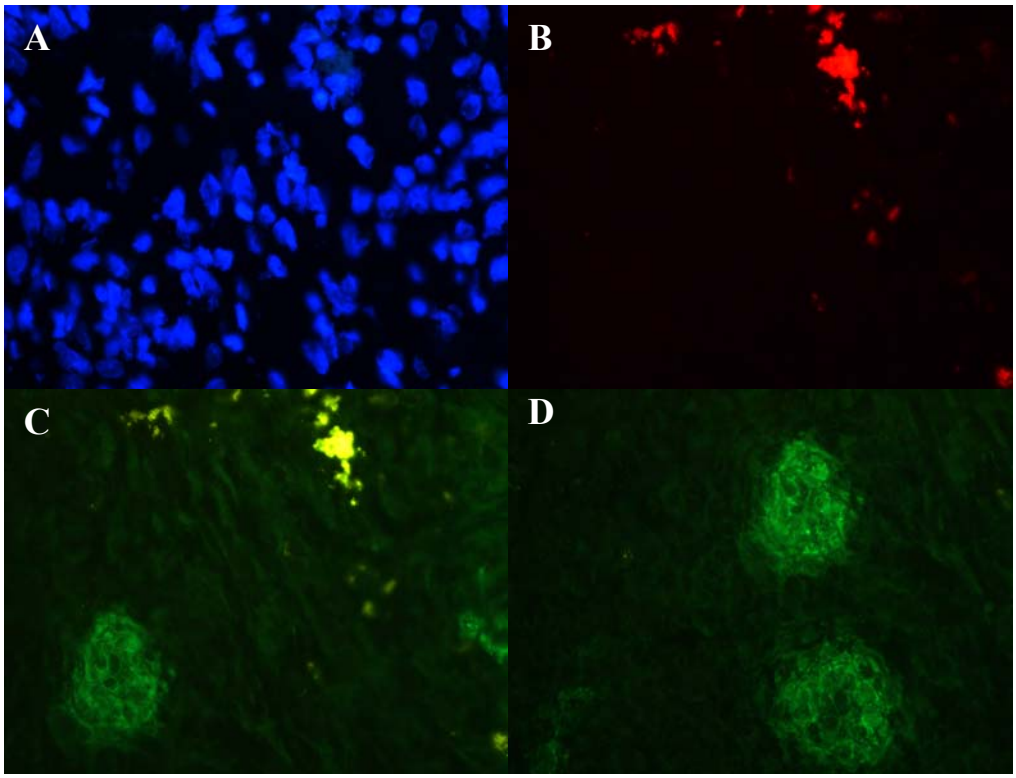


Figure 4.3. Photomicrograph showing CD90 expression in 3day post PH liver sections that received MSC generated from normal rat BM (Magnification 20X). (A) DAPI, (B) DiI, (C) CD90-FITC and (D) another area for CD90-FITC positive cells

As seen from IF micrographs, the results showed that there were several CD90 positive cells scattered around DiI-labeled MSCs in the liver section of 3 day PH rat (Figure 4.3. C & Figure 4.3. D). Consistent with earlier observations no specific CD90 staining was observed for i) normal, ii) post 1 day or iii) post 5 day PH rats (data not shown). Surprisingly, we have not been able to co-stain the cells positive for CM-DiI with CD90. One explanation is that, DiI is a dominant dye and it may bleed through to the excitation range of FITC which results in masking of FITC specific staining. Another plausible explanation is that, these cells have down-regulated their surface CD90 (after injected through tail vein) when they reached

hepatectomized liver and since they are interacting with the surrounding cells if they are secreting certain mediators, it is possible to undergone certain phenotypic change and downregulate certain MSC specific markers while upregulating others (or secreting certain factors).

4.1.4. Flt3 Expression in Hepatectomized Livers

Flt3 is a receptor tyrosine kinase (RTK) and a well-known hematopoietic stem cell marker (Peterson, 2001; Agnes *et al*, 1994). Oval cells are playing pivotal role in progenitor-dependent liver regeneration and are known to express specific proteins at the surface among these FLT3 is one of these proteins (Alison M, 1998). Thus, we performed immunofluorescent staining against FLT3 (labeled with FITC) to check the existence of hepatic oval cells in DiI-labeled MSC administered hepatectomized livers. As expected, Flt3 positivity can be seen around DiI-labeled MSCs in the liver of post 3 day PH rat (Figure 4.4.). When Figure 4.4 is investigated in detail, it is clearly evident that around the DiI positive MSCs a bright green cell population is seen. When these images merged (please check Fig 4.4. D) FITC positivity is abundant around bright yellow areas where MSCs are residing.

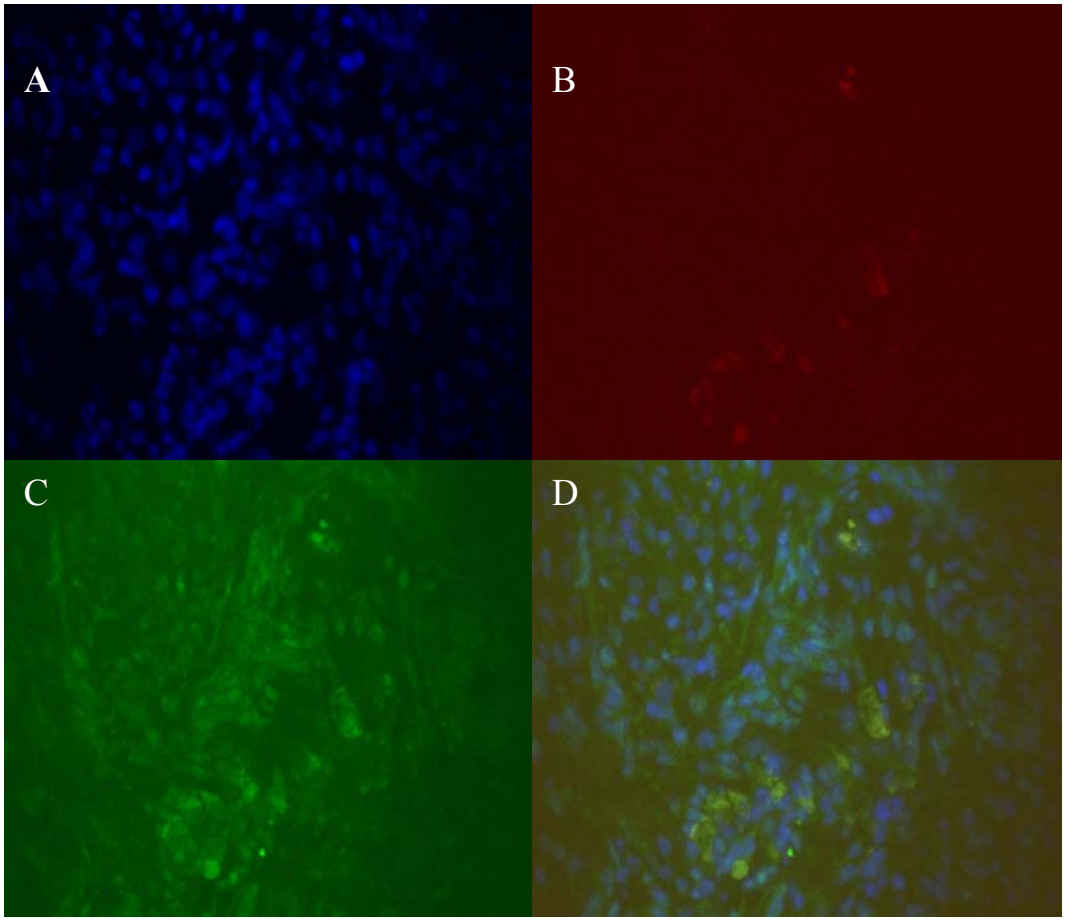


Figure 4.4. Photomicrograph showing Flt3 expression in 3day post PH liver sections that received MSC generated from normal rat BM (Magnification 20X). (A) DAPI, (B) DiI (C) Flt3-FITC and (D) Merged

4.2. Real Time RT-PCR Results

Our aim throughout this study is to establish the orchestral contribution of the MSC administration in addition to the expression profile of several TLRs during liver regeneration. As explained in the earlier sections, we have been able to detect MSC (labeled with DiI) in liver sections at post 3 day following hepatectomy. We were curious to link this phenomenon to TLRs. In this section results regarding the status of TLR expression profiles of several treatments will be covered.

For real-time RT-PCR studies the cDNA samples of sham (see appendix C), partial hepatectomized (PH), and MSC-administered PH groups were used. The real-time PCR reactions were performed as explained in section 3.7. Efficiency of CYC and TLR1 to 10 primers were tested, standard curves were derived and E values were calculated for all primer sets (Table 4.1)

Table 4.1 Efficiency values of primer sets used during real-time RT PCR studies

Primer set	**E value**
CYC	1,9
TLR1	1,94
TLR2	1,94
TLR3	1,98
TLR4	1,88
TLR5	1,86
TLR6	1,99
TLR7	2
TLR8	2
TLR9	2
TLR10	1,9

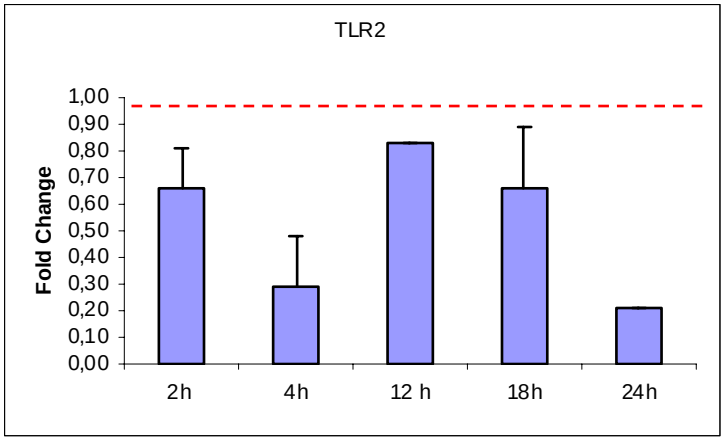
4.2.1. TLR Expression in Partial Hepatectomized Rats

The C_t values of normal liver, and PH groups (post PH at 2, 4, 12, 18 and 24 hours) for *TLR* genes and *CYC* were calculated. The C_t values of the PH groups were normalized according to their difference from the C_t value of normal liver sample. By using normalized C_t values, ΔC_t ($\Delta Ct_{TLR\ gene}$ / ΔCt_{CYC}) value for each TLR at indicated time of was calculated. Fold change in expression of all the tested TLR genes were calculated by using $2^{-(\Delta Ct_{PH} - \Delta Ct_{NL})}$ formula (which is specific to each *tlr* gene primer

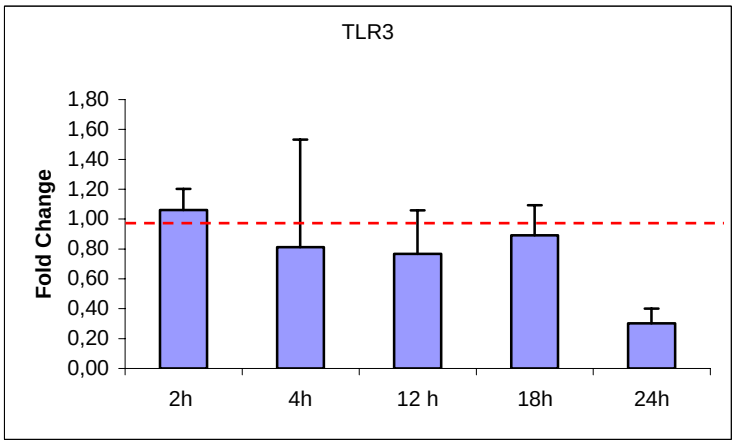
sets including cyclophilin set) (data not shown). From these values fold change in expression for each TLR is plotted (Fig 4. 5. A to F).

