CONTRIBUTION OF TOLL-LIKE RECEPTORS TO MESENCHYMAL STEM CELL DIFFERENTIATION AND IMMUNOMODULATION

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ABSTRACT

CONTRIBUTION OF TOLL-LIKE RECEPTORS TO MESENCHYMAL STEM CELL DIFFERENTIATION AND IMMUNOMODULATION

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Mesenchymal stem cells (MSC) are pluripotent progenitor cells harboring unique characteristics along with several stem cell features such as lineage dependent differentiation and self-renewal capacity. MSCs are known to induce immunomodulatory activity and homing capacity to damaged tissue sites. Such diverse capabilities of MSCs make them distinct from adult stem cells and can be harnessed in several therapeutic applications.

Toll-like receptors (TLR) can recognize conserved microbial byproducts and are mainly expressed by innate immune system cells as well as epithelial or endothelial cells. Recent findings suggest that *in vitro* generated MSCs express some of these pathogen recognition receptors. In our view, to broaden the breath of the therapeutic potential, TLR mediated activation of MSCs and demonstrate its impact on differentiation and immunomodulatory activity is critical.

First, bone marrow-derived MSCs were generated and characterized via their surface marker expression by FACS (CD90, CD106 and CD45) at protein level and their message transcripts by RT-PCR (CD11b, CD29, CD34, CD45, CD71, CD73, CD90 and CD166). The most abundant marker was found to be CD90 over several passages. Following determination of TLR expression profile by RT-PCR, contribution of TLR ligands addition (TLR2, TLR3, TLR7 and TLR9) to MSCs during adipogenic or osteogenic differentiation was studied. TLR3 was found to be the most abundant type over several passages. The adipogenic differentiation of rMSCs was found to be facilitated in the presence of TLR2 TLR3 and TLR7 ligands. Additionally, changes in the adipogenic and osteogenic markers (LPL, PPAR-g for adipogenesis, and ALP, OC-1, RUNX for osteogenesis) were analyzed by RT-PCR. While adipogenic markers upregulated osteogenic markers were downregulated in response to TLR ligand treatment.

The final part of this study was performed with mouse mesenchymal stem cells. In order to define the immunostimulatory/immunosuppressive potential of mouse MSCs, immunomodulatory character of MSCs were examined in the presence or absence of mouse spleen cells. Our data suggested that when mMSCs are primed with TLRL, a pro-inflammatory cascade as evidenced by increased IL-6 and IFN-γ secretion is initiated either alone or in co-culture with splenocytes.

In conclusion, TLR priming of MSCs augments their differentiation primarily into adipogenesis, and mainly these cells are immunostimulatory.

Keywords: Mesenchymal stem cells, Toll-like receptors, differentiation, immunomodulation.

ÖZET

TOLL BENZERİ RESEPTÖRLERİN MEZENKİMAL KÖK HÜCRE FARKLILAŞMASINA VE İMMÜNMODÜLASYONUNA KATKILARI

İBRAHİM FIRAT TAŞ
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Mezenkimal kök hücreler (MKH) değişik doku tiplerine ait hücrelere farklılaşabilen ve kendini yenileme gibi çeşitli kök hücre özelliklerinin yanı sıra özgün karakteristiklere sahip çok potansiyelli öncül hücrelerdir. MKH, immün düzenleyici aktiviteyi uyarma ve yaralı dokuya göç etme özellikleri ile bilinmektedir. Bu tür farklı kapasiteleri, MKH'yi, diğer yetişkin kök hücrelerden farklı kılmakta ve terapötik uygulamalarda kullanılabilinir hale getirmektedir.

Toll-benzeri reseptörler (TBR), korunmuş mikrobiyal yan ürünleri tanımakta ve başlıca doğal bağışıklık sistemi hücreleri yanı sıra endotel veya epitel hücreleri tarafından da ifade edilmektedirler. Son bulgular, *in vitro* ortamda geliştirilen MKH'nin bu patojen tanıyıcı reseptörlerden bazılarını ifade ettiğini ileri sürmektedir. Görüşümüze göre, terapötik uygulamalarını genişletmek için TLR aracılıklı MKH aktivasyonu ile bu durumun hücre farklılaşma ve immün düzenleyici potansiyeli üzerine olan etkilerini araştırmak kritiktir.

İlk olarak, kemik iliği kökenli MKH geliştirildi ve yüzey işaretçilerinin ifadesi (CD45, CD90 ve CD106) FACS ile protein seviyesinde; mesaj transkriptleri (CD11b, CD34, CD45, CD71, CD73, CD90 ve CD166) RT-PCR ile karakterize edilmiştir. Hücre pasajları boyunca CD90 en bol işaretçi olarak bulunmuştur. TBR ifade profillerinin RT-PCR ile belirlenmesinden sonra; TBR ulaklarının (TBR2, 3, 7 ve 9) MKH'nin adipojenik ve osteojenik farklılaşmasına katkısı çalışılmıştır. Farklı hücre pasajlarında TBR3 en yoğun bulunan tip olarak bulunmuştur. Sıçan MKH'lerin adipojenik farklılaşmasının TBR2, TBR3 ve TBR7 ulaklarının varlığında hızlandığı bulunmuştur. Ayrıca, adipojenik ve osteojenik değişimlere özgün işaretçilerin (adipojenez için LPL, PPAR-g ile osteojenez için ALP, OC–1 ve RUNX) mesaj düzeyleri RT-PCR ile analiz edilmiştir. TBR ulaklarının muamelesine karşılık olarak adipojenik işaretçilerin ifadesi artarken osteojenik işaretçilerin ifadesi azalmıştır.

Bu çalışmanın son kısmı fare MKH'leri ile gerçekleştirilmiştir. Fare MKH'sinin immün uyarıcı/baskılayıcı potansiyelini belirlemek için fare MKH'sinin immün düzenleyici karakteri fare dalak hücreleri varlığında ve yokluğunda araştırılmıştır. Verilerimiz, fare MKH'sinin TBR işlemesiyle, yalnız olarak veya dalak hücreleriyle ko-kültür ortamında, IL-6 ve IFN-γ salınımının tanıklığında pro-inflamatuvar bir akışın başlatıldığını önermektedir.

Sonuç olarak, MKH'nin TBR işlemesi öncelikle adipojenez olmak üzere hücrelerin farklılaşmasını artırmaktadır ve temelde bu hücreler immün uyarıcıdır.

Anahtar kelimeler: Mezenkimal kök hücre, Toll-benzeri reseptör, farklılaşma, immün modülasyon

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ABBREVIATIONS

ALP	Alkaline phosphatase
APC	Antigen presenting cell
bp	Base pairs
BCR	B-cell receptor
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CpG	Unmethylated cytosine-phosphate-guaniosine motifs
DC	Dendritic cell
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked-Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
g	Grams
GvHD	Graft-versus-host Disease
HSC	Hematopoietic Stem Cell
ICAM	Intracellular Adhesion Molecule
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IFN	Interferon
µg	Microgram
µl	Microliter
LBP	LPS-binding protein
LPL	Lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipotheicoic Acid
M	Molar
MALP	Mycoplasmal lipopeptide

MAP	Mitogen-activated protein
MCP	Monocyte Chemoattractant Protein
MDP	Muramyl dipeptide
MHC	Major Histocompatibility Complex
mDC	Myeloid dendritic cells
MSC	Mesenchymal stem cell
MyD88	Myeloid Differentiation Primary Response gene 88
NaCl	Sodium Chloride
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NLR	NOD-like receptors
NOD	Nucleotide binding oligomerization domain
OC-1	Osteocalcin-1
OD	Optical Density
ODN	Oligodeoxynucleotide
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
p(I:C)	polyinosinic acid:cytidylic acid
PNPP	Para-nitrophenyl pyro phosphate
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PRR	Pattern recognition receptors
RIG	Retinoic acid-inducible gene
RLH	Retinoic acid-inducible gene-like helicases
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
RUNX	Runt-related transcription factor
SA-AKP	Streptavidin Alkaline-phosphatase
ssRNA	Single-stranded RNA
TAE	Tris Acetate EDTA

TGF-β	Transforming Growth Factor beta
TIR	Toll/IL-1 receptor
TIRAP	Toll/IL1 receptor-associated protein
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF-associated factor
TRIF	TIR domain containing adaptor inducing IFN-β

1. INTRODUCTION

1.1. Mesenchymal Stem Cells

The history of mesenchymal stem cells (MSCs) can go back to mid 60's. The first efforts for describing a stem cell population in bone marrow brought to attention after observation of osteogenic differentiation of fully and partially transplanted bone marrow into mice (Friedenstein et al., 1966). The discovery of MSCs was done by Friedenstein and colleagues in 1966. However, at that time it was not possible to fully describe this cell type. Later intensive studies revealed that there is a fibroblast-like cell population in bone marrow able to form colonies in culture (Friedenstein et al., 1970). As the studies concentrated on establishing the possible cell population present in bone marrow with osteogenic differentiation capacity, information on the MSCs has continued to accumulate up to now. Despite all the studies performed about MSC biology, several issues are still unresolved and inconsistencies present in the literature.

1.1.1. Characterization of Mesenchymal Stem Cells

For any given cell type, characterization of that particular cell is an essential and first step for studying this cell population. In MSC case, unfortunately due to variations in common practice while culturing BM cells to yield MSC common "gold standard" markers are not available. Today, with every paper published, there is a different statement claimed for MSC characterization. These discrepancies preclude an acceptable consensus on the establishment of standard method for MSC characterization. Among many, one approach used in common to characterize MSC generation is to demonstrate their capacity to differentiate tissue-specific cells. MSCs are now accepted as multipotent cell populations that can differentiate into three main mesenchymal lineages; adipocytes, osteocytes and chondrocytes (Pittenger et al., 1999). With their unique multipotential ability, at least these stem cells are distinguished from other stem cell types co-existing in the bone marrow niche. However, one method would not be sufficient to fully annotate a cell as MSC. Other methods applied to characterize is the surface marker expression profiles. Since there are controversial views about rate and type and level of expression of certain genes

as markers of MSCs among the species, still a handful of CD markers do present on MSCs regardless of the type of the host. These are CD90, CD73, CD105 according to International Society for Cellular Therapy, ISCT (Dominici et al., 2006). In addition to these positive markers, CD11b, CD34, CD45 or CD14 and HLA-DR surface molecules were regarded as negative markers of MSCs. The third and the most convenient method is the plastic adherence property of these cells. This method is a "gold standard" nearly for 40 years beginning with the first identification of MSCs (Friedenstein et al., 1966). Since, hematopoietic stem cells and erythrocytes do not attach to plastic surfaces; MSCs can be easily isolated from the rest of the cells with this primitive feature during bone marrow culturing. Today, these three main methods are widely used and accepted for MSC isolation and characterization. Nevertheless, the scientists are still investing considerable effort in order to describe more common, universally acceptable MSC markers.

1.1.2. The Mesenchymal Stem Cell Niches in Body

The main and the most abundant niche of MSC is bone marrow. Besides MSCs, bone marrow consists of a variety of other cell types including progenitors for blood cells, and other stromal cells (Dorshkind, 1990). Within this microenvironment, MSCs makes up the 0.001%-0.01% of the whole cell population. This ratio was determined with Percoll density gradient centrifugation (Pittenger et al., 1999). The efforts for isolation of MSCs from tissues other than bone marrow are due to the need for finding better non-invasive sites. In the search for such an alternative source of MSC, there are a number of other tissues/sites where MSCs were successfully isolated. These include but not limited to umbilical cord blood (Romanov et al., 2003), adipose tissues (da Silva Meirelles et al., 2009; De Ugarte et al., 2003; Nathan et al., 2003) and umbilical cord matrix (Zeddou et al., 2010). In addition to these tissues, trabecular bone (Noth et al., 2002), synovium (Fickert et al., 2003), vascular wall (Abedin et al., 2004) and periosteum (Nathan et al., 2003) are found to be consisting MSCs in adults. What's more, during development processes, MSCs were also isolated from liver (Anker et al., 2003; Paniushina et al., 2004), heart (Warejcka et al., 1996), spleen and lung (Anker et al., 2003), derma (Chunmeng and Tianmin, 2004) and pancreas (Hu et al., 2003).

1.1.3. Differentiation Capacity of Mesenchymal Stem Cells

The first defined potential of MSC was in the direction of differentiation into osteocytes (Friedenstein et al., 1966). Within years, standardization was brought for characterization of MSCs in terms of differentiation capacity. Recently, MSCs are accepted as having capacity to differentiate at least into three lineages; adipocytes, osteocytes and chondrocytes (Pittenger et al., 1999). However, studies revealed that MSCs have much more capacity than previously stated. For instance, these stem cells have potential to develop into ligaments and tendons when embedded in collagen and subjected to mechanical force (Altman et al., 2002). MSCs were also reported to differentiate into endotheliocytes both *in vitro* (Campioni et al., 2003) and *in vivo* (Silva et al., 2005). In addition to these observations, it is also reported that these stem cells could be differentiated into cardiomyocytes (Xu et al., 2004). It is expectable that MSCs could differentiate into cells of mesenchyme origin. However, there are studies also illuminating that MSCs have capacity to differentiate into cells of endodermal and ectodermal lineages such as neuron-like cells (Hermann et al., 2004) and hepatocytes (Wang et al., 2004). The most widely studied differentiation processes of MSCs would be investigated in Figure 1.1.

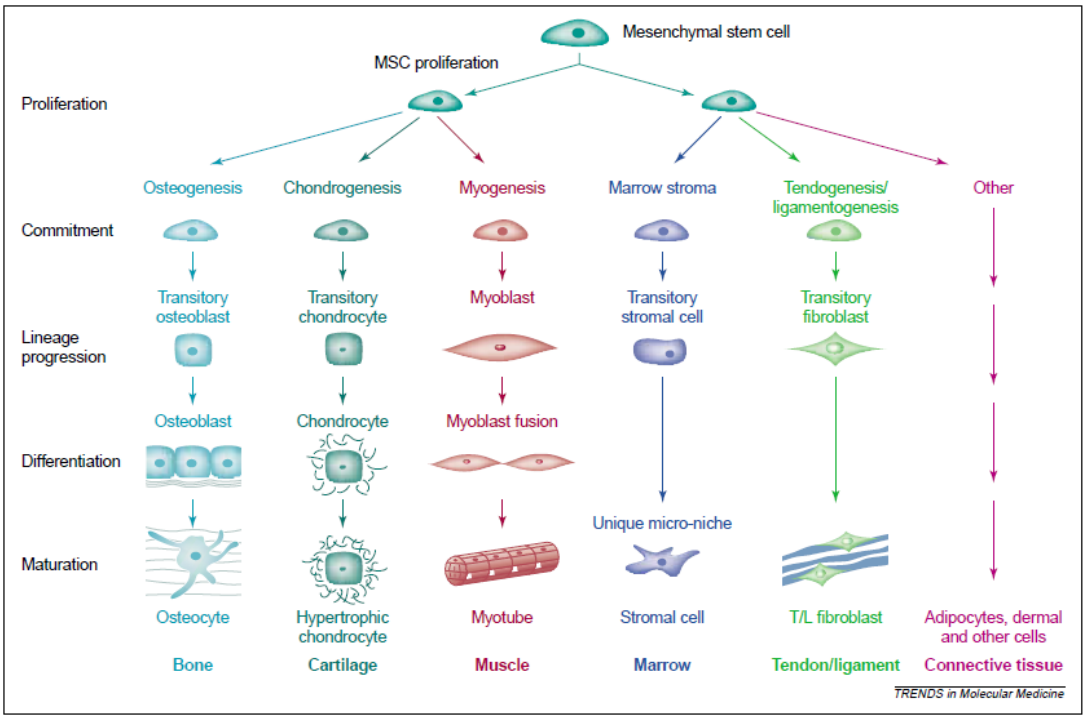


Figure 1.1 - Multipotency of MSC and differentiation pathways (Adopted from Caplan and Bruder, 2001 with permission)

1.1.4. Immunomodulatory Effects of Mesenchymal Stem Cells

For the characterization of mesenchymal stem cells, functional studies were carried out with these cells both *in vitro* and *in vivo*. During animal studies, MSCs were discovered to be suppressing immune system. Further studies demonstrated that these cells have immunosuppressive capacity. Moreover, these cells were observed to escape from immune system resulting in no immune response even interspecies engraftment of these cells (Nauta and Fibbe, 2007; Rasmusson, 2006). The immunosuppressive property of these cells was related to the expressed receptors and ligands for specific receptors. MSCs express growth factor receptors like TGF-β and Wnt (Mishra et al., 2005), adhesion proteins like vascular cell adhesion molecule (VCAM), intracellular adhesion molecule (ICAM) and CD166 (Ruster et al., 2006), several cytokine and chemokine receptors such as IL-6R, IFN-γR, TNF-α1R, CXCR4R, CXCR9 (Honczarenko et al., 2006; Ji et al., 2004), toll-like receptors like TLR2 (Pevsner-Fischer et al., 2007), TLR3 and TLR4 (Liotta et al., 2008) and also these cells can secrete ligands for natural killer cell receptors (Spaggiari et al., 2006). A comprehensive view of immunosuppresion and how it is directed by MSCs for several immune cells is summarized in Figure 1.2 (Rasmusson, 2006).

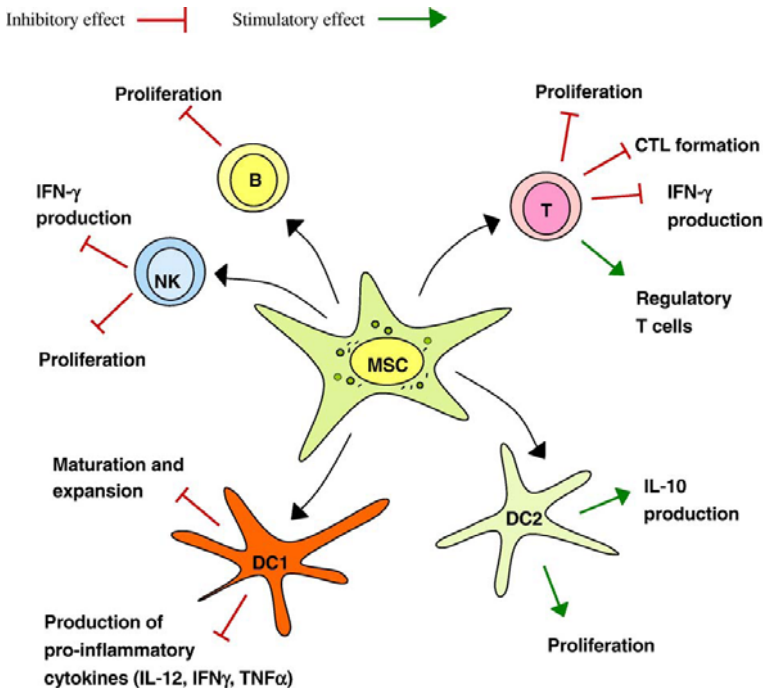


Figure 1.2 - Immunomodulatory effects of MSCs on immune system cells (Adopted from Rasmusson, 2006 with permission)

Especially, immunosuppresion is mediated via these singular interactions. At this point, interactions of MSCs with specific immune cells play the key role for inhibiting the stimulatory effects of immune system. Since MSCs show the expression of several receptors and ligands, it is inevitable that these cells are interacting with other cells individually. As a specific point for immunosuppresion, MSCs can inhibit the T lymphocytes. The inhibitory action is delivered by hampering the maturation of T lymphocytes via a soluble factor, indoleamine 2, 3-dioxygenase (IDO), which converts the tryptophan required for T lymphocyte maturation into kynurenine (Meisel et al., 2004). As in the suppression of T lymphocytes, other immune system cells are controlled by MSC with a different mechanism or a similar one with T lymphocyte case.

1.1.5. Potential subpopulations of Mesenchymal Stem Cells

It is a well-known fact that bone marrow microenvironment consists of various cell types forming a heterogeneous residing place for many cell populations. Considering this heterogeneous environment, without no doubt, any cell population removed from this tissue would be "contaminated" with neighboring cell types. To get a more homogeneous cell group, special isolation processes are performed following the bone marrow extraction. However, such isolation procedures would not be sufficient to obtain a pure cell population. Recent studies demonstrated that even at the end of 14 day isolation procedure, mesenchymal stem cells do not display a homogeneous cell population (Harting et al., 2008). Moreover, stimulation of these cells with different ligands gives different cell profiles (Waterman et al., 2010). It is even possible to classify two distinct populations of MSCs according to immuno-phenotyping (Battula et al., 2009; Buhring et al., 2009). What is more important is, these two studies revealed that subpopulations have very different characteristics in terms of immunosuppresion. The former study has clearly stated that upon priming with different TLR ligands, MSCs were directed into two distinct phenotypes: an immunosuppressive and other pro-inflammatory phenotype (Battula et al., 2009). Such a pro-inflammatory MSC phenotype was reported previously with IFN-γ priming studies of MSCs (Chan et al., 2006; Romieu-Mourez et al., 2009; Stagg et al., 2006). These and other studies establish the immune plasticity concept for MSCs.

Recent reports also stated the presence of a subpopulation defined by high osteogenic properties (Leonardi et al., 2009; Liu et al., 2009; Tormin et al., 2009).

1.1.6. Clinical Applications of Mesenchymal Stem Cells

As stated in previous sections, the versatile potentials of MSCs in terms of differentiation and migration capacity to the injury site make these cells a valuable tool for regenerative medicine and delivery vehicle. The idea of using MSCs in the regenerative medicine developed with the discovery of multilineage differentiation potential of these cells. These cells were administered following hematopoietic stem cell (HSC) transplantation in order to enhance the engraftment of HSCs in patients who has hematological disorders (Koc et al., 2000; Lazarus et al., 2005). In addition to such benefits of MSCs, these cells are used in tissue repair with patients suffering bone and cartilage disorders (Horwitz et al., 2002; Horwitz et al., 1999; Turgeman et al., 2002). In addition to these applications, MSCs were also administered to patients who had myocardial infarction and significant improvements with the heart function were recorded (Stamm et al., 2003). Beside such studies, MSCs were also used for their immunosuppressive properties. In order to prevent the engrafted tissue rejection, MSCs were also administered along with or following the tissue transplantation. Such studies demonstrated that graft-versus-host disease (GvHD) which results following tissue transplantation was prevented via immunosuppressive feature of MSCs. (Le Blanc et al., 2008; Le Blanc et al., 2004). Additionally, MSCs were benefited as a delivery vehicle to desired tissue using their migration capacity. Such studies generally include the cancer therapy and in these studies MSCs were loaded with anti-cancer drugs (Nakamizo et al., 2005; Studeny et al., 2004).

1.2. Mesenchymal Stem Cells and Toll-Like Receptors

Recently, therapeutic applications of mesenchymal stem cells had a broad range in different disease states. These applications do not include only regenerative medicine but also in cancer therapy. MSCs provide a safe biological delivery tool. However, these stem cells have some unique properties that put them in a different position than only being multipotent stem cell. These cells express several cytokine (Honczarenko et al., 2006; Ji et al., 2004) and other immune system receptors,

especially TLRs (Pevsner-Fischer et al., 2007). What's more, MSCs can behave differently under unfavorable conditions such as hypoxia (Cho et al., 2006) and inflammation (Raicevic et al., 2010). Defining the behavioral changes in MSCs under these conditions carries significant information in terms of suitable and reliable administration of these cells.

In the recent years, it is proposed that, TLRs can mediate the immunomodulatory potential of MSCs, yet there are several unresolved and contradicting issues on this topic. These modulations are in the way of stimulating down-stream signaling of TLRs and thus providing MSCs a more immunostimulatory character (Romieu-Mourez et al., 2009). Additionally, stimulation of TLR with the proper ligands causes an increase in the migratory capacity of MSCs (Tomchuck et al., 2008). Moreover, some studies have shown that TLR ligand priming had resulted in changes with the differentiation capacity of MSCs. For instance, MSCs stimulated with TLR3 and TLR4 ligands had an increased osteogenic differentiation capacity (Lombardo et al., 2009). Parallel to the stated effects of TLR3 and TLR4, these receptors have been shown to inhibit the modulatory activity of T lymphocytes by means of hampering Notch signaling (Liotta et al., 2008). Along with these studies, in a recent study it is stated that lipopolysaccharide which is a ligand for TLR4 had increased the proliferation rate of MSCs (Wang et al., 2009). Like in the characterization problem of MSCs, today there are a number of papers stating inconsistent results in similar experimental systems. Such controversial findings raise a new concept about MSCs. Do they possess opposing suppopulations? A recent paper claimed that stimulation with two different TLR ligands has resulted two converse MSC types (Waterman et al., 2010). Without doubt, in the near future there will be more findings supporting this view of differential contribution of TLR engagement and its involvement on MSC immunobiology.