From these plots, it was apparent that specifically three genes (tlr2, tlr3, and tlr9) gave unchanged expression profile over the course of 24h post PH (Fig 4.5A, 4.5 B, and 4.5 F).These genes, are triggered by RNA, CpG DNA, and a well known bacterial byproduct peptidoglycan, and is reported to be triggered via endogenous host ligands after injury. We have selected these genes to monitor the fold change in expression upon MSC administration.

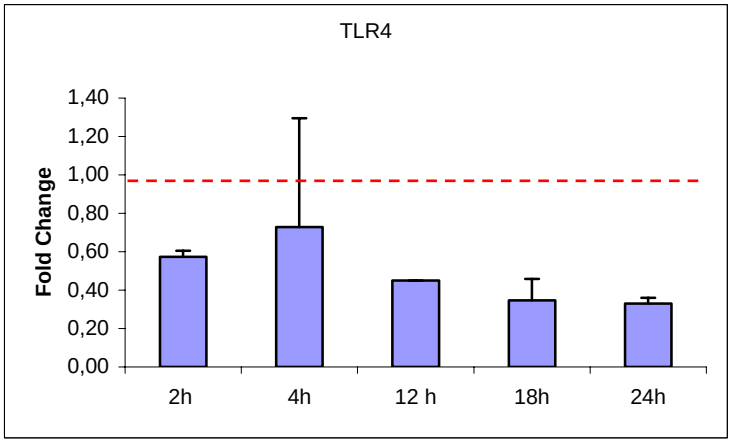
A)



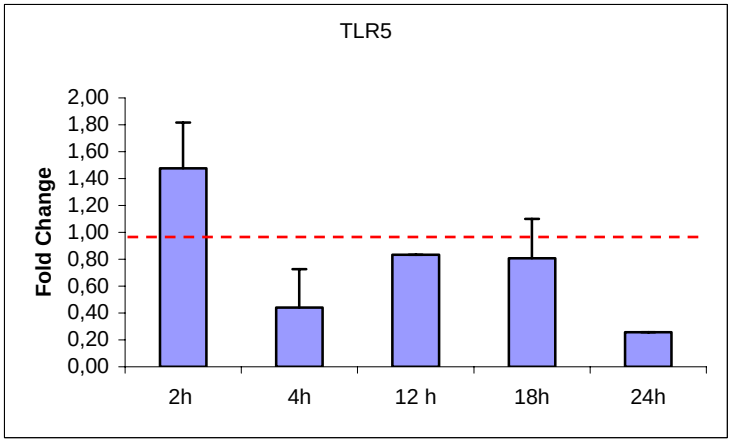
B)



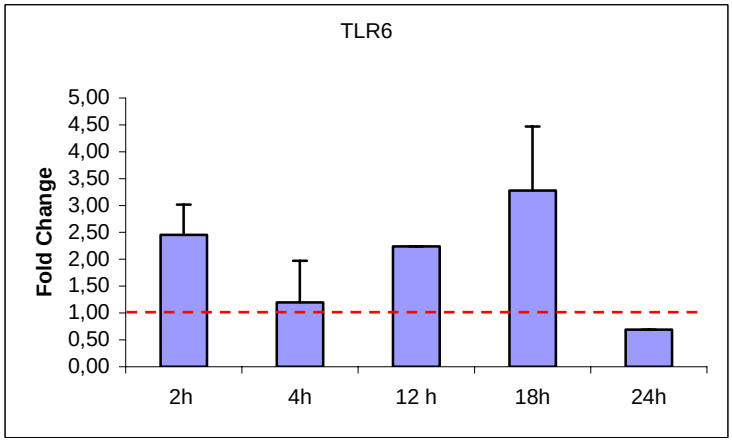
C)


TLR4
Fold Change
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
2h
4h
12 h
18h
24h

D)


TLR5
Fold Change
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
2h
4h
12 h
18h
24h

E)


TLR6
Fold Change
5,00
4,50
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
2h
4h
12 h
18h
24h

F)

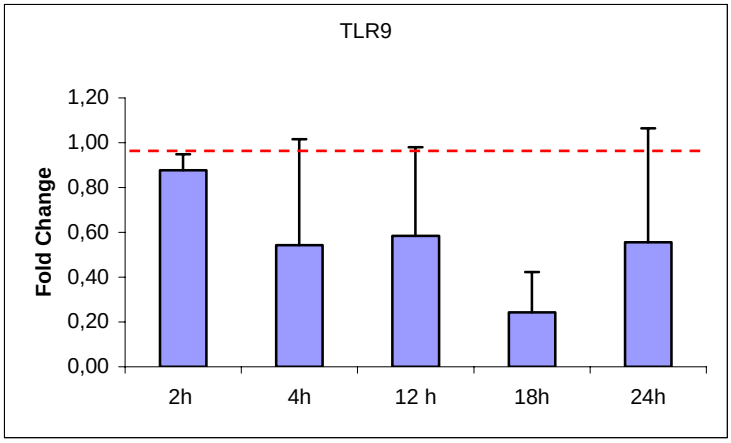


Figure 4.5 Fold Change in (A) TLR2, (B) TLR3, (C) TLR4, (D) TLR5, (E) TLR6 and (F) TLR9 expression in different time points post PH.

4.2.2. Expression of TLRs in MSC Administered PH Groups

The C_t values of PH groups (normal liver vs. 3 and 7 days) and MSC-administered PH groups (normal liver vs. 3 days) for TLR2, 3, and 9 genes and *CYC* were calculated. The C_t values of the PH and SH groups were normalized according to their difference from the C_t value of normal liver sample. By using normalized C_t values, ΔC_t ($\Delta Ct_{TLR\ gene} / \Delta Ct_{CYC}$) value for each group was calculated. Fold change in expression of all genes were calculated by using the formula given below (Table 4.2, 4.3, 4.4):

$$\text{Fold Induction of gene x} = \left(E_{\text{TLR gene}}^{\Delta Ct(Ct\ NL - Ct\ samples)} / E_{\text{CYC}}^{\Delta Ct(Ct\ NL - Ct samples)} \right)$$

Where *Ct NL* is Ct values for *normal rat*, and Ct *samples* are Ct values for different *PH groups*

Table 4.2 TLR2 real time RT-PCR data for PH and MSC administered PH groups

Group Names	Ct_{TLR2*}	ΔCt_{TLR2}	$E^{(\Delta CtTLR2)}$	Ct_{CYC}	ΔCt_{CYC**}	$E^{(\Delta CtCYC)}$	$E^{(\Delta CtTLR2)}/E^{(\Delta CtCYC)}$
1 day PH	25.9	1	1.94	15.5	0.6	1.48	1.31
1 day PH+ MSC from normal rat	25.2	1.7	3.09	16.8	-0.7	0.63	4.89
3 day PH	27.1	-0.2	0.88	16.8	-0.7	0.63	1.39
3 day PH+ MSC from normal rat	24.2	2.7	5.99	16.3	-0.2	0.88	6.83
5 day PH+ MSC from normal rat	25	1.9	3.52	16.8	-0.7	0.63	5.58
Normal rat +MSC from normal rat	26.5	0.4	1.30	16.7	-0.6	0.67	1.93
3 day PH+ MSC from 1 day PH rat	24.7	2.2	4.30	16.1	0	1.00	4.30
Normal rat+ MSC from 1 day PH rat	26.7	0.2	1.14	16.6	-0.5	0.72	1.59
3 day PH+ MSC from 3 day PH rat	26.8	0.1	1.07	17.2	-1.1	0.49	2.20
Normal rat+ MSC from 3 day PH rat	26.7	0.2	1.14	17.1	-1	0.52	2.20

* Ct_{TLR2} value for normal liver is 26.9.

** Ct_{CYC} value for normal liver is 16.1.

Table 4.3 TLR3 real time RT-PCR data for PH and MSC administered PH groups

Group Names	Ct_{TLR3*}	ΔCt_{TLR3}	$E^{(\Delta CtTLR3)}$	Ct_{CYC}	ΔCt_{CYC**}	$E^{(\Delta CtCYC)}$	$E^{(\Delta CtTLR2)}/E^{(\Delta CtCYC)}$
1 day PH	24.9	-1	0.51	15.5	0.6	1.48	0.34
1 day PH+ MSC from normal rat	24.2	-0.3	0.81	16.8	-0.7	0.63	1.29
3 day PH	25.4	-1.5	0.36	16.8	-0.7	0.63	0.57
3 day PH+ MSC from normal rat	23.6	0.3	1.23	16.3	-0.2	0.88	1.40
5 day PH+ MSC from normal rat	23.9	0	1.00	16.8	-0.7	0.63	1.58
Normal rat +MSC from normal rat	23.4	0.5	1.41	16.7	-0.6	0.67	2.09
3 day PH+ MSC from 1 day PH rat	24.7	-0.8	0.58	16.1	0	1.00	0.58
Normal rat+ MSC from 1 day PH rat	23.9	0	1.00	16.6	-0.5	0.72	1.39
3 day PH+ MSC from 3 day PH rat	24.9	-1	0.51	17.2	-1.1	0.49	1.04
Normal rat+ MSC from 3 day PH rat	23.5	0.4	1.31	17.1	-1	0.52	2.54

* Ct_{TLR3} value for normal liver is 23.9.