1.3. The Immune System

The main goal of immune system of an organism is to distinguish the foreign particles from the self-particles. This "self *vs* non-self" distinguishment of immune system is the first strategy in the way of protection mechanism. It is crucial to perform this distinguishment properly. For a proper distinguishment, the immune

system is “trained” from the first development stages of an organism. For instance, human immune system is “educated” in the womb against the foreign particles, antigens, of mother. Any mistake made in the defining of self and non-self antigens would result in problems with the immune system like autoimmune diseases, chronic diseases or even with death. Thus, this process has a pivotal importance in the immune system. In addition to “training” process of immune system, this system has also other strategies to recognize foreign particles. The immune system can be categorized into two different subtypes; innate and acquired (adaptive) immune system. The innate immune system has evolutionarily conserved universal mechanisms to recognize foreign particles whereas adaptive immune system has unique property to “learn” and “remember” previously encountered antigens with specificity (Akira et al., 2001). Beside adaptation and memory of adaptive immune system has also some disadvantages like allergy and rejection of tissues following transplantation (Medzhitov and Janeway, 1998).

1.3.1. The Innate Immune System

The innate immune system has been universally found in a very wide range of organisms from different levels. The innate system is composed of mucosal epithelia, being the first line of host defense, and several cell types, like natural killer cells, phagocytes, expressing similar receptor profiles. Since the innate system is an ancient way of pathogen recognition, this system has limited germline-encoded receptors in number. Corresponding with the receptor limitation, this system has such a limit in the particle recognition. Only the universally conserved structures are recognized by this system (Janeway and Medzhitov, 2002). Conversely to this system, adaptive immune system has an unlimited antigen recognition system. However, without the innate immune system, the adaptive immune system cannot provide the protection against foreign particles since these two systems function in harmony. Actually, the innate immune system takes part in the first branch of recognition and with the response given by innate immune system, adaptive immune system settles a more vigorous further response (Medzhitov et al., 1997). Such situations take place when the innate immune system is unable to challenge the pathogen or recognize the pathogen. At this point, adaptive immune system gets into action and “generate” receptors for the recognition of pathogen. These receptors are

expressed with the somatic recombination of the gene segments which provides a variety of receptor types (Schatz et al., 1992). However, the innate immune system, as previously mentioned, has a limited number of receptors for fighting off the pathogens. These receptors are called pattern recognition receptors (PRRs) and recognize the pathogen associated molecular patterns (PAMPs) (Akira et al., 2006).

1.3.1.1. Pattern Recognition Receptors

These receptors are found on innate immune system cells and implement their functions through recognizing the non-self pathogen-associated molecular structures that build up the main structures of microorganisms. These receptors are germ-line encoded meaning that in any given cell type of innate immune system, all cells express these receptors. Contrary to adaptive immune cells, T and B-lymphocytes, the cells expressing these receptors do not have immunological memory or specificity. What's more, each PRR can recognize a specific PAMP regardless of the development stage of related microorganism (Akira et al., 2006). PRRs have distinct mechanisms of serving their functions. The most prevalent one is the activation of proinflammatory signaling pathways. Again the widely activated proinflammatory pathway is the nuclear factor-kappa B (NF-κB) pathway and to a lesser extent mitogen activated protein (MAP) kinase pathway (Clemens and Elia, 1997). Other mechanisms include the induction of apoptosis, phagocytosis and opsonization.

There are three main classes of PRRs: nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG)-like helicases (RLHs) and Toll-like receptors (TLRs) (Kawai and Akira, 2006).

1.3.1.1.1. Toll-Like Receptors as Pattern Recognition Receptors

The Toll-like receptor name was given after the discovery of a homologue Toll protein in human that was first defined in *Drosophila melanogaster*. Just like the function in *D. melanogaster,* human homologue of Toll protein was inducing the innate immune system through the NF-κB signaling pathway as well. By cloning and characterization studies, TLRs were defined as type I transmembrane receptors with leucine-rich repeats (LRR) containing extracellular domains and an intracellular

Toll/IL-1 receptor (TIR) homology domain (Medzhitov et al., 1997). As TLRs are found to be a part of innate immune system, they were also characterized with the evolutionary conservation from *Caenorhabditis elegans* to higher vertebrates, humans (Roach et al., 2005). From the identification of TLRs for the first time, up to now, 10 TLRs in humans and 13 in murines have been identified. There are a number of differences among the TLRs such as ligand specificities, expression profiles, target genes that are induced by these receptors and the signaling pathways as well (Kumagai et al., 2008).

1.3.1.1.2. Toll-Like Receptors in Innate and Adaptive Immunity

Defined as one of the main functioning units for innate immune system, TLRs are expressed on a variety of innate immune system cells such as dendritic cells and macrophages. Although, TLRs are characterized as PRRs of innate immune system cells, these receptors are also expressed on B cells and some specific types of T cells from adaptive immune system. Moreover, the expression profiles of these receptors can include the fibroblasts and epithelial cells (Kumagai et al., 2008). Interestingly stem cells are also found to be expressing these receptors (Pevsner-Fischer et al., 2007; Scumpia et al., 2010). Besides their wide expression range, these receptors are also found to be taking role in directing the innate immune system response and linking innate immune responses with adaptive immune system (Majewska and Szczepanik, 2006; Takeda et al., 2003). Most of the time, flow of information from innate immunity to adaptive immunity is provided by dendritic cells. Recognition of pathogens by DCs via TLRs causes the expression of co-stimulatory molecules, like CD80/CD86. This induction is crucial for activation and survival of T cells. Along with this action, pathogen recognition by DCs ends up in the induction of inflammatory signals and as a result of this induction, inflammatory cytokines are produced (Akira et al., 2001).

1.3.1.1.2.1. The Toll-Like Receptor Family Members

In mammals, 10 members of TLRs are identified up to now (Rakoff-Nahoum and Medzhitov, 2009) and in mice 13 TLRs are identified (Takeda and Akira, 2007). Among these TLRs, the first characterized one was the human TLR4 (Poltorak et al.,

1998). Within such diverse TLR member family, it is possible to classify these receptors according to different properties possessing. However, the localization of these receptors in a cell supplies the most convenient way of categorizing. Thus, TLR1, 2, 4, 5, 6 and 11 make a TLR subtype located on the cell membrane while TLR3, 7, 8 and 9 forms another subtype existing in the intracellular endosomal and/or endoplasmic reticulum parts (Iwasaki and Medzhitov, 2004). Moreover, the former subtype presumably specialized in the recognition of main bacterial structures whereas the latter subtype recognizes the viral and bacterial nucleic acids specific to these microorganisms (Rakoff-Nahoum and Medzhitov, 2009) (Figure 1.3).

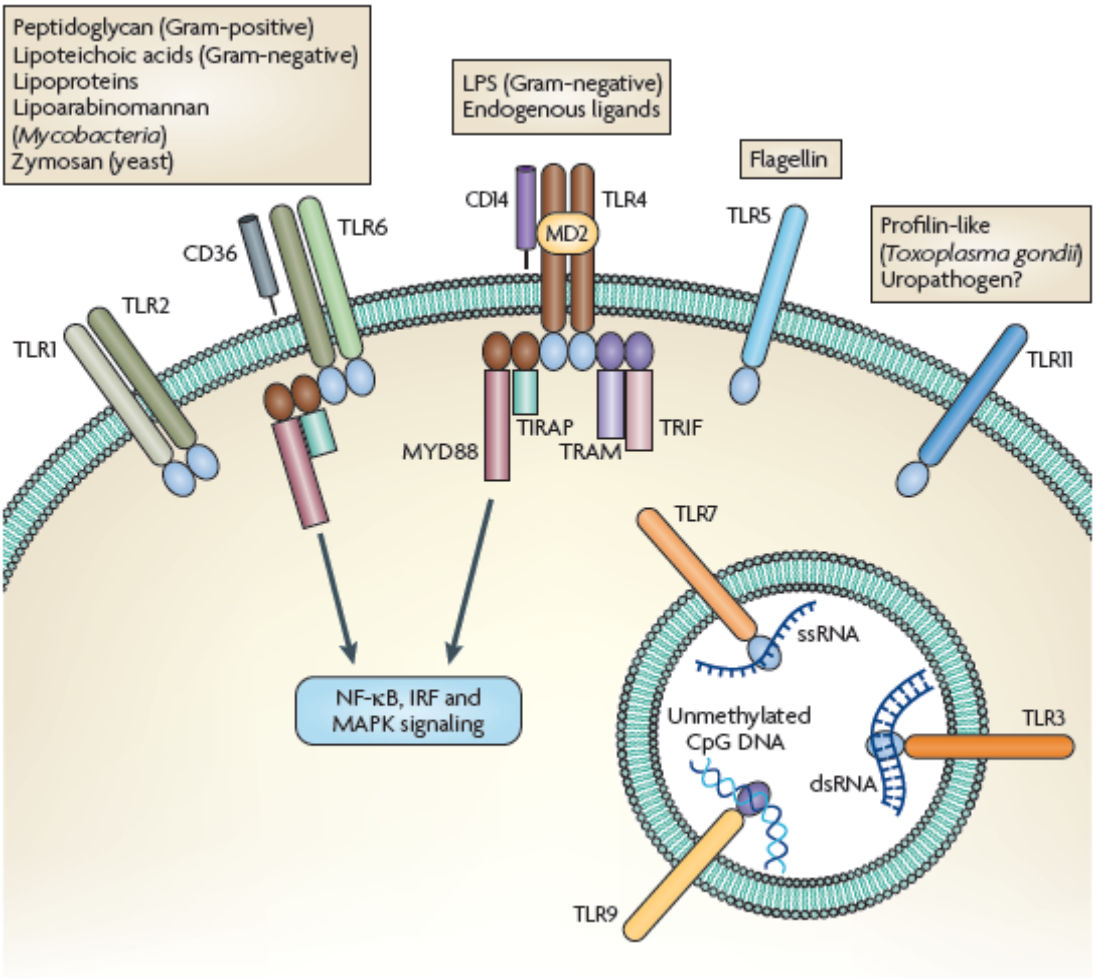


Figure 1.3 - Localizations of Toll-like receptors and related ligands for each TLR (Adopted from Rakoff-Nahoum and Medzhitov, 2009 with permission).

As depicted in the Figure 1.3, TLRs can function alone or in dimers with another TLR or self. The dimerization is necessary for the induction of proper signaling pathways. Another reason for dimerization is to recognize specific pathogen particles,

which will be explained in detail in each TLR section. For such a recognizing pattern, TLR2 forms heterodimer with TLR1 and TLR6. That's why, these three TLRs will be investigated under one topic.

1.3.1.1.2.1.1. TLR1,2 and 6

TLR2 is the most extensively studied among these three TLRs. Since TLR2 is known to recognize a wide range of microbial particles such as peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, lipoproteins from various microorganisms, glycosylphosphatidylinositol anchors of protozoans like *Trypanosoma cruzi,* zymosan from fungi, a phenol-soluble modulin from *Staphylococcus epidermis* and glycolipids from *Treponema maltophilum* (Takeda et al., 2003). Beyond these pathogen particles, TLR2 can recognize the lipopolysaccharide (LPS) preparations of *Helicobacter pylori* (Smith et al., 2003). However, wide spread function of TLR2 cannot be attributed to only TLR2. Dimerization of TLR2 with TLR1 and 6 provides its multiple pathogen particle recognizing talents. For instance, TLR2 cannot distinguish diacyl or triacyl lipopeptides by itself. Dimerization of TLR2 with TLR1 provides the diacyl lipopeptides recognition whereas dimerization with TLR6 ensures the triacyl lipopeptides recognition (Takeuchi et al., 2001; Takeuchi et al., 2002). Beyond dimerization with TLRs, TLR2 can also function with other kind of receptors. For fungal-derived component recognitions like β-glucan, TLR2 collaborate with lectin receptor family, herein dectin-1 (Gantner et al., 2003).

The expression level of TLR2 is regulated positively by pro-inflammatory signals like TNF-α and negatively by anti-inflammatory signal molecules such as glucocorticoids (Hermoso et al., 2004). Correlated with its expression level, TLR2 activation is also regulated with respect to inflammatory signals.

1.3.1.1.2.1.2. TLR3

TLR3 recognizes the double-stranded (ds) RNA molecule of viruses. dsRNA is generated by a wide range of viruses during their life cycle. By finding out that dsRNA is recognized by TLR3, it was no doubt that TLRs have roles in host defense

against viral components as well as bacterial components (Alexopoulou et al., 2001). Upon dsRNA recognition, antiviral and immunostimulatory signals are activated. For antiviral alerts, type I interferons, IFN-α/IFN-β, are induced and synthesized. Besides, NF-κB signaling pathway is also activated in order to enhance immune response. TLR3 is found to be expressed by various immune system cells. For instance, dendritic cells, macrophages and even some epithelial cells are known to be expressing TLR3. However, as the most important director of antiviral responses, natural killer (NK) cells are the number one expressing TLR3. In addition to dsRNA, NK cells are responsive to polyriboinosinic polyribocytidylic acid (poly(I:C)) which is a synthetic dsRNA and induces the production of IL-6 and IL-8 as proinflammatory cytokines, IFN-γ as antiviral cytokine (Schmidt et al., 2004).

1.3.1.1.2.1.3. TLR4

Lipopolysaccharide (LPS), the major part of Gram-negative bacteria outer membrane, is the ligand for TLR4, the first defined human TLRs. The ligand for TLR4 is revealed out by the hyposensitivity studies to LPS when point mutations were introduced to this receptor (Hoshino et al., 1999). Within the last decade, it was found out that like TLR2, TLR4 has more ligands than one. For instance, taxol from *Taxus brevifolia* (Kawasaki et al., 2000) and respiratory syncytial virus fusion protein (Kurt-Jones et al., 2000) are other ligands for TLR4. What's more, endogenous ligands, like heat shock proteins (HSP60, HSP70) at high concentrations were also activating TLR4 signaling pathways (Cohen-Sfady et al., 2005; Takeda and Akira, 2005).

Just like TLR2 functioning, TLR4 has to cooperate with some accessory molecules in order to recognize LPS. LPS cannot directly bind to TLR4. First, it has to bind LPS-binding protein (LBP) which is found in the serum. Then, this serum protein transfers the subunits of LPS to CD14, which functions as a co-receptor of TLR4. Finally, TLR4 comes next to CD14 to recognize LPS particles and start down-stream signaling with the help of MD-2 (Shimazu et al., 1999; Wright et al., 1989).

1.3.1.1.2.1.4. TLR5

Besides cell wall or genetic elements of bacterial organisms, some kinds of bacteria require flagellum for mobility. Therefore, subunits of flagellum, flagellin, provide a good recognizing agents for host defense. TLR5 does the flagellin recognizing duty of the innate immune system (Smith et al., 2003). The flagellin recognition induces inflammatory cytokine production such as TNF-α and IL-8.

1.3.1.1.2.1.5. TLR7 and 8

TLR7 and TLR8 have a close relativity since they are structurally conserved relative to each other. The discovery of ligands for these TLRs came during the approved treatment of viral infections. The synthetic compounds imidazoquinolines were the first to be defined as causing anti-viral activity upon administration (Hemmi et al., 2002). It was shown that both TLRs were expressed; however, TLR8 was unresponsive to ssRNA in mice (Akira et al., 2006). Upon induction of TLR7 and 8 with imidazoquinolines and ssRNA, antiviral cytokines was found to be produced. These cytokines are synthesized upon activation of interferon regulatory pathways like IRF3 and IRF6 (Ito et al., 2005; Kawai et al., 2004).

In the last decades, it was discovered that host cells also produces ssRNA. However, these ssRNAs, miRNA and siRNA, did not possess any danger signal for the host cells. This may explain why nucleic acid recognizing TLRs are localized inside the cell, on the endosomal membranes since self-derived ssRNAs are not delivered to endosomes (Lund et al., 2004; Takeda and Akira, 2007).

1.3.1.1.2.1.6. TLR9

One pivotal difference between bacterial genetic elements and mammalian genetic elements is the CpG dinucleotides. Although two groups have these sequences, bacterial DNA contains these CpG motifs in unmethylated form, which provides a basis for self/non-self discrimination of the immune system. TLR9 takes advantage of this difference and recognizes unmethylated bacterial CpG motifs. In vertebrates, the methylation of CpG motifs at cytosine residues ensures the self recognition and

does not lead to an immunostimulatory effect (Krieg et al., 1995). In TLR9-KO mice, CpG motifs from bacterial DNA do not cause any immunostimulatory response supporting the function of TLR9 in recognizing unmethylated CpG DNA (Hemmi et al., 2000). TLR9 expression has been found on B cells, natural killer cells and dendritic cells mostly. The expression of TLR9 is seen during the maturation, proliferation and cytokine secretion of related cell types (Krieg, 2000). In nature, two types of CpG DNA was discovered: CpG-B type and CpG-A type. Two types have differential effects and immunostimulatory potential on plasmacytoid dendritic cells (pDC) and B cells. The first identified CpG type is conventional CpG-B type which has the ability to induce pro-inflammatory cytokines like IL-12 and TNF-α. Differently from A type of CpG, conventional CpG type has phosphorothioate backbone without a poly (G) tail. Conversely, CpG-A type has its 3' and 5' ends linked to a poly (G) tail and build up with a phosphodiester/phosphorothioate mixed backbone. Another difference of these two types is the potential ability to induce IFN-α and IL-12 from pDCs. CpG-A type has a greater IFN-α induction potential, however it cannot induce as much IL-12 as CpG-B type could induce. In addition to this, CpG-A type cannot stimulate B cells (Gursel et al., 2002; Honda et al., 2005; Verthelyi et al., 2001). The potentiality differences between CpG types are due to the characteristics of target cells. Recently, pDCs are found to be expressing CXCL16 which serves as a co-receptor along with the TLR9 in recognition of CpG-A type. On the contrary to pDCs, B cells do not express CXCL16 which results in no stimulation upon CpG-A type induction (Gursel et al., 2006).

Collectively, it is possible to analyze the ligands for each TLR in Table 1.1.

Table 1.1 – Toll-like receptors and their ligands (Adopted from Takeda and Akira, 2007 with permission)

TLR[a]	Ligands[b]
TLR1	Triacyl lipopeptides
TLR2	Peptidoglycan
	Lipopeptides, lipoteichoic acid, lipoarabinomannan, GPI anchors, phenol-soluble modulin, zymosan, glycolipids
TLR3	dsRNA
TLR4	LPS, taxol, RSV fusion protein, MMTV envelope protein, endogenous ligand (HSPs, fibronectin, hyaluronic acid)
TLR5	Flagellin
TLR6	Diacyl lipopeptides
TLR7	ssRNA, imidazoquinolines
TLR8	ssRNA, imidazoquinolines (only in humans)
TLR9	CpG DNA
TLR11	Profilin

[a]Ligands for missing TLRs (i.e., TLR10, 12, and 13) have not been identified.
[b]All mouse and human TLRs, except TLR8, recognize these ligands.

1.3.1.1.2.2. Toll-Like Receptor Signaling Pathways

TLRs bind specific ligands and signaling pathways are triggered. Through these signaling pathways, genes that are related with the inflammatory machinery starts to be expressed. Between ligand recognition and gene expression, there is an adaptor protein, myeloid differentiation primary response gene (88)-MyD88, which takes part in the TLR signaling. After ligand binding, intracellular domain of TLRs, called as TIR, associates with MyD88 and signals are transduced. The interaction of MyD88 is provided by the TIR domain of this molecule (Hemmi et al., 2002; Takeuchi et al., 2000). However, the signal transductions of TLRs are not always MyD88-dependent. For instance, TLR4 signaling can also occur via an alternative pathway; TRIF-dependent pathway. TIR-domain containing adaptor inducing IFN-β, TRIF, protein takes the role of MyD88 in this pathway (Youn et al., 2005). TLR3

and TLR4 can alternatively use this MyD88-independent/TRIF dependent pathway (Brint et al., 2002).

As demonstrated in Figure 1.4, regardless of MyD88 dependency, stimulation of each TLR results in activation of NF-κB pathway, which in turn starts an inflammatory signaling cascade.

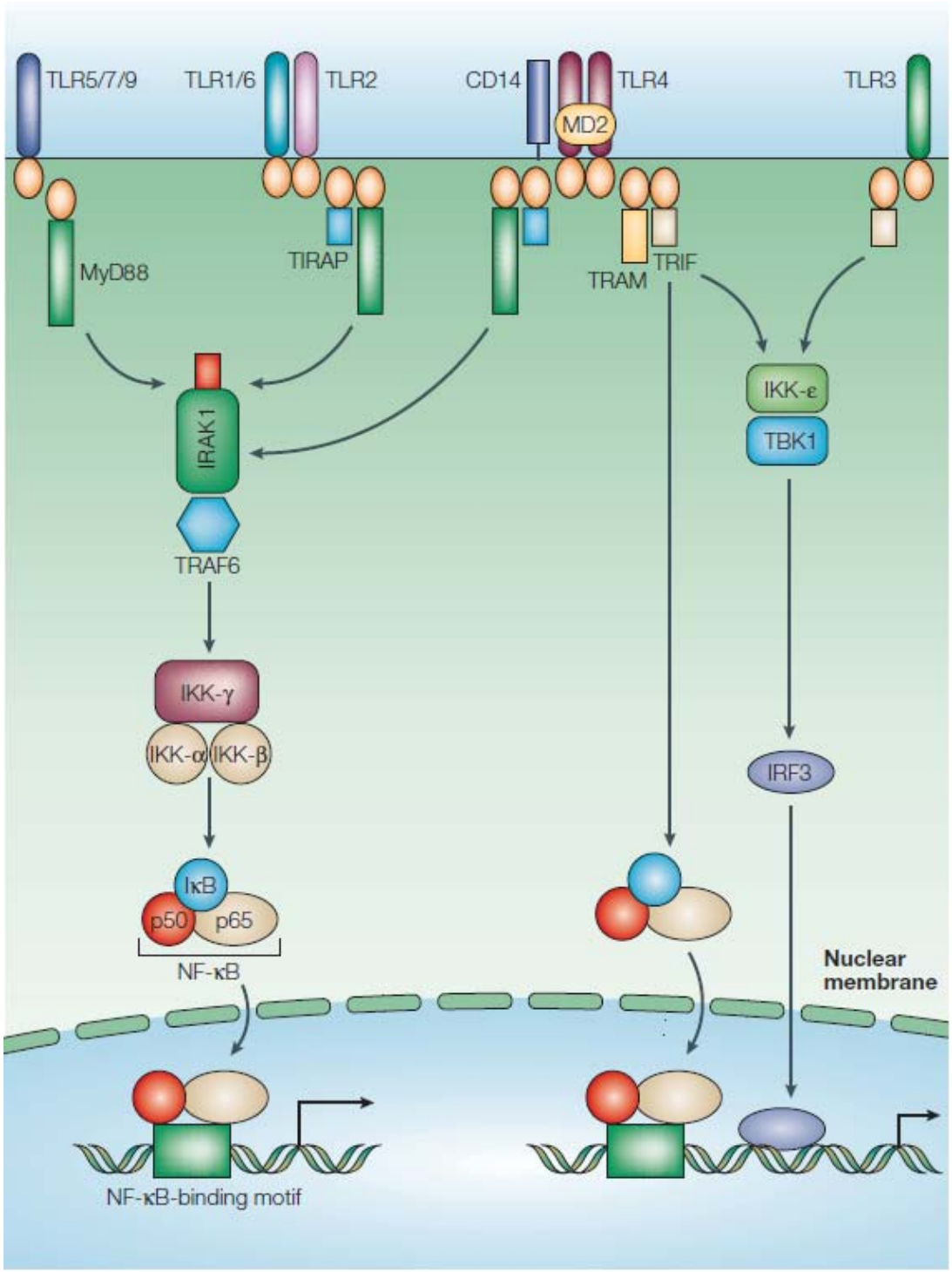


Figure 1.4 - TLR signaling pathways (Adopted from Akira and Takeda, 2004 with permission).