** Ct_{CYC} value for normal liver is 16.1.

Table 4.4 TLR9 Real time RT-PCR data for PH and MSC administered PH groups

Group Names	Ct_{TLR9*}	ΔCt_{TLR9}	$E^{(\Delta CtTLR9)}$	Ct_{CYC}	ΔCt_{CYC**}	$E^{(\Delta CtCYC)}$	$E^{(\Delta CtTLR2)}/E^{(\Delta CtCYC)}$
1 day PH	24.9	0.6	1.52	15.5	0.6	1.48	1.02
1 day PH+ MSC from normal rat	24.2	1.3	2.46	16.8	-0.7	0.63	3.90
3 day PH	25.4	0.1	1.07	16.8	-0.7	0.63	1.70
3 day PH+ MSC from normal rat	23.6	1.9	3.73	16.3	-0.2	0.88	4.26
5 day PH+ MSC from normal rat	23.9	1.6	3.03	16.8	-0.7	0.63	4.80
Normal rat +MSC from normal rat	23.4	2.1	4.29	16.7	-0.6	0.67	6.36
3 day PH+ MSC from 1 day PH rat	24.7	0.8	1.74	16.1	0	1.00	1.74
Normal rat+ MSC from 1 day PH rat	23.9	1.6	3.03	16.6	-0.5	0.72	4.21
3 day PH+ MSC from 3 day PH rat	24.9	0.6	1.52	17.2	-1.1	0.49	3.12
Normal rat+ MSC from 3 day PH rat	23.5	2	4.00	17.1	-1	0.52	7.72

* Ct_{TLR9} value for normal liver is 25.5.

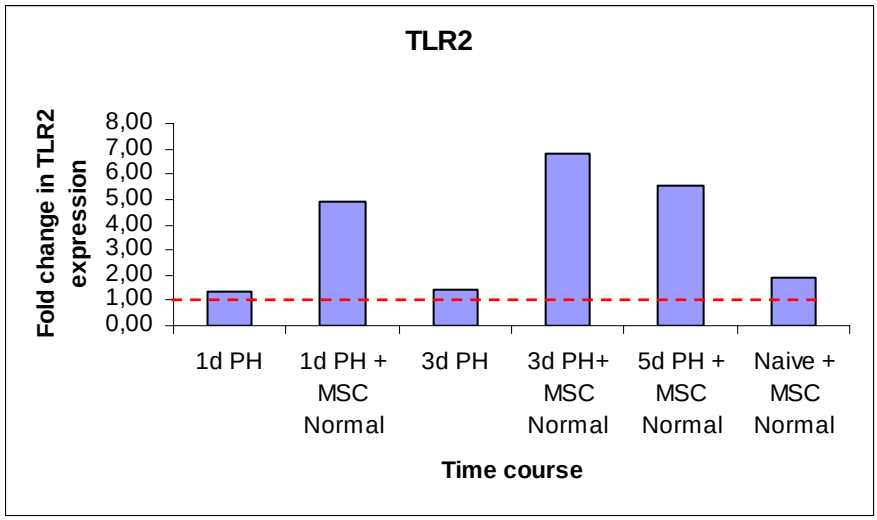
** Ct_{CYC} value for normal liver is 16.1.

Of three TLR genes tested, TLR3 gave the lowest fold change in message (Figures 4.6.). 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 4.2., 4.3., 4.4. and Figure 4.6. A, C, and E). The state of recovery does not influence this feature (*i.e.* whether the animal is day 1 or day 5 post operation). For PH animals after MSC (generated from normal rat) treatment, TLR2 and TLR9 fold change in expressions were significantly higher to that of TLR3 expression change (ca. 7 fold to 2 fold). It is fair to generalize that upon administration of MSCs (irrespective of MSC source) all three TLR expression levels increased for hepatectomized rats and decreased or remained unchanged for normal rat livers (compare Figs 4.6. B, D, F).

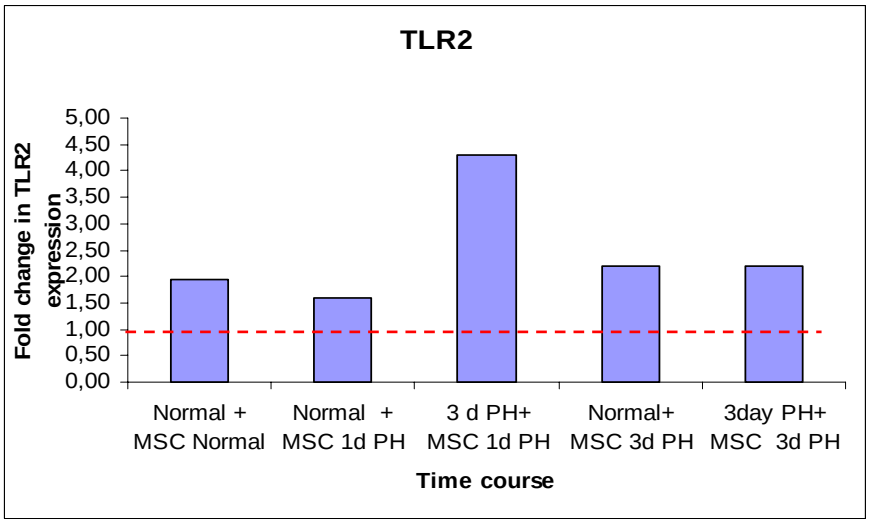
These data suggest that upon MSC injection *tlr gene* expressions dramatically increase in PH animals. This increase is not dependent on the hepatectomy but rather depends on the mediators/factors secreted from MSCs. When microscopic and PCR

data are taken together, MSC effect on TLR expression could be either *trans* or *cis*, since in the absence of MSCs residing at that site, of injury (lack of DiI signal) we still detect upregulation at day 1 and 5 post PH treatment.

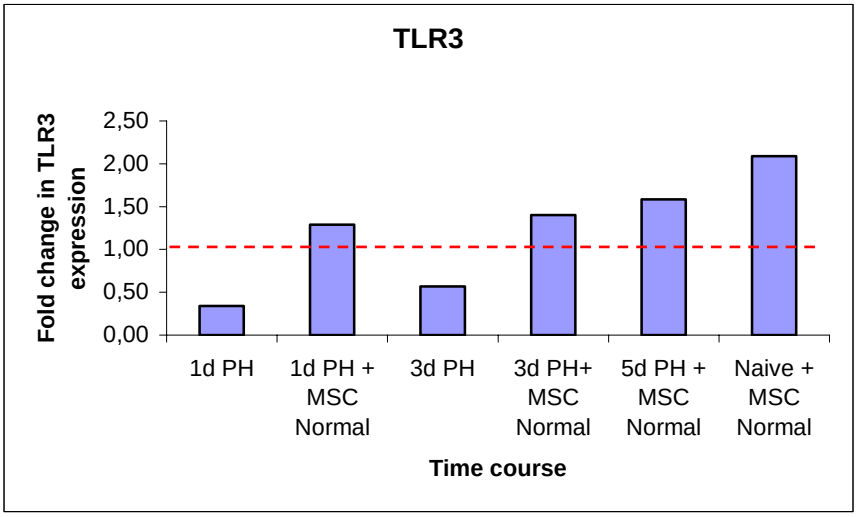
A)



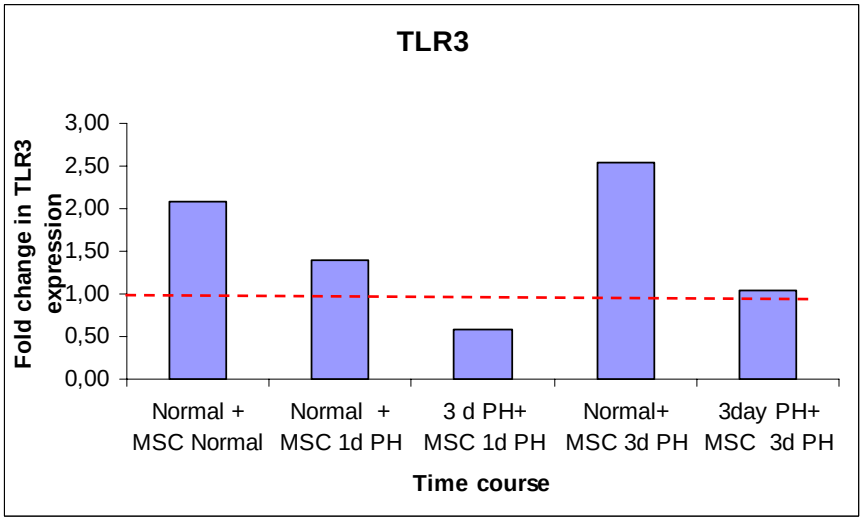
B)



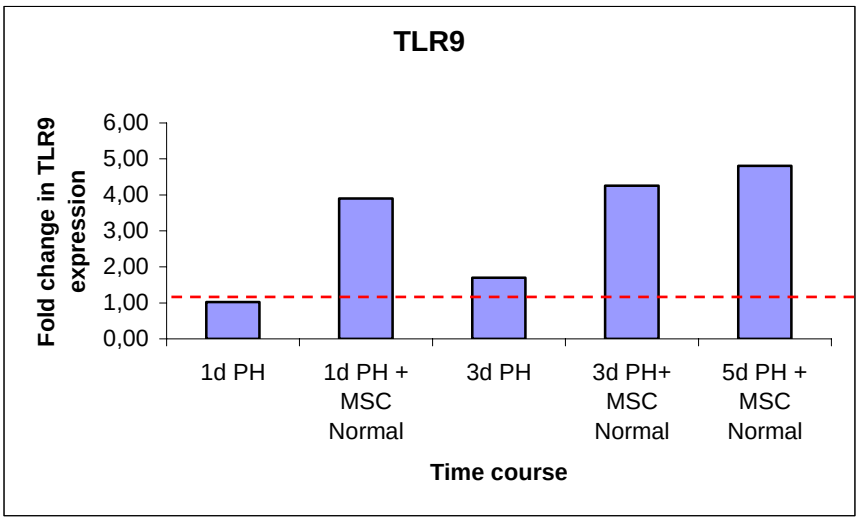
C)


TLR3
Fold change in TLR3 expression
2,50
2,00
1,50
1,00
0,50
0,00
1d PH
1d PH + MSC Normal
3d PH
3d PH+ MSC Normal
5d PH + MSC Normal
Naive + MSC Normal
Time course

D)


TLR3
Fold change in TLR3 expression
3,00
2,50
2,00
1,50
1,00
0,50
0,00
Normal + MSC Normal
Normal + MSC 1d PH
3 d PH+ MSC 1d PH
Normal+ MSC 3d PH
3day PH+ MSC 3d PH
Time course

E)



F)