2. AIM

Mesenchymal stem cells (MSC) are special progenitor cells and are distinct from adult stem cells in many ways. It was suggested by many studies that these cells undergoes lineage dependent differentiation depending on their micro-niche as well as self-renewal and homing capacity to damaged tissue sites. MSCs are also known to induce immunomodulatory effects and were indicated as a suitable therapeutic cell in GVHD capacity. Such diverse properties make MSCs a powerful tool in several therapeutic applications.

Toll-like receptors (TLR) are expressed by the cells of the immune system that are evolutionarily selected to recognize conserved microbial byproducts. Recent findings suggest that *in vitro* generated MSCs express some of these pathogen recognition receptors raising the debate with regard to their immunosuppressive character. We reasoned that in order to broaden the therapeutic potential in addition to better understand MSCs biology, there is a need to describe the interplay between MSC and TLR interaction.

In this context, present study was designed to pursue TLR mediated activation of MSCs and understand its impact on differentiation and immunomodulatory potential.

This study is planned to be conducted in three main parts: In the first part, our goal was to determine the expression levels of TLRs by MSCs. Next the effect of TLR ligand addition on the adipogenesis was assessed. In the second part, migration capacity of these stem cells in the presence or absence of TLR ligands as well as their proliferation was investigated. Finally, immunomodulatory potential of MSCs either alone or in combination with spleen cell co-cultures in the presence or absence of different ligand was investigated.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Reagents

MesenCult® (STEMCELL Technologies, Canada) was the main culture media used during the mesenchymal stem cell isolation procedure and culturing. In addition to this, RPMI1640 and low glucose DMEM and supplementary solutions of these media were all from Hyclone (USA).

TRI Reagent (Trizol®), used for manual RNA isolation, was from Invitrogen (USA). *DyNAmoTM* cDNA Synthesis kit, *DyNAzyme™ II PCR Master Mix* for PCR was obtained from Finnzymes (Finland). All primers for PCR (designed), were purchased from Alpha DNA (Canada). 50 bp DNA ladder was purchased from Jena Bioscience (Germany).

Unlabeled or biotinylated monoclonal antibodies against IL-6 or IFN-γ were purchased from Thermo Scientific (previously known as Endogen, Pierce (USA). Recombinant cytokines used for standard curve preparations during cytokine quantitation and streptavidine-alkaline phosphatase (SA-AKP) were purchased from Endogen, Pierce (USA). Substrate for alkaline phosphatase *p*-nitrophenyl phosphate disodium salt (PNPP) was purchased from Thermo Scientific (USA).

3.1.2. Toll-Like Receptor Ligands and Oligodeoxynucleotides

Throughout this study, during the stimulation assays of co-cultured splenocytes or induction of MSCs to undergone differentiation following TLR ligands were used. Their sources and specifications were listed below: i) peptidoglycan (PGN, TLR2L or TLR2/6L) isolated from *B. subtilis* were from Fluka, (Switzerland), ii) lipopolysaccharide (LPS; TLR4L) isolated from *E. coli* were from Sigma, (USA), iii) poly inosinic acid:cytidylic acid (p(I:C), TLR3L) were received from Amersham, (UK), iv) a modified adenine base, R848, known as imiquimod™ that was developed by 3M company (TLR7/8L) was purchased from Invivogen (USA) and v)

immunostimulatory CpG motif expressing single stranded short ODNs (TLR9L), immunosuppressive telomeric repeat unit expressing ODNs (as a TLR antagonists) or control ODNs (flip CpG dinucleotides) were purchased from Alpha DNA (Canada), and some sequences (i.e. A151) were kindly provided by Dennis M. Klinman (NCI/NIH, USA).

3.1.3. Standard Solutions, Buffers, and Culture Media

The detailed information about the ingredients and preparations of common laboratory used buffers, sterile standard solutions and several other culture media used in the present work is presented in Appendix A (please refer to get further details).

3.2. METHODS

3.2.1. Maintenance of animals

This M.Sc. thesis work was carried out using two different experimental animals, namely, rat and mice. Throughout the animal experiments, different organs of adult male Sprague-Dawley rats (6-9 months old) and adult male C57BL/6 mice (3-6 months old) were used. These animals were sustained under controlled ambient conditions (22±2°C) with a cycle of 12 hrs light/dark cycles. They were fed *ad libitum*. Animal procedures employed throughout this thesis were approved by Bilkent University Animal Ethical Committee (Bil-AEC).

3.2.2. Isolation and culture of Rat & Mouse Mesenchymal Stem Cells

For bone marrow (BM) isolation, the animals were sacrificed by cervical dislocation. Next, the femur and tibia bones of both legs were removed and cleaned off muscles and adipose tissues. The ends of bones were cut with a bone cutting forceps and BM was flushed with pre-warmed DMEM media supplemented with 5-10 % FBS and 1% penicillin/streptomycin (Hyclone, USA). The flushing of BMs was done using a 10 ml syringe in the case of rat bone marrow and a 5 ml syringe during mouse BM isolation. After collecting BM cells in a 50 ml falcon, they were centrifuged at 1500

rpm for 8-10 mins at RT. Then, the supernatant was sucked with glass pasteur pipette travelling the inner wall of falcon tube in order to remove any connective or adipose tissue. The pellet was mildly dislodged with the help of a pin rack and washed 3x with warm DMEM. At the end of washing steps, BM cells were counted and seeded into T75 flasks with a concentration of not more than $25x10^6$ cells/flask (2-$2.5x10^6$/ml).

3.2.3. Cell Culture

3.2.3.1. Preparation of Single Cell Suspension of Mouse Spleen Cells

Male C57BL/6 were sacrificed by cervical dislocation and spleens were removed in aseptic conditions and the whole organ was transferred into 2-3 ml warm DMEM (or in some cases RPMI media) containing 5-10% FBS in 6-well plates. In order to obtain single cell suspension, under lamin-air hood, spleens were first smashed with a sterile syringe plunger back moving in circular directions. They were then collected in 15 ml falcon tubes. The cells were centrifuged at 1500 rpm for 8-10 mins, washed twice with warm media and were resuspended in 5% oligo FBS supplemented RPMI-1640 medium (Hyclone, USA). Single cell suspensions of splenocytes were counted and used for further assays.

3.2.3.2. Adjusting the Cells Following Counting

A sample of 10 µl volume of single cell suspension of any intended cell source was transferred on a Neubaer cell counting chamber and covered by the help of a cover slip (please see figure 1 for the counting and cell number estimation strategy). Cells were counted, and the total cell number was computed. Based on the total cell number extracted after each procedure, cell populations were adjusted to required cell concentrations. Normally, for proliferation or of cytokine secretion detection assays $2x10^6$/ml cells were harvested.

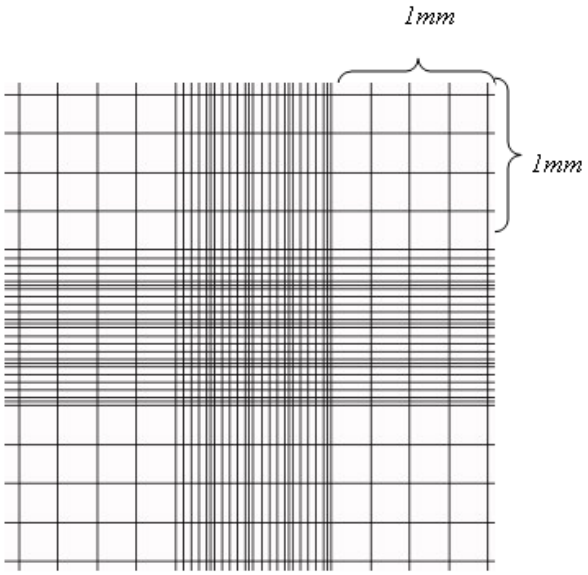


Figure 3.1 - Neubaer cell counting chamber

Since 1 mm^2 area of this chamber has a volume of 0.1 mm^3, the total number of cells in 1 ml was calculated as follows.

(Cell number in 4 little square x 4) x 10^4 x Dilution factor = Total cell number/ml

Cell number in 0.1 mm^3 volume

If any

3.2.3.3. Stimulation with Toll-Like Receptor Ligands

The final concentrations of TLR ligands for stimulation assays were presented in Table 3.1.

Table 3.1 - The final concentrations of TLR ligands and ODNs used

TLR Ligands and/or ODNs	Final concentrations
PGN : TLR2L or TLR2/6L	5 µg/ml
p(I:C) : TLR3L	20 µg/ml
LPS : TLR4L	5 µg/ml
R848 : TLR7/8L	5 µg/ml
CpG DNA : TLR9L	1 µM
Control ODN : CpGflip ODN	1 µM
A151	1 µM

3.2.4. Quantification of Gene Expression at Transcript Level

3.2.4.1. Total RNA Isolation and Quantification

In cell culture if adherent, cells were trysinized, collected and centrifuged at 1500 rpm for 5 mins. After sucking supernatant, 1 ml TriZol® reagent (approx. over 10^7 cells) was added onto the cell pellet, gently pipetted until viscous, pink milkish homogenous solution appears. It was immediately transferred into an eppendorf tube. At this step, dissolved cell pellet in TriZol® reagent could be stored up to a month at -80°C if immediate RNA isolation was not necessary. Alternatively, RNA isolation could be continued by the of 0.2 ml chloroform. At this stage the tube must be hand-shaken vigorously for 15 secs. Following incubation at RT for 3 mins it was centrifuged at 13200 rpm for 17 mins at 4°C. At the end of centrifugation, the mixture was separated into three different layers. RNA presents at the top transparent liquid phase. With great care, this phase (>600 µl) was transferred into a new tube and 500 µl isopropanol was added and the solution was mixed by gentle up/down movements. It was incubated at RT for 10 mins and centrifuged again at 13200 rpm for 17 mins at 4°C. Supernatant of this mixture was sucked and 1 ml 75 % ethanol was added, gently mixed to dislodge RNA pellet and spin down at 8000 rpm for 7 mins. After removal of the supernatant, second washing was performed with 1 ml of 99.9 % ethanol. Centrifugation was repeated as done for the first wash and the liquid was discarded. The RNA pellet was left for drying at under lamin-air flow hood. Air-dried pellets were dissolved in 40 µl nuclease-free ddH_2O (Hyclone). The dissolving process should be done by pipetting for several times in order to obtain a homogenous RNA solution. The OD measurement of RNA was done with the spectrophotometer NanoDrop® ND-1000 (NanoDrop Technologies, USA) with 1 µl of RNA solution. The quality of RNA was determined using OD_{260}/OD_{280} ratio that should be between 1.8 and 2.0. RNA solution outside of this OD measurement range indicates contamination (possibly coming from DNA, protein or phenol) these samples must be either re-purified or discarded. The isolated RNA samples were stored at -80°C until for further use.

3.2.4.2. cDNA Synthesis

cDNAs were synthesized from isolated RNA samples with the cDNA synthesis kit according to the manufacturer's protocol. 1 µg of total RNA and 1 µl Oligo (dT) primer was completed to 8 µl with ddH_2O. This mixture was pre-denatured at 65°C for 5 mins in order to get rid of the secondary RNA structures. After pre-denaturation the mixture was incubated on ice for 3 mins. Then, i) 10 µl RT Buffer (supplemented with dNTP mix and 10 mM MgCl2) and ii) 2 µl M-MuLV RNaseH RT (it also contains RNase inhibitor) were added. The reaction mixture, in 0.2 ml PCR tube was placed in MJ Mini Thermocycler, (BIORAD, USA) and incubated at 25°C for 10 mins, 40°C for 45 mins, 85°C for 5 mins and 4°C for 10 mins. The synthesized cDNA samples were stored at -20°C for further use.

3.2.4.3. Primers Designed for RT-PCR Studies

All the primers used in this study were designed using Primer3 Input v.0.4.0 program (http://frodo.wi.mit.edu/primer3/) and the related gene sequences of rat and mouse were retrieved from Ensembl™ and Pubmed databases. In Table 3.2, rat, and mouse primer sequence details of TLRs and their PCR product sizes are presented.

Table 3.2 - Rat* and mouse* primer set sequences and their expected product sizes

Primer Name	**Direction**	**Sequence**	**Product Size**
rGAPDH	F R	5'-AGACAGCCGCATCTTCTTGT-3' 5'-CTTGCCGTGGGTAGAGTCAT-3'	207 bp
rTLR1	F R	5'-ACCTTCCTGGATGTGGAGCC-3' 5'-CCTGCAGTGGGGTTCCCTTA-3'	261 bp
rTLR2	F R	5'-CAAACTGGAGACTCTGGAAG-3' 5'-CTAAGAGCAGGATCAACAGG-3'	246 bp
rTLR3	F R	5'-GGGTCAACTCAGGATACTTG-3' 5'-AAGTCCTCGTTCAGGTTGG-3'	285 bp
rTLR4	F R	5'-CTAGCCGTCTTCAATCTGAC-3' 5'-ACACTGACCACCGATACACT-3'	215 bp

rTLR5	F R	5'-AGAGGCTCCTACTCAGCTTC-3' 5'-AGAGTCCACAGTCAAACAGC-3'	249 bp
rTLR6	F R	5'-GAGCCTTCAGTAGCCTTTCT-3' 5'-GCTGTCTGGGTAATCACATC-3'	224 bp
rTLR7	F R	5'-CCCTTGAGAGAGCTGCGGTA-3' 5'-CTTTCCATGGTCCTGCTGGC-3'	236 bp
rTLR9	F R	5'-CATGGTCAGGTGTAAGAACC-3' 5'-CAGGTCCAGCTTGTTATAGG-3'	208 bp
rTLR10	F R	5'-GGCTGCTAACTTCTGGGTGC-3' 5'-TGTGGTCCCGACTAGCCATC-3'	272 bp
mGAPDH	F R	5'-GTATGCCTCGGTCGTACCA-3' 5'-CTTCTGCATCCTGTCAGCAA-3'	450 bp
mTLR1	F R	5'-TTTGGGGGAAGCTGAAGACATC-3' 5'-CTTCGGCACGTTAGCACTGAGAC-3'	410 bp
mTLR2	F R	5'-TCTCTGGGCAGTCTTGAACATTTG-3' 5'-CGCGCATCGACTTTAGACTTTG-3'	320 bp
mTLR3	F R	5'-GGGGCTGTCTCACCTCCAC-3' 5'-GCGGGCCCGAAAACATCCTT-3'	250 bp
mTLR4	F R	5'-TGCCGTTTCTTGTTCTTCCTCT-3' 5'-CTGGCATCATCTTCATTGTCCTT-3'	240 bp
mTLR5	F R	5'-TGGGGCAGCAGGAAGACG-3' 5'-AGCGGCTGTGCGGATAAA-3'	380 bp
mTLR6	F R	5'-GCCCGCAGCTTGTGGTATC-3' 5'-GGGCTGGCCTGACTCTTA-3'	650 bp
mTLR7	F R	5'-TTAACCCACCAGACAAACCACAC-3' 5'-TAACAGCCACTATTTTCAAGCAGA-3'	700 bp
mTLR9	F R	5'-GATGCCCACCGCTCCCGCTATGT-3' 5'-TGGGGTGGAGGGGCAGAGAATGAA-3'	430 bp

***** *"**r**" stands for rat and "**m**" stands for mouse primers.*

For rat characterization of MSCs, PCR products of several CD markers were studied. The primer sequences of CD markers for and their PCR product sizes are given in Table 3.3.

Table 3.3 - Rat CD marker primer set sequences and their expected product sizes

Primer	Direction	Sequence	Product size
rCD11b	Forward Reverse	5'-GCTGGGAGATGTGAATGGAG-3' 5'-TGATGCTGGCTACTGATGCT-3'	113 bp
rCD29	Forward Reverse	5'-ACTTCAGACTTCCGCATTGG-3' 5'-GCTGCTGACCAACAAGTTCA-3'	190 bp
rCD34	Forward Reverse	5'-TGTCTGCTCCTTGAATCT-3' 5'-CCTGTGGGACTCCAACT-3'	281 bp
rCD45	Forward Reverse	5'-ATGTTATTGGGAGGGTGCAA-3' 5'-AAAATGTAACGCGCTTCAGG-3'	175 bp
rCD71	Forward Reverse	5'-ATGGTTCGTACAGCAGCAGA-3' 5'-CGAGCAGAATACAGCCATTG-3'	182 bp
rCD73	Forward Reverse	5'-GAACTTGGGAGGGAGGAGAG-3' 5'-CATTGGCAGGAAGAGAGGAG-3'	282 bp
rCD90	Forward Reverse	5'-CCAGTCATCAGCATCACTCT-3' 5'-AGCTTGTCTCTGATCACATT-3'	374 bp
CD105	Forward Reverse	5'-CGGGAGGTGTTTCTGGTCT-3' 5'-GTGTCTGGGTTCGTGGTTG-3'	331 bp
rCD166	Forward Reverse	5'-CTTTGTTCTGGGAGTGGCTG-3' 5'-GGTGTTGCCGTATGTGTTTG-3'	303 bp

3.2.4.3.1. PCR Studies for Rat and Mouse

Throughout this work, in order to assess the changes in the gene levels of several related procedures, PCR analyses were extensively used. These PCR set-up conditions and reactions were summarized in Tables 3.4 and 3.5 (please review for details).

Table 3.4 - PCR Reaction Ingredients and Amounts

Reaction Ingredients	**Volume**
cDNA	1 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
Nuclease-free ddH2O (Hyclone)	9.5 µl
Total volume	**25 µl**

***** 2x *DyNAzyme™ II PCR Master Mix* includes 0.04 U/µl DyNAzyme™ II DNA Polymerase, 20 mM Tris-HCI (pH 8.8 at 25°C), 3 mM MgCl2, 100 mM KCl, stabilizers and 400 µM of each dNTP.

Table 3.5 - PCR conditions

PCR step	**Rat TLR primers**	**Rat CD marker primers**	**Mouse TLR primers**
Initial Denaturation	95°C, 5’	95°C, 10’	95°C, 5’
Denaturation	94°C, 30’’	94°C, 40’’	94°C, 30’’
Annealing	55°C, 30’’	60°C, 30’’	60°C, 30’’
Extension	72°C, 30’’	72°C, 40’’	72°C, 1’
Final Extension	72°C, 5’	72°C, 10’	72°C, 10’
Number of cycles	30 cycle	23 cycle	30 cycle

3.2.4.4. Agarose Gel Electrophoresis and Quantification of Band Intensities

1.5% agarose gel was prepared during gel electrophoresis with 1x TAE buffer and 1 mg/ml ethidium bromide solution was used. 25 µl of PCR samples were mixed with 5 µl loading dye and 10 µl of this mixture were loaded to each well. To serve as a marker, 2 µl of low range DNA ladder (50-500 bp, Jena Biosciences, Germany) was loaded along with the gene products. Running conditions were set at 90 V power for a duration of 45 mins. Gel band intensities were visualized under UV

transilluminator (Vilber Lourmat, France) for a period of 5-10 msec. The gel images were stored by Chemicapture software (Vilber Lourmat, France).

3.2.5. Flow Cytometry

3.2.5.1. Fixation of Cells

The analysis of cell surface markers were studied from fixed MSCs collected at different passages. In order to fix MSCs, the cells in T75 flasks were first washed with 1X PBS w/o magnesium and calcium (Hyclone, USA) for once and then trypsinized and left at 37°C for 1-2 mins. The trypsinization was blocked with 2% FBS supplemented DMEM, washed twice, and pooled in falcon tubes following centrifugation at 1500 rpm for 5 minutes. The remaining cell pellet was dislodged by the help of a pin rack, while vortexing (15 secs) 100 µl fixation medium (Fix & Perm®, Caltag Lab, USA) was added at RT. The fixation was terminated at the end of 15 mins by the addition of 2 ml PBS-BSA-Na Azide solution (20x fold of initial fixation solution amount). The cells were centrifuged at 1500 rpm for 5 mins, supernatants were sucked and onto the pellet fresh 1 ml PBS-BSA-Na Azide was added to resuspend the cell pellets. They were kept at 4°C until for further analysis by FACS.

3.2.5.2. Cell Surface Marker Staining

Freshly or previously fixed cells were spun down at 1500 rpm for 5 minutes and were distributed into different tubes planned for specific staining at a 100 µl aliquots. Onto cell pellet following centrifugation and removal of the residual supernatants, 1 µl antibody of CD90-FITC (Abcam, USA), CD45-PE/Cy5 (Abcam, USA), CD29 (Chemicon, USA), CD71 (Santa Cruz, CA, USA) and CD106 (Santa Cruz, CA, USA) as well as their proper isotype control abs, was added and tubes were labeled. At the end of 30 mins incubation in dark at RT, cells were washed twice with 2 ml PBS-BSA-Na Azide and centrifuged at 1500 rpm for 10 minutes. Finally, cells were resuspended in 400 µl PBS-BSA-Na Azide in FACS tubes and analyzed in FACS Calibur instrument (Beckon Dickinson, San Jose, USA).

3.2.5.3. Flow Cytometric Analysis of Cells

The analysis of cells was done according to 20,000 event count with the FACS Calibur instrument (Beckon Dickinson, San Jose, USA). The channel choice, gating and compensation adjustments were done related to sample and staining properties. The analysis of acquired data was carried out using CELL QuestPro software (Becton Dickinson Immunocytometry Systems, San Jose, USA).

3.2.5.4. Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Assay

The CFSE staining method is adopted from a previously published protocol paper (Parish et al, 2009). Cells were collected into a falcon tube and centrifuged at 1500 rpm for 5 minutes. Cell concentration was adjusted to 10^6 cells/ml and resuspended in warm 1x PBS. CFSE, in 1x warm PBS, was adjusted to a final concentration of 5 µM CFSE. For homogenous distribution of CFSE, special care was taken to pipette the solution following CFSE addition. The cells were incubated at 37°C for 10 mins. In order to stop the reaction, 10 ml of warm 10% low glucose DMEM was added, cells were centrifuged at 1500 rpm for 5 mins and supernatant was sucked. The desired amounts of cells were reseeded in flasks and stimulations were done according to regular stimulation protocol of TLR ligands in MesenCult® medium (Please see section 3.2.3.3 for more details). The cells were then left for 3 days of incubation at 37°C in CO_2 incubator. The cells were then, fixed and studied for their proliferation rate in FACS Calibur instrument (Beckon Dickinson, San Jose, USA) and data were analyzed by CELL QuestPro software (Becton Dickinson Immunocytometry Systems, San Jose, USA).

3.2.6. Differentiation of rat Mesenchymal Stem Cells with or without Toll-Like Receptor Ligands

In order to differentiate the role of supplementing the conventional differentiation media with various TLR ligands, modified assay conditions were planned to include specific ligands. To address this effect, adipogenic or osteogenic media were supplemented with different ligands according to the concentrations stated in Table

3.1. These different treatments were then stained for specific differentiations, positive cells were counted, and photomicrographs were recorded at different time intervals. Recordings were carried out by a Nikon Coolpix 4500 digital camera attached to Nikon Eclipse TS100 light microscope.

3.2.6.1. Adipogenic Differentiation Alone or in the Presence of Toll-Like Receptor Ligands

At the end of MSC induction protocol, MSCs (at P0) in T75 flasks were trypsinized (0.025%, w/ EDTA) and reseeded in 24-well plates at a $7x10^4$ cells/well. After 24 h MesenCult medium was removed and replaced with adipogenic induction medium (this includes, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 100 µM indomethacin and 10 µg/ml insulin (all from Sigma-Aldrich) in 10% high glucose DMEM medium). Similarly, wells containing adipogenic induction medium plus i) TLR2, ii) TLR3, iii) TLR7, iv) TLR9, and their proper control ligands were incubated (as the treatment groups) and the induction medium was replenished every other day. Adipogenesis was followed daily by microscopic investigations and staining was performed for a period of 3 weeks (at specific time intervals) with Oil Red O.

3.2.6.1.1. Oil Red O Staining for Adipogenic Differentiation Assessment

At specified time points, 24-well plate was brought to bench and media was aspirated. The fixation of cells was done in 4% paraformaldehyde solution (Carlo Erba, Italy) for 40 mins at RT. After fixation, cells were washed gently with adequate amount of 1X PBS for three times and rinsed with ddH_2O for two times with great care. The cells were incubated in sufficient amount of Oil Red O Solution (Chemicon, CA, USA) and left at RT for 50 mins. At the end of 50 mins, the Oil Red O Solution was aspirated and cells were rinsed gently with for ddH_2O three times. The cell nuclei were counterstained with Mayer's Hematoxylin Solution (Sigma-Aldrich, USA) for two minutes. Following counterstaining, cells were rinsed with ddH_2O for two times and left in 1-2 ml ddH_2O for picture capturing.

3.2.6.2. Osteogenic Differentiation Alone or in the Presence of Toll-Like Receptor Ligands

The isolation and seeding of P0 MSCs was performed as described above (Section 3.2.6.1.). The osteogenic induction medium consists of 10^{-7} M dexamethasone, 0.25 mM ascorbic acid and 0.45 mM glycerophosphate-disodium pentahydrate (all from Sigma-Aldrich) in 10% high glucose DMEM medium. Similarly, TLR ligands mentioned in the section 3.2.6.1., were included in the osteogenic media, and osteogenesis was followed daily by microscopic investigations and staining was performed for a period of 2 weeks (at specific time intervals) with Alizarin Red.

3.2.6.2.1. Alizarin Red Staining for Osteogenic Differentiation Assessment

At specified time points, 24-well plate was brought to bench and media was aspirated. The fixation of cells was done in ice-cold %70 EtOH for 1 h at RT. After fixation, cells were rinsed with adequate amount of ddH2O for two times. For staining, sufficient amount of Alizarin Red Stain (Chemicon, CA, USA) was added each well and left at RT for 30 mins. At the end of 30 mins, the stain was aspirated and cells were rinsed with ddH_2O for four times. As a final step, 1-2 ml distilled water was added to each well and immediate analysis for osteogenesis was done.

3.2.7. Migration Assay

Rat mesenchymal stem cells at P3 were seeded at a confluency of nearly 80% into 6-well plates in triplicates. The plates were checked every day and when cells reached near 100% confluency, it is crucial that cells must not be over confluent and a single layer of cells should be formed, with the help of a P1000 pipette tip “wounds” were generated. In order to have regular wounds, a ruler was used while generating wounds. Following wound generations, pictures of each group in triplicate experiment design were taken at 0 h just over the previously drawn horizontal lines at the back of the plates. Then, rMSCs were incubated in MesenCult® medium containing TLR ligands at concentrations previously calculated (see Table 3.1). After 24 h incubation at 37°C in CO_2 incubator, pictures of each group were taken in

triplicates for the same areas and medium containing proper TLR ligands were replenished. The plates were left for another 24 h incubation. At 48 h, pictures were taken for the same areas again and the experiment was terminated. The pictures were taken with Nikon Coolpix 4500 digital camera attached to Nikon Eclipse TS100 light microscope. The migration distances of rMSCs were calculated with ImageJ software (NIH, USA) by taking several measurements in the wound regions. The overall migrations for each group were given relative to the naive group containing only MesenCult® medium.

3.2.8. Co-culturing of Splenocytes and mouse Mesenchymal Stem Cells with or without Toll-Like Receptor Ligands

The TLR ligand and ODN stimulations were done according to the concentrations mentioned in Table 3.1.

The co-culturing of MSCs with splenocytes was done in an increasing titration of splenocytes where MSC ratio but not the number was kept constant comparing to splenocytes. The counting of splenocytes and MSCs were done as described in section 3.2.3.2. and cells were distributed in 96 well plate with the starting splenocyte and MSC cell number being 25 x 10^4. The titrations of MSC to splenocyte were from 1:1 to 1:10^3 in triplicate for each group. Moreover, splenocyte alone and MSC alone groups were also studied as two groups with 25 x 10^4 and 5 x10^5 cell numbers in triplicate.

In addition to co-culturing of splenocytes and MSCs, MSC alone titrations were studied in 96 well plates in triplicate in the presence of TLR ligands. MSC titrations were from 2.5 x 10^2 to 5 x 10^5.

3.2.9. Enzyme Linked Immunosorbent Assay (ELISA)

In order to check the early and late immunological responses in the case of cellular responses raised against TLR ligand inductions, (splenocyte alone, MSC alone, or co-culture experiments) IL-6 and IFN-γ cytokines were studied by in house developed sandwich ELISA.

3.2.9.1. Collection and Storage of Cell Supernatants Following Treatment with Toll-Like Receptor Ligands

At the end of 40 h incubation time, 96 well plates were centrifuged at 1275 rpm (200 g) for 6 minutes and approximately 180 µl supernatant from each well was collected into a sterile 96 well cell culture plates. Cell supernatants were frozen down and stored at -20°C until for further use.

3.2.9.2. Cytokine ELISA

Coating of Immulon 2HB plates (Thermo Labsystems, USA) were done with monoclonal antibodies against mouse cytokines IL-6 and IFN-γ. The concentrations of antibodies were adjusted to 10 µg/ml in 1x PBS and 50 µl of the antibody solutions were distributed in each well. For coating, incubations were done either at +4°C overnight or 4 h at RT. Following coating, anti-cytokine solution was decanted and blocked with 200 µl blocking buffer for 2 h at RT (alternatively at +4°C overnight). Washing step was done with ELISA wash buffer 5x with 5 mins incubation intervals after each wash. At the end of washing, plates were rinsed with ddH_2O for twice and dried by tapping. Next, collected supernatants were brought to RT by thawing on the bench and layered on the 96 well plate at a volume of 50ul/well for each cytokine. Corresponding recombinant cytokines were serially distributed. Normally for a cytokine standard 1000 ng/ml concentration was diluted serially by two-fold with 1x PBS for 11 times. Plates were left at RT for 2 h or at +4°C overnight. After this step, plates were washed and dried as described before. Subsequently, biotinylated anti-cytokine antibodies were prepared in T-cell buffer (at a ratio of 1:1000 dilution) and distributed over the wells 50 µl to each well and incubated for another 2 h. Washing steps repeated and streptavidin alkaline-phosphatase (SA-AKP, Pierce, IL, USA) was (in T-cell buffer, 1:1000) added to the plates as 50 µl/well. After 1 h incubation at RT, washing and drying steps were repeated. Finally, PNPP substrate (Pierce, USA) was prepared as 1 tablet/4 ml ddH_2O + 1 ml buffer and layered over the wells as 50 µl portions. Yellow color development was followed continuously and multiple OD readings (at 405 nm) were recorded by ELISA reader (Molecular Devices). The OD measurements were

analyzed by SoftMax Pro v5 software. The color development was terminated when 4-parameter standard curve was obtained.

3.2.10. Statistical Analysis

Statistical analysis for the treatment groups were conducted by SIGMA-STAT anaysis software. Student`s t-test between control (or naïve) vs each treatment group (two-tailed unpaired comparison) was done for assays. $P<0.05$ was considered significant throughout these studies.

4. RESULTS

4.1. Rat Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are localized in the bone marrow along with other stem cell types such as hematopoietic stem cells. Thus following isolation, characterization of these mesenchymal stem cells is a strictly essential step for studying these stem cells. Characterization could be done with a number of methods. One of these methods is following the morphological changes of these cells during the isolation period. MSCs have fibroblast-like morphology at the end of 14 days. Moreover, these stem cells are found in colonies in flasks and frequently proliferate from a single progenitor cell and finally form a colony. Another characterization method is the surface marker expression analysis. For certain CD markers these stem cells are positive whereas for some they are negative. Last but the most primitive characterization method is the property of plastic adherence. Contrary to hematopoietic stem cells or erythrocytes which are present in the bone marrow, mesenchymal stem cells are adherent and could adhere to plastic. On the basis of these three criteria stated by the (Dominici et al, 2006) rat and mouse mesenchymal stem cells were characterized during and/or after the isolation process from bone marrow of related animal.

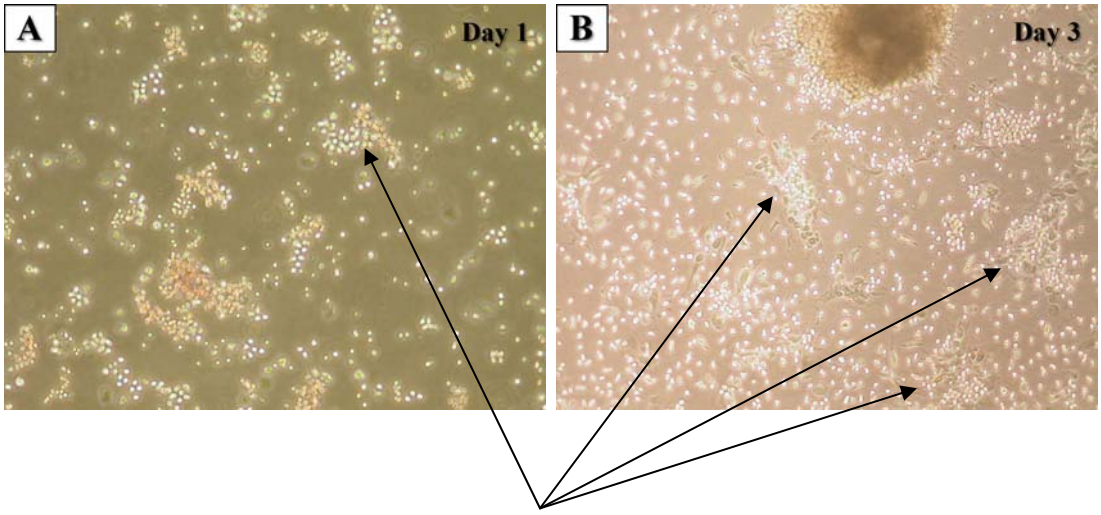
4.1.1. Characterization of Rat Mesenchymal Stem Cell

Isolation and generation of rat mesenchymal stem cells were performed as stated in Section 3.2.2. The characterization of rat mesenchymal stem cells was done according to their morphological changes during the isolation period, their surface marker expression was analyzed by RT-PCR and finally by flow cytometry, surface markers were analyzed.

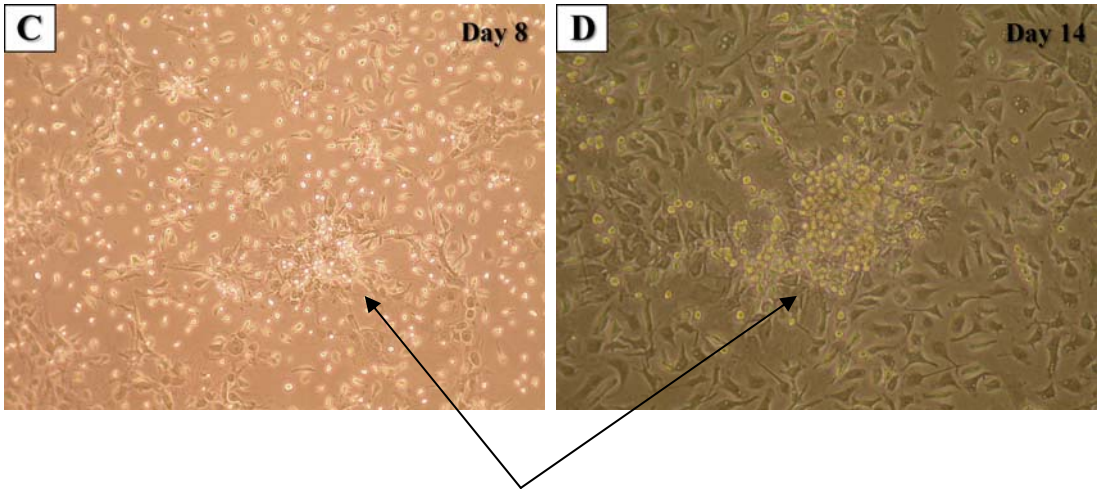
4.1.1.1. Transition from Bone Marrow to Mesenchymal Stem Cells during Culturing

Since mesenchymal stem cells have the plastic adherency, in the first day of isolation, media was replenished to get rid of the swimming concomitant cell types like

hematopoietic stem cells and erythrocytes. After then, twice a week, media was replenished with fresh media until the end of 14 day isolation period.



Progenitor cells potentially committed to undergo MSC generation at day 1 (A), day 3 (B)

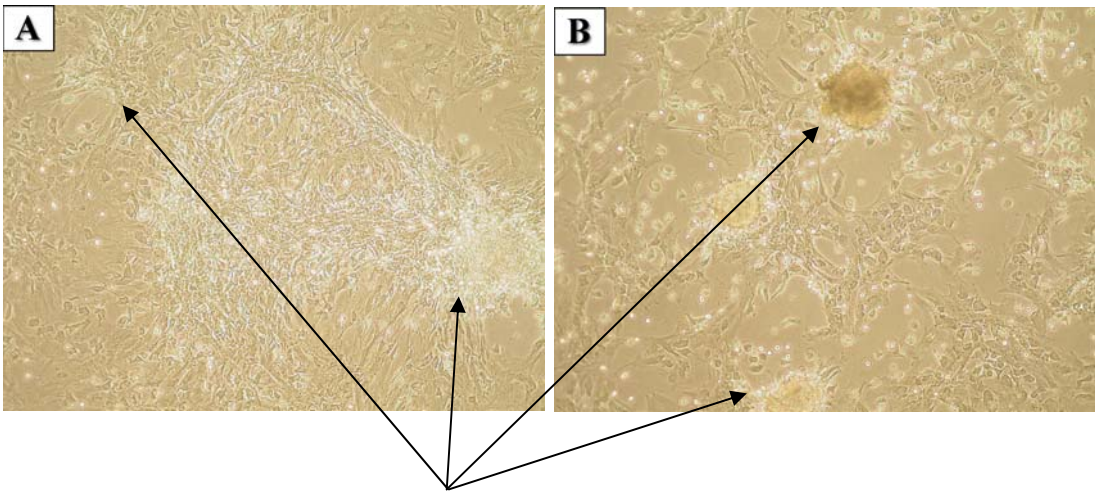


As the isolation period proceeds, fibroblast-like MSCs and their colonies are able to be clearly observed at day 8 (C), day 14 (D)

Figure 4.1A-D - Photomicrographic investigations of rat bone marrow in isolation phases (Mag = 10X)

In the Figure 4.1, the phases of mesenchymal stem cell generation from rat bone marrow cells could be seen during the isolation period. From the first day of isolation, cells with stem cell potential propensity start to form colonies and in the following days of isolation, these cells start to get a much more fibroblastic shape. By the way, in the first day picture, erythrocytes could be observed in pinkish color as small groups. With the media replenishment, this contaminating cell group and others are eliminated with the plastic adherency property of MSCs.

However, at the end isolation procedure, the remaining cell population is never a pure mesenchymal stem cell population. This is why, there are also cells present in bone marrow that can adhere to plastic. In order to diminish these cell types as much as possible, passaging of mesenchymal stem cells was done. In the Figure 4.2, passage 1 (from now on, passage no will be stated as P#, P2 e.g.) and passage 2 mesenchymal stem cell populations could be observed.



MSC colonies during different passages.

Figure 4.2A and 2B – Appearance of MSC colonies at different passages; left picture shows P1 MSCs, right one P2 MSCs. (Mag = 10X)

4.1.1.2. PCR Results and Gel Images Over Passages

4.1.1.2.1. CD Marker Expression Panels

It is clear that having a pure MSC population is necessary whilst contaminating cells would affect the potential and function of these cell populations. Along with this, passaging over and over would have side effects like stem cells would lose some of their stem cell-like potential (Wall et al., 2007; Yu et al., 2010). At this point, characterization of mesenchymal stem cells would be done with surface marker expression analysis to see how pure your cell population is. For mesenchymal stem cells, CD90, CD71 and CD105 surface marker expressions must be positive whereas CD11b as macrophage marker, CD34 and CD45 as hematopoietic stem cell marker expressions must be negative. Besides, it is a hard issue to differentiate MSCs from other cell types so recently numerous candidate MSC marker proteins were published in literature. Since it is sufficient to check for certain markers which are widely used

in the literature for MSC characterization, we also checked for the positive expression of CD29, CD71, and CD166 over the different passages of MSC populations.

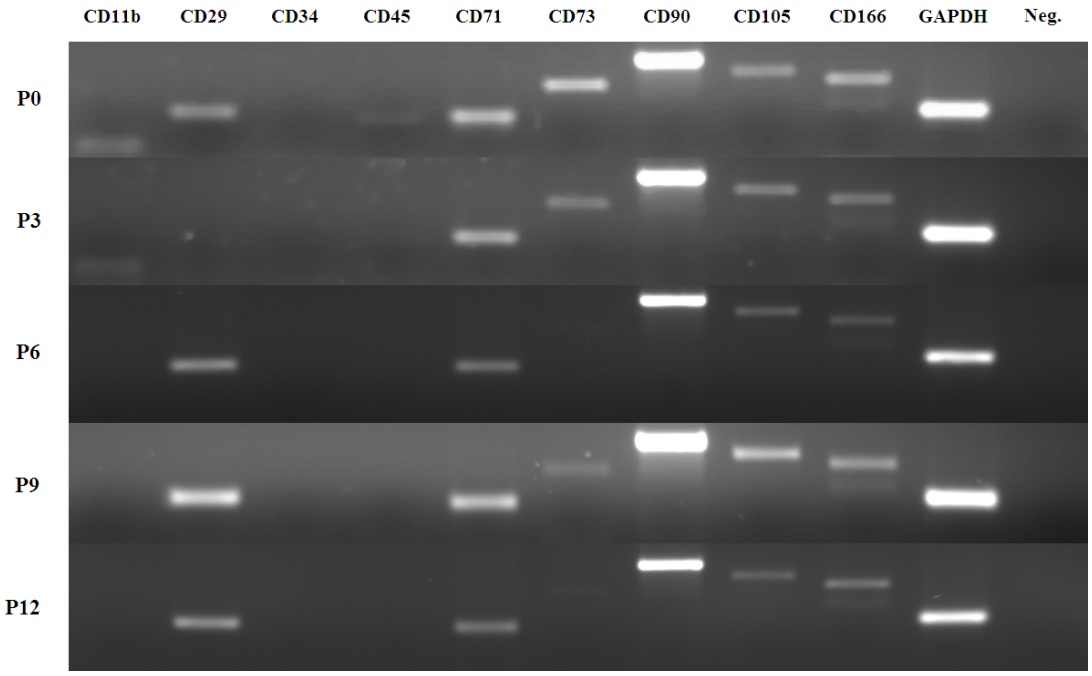


Figure 4.3 - CD marker expression panel over passages for characterization

In Figure 4.3, CD marker expression panels of P0, P3, P6, P9 and P12 MSCs are seen. CD90 is a reliable stem cell marker and throughout the passages, it is the most strongly expressed CD marker. There is a faint CD45 band observed in P0 MSC which shows that MSC population is still heterogeneous at the end of isolation procedure. However, with the passaging, this band goes off. This situation could also be observed in CD11b expression. Besides, positive markers for MSCs are expressed with a high stability. Throughout the passaging; expression of markers CD29, CD71, CD73, CD90, CD105 and CD166 could be observed with slight exceptions.

4.1.1.2.2. Toll-Like Receptor Expression Panels

During all the studies carried out in this thesis, two main materials were the key players. Toll-like receptors (TLRs) are one of them and so it is important to report the TLR expression of mesenchymal stem cells. In the Figure 4.4, TLR expression of some MSC passages could be observed.

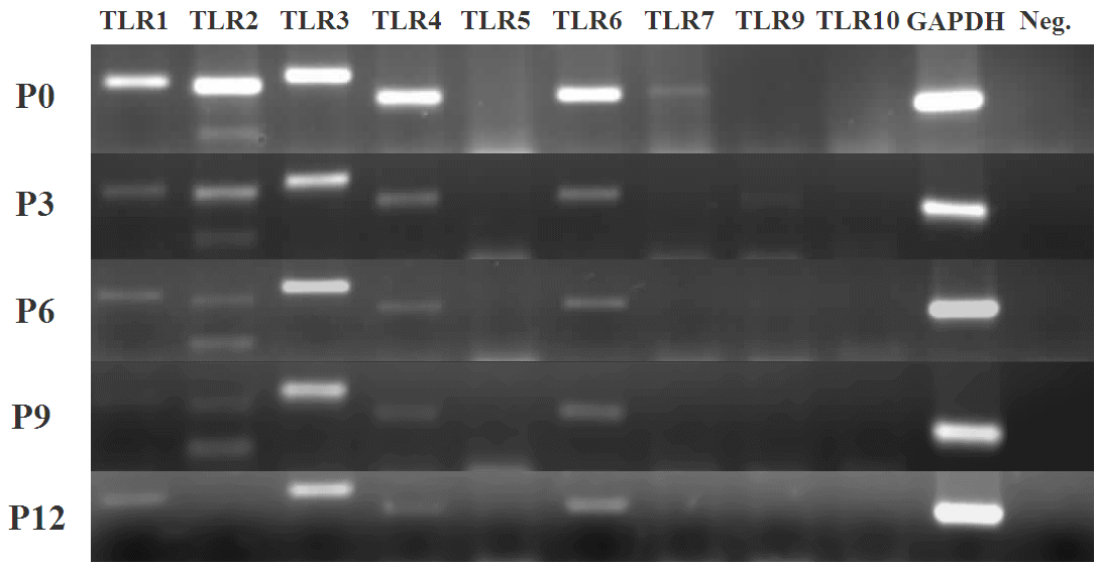


Figure 4.4 - TLR expression panel over different MSC passages

TLR expression profiles of MSC passages do not show fluctuations except P0 MSC. The difference in TLR expression of P0 among other passages should be a side effect of contaminating cell populations present in P0 MSC. In general, TLR3 expression is the strongest between TLRs while others show a basal expression and all together, TLRs do not show significant change over passages.

4.1.1.3. Flow Cytometry Analysis

Although PCR analysis of MSC characteristic genes provided valuable information with regard to their conversion to MSC from progenitor BM cells, detailed protein expression of the signature CD markers at the MSC surface by FACS analysis leaves very little doubt about the status of the MSC population recovered after successive passages.

Thus, along with RT-PCR expression of CD markers (CD90, CD45 and CD106) by flow cytometric analysis including their proper isotype controls were conducted for MSCs at different passages. It was decided to use CD90 as the positive marker and CD45 and CD106 as a negative marker.

As presented in Figure 4.5, CD marker expressions at P4 as expected, demonstrated that BM derived MSCs in culture is highly positive for CD90 and negative for CD45 and CD106, confirming previous PCR findings. In Table 4.1, % positivity for each tested surface expressed CD markers at different MSC passages is summarized. Table 4.2 provides the MFI of each CD marker studied at different MSC passages.