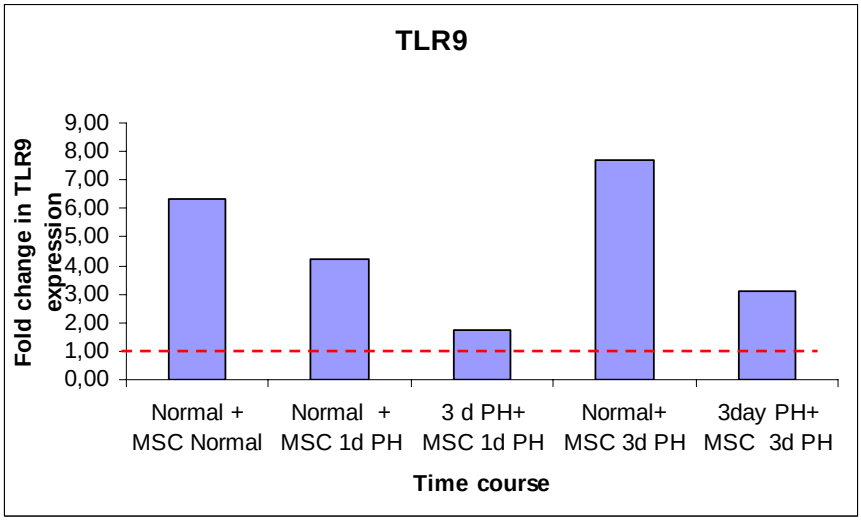


Figure 4.6. Fold change in (A, B) TLR2, (C, D) TLR3 and (E, F) TLR9 expression in PH and MSC-administered PH groups

4.3 Efforts to Delineate MSC Functions

Besides regeneration and tissue repair, MSCs are suggested to have role in immuneregulation (Ramasamy *et al*, 2007). It is shown both *in vivo* and *in vitro* that MSCs are not inherently immunogenic. In addition, they are called 'universal suppressor of immune system' having suppressive effect on different subsets of

immune cells (Maccario *et al*, 2005). Although they are claimed to be non-immunogenic, murine MSCs have shown to express several TLR receptors (Pervsner-Fischer *et al*, 2007). This paradoxical evidence suggests that MSCs could recognize several microbial by products collectively known as pathogen-associated molecular patterns. Considering accumulated data on immunosuppressive action of MSCs, the recent evidence suggest that either the TLRs are not functional or do not respond to their corresponding ligands even though they are present on MSCs or MSCs possess subpopulations that are either positive or negative for different TLRs. Until now the extent of TLR responsiveness on MSC at `passage 0` was not explored in detail by others. We aim to understand the molecular mechanism of the immuneregulatory function of mesenchymal stem cells (MSCs). Different from the previous sections, instead of rat we shift to mouse for these studies. The major reason for this change is because our stimulation and detection assay reagents are well established for mouse system.

4.3.1. TLR Expression Pattern of Mesenchymal Stem Cells and Spleen Cells

In this study; mouse MSCs were generated from bone marrow cells and incubated for two weeks in mesencult media.In all experiments the cells were used at passage 0 unless otherwise stated.

We first wanted to identify the TLR expression profiles of generated naive MSCs. As seen in Figure 4.7. MSCs at the end of 14 days of incubation were harvested and studied for indicated TLR genes from the purified total RNA we observed that they are expressing all known TLR mRNA as evidenced by PCR.

This piece of evidence can only establish that MSCs as well as mouse spleen cells (known to express several TLRs) have endogenous TLR message. Furthermore, different from naive spleen, MSCs are expressing TLR 1, 2, 3, 4, 5 (albeit at low level), 6, 7 and 9, whereas under the tested conditions we were unable to detect basal, tlr5 , tlr7 and tlr9 message from splenocytes.

Table 4.5. Product size of mouse TLRs and β actin

Gene Name	Product Size (bp)
mTLR1	410
mTLR2	320
mTLR3	250
mTLR4	240
mTLR5	380
mTLR6	650
mTLR7	700
mTLR9	430
m-β actin	450

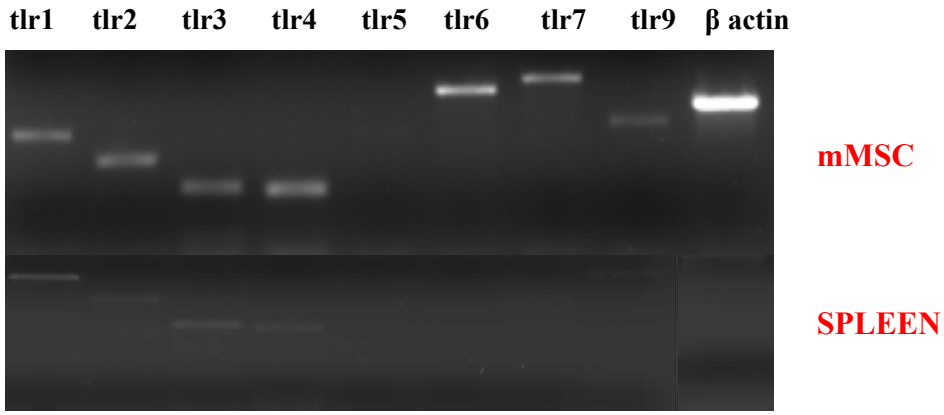


Figure 4.7. Variations in the TLR expression profile between mouse MSC (top panel) and spleen cells (bottom panel). Total RNA from murine splenocytes and MSCs were subjected to RT-PCR and *tlr1* to *tlr9* specific bands were visualized on agarose gel.

4.3.2. Immunoregulatory Effects of Mesenchymal Stem Cells

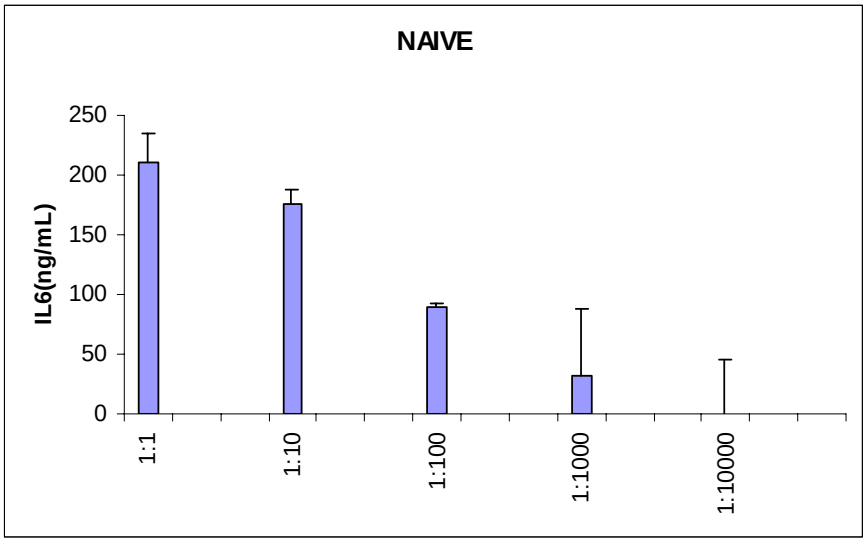
The detection of message is not directly indicative of the functionality of the receptor. There are several ways to test this. Since immunosuppressive action of MSCs are well established we reasoned that if we treat spleen cells with the corresponding TLR ligands, and co-culture with varying numbers of MSCs (even

though MSCs seem to possess TLR message) the stimulatory action of these ligands could be suppressed. Furthermore depending on the titrated MSC number this suppression may subside, thus implicate us that MSCs are immunosuppressive.

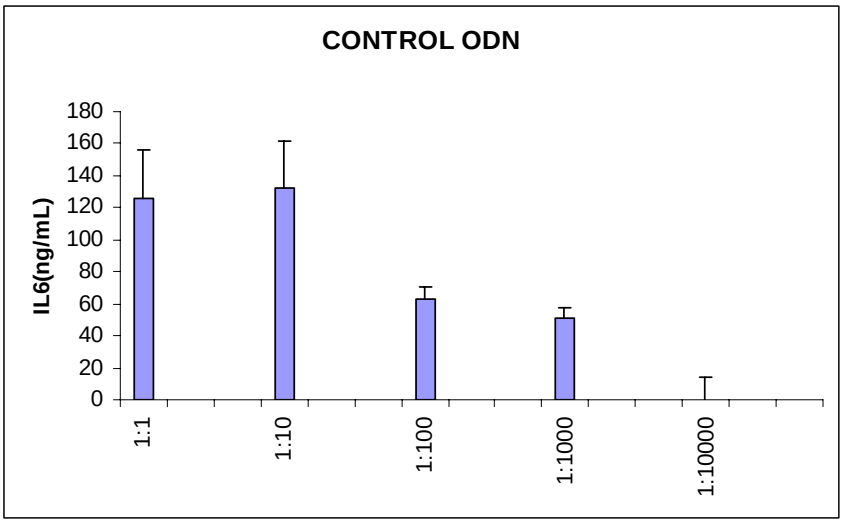
Splenocytes were plated as described in section 3.4.4. and following the addition of MSCs (cell number ratio ranged from 1:1 to 1:10000 MSC to Spleen cells) were stimulated with the indicated TLR ligands (i.e. for TLR2/6: PGN (5 µg/ml), TLR3: dsRNA (20 µg/ml), TLR4: LPS (1 µg/ml), TLR7/8: ssRNA or imiquimod (1 µg/ml) and TLR9: CpG DNA (1 µM)). The cells were stimulated for 48 h and supernatant was collected. Sandwich ELISA to detect IL6 production was studied from the supernatants (Figure 4.8.).

Unexpectedly, there was no significant suppression of the IL6 level. On the contrary, inclusion of MSCs in the spleen culture augmented cytokine secretion from spleen cells. This led us to test MSC only and spleen only induction and compare their IL6 production to that of co-culture experiments. As seen in Figure 4.8, MSCs by themselves when triggered by ligands were able to secrete IL6 in many cases at much higher level than spleen alone group (compare the induction levels of pI:C, LPS, R848 between MSC only and Spleen only groups in Figure 4.8. H and I responses). In particular, IL6 levels in response to pI:C and LPS stimulation synergize with increasing number of MSCs included in the splenocyte culture.