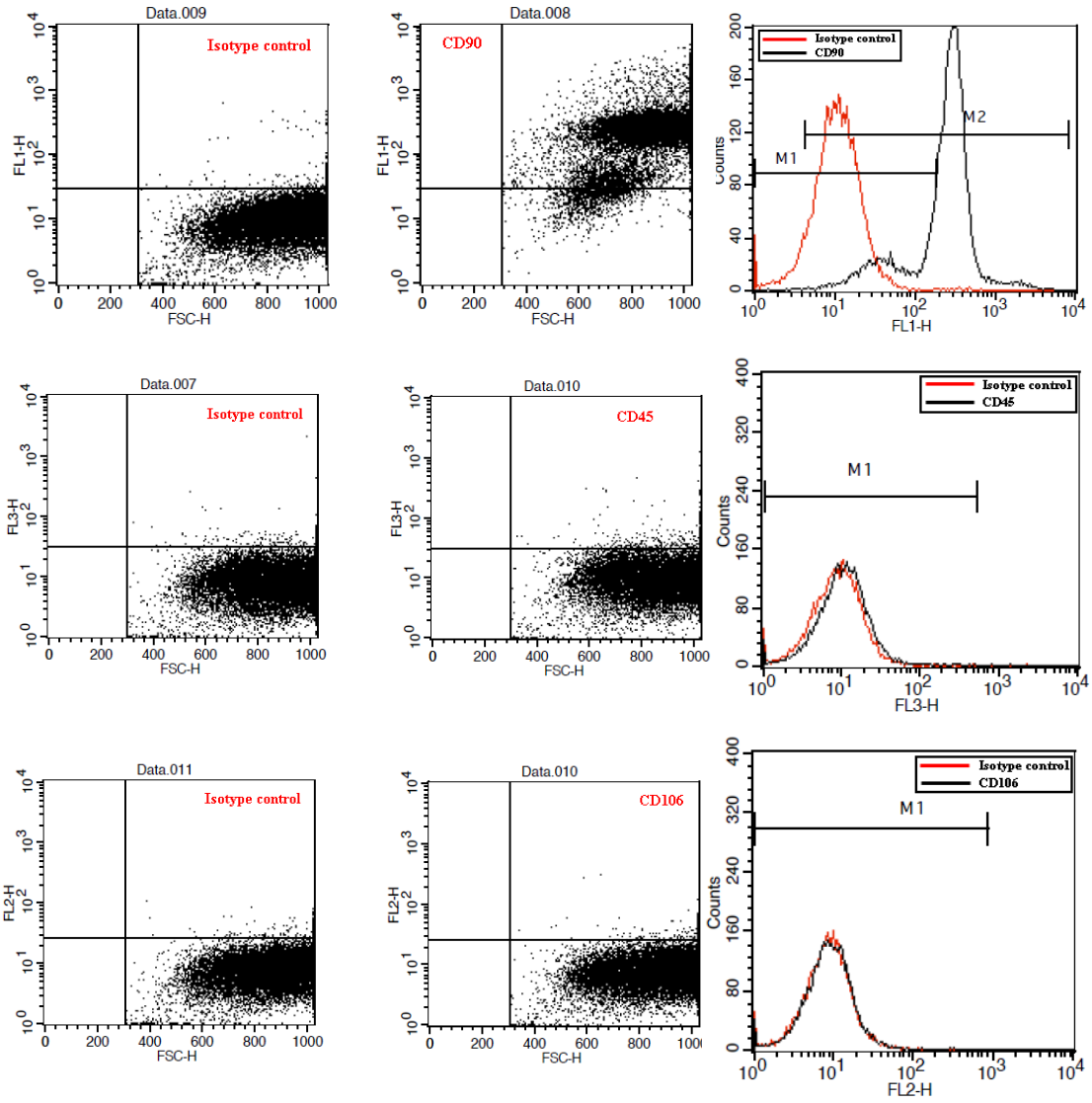


Figure 4.5 - Representative analysis plots of CD markers for P4

Table 4.1 % positive of CD marker expressions at different passages

	CD90*		CD45**		CD106**	
	Isotype	**Cell**	**Isotype**	**Cell**	**Isotype**	**Cell**
P0	1.0±0.3	30.2±15.4	0.3±0.2	0.5±0.3	0.9±0.2	1.1±0.4
P2	1.4±0.2	56.5±10.3	0.6±0.4	1.9±0.9	0.6±0.5	0.7±0.6
P3	1.9±1.0	79.6±4.9	1.0±0.03	4.3±0.6	1.0±0.01	1.1±0.1
P4	3.0±1.0	88.6±3.2	2.7±0.03	3.4±0.6	2.1±0.3	3.2±1.4
P6	3.0±1.0	78.6±5.3	3.0±1.0	3.0±0.9	2.9±0.9	3.3±1.9
P8	2.6±1.2	71.9±27.5	2.3±1.2	4.7±0.7	1.8±0.3	2.3±0.1
P10	2.6±1.2	71.9±8.5	2.7±0.03	3.4±0.6	2.1±1.1	2.8±1.3

** indicates positive marker, ** indicates negative markers for rat MSCs.*

Table 4.2 – Mean Fluorescence Intensity (MFI) values of CD markers obtained by FACS analysis at different passages

	CD90*		CD45**		CD106**	
	Isotype	**Cell**	**Isotype**	**Cell**	**Isotype**	**Cell**
P0	8.4±2.3	257.7±190	7.8±4.2	8.7±4.9	15.7±12.0	16.4±11.7
P2	9.8±1.6	212±41.4	10.2±3.0	11.7±3.0	23.6±12.8	10.8±1.3
P3	10.9±1.2	152±10.5	7.8±1.6	11.4±1.0	9.2±0.2	9.4±0.3
P4	14.51±2.1	260.43±20	13.19±2.1	15.5±2.0	7.64±2.1	12.7±2.8
P6	15.21±2.2	180.16±15	12.9±2.01	14.8±1.01	7.05±1.5	8.74±1.23
P8	8.0±0.7	127.9±38	4.8±1.1	6.6±0.8	5.5±0.7	6.5±0.3
P10	8.87±1.0	133.69±6	4.65±0.67	5.18±0.87	6.2±0.55	7.3±0.4

** indicates positive marker, ** indicates negative markers for rat MSCs.*

When taken together, Table 4.1 and 4.2 established that the characteristic surface marker expression (CD90+) increased substantially from P0 to P6 (up to ~ 90% positivity). These results suggested that the MSC generation protocol from rat BM indeed converted the progenitor cells into MSCs.

The MFI measurement is an indication of the extent of CD90 protein expression at the MSC surface. As seen in Table 4.2 these values are consistent with % expressed surface CD marker values. The findings in this section revealed that MSC conversion,

as judged by their +ve and -ve CD marker expression from rat BM took place and their MSC characteristics were retained after successive passages. This is one of the hallmarks to obtain pluripotent MSC population and could be harnessed to differentiate into organ specific cell types (i.e. adipocytes, osteocytes, myocytes, and neurons) when culturing them in selective differentiation culture medium.

4.2. Differentiation of Rat Mesenchymal Stem Cells in the presence or absence of Toll-Like Receptor Ligands

4.2.1. Adipogenic Differentiation Studies with rat Mesenchymal Stem Cells at Passage 0

One of the unique characteristics of mesenchymal stem cells is their ability to differentiate into different lineages. Differentiation of MSCs requires selective media supplemented with several compounds at specific concentrations. The incubation period is lengthy reaching up to several weeks for certain cells making this process not only lengthy but very expensive. Moreover, the conversion yields are not at the appreciable levels. Description of new media components that would significantly reduce differentiation time while increasing conversion yield is a critical issue in MSC field. Since earlier observations established that MSCs express certain TLRs (Figure 4.4 for details) we postulated that supplementing specific differentiation media with some of these TLR ligands might contribute to induce better MSC differentiation (i.e. reduced culture time as well as increased conversion yield). In this part of the present study, we checked the effect of TLR2L, TLR3L, TLR7L and TLR9L addition on adipocyte and osteocyte generation. The differentiation studies were performed with different passages of rMSCs. This is to understand if the passage number has any effects on the differentiation capacity of MSCs in the presence and absence of TLR ligands. Adipocyte induction was continued with freshly made medium and replenishments were performed at two days intervals (with or without TLRL) throughout the course of three weeks.

Under normal differentiation conditions, MSC to adipocyte conversion takes three weeks. By the fifth day of induction, cells grown under TLRL supplemented medium started to deposit small and shiny lipid droplets (data not shown) whereas these

inclusions were barely visible for the MSCs cultured in regular adipocyte medium. As the induction period continued, the numbers and the sizes of lipid droplets increased and filled up the cell cytoplasm (compare Figure 4.6 vs Figure 4.7).

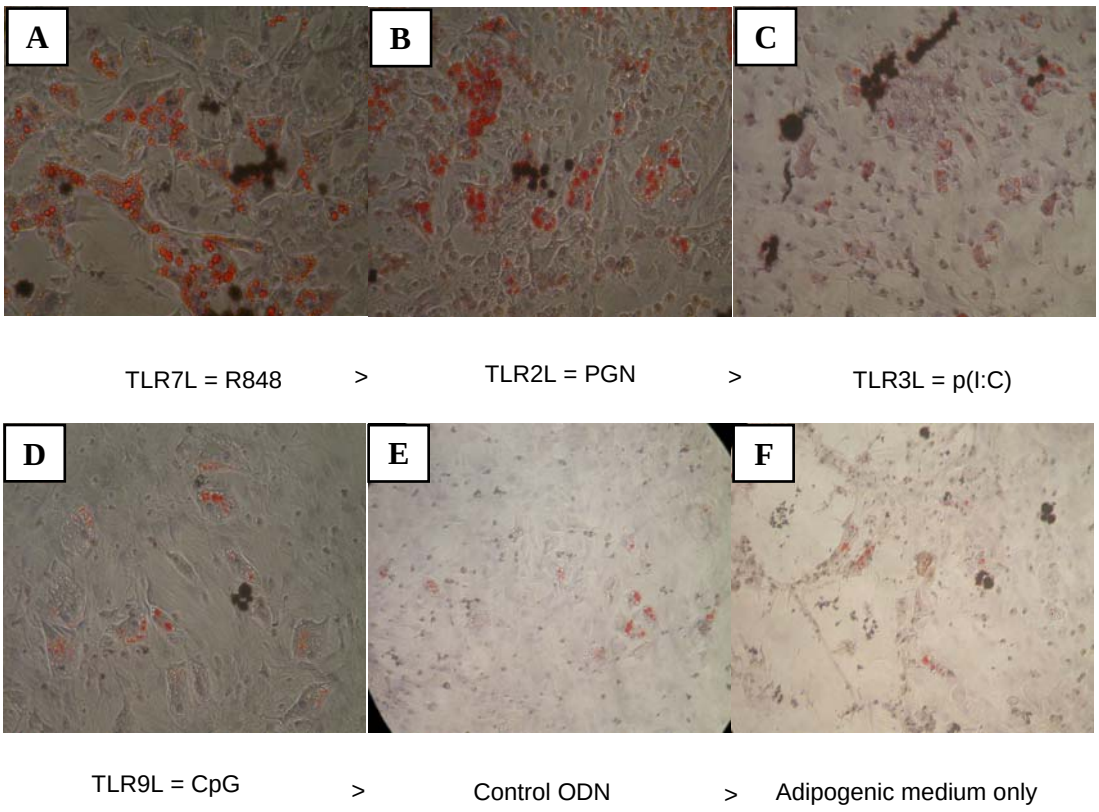


Figure 4.6 - P0 rMSC Oil Red O staining at the end of 1st week (Mag = 20X)

In the Figure 4.6, P0 rMSCs Oil Red O stainings are given at the end of week 1. These photomicrographs are presented based on the adipogenesis rates of each treatment. The quantitation of how much adipocyte was induced in the presence or absence TLR ligands were done by counting cells positive for Oil Red O stained lipid inclusions from four different areas representing the overall adipocyte generation for that well. According to these criteria, at the end of first week of induction, R848 (Fig 4.6A) gave the most adipogenic outcome and was followed by PGN, p(I:C), CpG and Control ODN (Fig 4.6B-E) as it can be seen normal adipogenic media (Fig 4.6F) performed the weakest of all. This feature seen for the adipogenic media is not surprising, since it requires 3 weeks to convert MSCs into adipocytes.

In Figure 4.7, the second week results of P0 rMSC adipogenic induction were shown. Comparing these results with the first week adipogenesis profiles, it is fair to say that PGN treated group significantly improved and led to near complete differentiation of the MSCs within two weeks (Figure 4.7, top left panel).

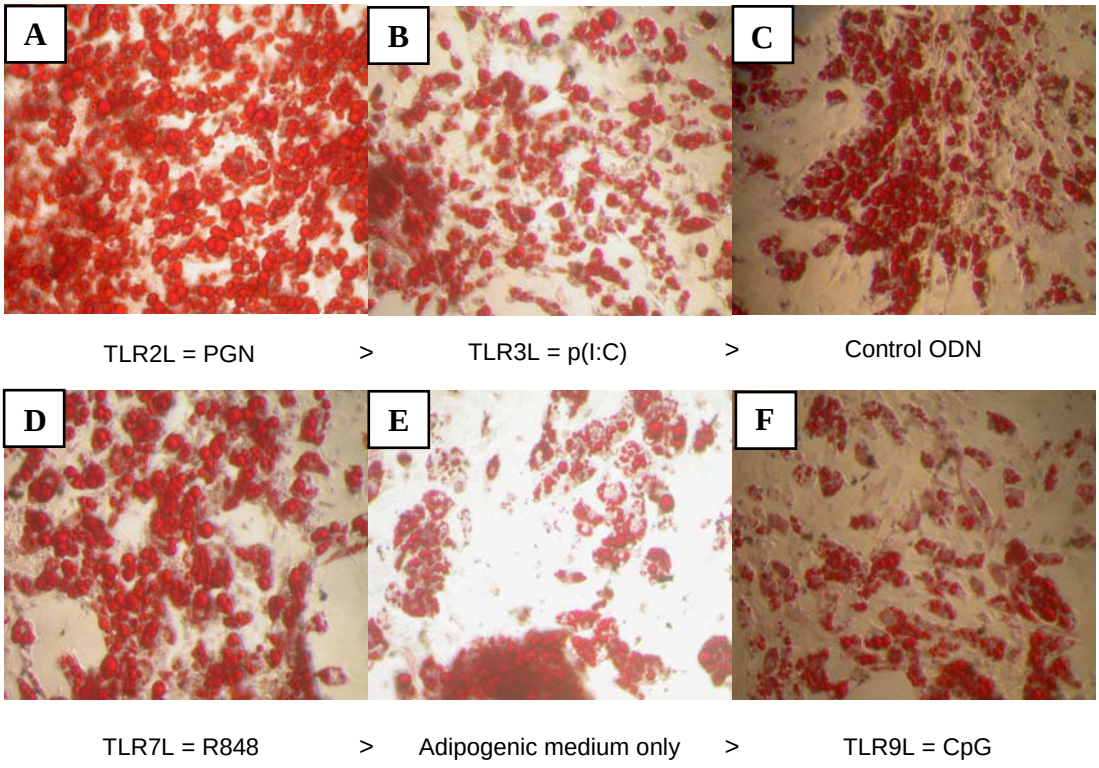


Figure 4.7 - P0 rMSC Oil Red O staining at the end of 2nd week (Mag = 20X)

Adipogenic induction medium was the weakest of all. Next to PGN, p(I:C), R848 and Control ODN treated groups induced significantly better differentiation than adipogenic induction medium only group at the end of two weeks. The continuation of the experiment for another week was unnecessary, thus this set of experiment was decided to be terminated at week 2. Figure 4.8 presents contribution of different TLR ligand supplement into adipose generation medium at the end of two weeks. As seen in this figure and supported by the photomicrographs presented in Figures 4.6 and 4.7, several TLR ligands (PGN, p(I:C), and R848) significantly boosted adipogenesis rate and efficiency, compared to gold standard medium conventionally utilized to obtain adipose differentiation.

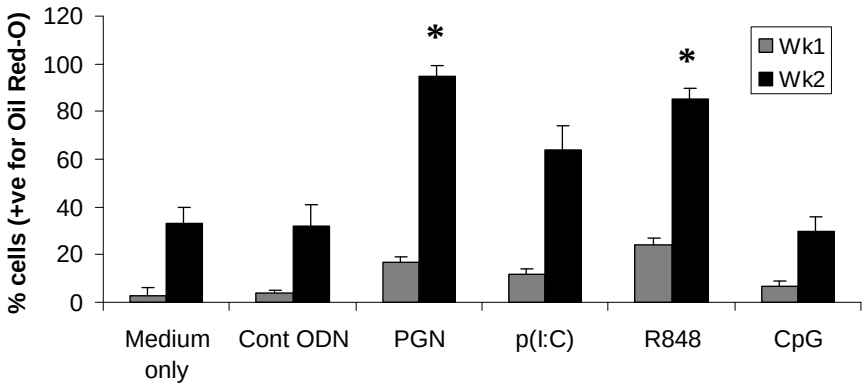


Figure 4.8 - MSC differentiation (@P=0) in the presence or absence of TLR ligands. Culture media replenishment in two days intervals was conducted and adipogenesis was monitored by counting the cells positive for Oil Red O (** p < 0.05, comparing to "Medium only").*

4.2.2. Adipogenic Differentiation Studies with rat Mesenchymal Stem Cells at Passage 3

In order to further delineate the kinetics of adipogenesis in the presence of TLR ligands, another set of experiment using rMSCs at P3 was initiated. The stainings were selected to include shorter time intervals (i.e. d5, d8, d11 and d14). The detailed photomicrographs are presented in Appendix B-2 (please see page 93 for more details).

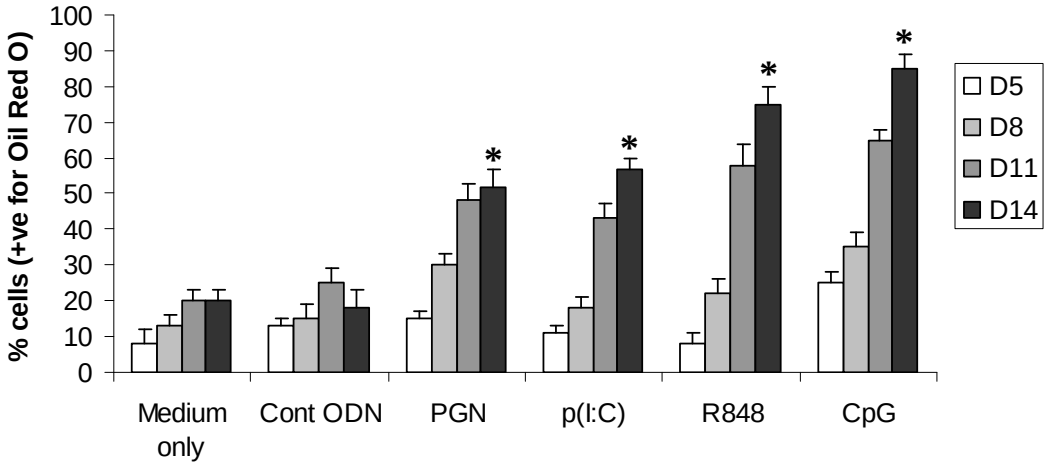
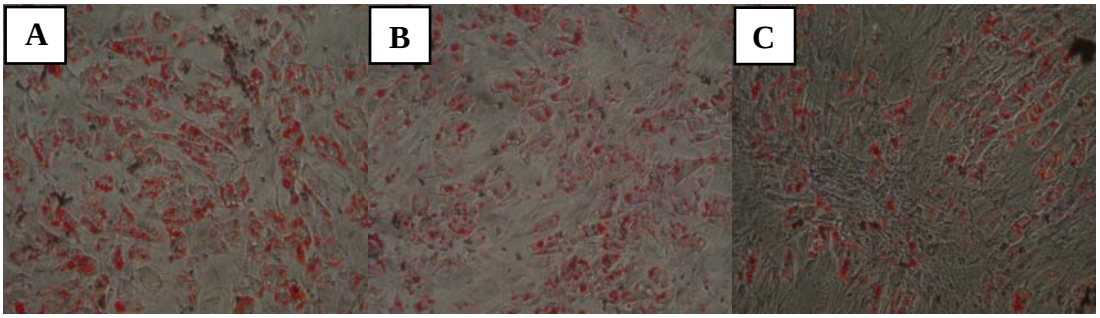


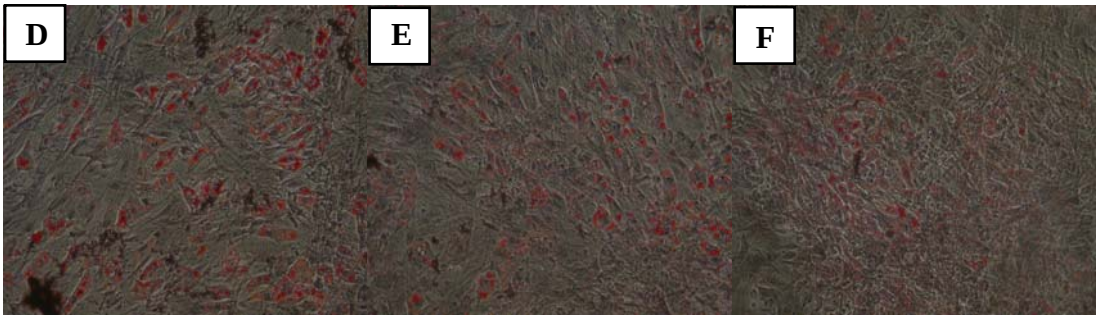
Figure 4.9 - Kinetics of rMSC differentiation (@P3) into adipocytes in the presence or absence of TLR ligands (see legend for Fig 4.8 for more details) (** p < 0.01, comparing to "Medium only").*

Over the course of two weeks, data from Figure 4.9 revealed that R848 and CpG ODN supplemented media induced the highest number of adipogenesis from MSC started at P3. As seen in the Figure 4.10, cells at the end of week 1 that was cultured

in the presence of PGN gave the highest conversion of Oil Red O positive cells, but this trend changed by the end of week 2. Starting with P3 MSCs, R848 together with CpG ODN performed better than PGN (Figure 4.11). Consistent with to previous

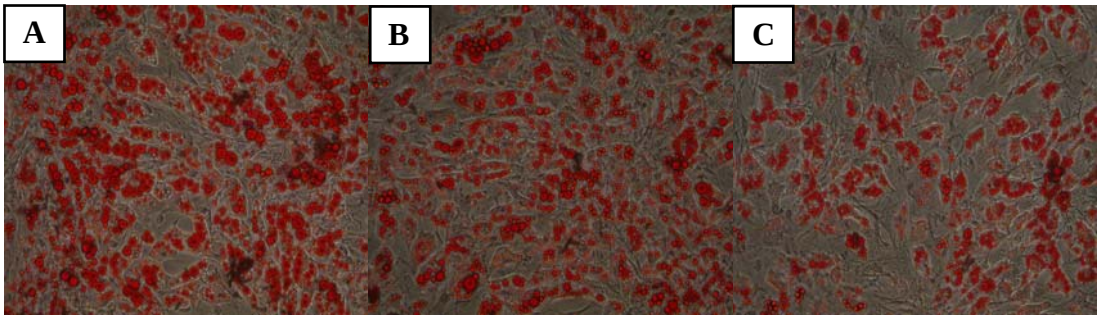


TLR9L = CpG > TLR2L = PGN > TLR7L = R848

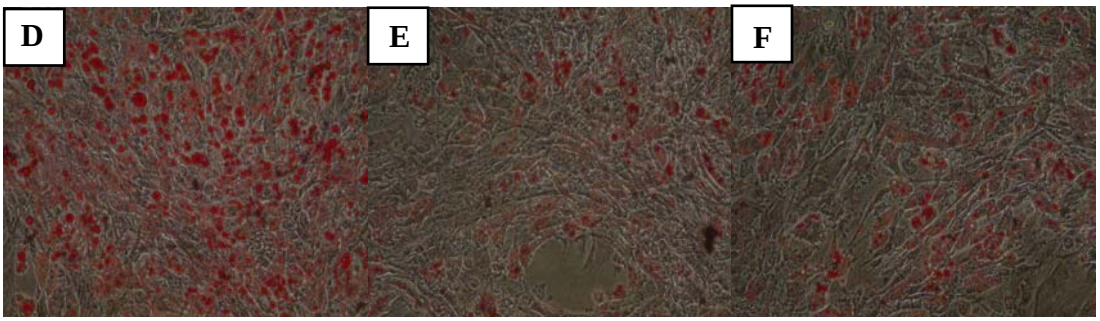


TLR3L = p(I:C) > Control ODN > Adipogenic medium only

Figure 4.10 - Photomicrographs of rMSC (@P3) differentiated to adipocytes at D8 (Mag = 20X)



TLR9L = CpG > TLR7L = R848 > TLR3L = p(I:C)



TLR2L = PGN > Control ODN => Adipogenic medium only

Figure 4.11 - Photomicrographs of rMSC (@P3) differentiated to adipocytes at week 2 (Mag = 20X)

finding, adipose medium alone treated MSCs performed worst of all the tested groups at all times (compare Figures 4.8 with 4.9).

Taking together, the findings obtained from P0 and P3 MSC revealed that Control ODN and adipose medium alone treated groups did not perform better than any of the tested four different TLR ligand throughout adipogenic inductions. These two adipogenesis experiments implicated that R848 is the best performing ligand. If one tries to rank the performances of these ligands (average of P0 and P3 studies) based on their induction capacity, the list appear like below:

1 - R848=TLR7 Ligand
2 - PGN= TLR2/6 Ligand
3 - CpG ODN= TLR9 Ligand
4 - p(I:C)= TLR3 Ligand

Another observation worth mentioning is that the rate of adipocyte generation at the end of each week for both studies suggested that at the end of week 1 R848 induction was better if P3 MSCs are used rather than P0 MSCs (35% vs 18%, compare week 1 values of Figures 4.8 and 4.9). This increased rate of conversion however, is lost by the end of week 2 and is not significant between different passages (~85% for both trials).

4.2.3. Adipogenic Differentiation Studies with rat Mesenchymal Stem Cells at Passage 6 and Passage 9

The adipogenic differentiation study in the presence or absence of different TLR ligands was designed to uncover two issues; i) rate and ii) efficiencies of adipogenesis at different MSC passages. The initial studies were carried out at P0 and P3, we also aimed to follow adipogenesis of MSCs after several passages. In TLR expression studies, we observed that TLR expression wanes at later stages of MSC passages. To understand the effectiveness of ligand supplementation during adipocyte differentiation, MSCs at P6 and P9 were also followed (please see Figure 4.12). The photomicrographs of adipogenic differentiation along time are presented in Appendix B-2 (please see page 94 for more details).

As seen in Figure 4.12, p(I:C) led the highest induction at the end of two weeks of differentiation culture. Recalling the TLR gene expression profiles of MSCs at different passages, one would notice that by P6 receptor for p(I:C), (i.e. TLR3) expressed at much higher level than any other TLRs, supporting the data presented in Figure 4.12.

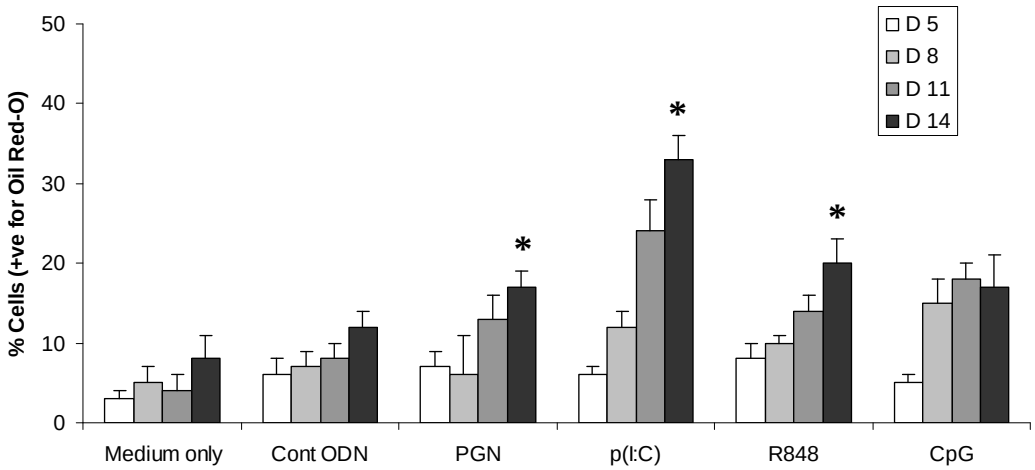


Figure 4.12 - Kinetics of MSC differentiation into adipocytes at P6 in the presence or absence of TLR ligands (see Fig 4.8 legend for more details) (** $p < 0.05$, comparing to "Medium only").*

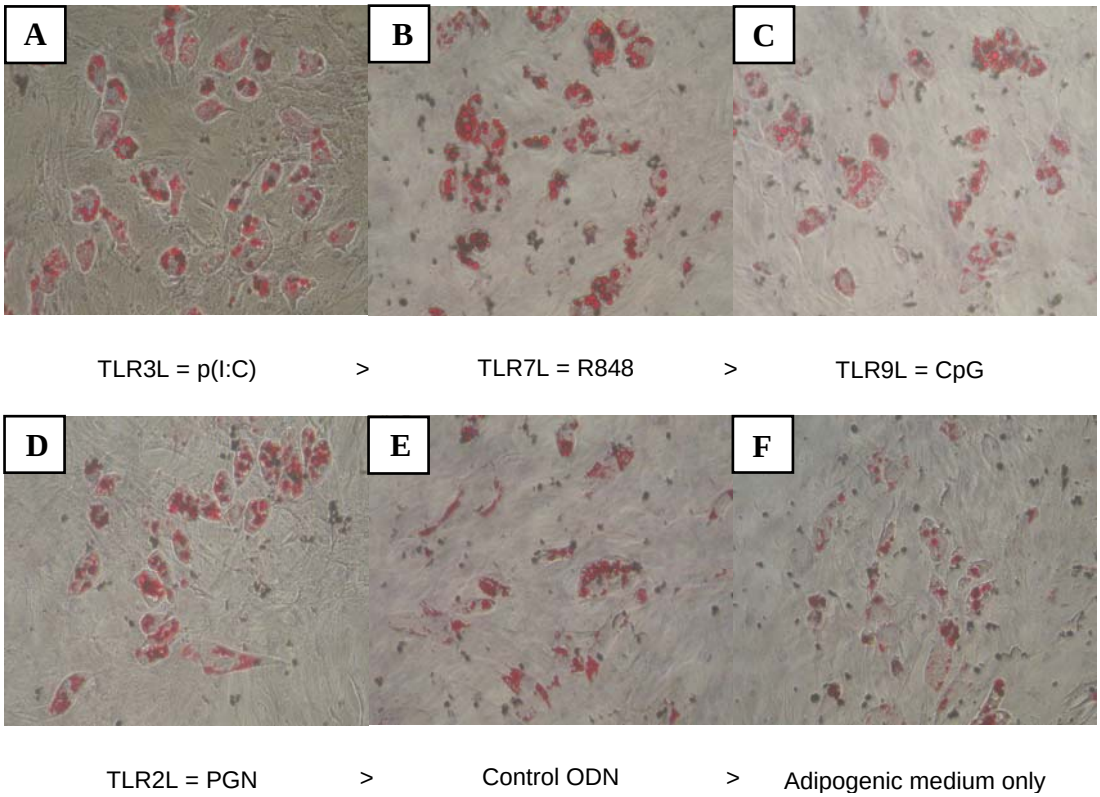


Figure 4.13 - Photomicrographs of rMSC at P6 differentiating into adipocytes at week 2 (Mag = 20X)

The Oil Red O staining of differentiated MSCs at P6 at the end of two weeks is presented in Figure 4.13. At P6 TLRL 2, 7, and 9 performed similar to each other but lower than p(I:C). Again, control ODN or adipose medium alone is very weak at the induction potential.

Lastly, in this part of the study we investigated the adipogenic performance of the MSCs at P9. Among the MSC passages studied so far, this passage showed the slowest rate of differentiation, therefore, we have decided to extend the induction period up to three weeks. As seen in the Figures 4.14 and 4.15, the rates of conversion into adipocytes were very low compared to previous passages (compare Fig 4.8, 4.9, 4.12, with 4.14).

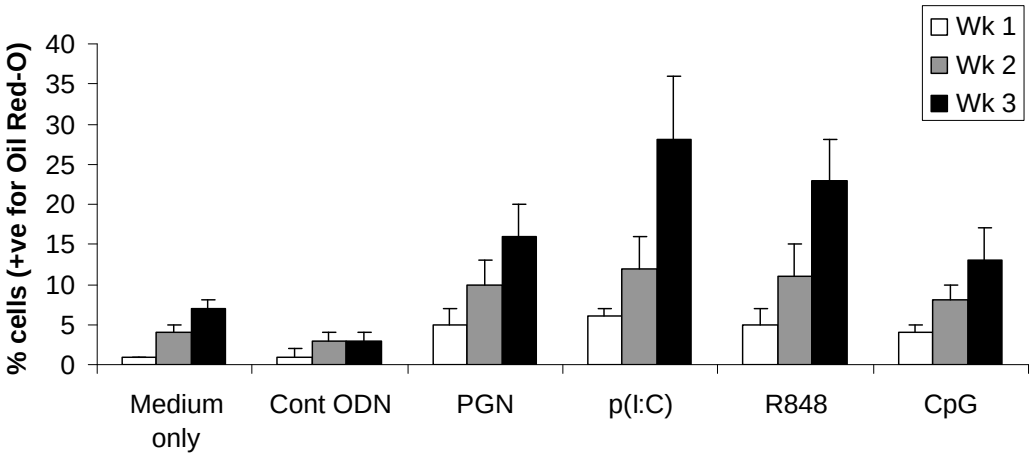


Figure 4.14 - Kinetics of MSC differentiation into adipocytes at P9 in the presence or absence of TLR ligands (see Fig 4.8 legend for more details)

As can be seen from Figure 4.14, the trend reached at the end of week 3 is only comparable to the differentiation rate seen at week 2 of MSCs used at P6. The induction rate of the MSCs at later passages is significantly delayed. Consistent with P6 findings MSCs at P9 initiated the highest adipogenic conversion when the medium is supplemented with TLR3 ligand (p(I:C)).

The overall summary of the differentiation in the presence or absence of TLR ligands at the end of the induction period is summarized in the Table 4.3

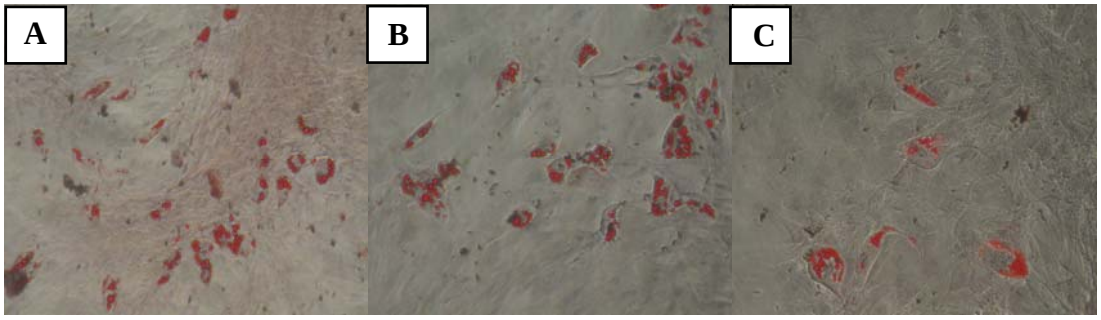
Table 4.3 - Overview of the adipogenesis from MSCs treated with different TLR ligand containing adipose differentiation medium

Rx Groups	**P0***	**P3***	**P6***	**P9****
Medium Alone	33±7	20±3	8±2	7±2
+ Control ODN	32±9	18±5	12±2	3±1
+ PGN (TLR2L)	95±4	52±5	17±2	16±4
+ p(I:C) (TLR3L)	64±10	57±3	33±3	28±8
+ R848 (TLR7L)	85±5	75±5	20±3	23±5
+ CpG ODN (TLR9L)	30±6	85±4	19±4	13±4

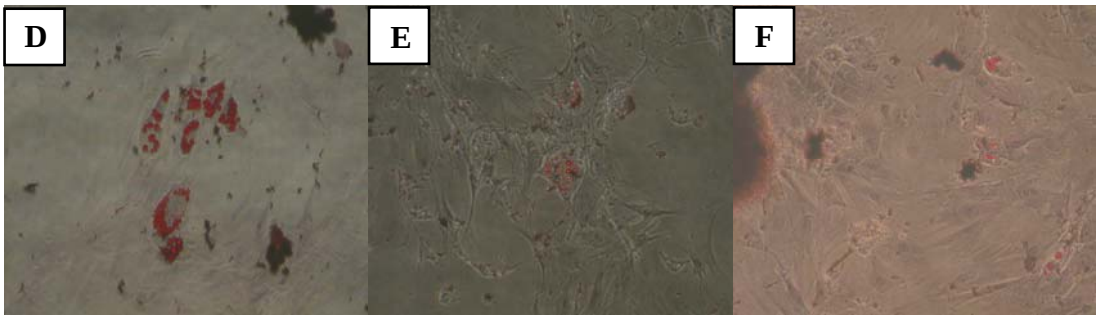
:

** T= Week 2, ** T= Week 3*

These set of experiments revealed that passage number is critical during adipocyte differentiation from MSCs. Furthermore, inclusion of certain TLR ligands in the differentiation media significantly facilitates adipogenic development at P≤3. Our results strongly suggested that, it is possible to accelerate as well as increase the rate and efficiency of MSC adipogenesis by the addition of either TLR2 ligand, (a member of cell surface expressed TLR family), or TLR7 ligand, (a member of endosome associated TLR family) during early passages. For MSCs (i.e. P≥6) undergoing differentiation, supplementing media with p(I:C) proved to be important.



TLR3L = p(I:C) > TLR7L = R848 > TLR2L = PGN



TLR9L = CpG > Control ODN > Adipogenic medium only

Figure 4.15 - Photomicrographs of rMSC at P9 differentiating into adipocytes at week 3 (Mag = 20X)

4.3. RT-PCR Analysis of Differentiation Experiments

4.3.1. RT-PCR Analysis of Lineage-Specific Genes

While Oil Red O staining of MSCs at various time of incubation and visualizing their induction patterns under microscopic investigation is a substantial evidence for the generation of adipocytes, it is also necessary to demonstrate the expression of several key gene expression specific for different differentiation. During this study, differentiations in the adipogenic and osteogenic directions were assessed in the presence or absence of TLR ligands. Commitment of MSCs in the direction of organ-specific differentiation was followed by RT-PCR. For adipocytes, i) *lpl* (lipoprotein) and ii) *ppar-γ* (peroxisome proliferator-activated receptor gamma) transcripts were selected as the lineage specific genes. For osteogenesis, i) *alp* (alkaline phosphatase), ii) *oc-1* (osteocalcin-1) and iii) *runx* (Runt-related transcription factor 1) were selected as the lineage-specific marker genes.

In the Figure 4.16, agarose gel images of the effect of TLR ligand addition on LPL and PPAR-γ message expression during adipocyte differentiation of P0 and P3 MSCs are presented. During MSC to adipogenic differentiation at P0, from the gel images an increased level of LPL and PPAR-γ expression is observed. This is mainly mediated by R848 and CpG treatment (please see Figure 4.16, top panel).

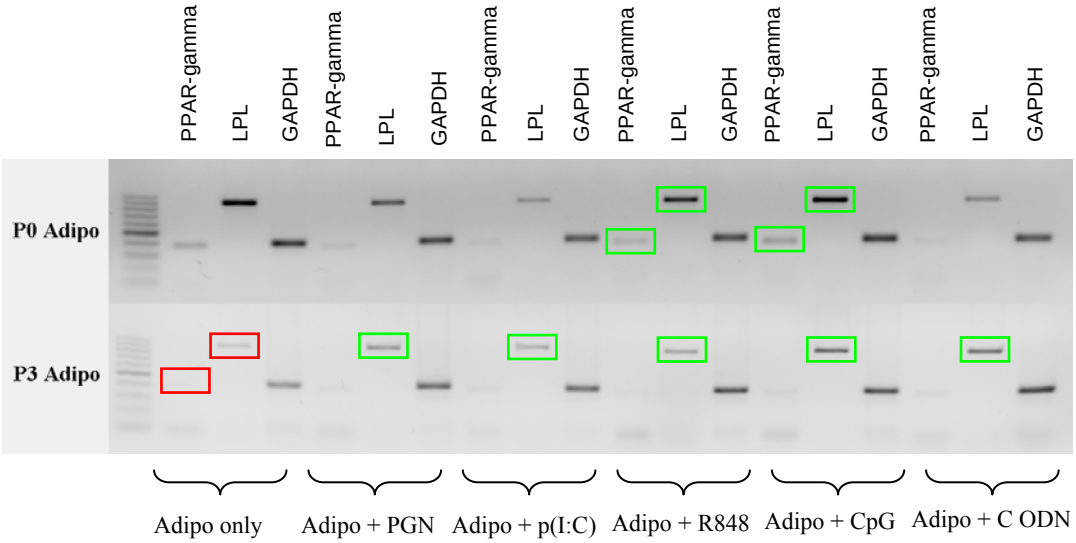


Figure 4.16 - Adipocyte specific gene expression panels of P0 and P3 rMSCs supplemented with different TLR ligands

Although a strong *lpl* and weak *ppar-γ* expressions were observed during P0 MSC commitment into adipogenesis when incubated in adipose only medium, the expressions of these genes were significantly very low when P3 MSC were utilized (compare upper left panel with lower left in Figure 4.16). Involvement of TLR ligands in the adipose generation medium significantly improved *lpl* expression and also low but detectable level of *ppar-γ* expression was induced. This evidence indicated that TLR ligands positively impact adipogenesis at P3 (please see lower panel for details).

In this part of the thesis, in addition to adipogenesis, osteogenic differentiation of MSCs were also studied. The lineage-specific gene expressions from TLR ligand treated groups were analyzed by RT-PCR for MSCs at P0 and P3 passages. Results demonstrated that at P0 addition of TLR ligands (except p(I:C)) induced substantial but non-significant improvement of the *alp, oc-1* and *runx* transcripts compared to osteogenic induction medium group (please see Figure 4.17 for more details)

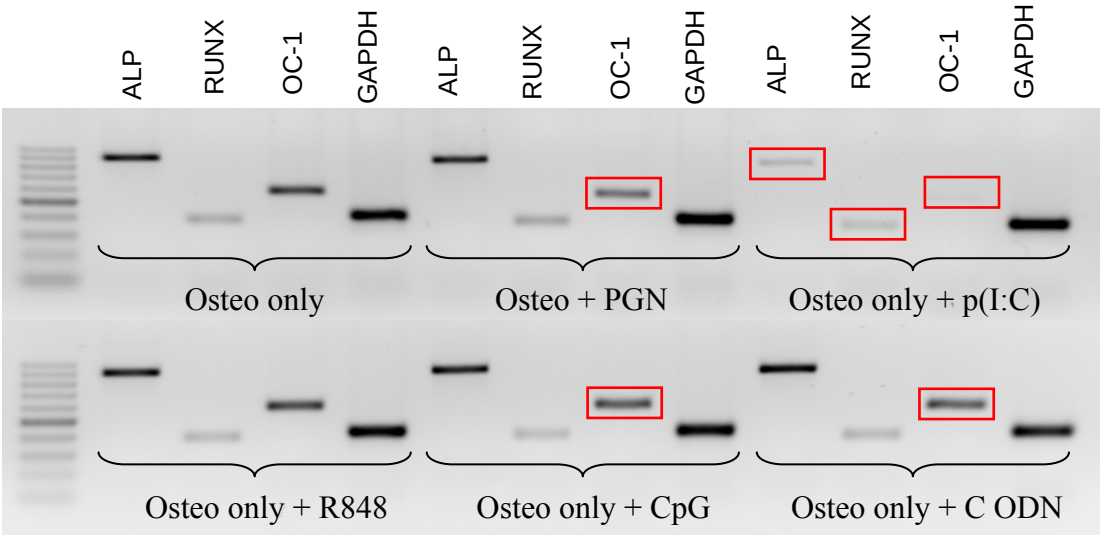


Figure 4.17 - Osteocyte specific gene expressions for P0 MSCs

Osteogenesis in the presence and absence of TLR ligand supplemented differentiation medium was also assessed for the MSCs at P3 by RT-PCR (Figure 4.18). Among tested ligands, only PGN slightly improved gene transcript levels. Contrary to results seen during adipogenic differentiation, addition of several other TLR ligands did not mediate any added effect during osteogenic differentiation process. Results revealed that PGN slightly improved *alp*, *oc-1* and *runx* message

RNA expressions. The other ligands such as p(I:C), R848, and CpG ODN reduced expressions of these gene transcripts. Only, p(I:C) treated group has shown a decreased osteogenic profile.

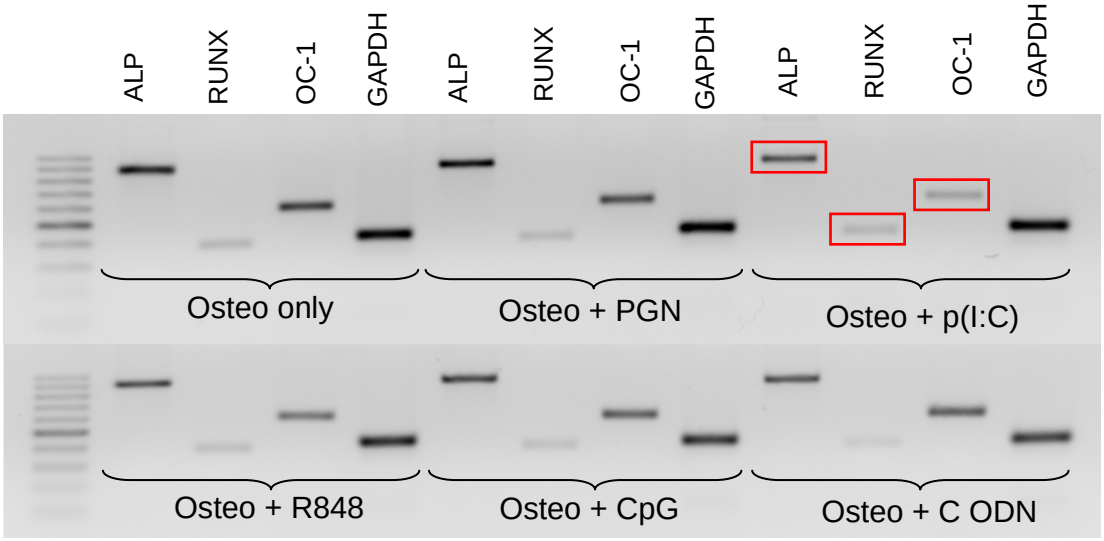


Figure 4.18 - Osteocyte specific gene expression panels of P3 rMSCs

The scenario of P0 rMSCs for the osteocyte differentiation is also applicable for P3 rMSCs in the terms of osteocyte specific gene expression levels. Again in the p(I:C) treated group, the osteocyte specific gene expressions were decreased but the other groups showed no significant difference in the positive or negative direction compared to naïve group.

4.3.2. RT-PCR Analysis of Toll-Like Receptor Panels

We and others have demonstrated that MSCs do express TLRs (Pevsner-Fischer et al., 2007; Schaffler et al., 2007) for TLR panels of specific rMSC passages see Section 4.1.1.2.2.). Our previous observations also revealed that when immune cells were triggered by a specific TLR ligand, many TLRs do change their expression levels (Tincer, G., M.Sc. thesis). In an attempt to understand the variation of gene expression of different TLRs following ligand treatment was followed using cells undergoing adipogenic or osteogenic differentiations at P0 and P3 passages. Figures 4.19 and 4.20 summarize the TLR panel obtained for P0 and P3 MSC undergoing adipogenic differentiation in the presence or absence of TLR ligand treatments.

Analysis of Figure 4.19, implicated that at P0 MSC undergoing in the presence of PGN upregulated TLR2, TLR6 and TLR9 gene transcripts more than adipose only medium group. Furthermore, CpG treatment upregulated its own receptor TLR9 (Figure 4.19). At P0, other TLR ligands did not show any increase but significant reduction of the TLR gene expressions.