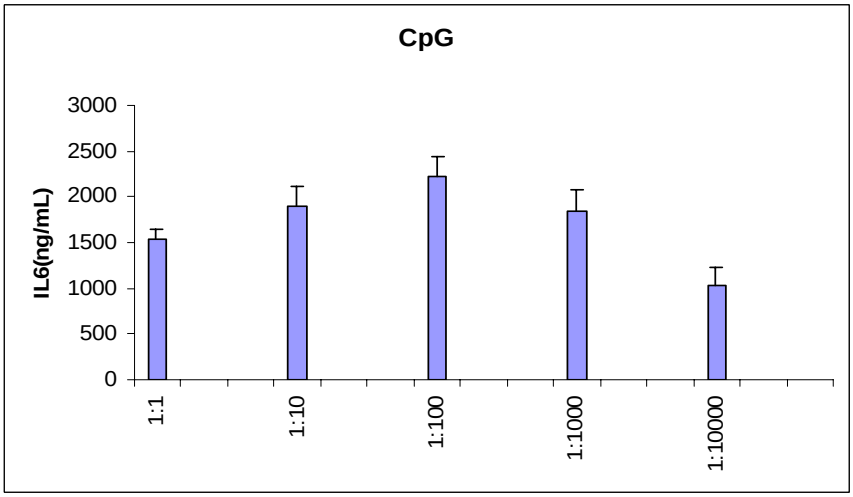
A)



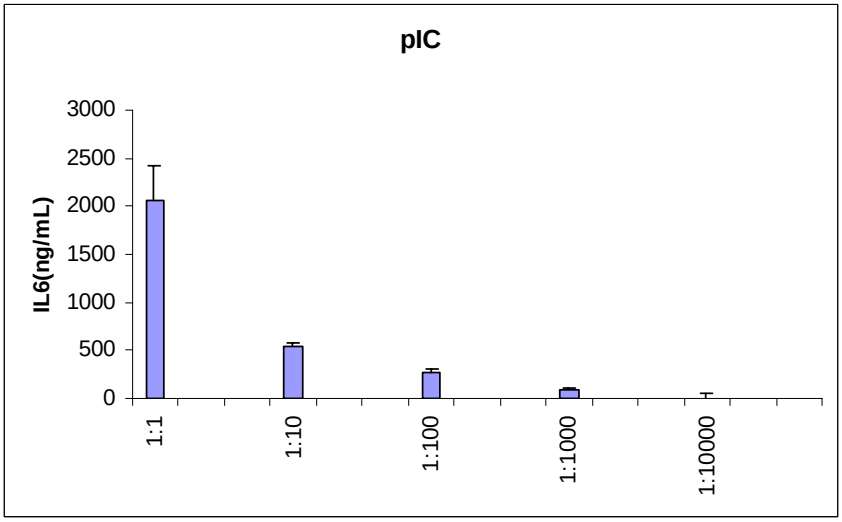
B)


CONTROL ODN
IL6(ng/mL)
180
160
140
120
100
80
60
40
20
0
1:1
1:10
1:100
1:1000
1:10000

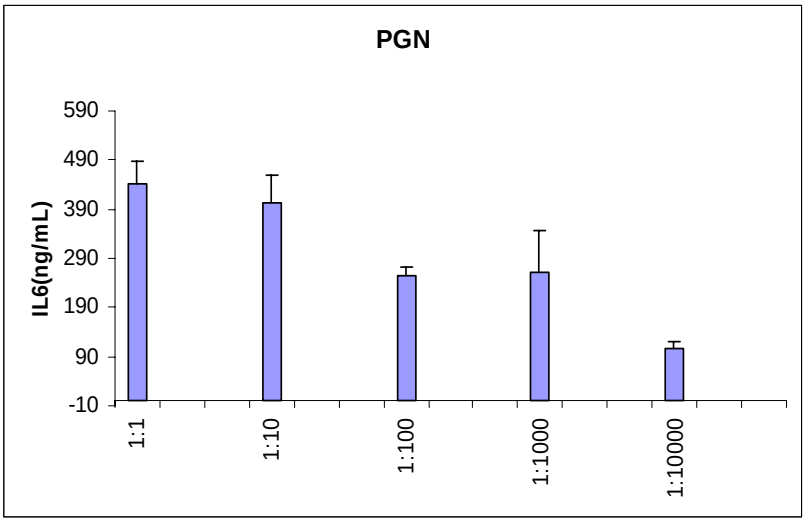
C)


CpG
IL6(ng/mL)
3000
2500
2000
1500
1000
500
0
1:1
1:10
1:100
1:1000
1:10000

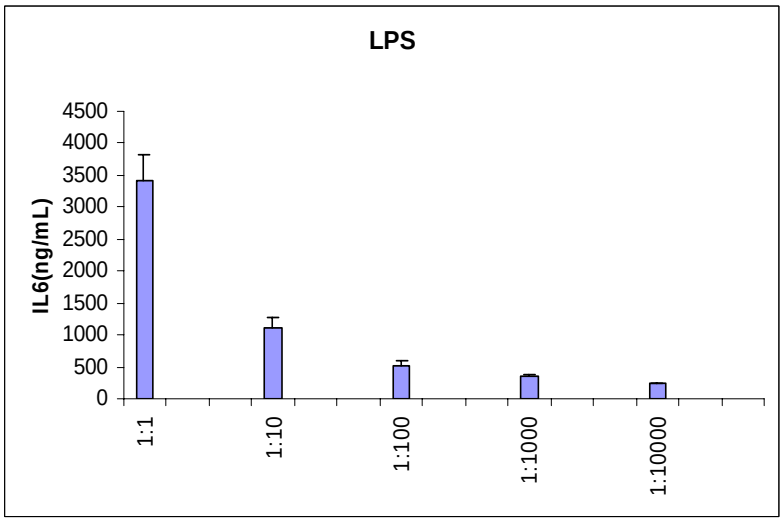
D)


pIC
IL6(ng/mL)
3000
2500
2000
1500
1000
500
0
1:1
1:10
1:100
1:1000
1:10000

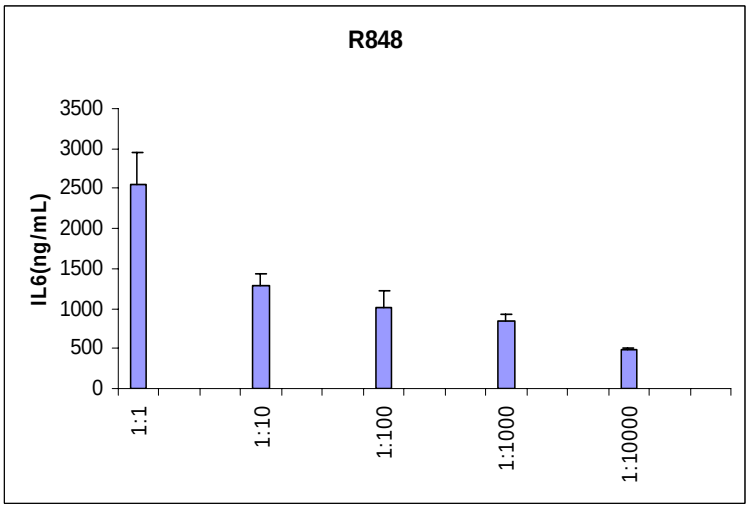
E)


PGN
590
490
390
290
190
90
-10
IL6(ng/mL)
1:1
1:10
1:100
1:1000
1:10000

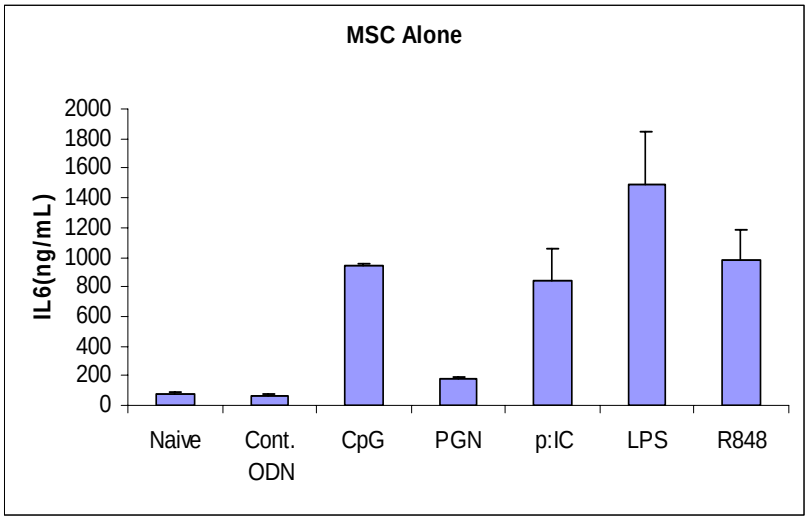
F)


LPS
4500
4000
3500
3000
2500
2000
1500
1000
500
0
IL6(ng/mL)
1:1
1:10
1:100
1:1000
1:10000

G)


R848
3500
3000
2500
2000
1500
1000
500
0
IL6(ng/mL)
1:1
1:10
1:100
1:1000
1:10000

H)



I)

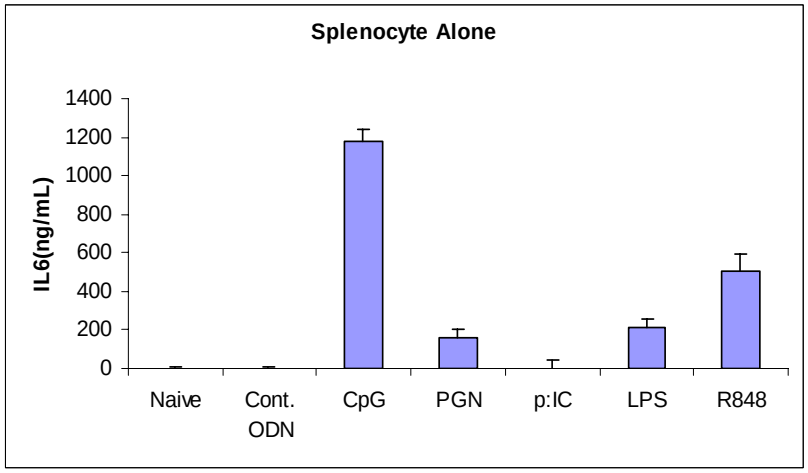


Figure 4.8. IL6 production after 48h stimulation of different ratios of MSC-Splenocyte co-culture with different TLR ligands. (A) Naive , (B) Control ODN: FlipCG1555 (1 µM), (C) TLR9: CpG ODN 1555 (1µM) , (D) TLR3: pI:C (20µg/mL), (E) TLR2/6: PGN (5 µg/mL), (F) TLR4: LPS (1 µg/mL), (G) TLR7: R848 (1 µg/mL), (H) MSC alone stimulated with all ligands and (I) Splenocyte alone stimulated with all indicated ligands.

4.3.3. Bone Marrow Cell Separation Before MSC Generation

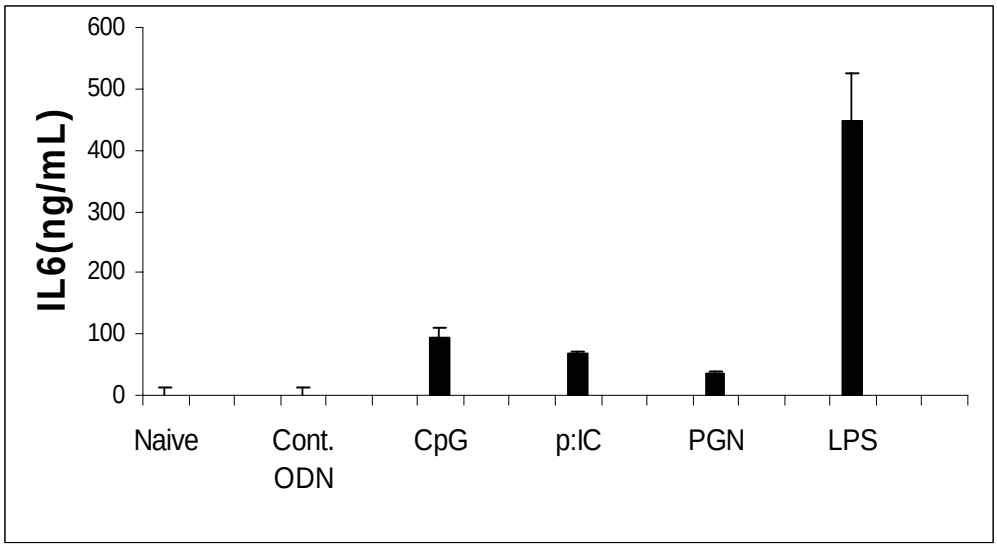
The immunostimulatory activity of MSCs could be due to contaminating cells remained in the culture; another possibility could be the existence of other MSC subsets within the population.

In addition to adherence method, after extraction of bone marrow cells one other approach is to separate BM cell population based on their CD34+ marker (separation of hematopoietic and non hematopoietic cells). We then wanted to understand what cell fraction is responsible from the immunomodulatory properties of the MSC.

In order to generate MSCs magnetic bead isolation method was selected. Cells were incubated with CD34 coated magnetic beads as described in section 3.15. After separation, cells were cultured in separate flasks as CD34- and CD34+ populations. At the end of 14 days in mesencult culture, as explained earlier (see section 3.4.4.) stimulation assay using several TLR ligands was conducted. IL6 levels of CD34-and CD34+ MSC cells were detected in response to PGN, pI: C, LPS, CpG ODN1555, and Control ODN (Figure 4.10 A and B).

We have expected to detect different IL6 production profiles from these sub populations but to our surprise both populations induced similar levels of IL6. Collectively, our results revealed that MSCs regardless of CD34 expression were secreting IL6 in response to indicated TLR ligands.

A)



B)

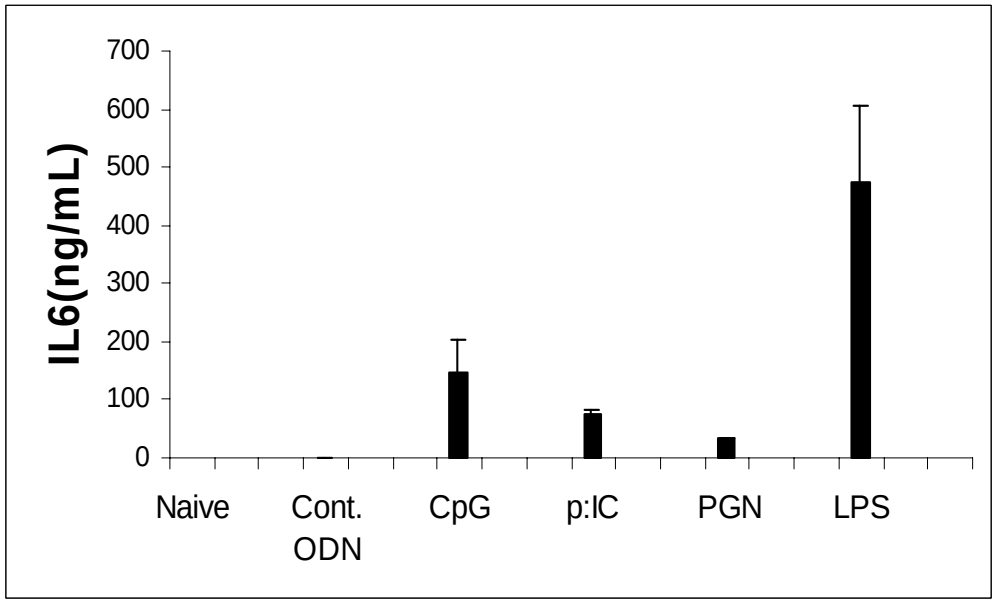


Figure 4.9. IL6 production after 5 days stimulation of (A) CD34- and (B) CD34+ cells with different TLR ligands.

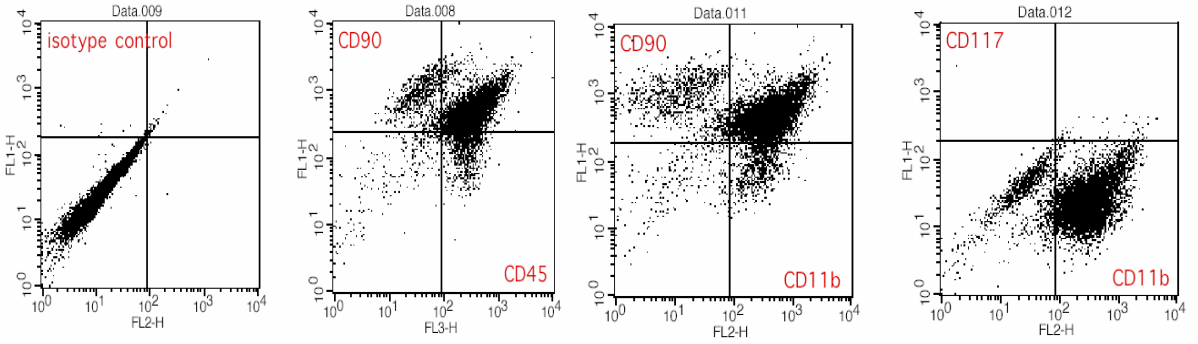
4.3.4. MSC Characterization by Flow Cytometry

When culturing MSCs, according to accumulating evidence; as passage number increases cell surface marker expression of the MSC population varies. While the percentage of expression of certain markers increases certain others become more negative among MSC cell population. Characterization of murine MSCs is still a huge challenge since limited numbers of cell surface markers are available. In that respect, ongoing studies are planned to determine whether certain candidate markers may be reproducibly used to characterize murine MSCs using flow cytometric approaches.

In order to analyze the percentage change of MSC specific markers and also delineate the change in the contaminating cell fraction proliferating along with MSCs we have decided to follow the expression of several positive and negative markers such as i) CD90 (Thy 1.2), ii) CD45 , iii) CD11b , and iv) CD117 by flow cytometry (Figure 4.11). Our results showed that the MSC population became more CD45 and CD11b positive and CD117 negative. (Figure 4.11., Table 4.6), CD90 expression decrease significantly at passage 3.

It is very unfortunate that there is no specific surface marker widely accepted as indicative of MSC generated from BM. At this stage we can not confidently say that we have generated MSCs of mixed population or there are contaminating cells that dominating the culture. But can only conclude that there are couple of markers expressed by these cells at the end of 14 day culture, and suggest that our cell population we have substantial amount of MSC.

A) Passage # 1



B) Passage # 3

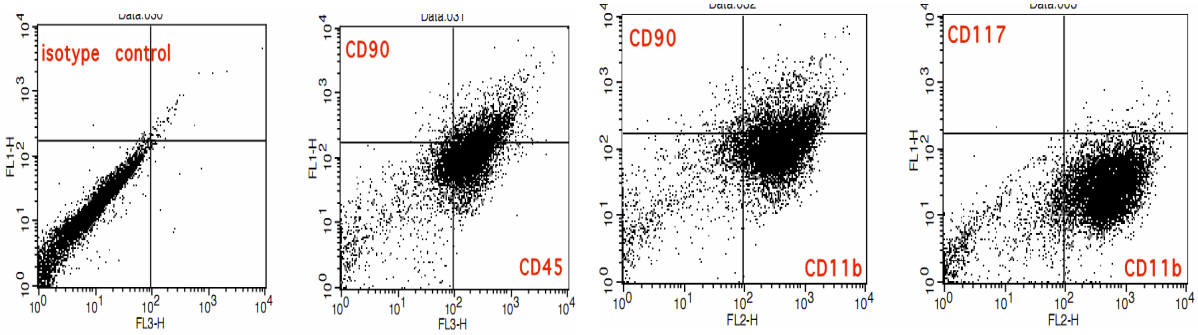


Figure 4.10. Surface marker expression change of different MSC passages. MSC from (A) passage number 1 and (B) passage number 3 were fixed and labeled with FITC-CD90, PE/Cy5-CD45, PE-CD11b and FITC-CD117.

Table 4.6. Expression of surface markers in different passages

Single or double positive % of cells	Passage # 1	Passage # 2	Passage # 3
CD90	87.8	91.2	22.6
CD45	89.4	91.6	80.6
CD11b	87.2	88.7	86.9
CD117	0,64	1.1	2
CD90-CD45	79.5	84.3	21.6
CD90-CD11b	79.6	84.9	20.6
CD117-CD11b	0.6	1	0.9

4. 5. TLR Mediated Proliferation and Morphological Changes of Murine Mesenchymal Stem Cells

In order to check effect of TLR ligands on the morphology of MSCs, we stimulated MSCs (10^6cells/well) with Control ODN, CpG, pI: C, PGN and Gardiquimod in every 3 days as described in section 3.4.4. TLR mediated morphological changes were observed at the end of day 12 of continuous stimulation (Figure 4.11.). In particular, TLR3 ligand p:IC seems to have detrimental effect on MSCs. Moreover, PGN and Gardiquimod seem to have a proliferative effect on MSCs.

In this study, we have found that CM-DiI labeled MSCs, generated from normal rat, are localized particularly in the liver of 3d post PH rats. Homing capacity of them are seemed to be decreased when MSC are generated form 3d post PH rats. Moreover, endogenous MSCs (CD90 positive) and hepatic oval cells (Flt3 positive) are found to be localized around CM-DiI labeled. Although mechanism is unclear, that observation is interesting in terms of attributing a role to MSCs in liver regeneration. Another intriguing observation is that MSC seems to increase TLR mRNA expression, particularly, TLR2, 3 and 9, in post PH rats. This may be another potential mechanism of MSC and also TLR contribution to liver regeneration process. Second part of my study concentrate on mostly characterization of MSCs since .We found that MSC express several TLRs and functionality of TLRs are confirmed by cytokine ELISA assay. In accordance with these data, change in the cell surface markers in different passages and different morphological and proliferative responses of MSCs to TLR ligand make us to think either there is a sub-population or contaminating cells in our MSC population.