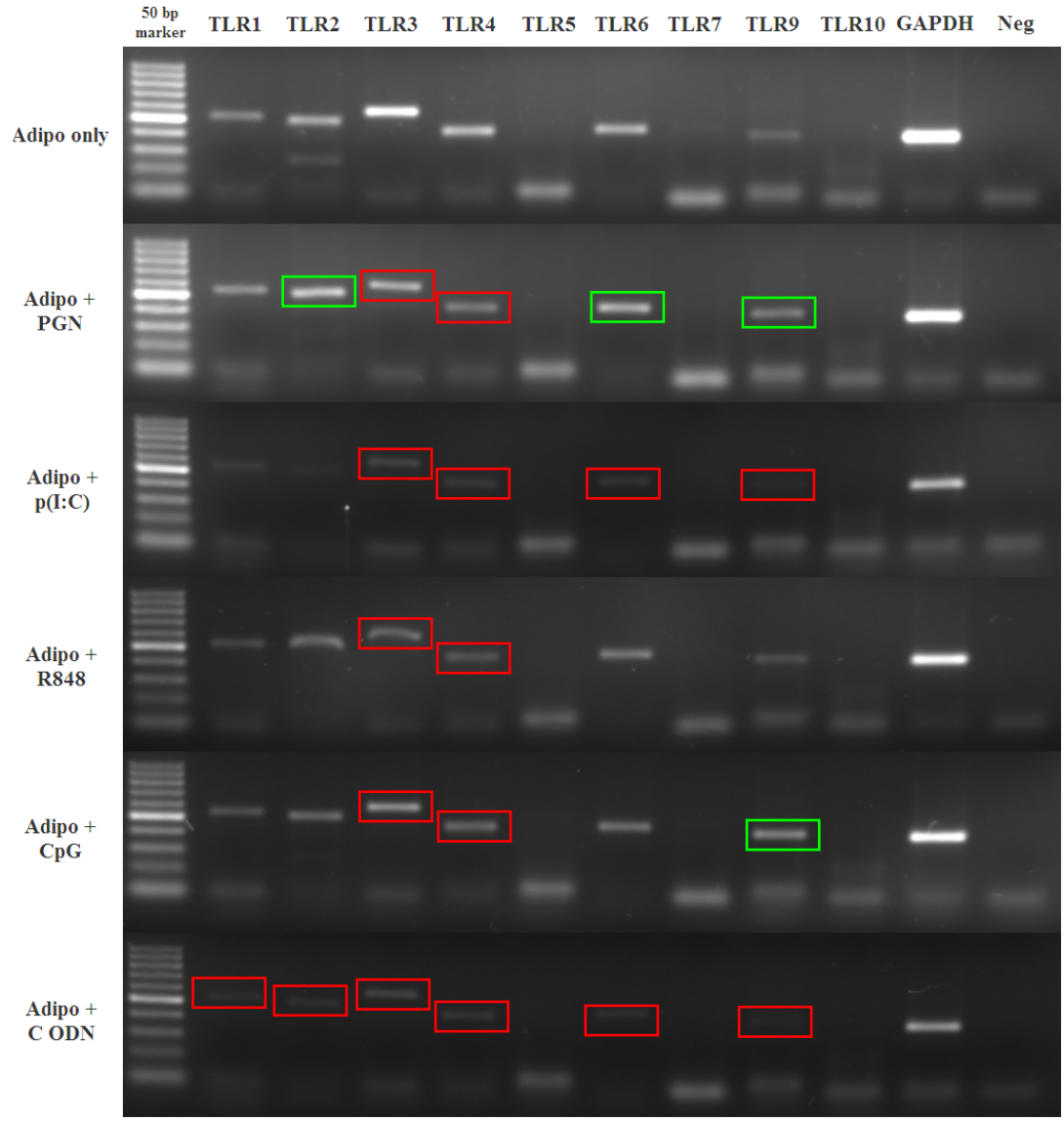


Figure 4.19 - TLR panels of P0 rMSCs during adipogenic differentiation

This trend is quite the opposite when adipogenesis by P3 MSCs were analyzed. As seen in the Figure 4.20, adipocyte differentiation medium alone retained expression of TLR1, 3, 4, and 6, albeit at low levels. Addition of TLR2L (PGN), TLR3L (p(I:C)), TLR7L (R848) and TLR9L (CpG ODN) significantly boosted all of these TLRs. The overview of TLR gene transcript variation is summarized in Table 4.4.

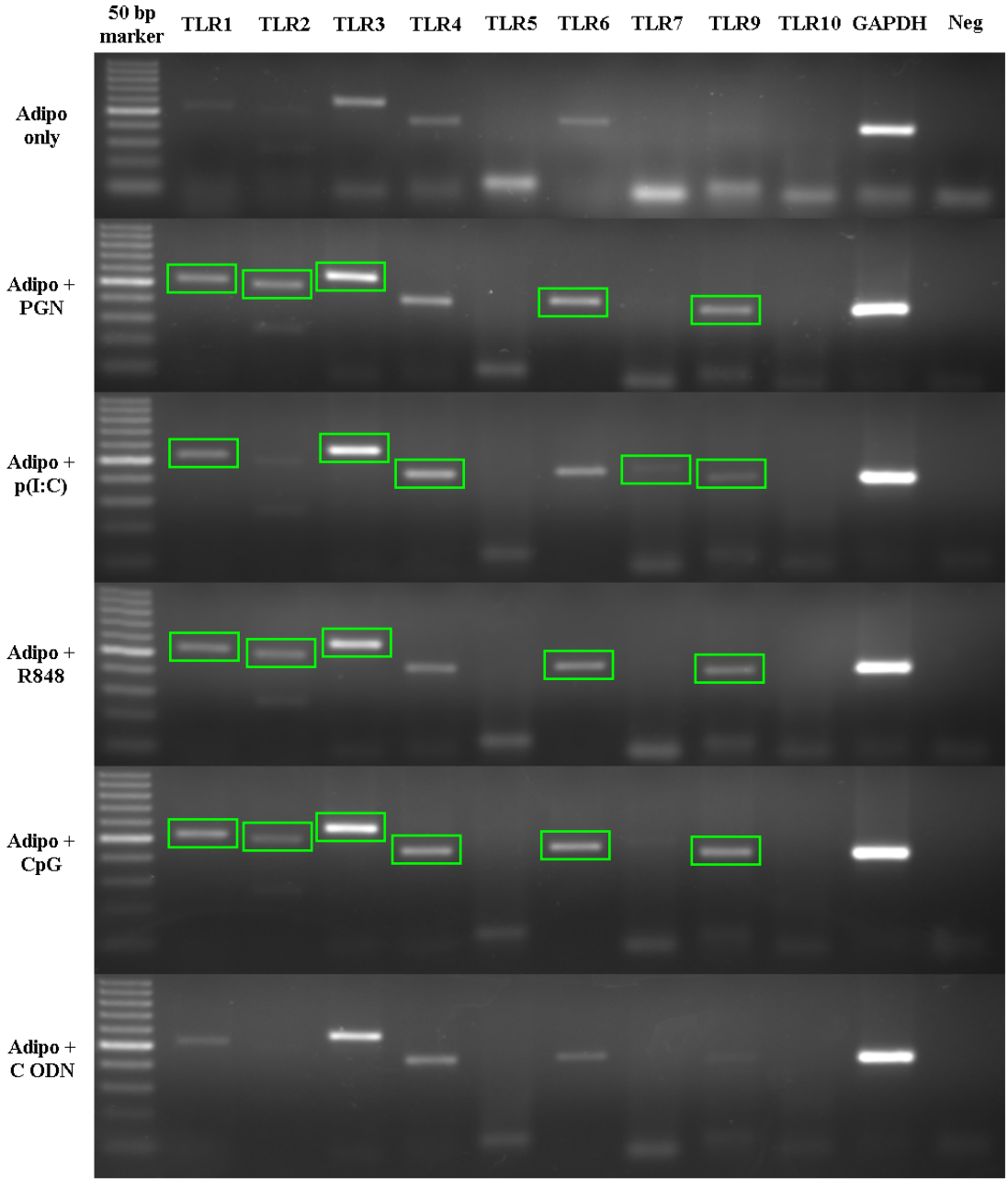


Figure 4.20 - TLR panels of P3 rMSCs during adipogenic differentiation

Table 4.4 - Effect of ligand treatment during P0 or P3 MSC differentiation in to adipocytes

MSC	Rx	TLR1	TLR2	TLR3	TLR4	TLR6	TLR7	TLR9
P0	Medium	*	**	****	**	**	UD	*
	PGN	*	***	**	**	***		**
	p(I:C)	UD	*	*	*	*	UD	
	R848	UD	*	*	*	*	UD	
	CpG	UD	*	**	*	*	UD	**
MSC	Medium	*	UD	**	*	*	UD	UD
P3	PGN	**	*	****	**	**	UD	**
	p(I:C)	**	UD	****	**	**	UD	**
	R848	*	*	***	**	**	UD	**
	CpG	**	*	****	**	**	UD	*

* *upregulated,* * *downregulated,* * *unchanged, UD undetected*

The effect TLR ligands on MSCs (at P0 and P3) undergoing osteogenesis were also analyzed by RT-PCR. Figures 4.21 and 4.22, as well as Table 4.5 provide gene expression profile analyses of these studies.

As clearly seen in this set of data, addition of TLR ligands upregulated TLRs both in P0 and P3 (compare gel images of Figure 4.21 and 4.22) during osteogenesis.

When taken together, upregulation of several TLRs throughout the course of differentiation has critical implications. Our findings strongly suggested that pluripotent MSCs when there is a microbial by product may become more strongly committed to undergo terminal differentiation possibly due to the inflammatory signals initiated by these ligands. These differentiated cells probably become more susceptible to triggering by the surrounding inflammatory signals (i.e. microbial by products present in that niche) and may lead to more robust immune modulatory responses. In order to further test this postulation additional experiments must be performed.

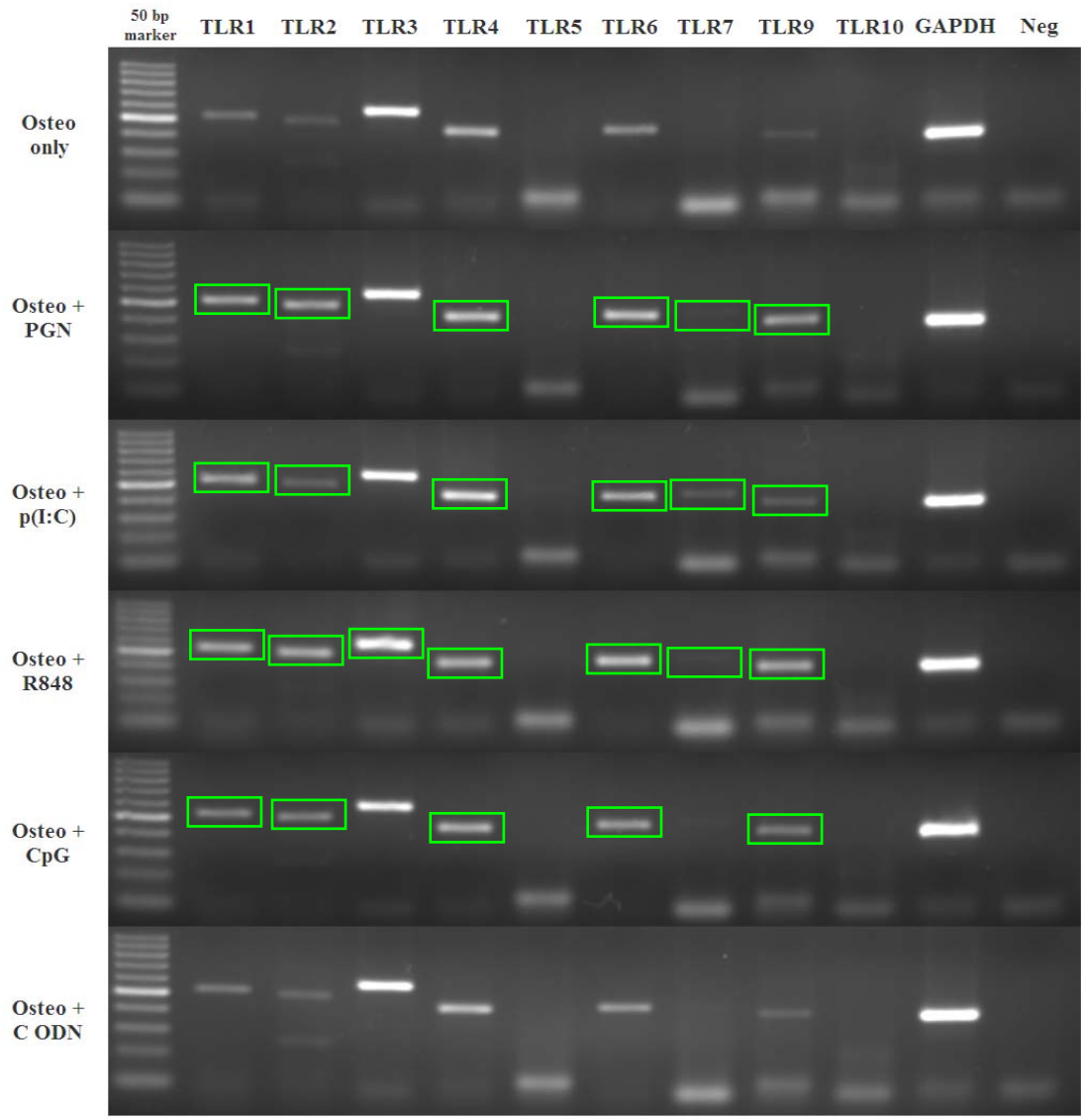


Figure 4.21 - TLR panels of P0 rMSCs during osteogenic differentiation

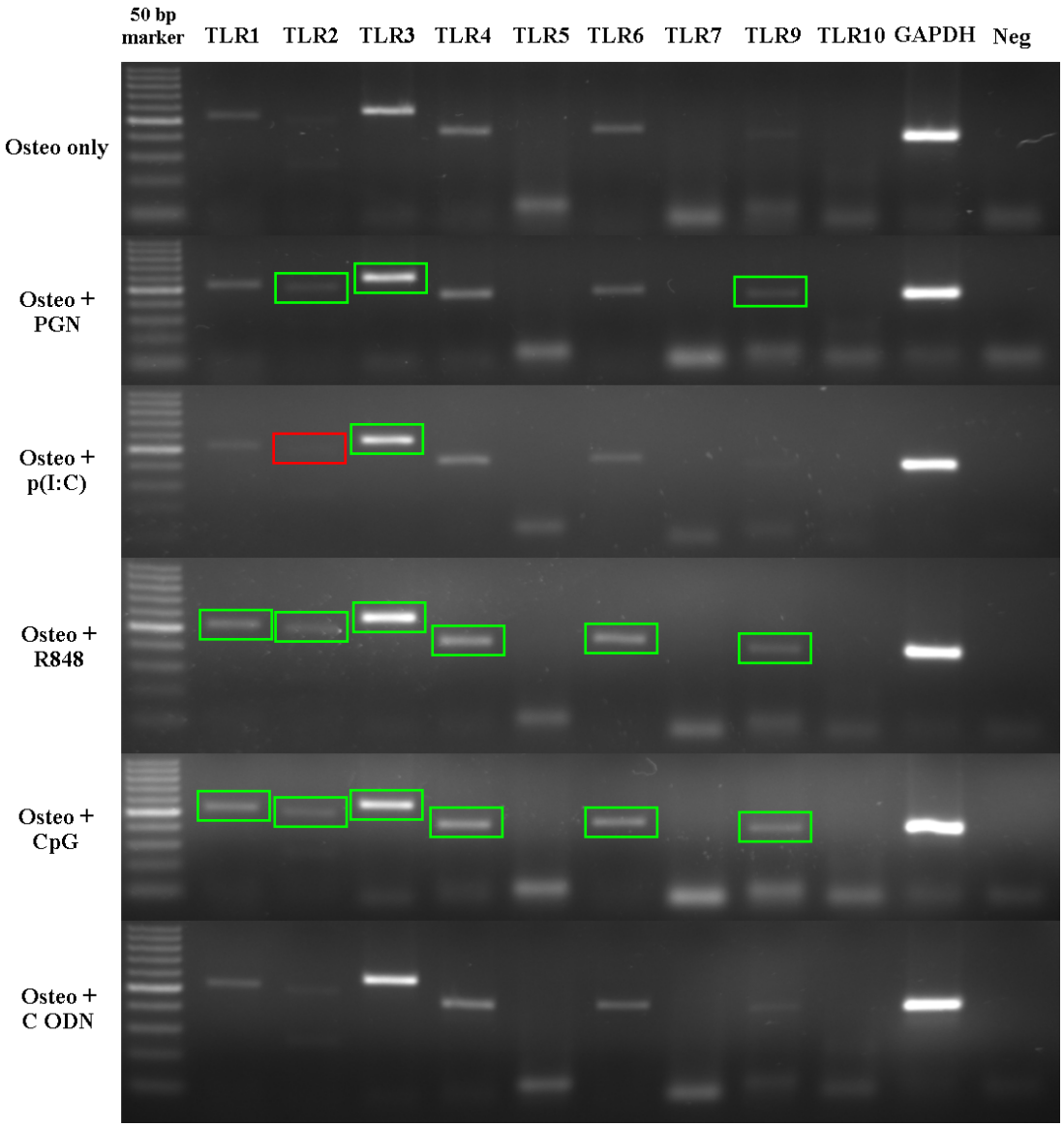


Figure 4.22 - TLR panels of P3 rMSCs during osteogenic differentiation

Table 4.5 - Effect of ligand treatment during P0 or P3 MSC differentiation in to osteocyte

MSC	Rx	TLR1	TLR2	TLR3	TLR4	TLR6	TLR7	TLR9
P0	Medium	**	*	****	***	**	UD	*
	PGN	***	***	****	***	***	*	***
	p(I:C)	***	**	****	****	***	**	**
	R848	***	***	****	***	***	*	***
	CpG	***	***	****	***	***	UD	***
MSC	Medium	**	*	****	***	**	UD	*
P3	PGN	**	**	*****	***	**	UD	**
	p(I:C)	**	*	*****	***	**	UD	*
	R848	**	**	*****	****	****	UD	**
	CpG	**	**	*****	****	****	UD	***

* *upregulated,* * *downregulated,* * *unchanged, UD undetected*

4.4. Migration Assay

Mesenchymal stem cells possess a very unique property known as “homing capacity” to the injured site. As described earlier, MSCs express TLRs, we reasoned that these TLRs may contribute to the “homing capacity” of the MSCs. In order to investigate this phenomenon, in vitro migration experiments were performed in the presence or absence of TLR ligands.

As demonstrated in the Figure 4.23, migration capacity of mesenchymal stem cells significantly facilitated upon treatment with TLR ligands. In our hand, compared to media alone and control ODN groups, addition of TLR ligands improved the migration capacity more than 100% within 24 hours (Figure 4.23). This phenomenon is independent of the proliferative capacity induced by ligand stimulation, since our CFSE assay resulted insignificant proliferation among treatment groups (for more information please refer to Appendix C).

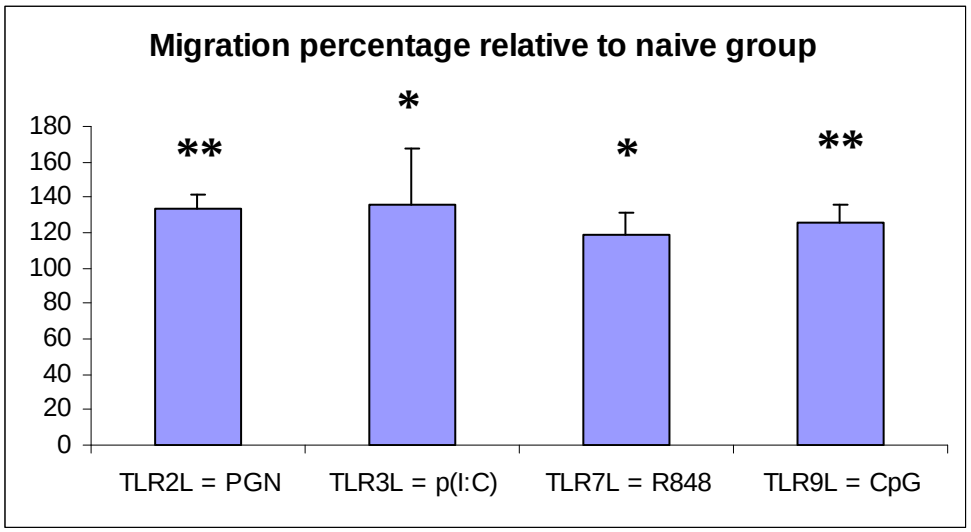


Figure 4.23 - Migration capacity of MSCs relative to MesenCult media and control group after 24 h *(* $p<0.05$, ** $p<0.001$, comparing to "Naïve group").*

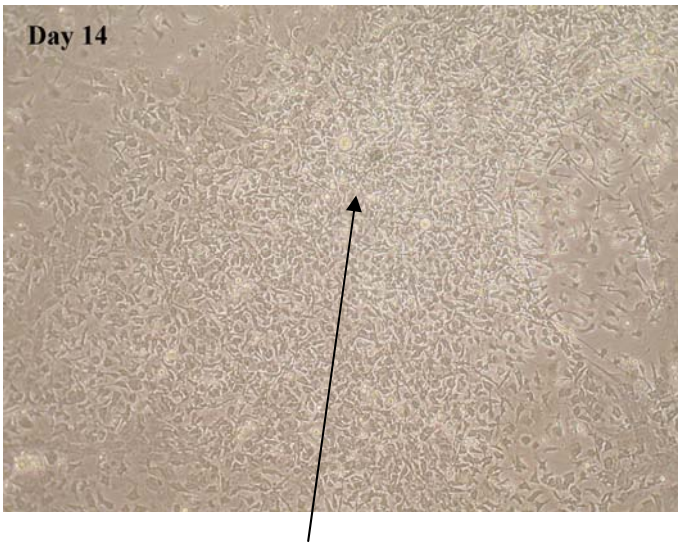
Of note, the data presented here, along with TLR expression data (please see Table 4.4 and 4.5 for details) strongly support the view of increased immunomodulatory capacity of MSCs due to TLR ligands induction. This implies that MSCs present in the microniche of tissues when exposed to microbial byproducts may be facilitated to terminally differentiate to organ specific cells. At the same time due to higher expression of TLRs they may become more immunomodulatory at that niche.

4.5. Mouse Mesenchymal Stem Cells

Although, in our laboratory substantial experience for rat mesenchymal stem cell generation was established, it was very challenging to conduct various assays due to limitations of the availability of rat specific consumables. However, historically considerable experience was accumulated in the lab utilizing mouse system. To further investigate whether MSCs initiate any immunomodulatory responses upon TLR ligand triggering we have decided to optimize mouse MSC generation from BM, and furthermore study these cells in the presence or absence of TLR stimulations. Lastly the interaction of MSCs with murine immune system cells in co-culture conditions. We assessed pro or inflammatory responses mediated by TLRs either from MSCs or their combination with splenocytes and determined cytokine secretion by ELISA.

4.5.1. Characterization of Mouse Mesenchymal Stem Cell

Since, cells types are same; they show nearly the same CD marker expression profiles and other mesenchymal stem cell properties. For instance, the isolation, generation and culturing of mouse MSCs are same as in rat mesenchymal stem cell system. Moreover, morphological structures and other physical properties are same for both mesenchymal stem cells. One difference which could be noticed by naked eye is the size between two cell types.



Fibroblast-like mMSCs form colonies at the end of isolation procedure

Figure 4.24 - Microscopic appearance of mouse mesenchymal stem cells at the end of 14 days of isolation period (Mag = 10X)

As seen in Figure 4.24, mMSCs are much smaller comparing to rMSCs. By the way, they have fibroblastic shape and form colonies as rMSCs did.

4.5.1.1. PCR Results and Gel Images

4.5.1.1.1. Toll-Like Receptor Expression Panels

In order to generate mouse mesenchymal stem cells similar procedure conducted to generate MSCs from rat BM was applied. We further aimed to establish that these cells are indeed express TLRs. To pursue this we checked the presence of these genes by RT-PCR. One important difference between rMSCs and mMSCs is their successful passages. Consistent with the literature, it is very difficult to achieve

several passages therefore, we only passaged them twice. In Figure 4.25, TLR expression profiles of P0 and P1 mMSCs are presented.