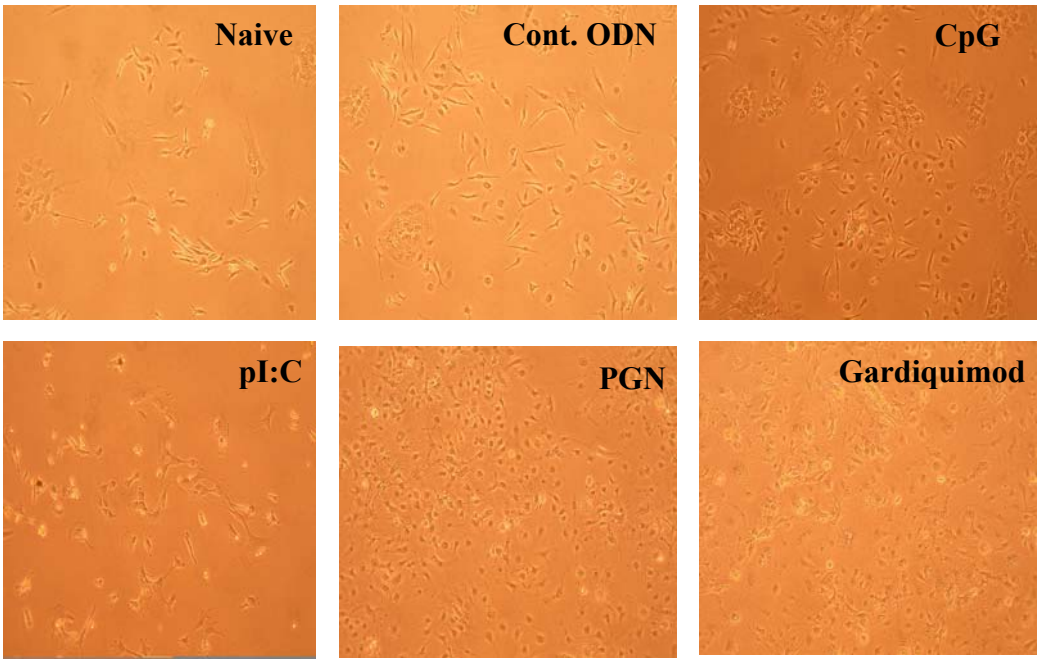


Figure 4.11. TLR mediated proliferation and morphological changes of murine MSCs. MSCs (10^6cells/well) were stimulated with indicated TLR ligands for 12 days (every 3^{rd} day ligands were replenished). Photos were taken after 4^{th} stimulation at 12^{th} day.

5. DISCUSSION

In this study, we aimed to understand whether liver regeneration have an effect on homing properties of bone marrow derived MSCs and also whether MSCs have a role in liver regeneration. Moreover, potential role of TLRs in both liver regeneration and their immunoregulatory properties are aimed to be elucidated.

Although Popp *et al.* (2007) reported that multipotent mesenchymal stromal cells from rat do not differentiate into hepatocytes *in vivo* when transplanted in regenerative conditions, Lee *et al.* (2004) has shown that bone marrow-derived mesenchymal stem cells from human differentiate into functional hepatocyte-like cells under defined conditions pointing out the potential for clinical applications. They have recently showed that these cells can effectively rescue experimental liver failure (upon CCl_4 administration) and contribute to liver regeneration. Thus, these can be used as an alternative therapy to organ transplantation for the treatment of liver diseases (Kuo *et al*., 2008). These data make us to think about the potential involvement of mesenchymal stem cells in experimental liver regeneration process using hepatectomized rat (PH) as a model. In order to investigate homing of MSCs in liver after PH, CM-DiI labeled MSCs were administrated to 1d, 3d and 5d PH rats. Our results revealed that only in 3 day PH rat, MSC were localized in liver. On the other hand, MSC localization was not observed in the liver of either 1d PH or 5d PH rats.

Homing property of MSCs is also investigated in MSCs generated from 1 day post PH and 3 day post PH bone marrow. Although MSCs obtained from 1 day post PH rats is also localized in the liver of 3 day post PH rat, localization of MSC could not be seen in liver of 3 day post PH rat when MSCs are generated from 3 day post PH rats. This observation leads to the question of whether there may be a negative contribution of PH in homing property of MSCs.

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. The IF data from CD90 immunostained liver sections revealed that the timing of the appearance of endogenous MSCs are similar to that of exogenous allogeneic MSCs. They were seen in the liver regeneration site and localized in liver of 3d post PH which is remarkably similar to that of exogenous allogeneic MSCs injection. Other emerged question is why allogeneic MSC do not express CD90. A possible answer may be that MSC, as shown by Lee *et al.* (2004), can be differentiated into hepatocyte-like cells, thus cell surface markers may change. Therefore one can speculate that, the answer of former question may be found in the probable answer of latter question. If exogenous MSC started to differentiate into hepatocyte, then they would reside in liver and drive endogenous MSC localization. However, this scenario remained to be elucidated in depth.

It is also interesting that, the marker of hepatic oval cells, Flt3 expression, was observed only in MSCs administrated into 3 day PH rats in the vicinity of labeled MSCs. Expression of Flt3 around this location suggests the onset of progenitor-dependent liver regeneration.

Several studies have shown that multiple pathways play role during liver regeneration. Faust *et al* (2006) proposed that three major pathways may regulate the circuitry required for liver regeneration. These are cytokine, growth factor and 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. In particular, IL6 and TNFα 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. Iimuro *et al.* showed that IL6 and TNFα 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-κB activation and IL6 secretion (Campbell *et al*, 2006; Seki *et al,* 2005). 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,

1996). Therefore, we decided to investigate the mRNA expression of TLR2, 4, 9 as well as TLR 3, 5, 6 in 0h, 2h, 4h, 12h, 18h and 24h after PH. Our results revealed that induction in the expression of TLR 2, 3, 4, 5 and 9 do not exceed 1 to 2 fold in all time points compared to the expression seen for normal animals at 0h. On the other hand, TLR6 mRNA expression increases 3, 5 fold in 18h PH group. Since TLR6 function requires dimer formation with TLR2 and no significant upregulation was seen for TLR2, we reasoned that this upregulation can not be physiologically meaningful, but could be a spike appeared due to assay procedure.

As we hypothesized that mechanism of MSC homing to injury site may be due to TLR expression ability of the organ in which injury occurs, we investigated the relationship between the MSC homing to liver and TLR expression profile in response to PH. We particularly choose TLR 2, 3 and 9 since we have found their expressions are relatively unchanged over the period of 24 hours after PH. Thus, any change in the expression could only be explained by the fact that the injected MSCs may contribute to these expression shifts. TLR 2, 3 and 9 expressions were investigated in 1d post PH and 3d post PH rats with or without MSC administration. Our results revealed that mRNA expression levels of TLR 2, 3 and 9 are increased in MSC-administrated 1d PH and 3d PH in comparison to that of liver samples that had no MSC injection. Our data suggest that the driving force of MSC homing is not related to the increase in TLR expression, rather, MSC homing led to increase in TLR gene expression in post PH rats.

When this data along with DiI-labeled MSC immunofluorescence study were taken together, the increase in TLR expression by injected MSCs is modulated either by *cis* (at day 3), or effects *trans* (at days 1 and 5). The absence of MSCs at post PH 1 and 5d at the liver sections suggest that even though we could not detected the labeled cells during fluorescence microscopy the injected MSCs might be still secreting certain factors into circulation and these factors are sufficient enough to drive the cells of injured liver to increase their TLR expression.

One could argue that another possibility for increased TLR expression could be due to the presence of the injected MSCs existing at the site rather than the liver cells. This is an impossible possibility since we only injected one million cells and the amount of tissue used during RNA extraction is in the order of several milligrams.

Characterization of MSC is the most challenging aspect of MSC studies. There are controversial data showing that MSCs are immunosuppressive but also exert immune response through TLR signaling pathways. Although MSCs are claimed to be non-immunogenic, Pevsner-Fischer *et al* (2007) have shown that they express TLR1-8. These observations led us to investigate the immunoregulatory properties of MSC which is extremely important for MSC based cell therapies. Our TLR expression results confirm Pevsner-Fischer data although we also found the expression of TLR9 in addition to others. Since mRNA expression may not mean functionality of the proteins, we decided to check the immunoregulatory property by MSC/splenocytes co-incubation experiments. Surprisingly, there seems no suppressive effect induced by MSCs on splenocytes, rather IL6 secretion increases with increasing ratio of MSC: splenocyte in response to several TLR ligand stimulation. This observation implied that our MSC population could be either composed of heterogeneous population or could contain a fraction of contaminating adherent cells of hematopoietic origin BM cells (that remained after adherence method separation procedure). We, therefore, further the surface marker expression of these MSCs by flow cytometry. Although there is no consensus as to which surface markers are useful for MSCs characterization, we used most widely accepted CD90 as a positive and hematopoietic stem cell marker (CD45 and CD117) and macrophage (CD11b) markers as negative controls.

Our results showed that percentage of surface marker expression changes with respect to the increasing passage number. In particular, we found that CD90 expression decreases significantly at passage 3. There may be two explanations for this observation. The population is either contaminated by other cells and these cells rather than MSCs, are proliferating and diluting the MSCs as passage progresses.

Secondly, there may be MSC subpopulations that are proliferating and dominating the population in later passages. If the latter explanation is the case, the characterization of these subpopulations may be very useful for understanding immunological properties of MSCs.

Despite the probability of subpopulations or contaminating cell, IL6 secretion of MSCs regardless of subpopulations is still interesting. As mentioned above, IL6 secretion by Kupffer cells were found to be required for initiation of liver regeneration after PH. This may be a potential mechanism of MSC having role in liver regeneration.

In conclusion, we found that intravenously injected MSCs localized in livers of 3d post PH rat independent of their original TLR expression. Moreover, our data strongly suggest that it is the MSC injection that drive increase in TLR 2, 3 and 9 expression in rats (including 1d, 3d and 5d post PH) but not the vice versa.

6. FUTURE PERSPECTIVES

Our studies revealed preliminary data on the timing of MSC localization during liver regeneration. Moreover, endogenous MSC and hepatic oval cells are observed after MSC administration to 3day PH rats. However, we do not know the factors that regulate the specific pattern of MSC localization in regenerated liver. Future studies to elucidate these factors (such as chemoattractant molecules) definitely will help to better understand liver regeneration process and provide very useful information for possible therapies.

In order to extent our observation that MSC injection increased TLR mRNA expression, TLR staining of the liver sections should be studied and the tissue distribution of TLR proteins in response to MSC administration should be demonstrated.

Tomchuck *et al* (2007) recently shown that TLR stimulation on human MSC drives their migration *in vitro*. In the light of this data, one can hypothesize that TLR expression profile on the MSC may contribute to homing of them through liver in response in PH. The TLR profile of MSC generated from normal, 1d post PH and 3d post PH rats can be a significant data for revealing the role of TLR in homing property. Moreover, these groups of MSCs may be injected to hepatectomized animals (with/without immunesuppressing agents) after stimulating with several TLR ligands.

Future studies should include significant effort to elucidate the existence of MSC sub-populations and possibly better surface marker characterization. It would be fascinating to identify a subset of MSCs that do not possess TLR and drive immunosuppressive effect as well as another subpopulation that do express and induce immunostimulatory action that can cooperate with innate immune cells present at the local inflammatory site.

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8. APPENDICES

8.1. Appendix A

Standard Solutions and Buffers

Blocking Buffer (ELISA)

500 mL 1XPBS

25 g BSA (5%)

250 µl Tween20 (0.025%)

Crystal particles of BSA should be dissolved very well, with magnetic-heating stirrer for 20-30 minutes. The buffer should be stored at -20˚C.

T-cell Buffer (ELISA)

500 mL 1X PBS

25 mL FBS (5%)

250 µl Tween20 (0.025%)

The buffer should be stored at -20˚C.

Wash Buffer (ELISA)

500 mL 10X PBS

2.5 mL Tween20

4.5 lt dH_2O

DEPC-Treated ddH_2O

1mL DEPC

1 lt ddH_2O

Stirred in a hood for 1 hour, autoclaved in order to inactivate the DEPC

10X PBS

80 g NaCl

2 g KCl

8.01 g $Na_2HPO_4.2H_2O$

2g KH_2PO_4

1 liter ddH_2O pH: 7.2

Working solution (1X PBS) was prepared by diluting 10X PBS by 10 times

50X TAE (Tris-Acetate-EDTA) Buffer

2M Tris Base (242 g)

57.1 mL Glacial Acetic Acid

50 mM EDTA

Add to 1 lt by ddH_2O

Working solution (1X TAE) prepared by diluting 50X TAE by 50 times

10X Agarose Gel Loading Dye

0.009 g BFB

0.009 g XC

2.8 mL ddH2O

1.2 mL 0.5M EDTA.

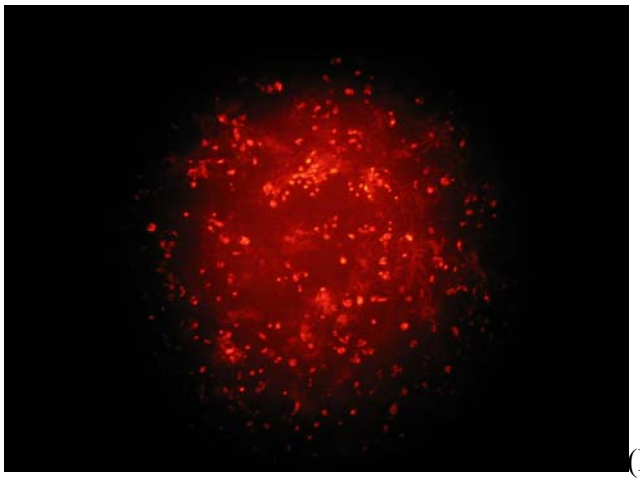
Total volume brought to 15 mL by adding glycerol, dilute 1:10 in sample prior to loading to electrophoresis gel

Blocking Solution for Immunofluorescent Staining

2% BSA

8.2. Appendix B

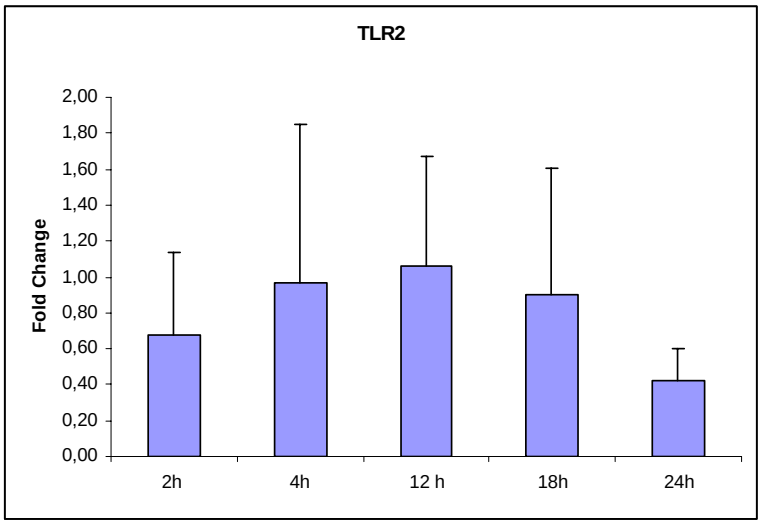
Photomicrograph of CM-DiI labeled MSCs before injection (4 days post staining in culture)

 (Magnification: 10X)

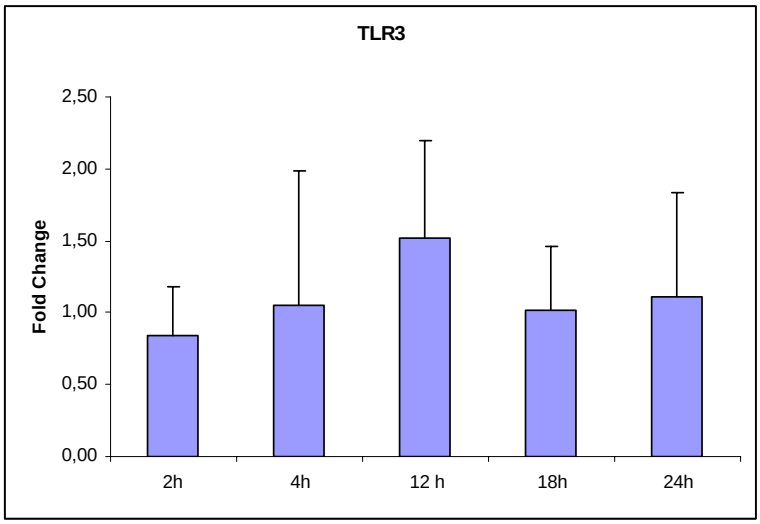
8.3. Appendix C

Fold Change in TLR2, TLR3, TLR4, TLR5, TLR6 and TLR9 expression in sham groups. Total RNA was recovered at different time points post PH.

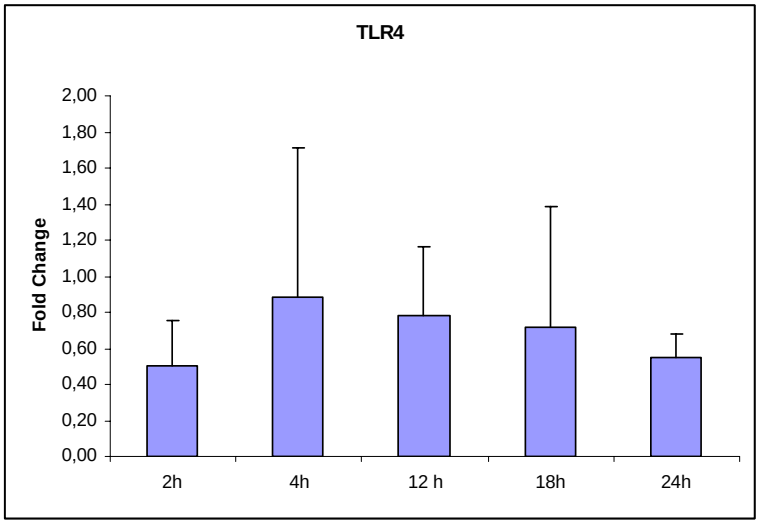
A)



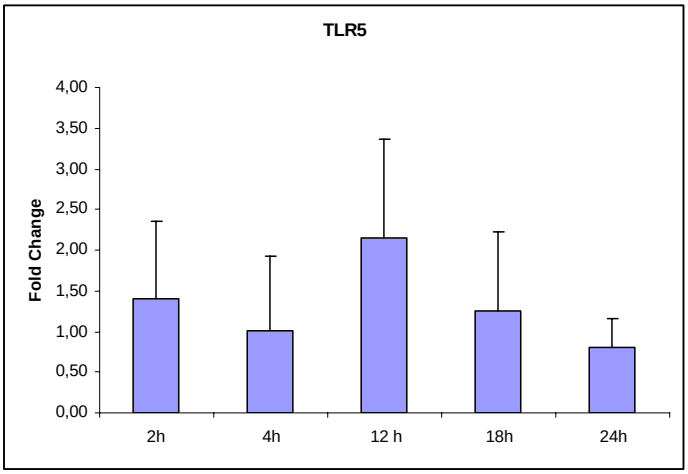
B)


TLR3
Fold Change
2,50
2,00
1,50
1,00
0,50
0,00
2h
4h
12 h
18h
24h

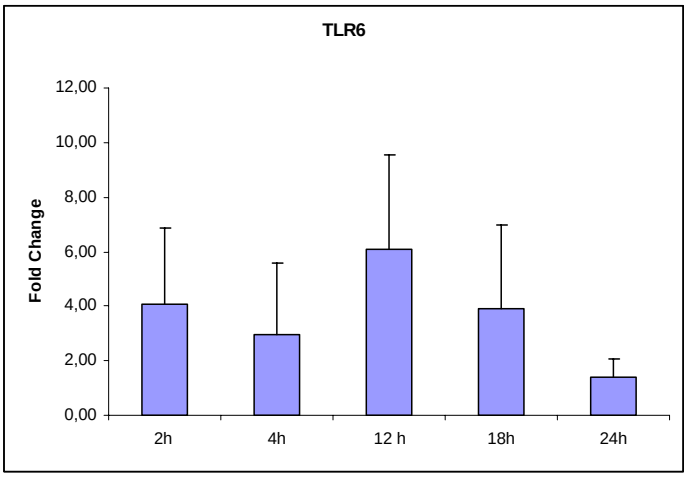
C)


TLR4
Fold Change
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
2h
4h
12 h
18h
24h

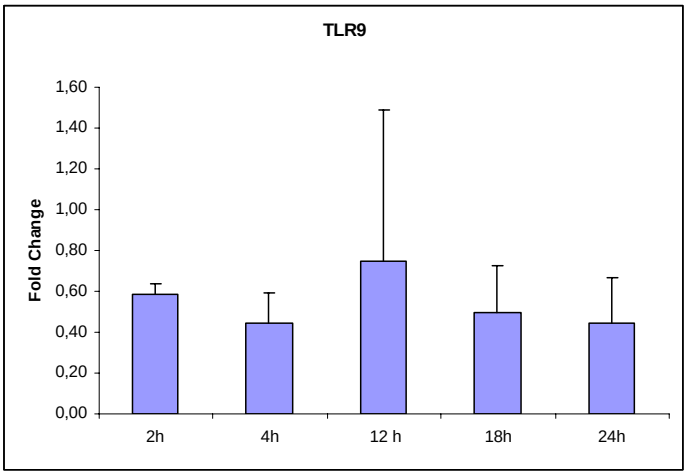
D)


TLR5
Fold Change
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
2h
4h
12 h
18h
24h

E)


TLR6
Fold Change
12,00
10,00
8,00
6,00
4,00
2,00
0,00
2h
4h
12 h
18h
24h

F)


TLR9
Fold Change
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
2h
4h
12 h
18h
24h