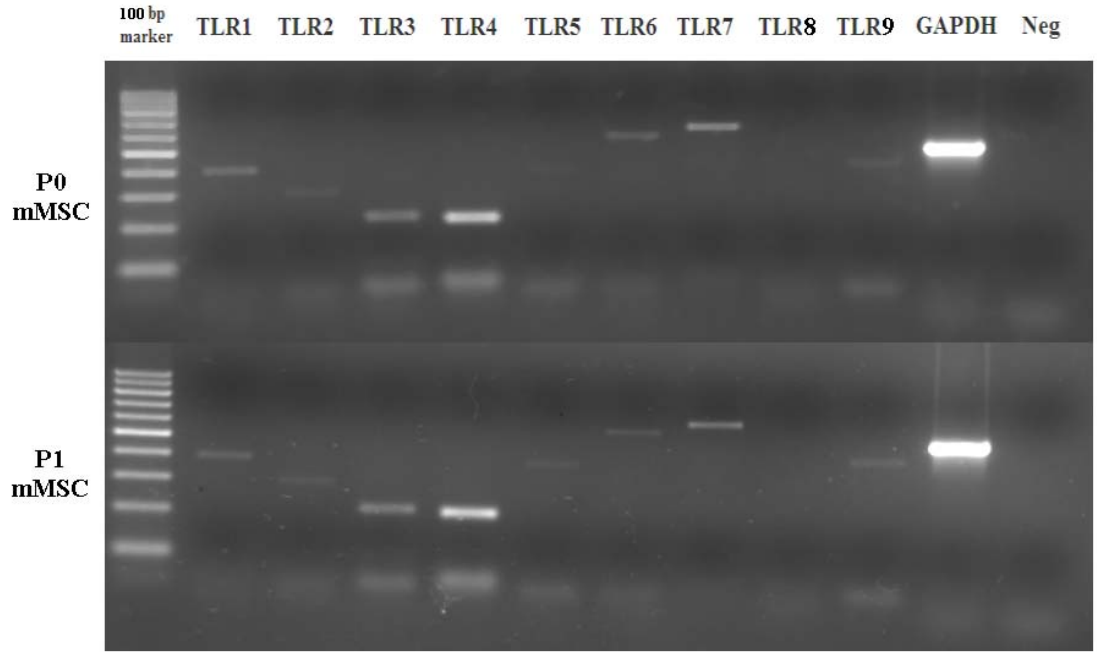


Figure 4.25 - TLR panels of P0 and P1 mMSCs

From this data two points worth mentioning, i) mMSCs do not express TLR5 (similar to rMSCs) but they do express substantial TLR7 transcript (contrary to rMSCs, please compare Figure 4.4 with Figure 4.25). Another discrepancy between rMSC is that here, TLR4 rather than TLR3 expressed at the highest level.

4.6. Ability of mouse Mesenchymal Stem Cells to Secrete Cytokine following Toll-Like Receptor Ligand Stimulation

After establishing that we can successfully generate mMSCs and these cells indeed expresses several TLRs we initiated immunostimulation assays and incubated cells with different TLR ligands (for the concentrations used please refer to Section 3.2.3.3., Table 3.1). Initial experiments involved serial dilution of MSCs between $2.5x10^2$ to $5x10^5$ cells. The aim of this experiment was to establish the optimum number of cells required to induce appreciable amount of cytokine secretion (Figure 4.26). As seen here, maximum level of IL6 production was attained at $2.5x10^5$ MSCs for all TLR ligands tested. Consistent with the PCR results, MSCs positive for TLR3, TLR4, TLR7 and TLR9 induced detectable and sufficient amounts of IL-6 production at the end of 40 hours of incubation in culture.

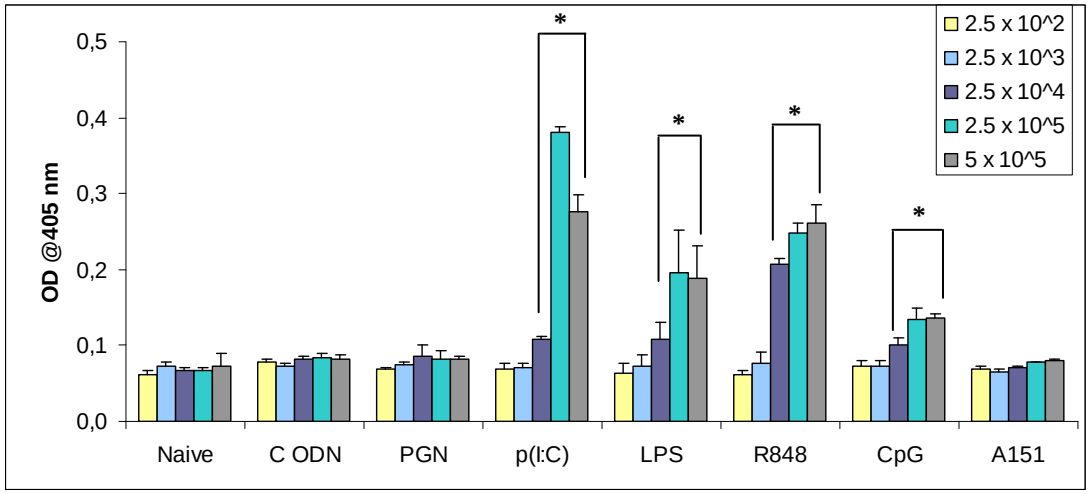


Figure 4.26 - IL-6 release results at different cell numbers for OD readings at 405 nm. (** $p < 0.05$, ** $p < 0.001$, comparing to "Naïve group", n=3).*

Another important point that should be mentioned is that A151 and Control ODN treated groups along with PGN (TLR2L) did not trigger any IL-6 release and behaved similar to the response seen for the untreated group.

4.7. Co-culture Studies of mouse Mesenchymal Stem Cells with mouse Splenocytes

Since the mesenchymal stem cells are regarded as "immunosuppressive", and implicated that they are potentially suitable for the graft vs host disease applications, we planed to check their immunomodulatory performance when co-incubated with spleen cells in the presence or absence of TLR ligands at different cell ratios. In these experiments as a baseline positive controls MSC alone and splenocyte alone groups at two different cell concentrations were included. From the supernatants IL-6 and IFNγ production was studied with sandwich ELISA assay.

As depicted in the Figure 4.27, contrary to expectations, from the IL-6 secretion levels there seems to be a synergy between spleen cells and MSCs when co-incubated together at 1:1 ratio (please compare blue, yellow and grey bars of each treatment). When this ratio is not met, spleen cells are suppressing the MSC mediated IL-6 secretion.

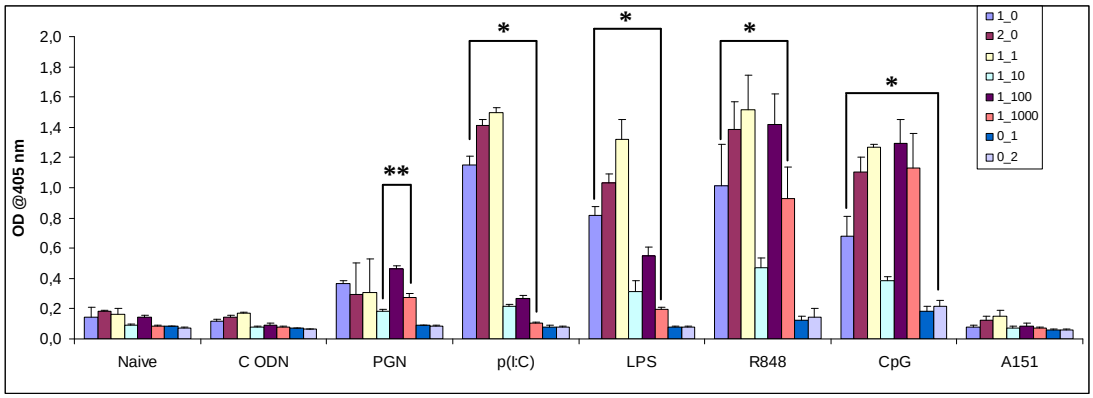


Figure 4.27 - IL-6 production profiles of MSC-Spleen cell co-culture at different ratio (* *p < 0.01, ** p < 0.001, comparing to "Naïve group", n=3*).

There are few publications describing the immunomodulatory potential of MSCs when generated in culture. Although this topic was not thoroughly investigated by others, reports suggesting that MSCs might act as immunosuppressive cells are abundant to date. In our hand mouse BM generated MSC induced IL-6 in response to TLR ligand stimulation. Furthermore, these cells were potent IFNg producing cells when triggered by several TLR ligands. Their cytokine production ability was much superior than spleen cell culture (even when 5 fold more spleen cells were used).

This observation has many important implications. One must be very cautious to consider using these cells in cases where immune suppressive effects by MSCs are desired.

Another interesting point is that when MSCs and spleen cells were co-cultured, there was a strong synergistic effect in cytokine production. When the co-culture was treated with TLR ligand such as i) p(I:C), ii) R848, iii) CpG and iv) LPS the synergy in cytokine production was very significant. From the culture supernatants, parallel to IL-6 production, IFNg levels were also checked. Figure 4.28, demonstrates the outcome of this set of data.

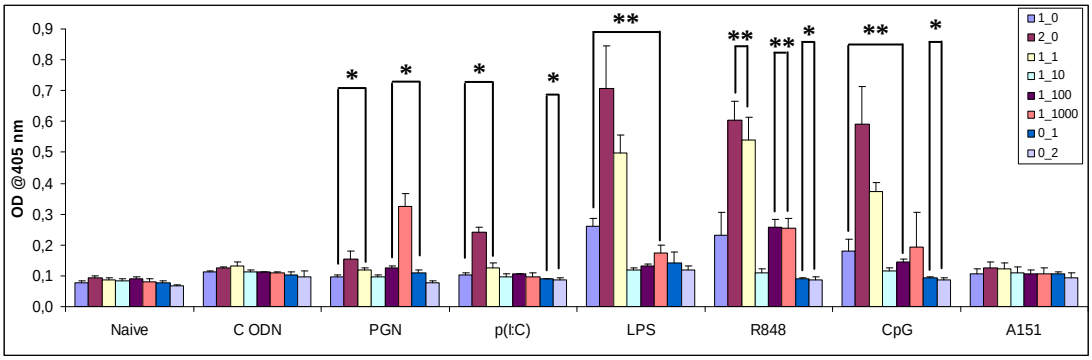


Figure 4.28 - IFNg production profiles of MSC-Spleen cell co-culture at different ratio (** $p < 0.05$, ** $p < 0.01$, comparing to "Naïve group", n=3*).

In this figure, results revealed that MSC only groups induced significantly high TLR mediated IFNg production (@P0). In the co-culture experiments, spleen cells reduced IFNg secretion levels produced by MSCs, and there is no synergy between these cells. On the contrary, there is a suppressive activity induced by spleen cells. Under the light of these findings, it is very complicated to dissect the interplay between MSCs and spleen cells. More detailed investigations are required in order to better describe the complex nature of this relation between MSC and other immune cells. One option is to co-culture purified B-, T-, Macrophage, dendritic cells with MSCs, and analyze the cytokine secretion characteristics of these mixed cells. May be then, one could describe which cells are synergizing with MSCs to increase cytokine production, or which cells are imposing suppressive effects to reduce the overall cytokine production. These and other related topics in the context of immunomodulation must be planned for the future studies.

5. DISCUSSION

Mesenchymal stem cells (MSC) and the events initiated following stimulation with different TLR ligands was the main theme of this thesis. Throughout this project, the contribution of TLR mediated signaling on i) lineage specific MSC differentiation rate and efficiency, ii) migration and proliferative potential, and iii) changes in immunomodulatory activities regulated by MSCs were investigated. In our opinion, it was important to study these parameters, because in order to broaden the breath of the therapeutic applications of MSCs as well as to better understand the underlying biological phenomena regulated by TLR activation would help to impact the clinical utility of these versatile cell populations.

In our study, characterization of mesenchymal stem cells was primarily pursued by checking CD90 expression as a positive marker and CD45 as a negative marker for rat MSC characterization. Along with these markers, CD106 was checked as a negative marker in rat system (Barzilay et al., 2009). At the end of 14 day isolation process, the immunophenotypic characterization revealed that the isolation process yielded impure population of rMSC. In order to reach homogeneous population, culturing of these cells over passages was performed. As a result the expression of CD90 rose from 30% to nearly 90% (Table 4.2) as expected CD45 and CD106 expressions were negligible (ca. ~3-4%). This set of findings were in accordance with the reports in the literature (Harting et al., 2008). While FACS analysis of the cardinal surface markers at protein level was conclusive, due to the unavailability of several other critical markers we decided to analyze their gene expression patterns via PCR.

In addition to surface marker analysis of mesenchymal stem cells by flow cytometry, the expressions of other negative and positive markers were checked at transcript level. Here, CD11b as a macrophage marker, and CD34 as a negative marker as well was included in the investigation. Moreover, other critical CD markers such as CD29, CD71, CD73, CD90, CD105 and CD166 were checked by PCR. According to these results, at P0 mesenchymal stem cells were still a heterogeneous population contaminated with other adherent/fibroblastoid cells. Separately, the cell population expressed CD73, CD105 and CD71. As expected, increasing the passage number

increased mesenchymal stem cells homogeneity. In P3, and in further passages, all negative CD marker transcripts were reduced to undetectable levels, where positive markers demonstrated an increase in gel band intensities. Although the transcript level analysis of CD markers reflects that we have a homogeneous population of related cell type, CD marker expressions should be further analyzed in the protein levels.

Next, we investigated TLR expression profiles of BM generated MSCs. In our hand, rat MSCs expressed high levels of TLR1, 2, 3, 4, 6, and moderate levels of 7 and 9. Surprisingly, irrespective of passages, no TLR5 or TLR10 was detected. Since it is previously established that TLR8 was not present in rats, we did not check for the expression of this receptor. However, the expression levels of these receptors have decreased profoundly following culturing with one exception-TLR3. The reduction in the levels of several TLRs as opposed to increased passage number suggested that there was a significant reduction of the contaminating cells form P3 and onwards. It was previously reported that macrophages (Delneste et al., 2007) and hematopoietic stem cells (Kondo et al., 2003) were also expressing some TLRs. Thus, decrease in the TLR expression levels would not be surprising with the elimination of "contaminating" macrophages and hematopoietic stem cells. Yet, albeit at low levels TLR1, 2, 3, 4 and 6 expressions were retained until P12. Of note, among these TLRs TLR3 was expressed at very high levels at all passages.

In this study, we have found that the TLR expression profile of mouse mesenchymal stem cells is different from previously reported (Pevsner-Fischer et al., 2007). We have consistently demonstrated that expression of TLR1, 2, 3, 4, 5, 6, 7, and 9 the present on mMSCs. One important note to point out is that, there is a distinct difference between rMSCs and mMSCs in terms of the pattern of TLR profiles.

One of the main focus points of this thesis was to explore TLR contribution on the lineage-specific differentiation of MSCs. We established that different TLR ligands accelerated as well as increased adipogenic differentiation efficiency of rat mesenchymal stem cells at different several passages. On the contrary to our results, previously it was reported that TLR ligands especially PamCys3 (a TLR2 ligand), had a negative effect on the adipogenic differentiation of mesenchymal stem cells

(Pevsner-Fischer et al., 2007). However, in this study, a different ligand, peptidoglycan, for TLR2, was used in their work, instead of rat they used mouse MSCs.. The facilitating effects of TLR ligands on differentiation rates prompted us to terminate the incubation time significantly shorter than the normal differentiation process. This is a very significant finding, since conventional adipogenic differentiation procedure requires at least three weeks. This point is the most profound contribution of TLR ligands on MSC differentiation as it is the subject of this thesis. The accumulated data suggested that TLRL for TLR2, TLR3 and TLR7 is critical elements facilitating fast MSC to lineage–specific commitment. However, it is not possible to state that for each passage the contribution of TLR ligands were at the same level. For instance, in P3 CpG as TLR9 ligand has the highest contribution in adipogenic differentiation among the other TLR ligands while same ligand has a worse contribution trend in P0. A very highly possible reason for such results would be the contaminating cell populations present in P0 MSCs. These fluctuations would also be dependent on the TLR expression profiles of MSCs since there is a correlation between TLR expression levels and responsiveness of MSCs to these ligands (Liang et al., 2007). Another observation about the differentiation rates of treatment groups was that passaging resulted a decrease in the adipogenic differentiation capacity of MSCs. For instance, in P0 nearly hundred percent of cells have differentiated into adipocyte at the end of second week; whereas in P9, group with the highest adipocyte generation percentage exhibited only 25% conversion at the end of third week (compare Figs 4.8, vs 4.14). This observation was correlated with the literature. In recent studies, it was reported that passaging (no TLR involvement in these findings are associated) had a negative effect on the adipogenic differentiation capacity of MSCs (Wall et al., 2007; Yu et al., 2010). Our results advanced the existing literature information about the adipogenic differentiation of MSCs for several reasons, i) higher rates could be attained at a much lesser time and ii) conversion efficiencies are improved by the ligand supplementation to the differentiation media.

It is inevitable that the most drastic effect of TLR ligands on MSC biology is the differentiation facilitating effects. This is a crucial question that why mesenchymal stem cells are differentiating faster into adipocyte when stimulated with TLR ligands? Here I would like to concentrate on the adipose tissue itself. Recently,

papers published in immunology journals are referring adipose tissue as an helper organ and part of the innate immune system (Schaffler et al., 2007). What's more, in the inflammation status, expression of TLRs were shown to be increased in MSCs (Raicevic et al., 2010). Combining these two data, our results may build a bridge between these two interesting statements. Whenever mesenchymal stem cells are exposed to TLR ligands, here mimicking inflammation environment, these cells respond in the adipogenic differentiation direction, here generation of adipose tissue as an immunological organ. Such an observation and linkage is not present in current literature. Thus, our results stated here are the first to link adipogenic differentiation capacity of mesenchymal stem cells in an inflammation-like micro environment created with the use of TLR ligands.

We have also analyzed the TLR expression profiles of different TLR ligand stimulated adipogenic and osteogenic differentiation groups. We have observed increases in the expression levels of specific TLRs in adipogenic and osteogenic differentiation groups (see Figure 4.24 and Figure 4.25). These results are again in concordance with the current literature (Liang et al., 2007; Raicevic et al., 2010).

Apart from differentiation studies, we have also checked the effects of TLR ligands on the migration capacity of mesenchymal stem cells. Migration capacities of mesenchymal stem cells in the presence of TLR ligands have increased as it was stated previously (Tomchuck et al., 2008). However, the migration capacities vary according to the TLR ligands stimulated with. The highest migrated group is p(I:C) which is TLR3 ligand and the least one is R848 which is TLR7 ligand. It is a hard question that what causes such a difference. Maybe the expression levels of related TLRs could be an answer to this question. Response levels to TLR ligands may correlate with the expression levels of related TLRs and as a result of this, migration capacities would exhibit such differences. All in all, migration capacities of mesenchymal stem cells have increased when stimulated with any TLR ligands.

In mouse mesenchymal stem cell studies, immunosuppressive capacities of mMSCs were investigated. The experiment design was including co-culturing MSCs with splenocytes in the presence and/or absence of TLR ligands. Contrary to expected, our results have demonstrated a pro-inflammatory MSC type regardless of the splenocyte

presence. Actually these results are in coherent with the similar studies. TLR stimulation has gained MSCs an immunostimulatory phenotype in previous studies in terms of IL-6 secretion (Pevsner-Fischer et al., 2007; Romieu-Mourez et al., 2009). The most immunostimulatory phenotype of MSCs was observed in the R848, CpG and LPS treated groups; TLR7L, TLR9L and TLR4L, respectively in terms of both IL-6 and IFN-γ secretion. It is interesting that PGN treated group did not respond at same levels as other TLR ligands did for IFN-γ secretion. This may be due to the triggered signaling pathways of TLR2 ligand. Since PGN, as TLR2L, is a bacterial component, it is not surprising that this ligand did not stimulate the anti-viral cytokine, IFN-γ, secretion. Besides these results, A151 as an immunosuppressive ODN has successfully inhibited the secretion of both IL-6 and IFN-γ cytokines in all groups. This inhibition is also a significant result in terms of immunosuppresion.

Since mesenchymal stem cells were said to be immunosuppressive, the presence of potential subpopulations is also a possibility and stands as an important issue in the way of MSC biology. As in our case, a potential pro-inflammatory phenotype of MSC would give such unexpected results. Surprising results of co-culturing experiments would be due to a MSC subpopulation with pro-inflammatory phenotype.

In conclusion, this thesis study clearly demonstrates that TLR ligands have significant effects on mesenchymal stem cell biology in terms of adipogenic differentiation, migration and stimulation of the immune system. Moreover, a potential subpopulation of MSC with immunostimulatory character is present and it is essential to isolate such subpopulations of MSCs for proper and reliable administration of these cells.

6. FUTURE PERSPECTIVES

This study has stated the TLR contribution to adipogenesis of rat mesenchymal stem cells and enhancement in the migration capacity of these cells. Moreover, the immunomodulatory effects of these cells in the presence and/or absence of TLR ligands were revealed out.

Unfortunately, the first thing to handle with MSC studies should be the standardization of the characterization issue of these cells. For our laboratory, more surface markers, both positive and negative ones, should be checked with proper controls. On the other side, differentiation protocols other than adipogenesis, like osteogenesis and chondrogenesis, should be established. With such differentiation protocols, it would be more reliable to introduce these cells as mesenchymal stem cells.

Even though the results mentioned here are striking, a similar study to this one should be carried out in other systems like mice. If the same trend should be seen in another system, without doubt it is possible to say that TLRs facilitate the differentiation process of mesenchymal stem cells. As previously stated in MSC niches section, there are several tissues for MSC residing. Other studies may be carried out with MSCs isolated from these tissues. It is highly possible that some kind of differences would be observed with these tissues. In addition to such studies, other differentiation processes should be investigated under TLR stimulations.

About the most dramatic findings of this study, the fundamental molecular mechanisms of adipogenesis should be deeply studied in order to delineate at which state, TLRs contribute to this process. For this reason, the underlying mechanisms of adipogenesis should be studied along with immune system since adipose tissue is "called" as an immune system organ (Schaffler et al., 2006). May be, it would be significant to study the mesenchymal stem cells of obese *vs* healthy mice in terms of TLR expression and adipogenic differentiation capacity.

The migration of mesenchymal stem cells is also a significant issue to study. The migration pace of these stem cells was increased when they were stimulated with

TLR ligands. Just like a previous study, migration capacity should be investigated in an *in vivo* system (Kocak et al, 2010, submitted to *J Hepatol*). However, this time TLR ligand pre-treated MSCs should be administered to the animal with a wound model. After proper time points, animals should be sacrificed and the wounded organ should be deeply studied for MSC localization differences in cell number. Such a study would be much more valuable in terms of deciphering TLR ligand effects on migration capacity of MSCs.

Another concept which should be further studied is the subpopulation characterization of these stem cells. If there are really two types of MSC populations according to their immunomodulatory phenotype, dissecting these two groups would have profound effect in terms of suitable MSC choose in therapeutic applications.

7. REFERENCES

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8. APPENDICES

8.1. Appendix A

Standard Solutions, Buffers, and Culture Media.

Blocking Buffer (ELISA)

- 500 ml 1x PBS
- 25 grams BSA (5%)
- 250 µl Tween20 (0,025%)

Store at -20°C.

T-cell Buffer [ELISA]

- 500 ml 1x PBS
- 25 ml FBS (5%)
- 250 µl Tween20 (0,025%)

Store at -20°C.

Wash Buffer [ELISA]

- 500 ml 10x PBS
- 2,5 ml Tween20
- 4,5 lt ddH2O

Prepare prior to usage and use immediately.

Loading Dye

- 0,009 grams Bromofenol blue
- 0,009 grams Xylen cyanol

- 2,8 ml ddH_2O
- 1,2 ml 0,5M EDTA
- 11 ml glycerol

PBS (Phosphate Buffered Saline) [10X]

- 80 grams NaCl
- 2 grams KCl
- 8,01 grams $Na_2HPO_4 . 2H_2O$
- 2 grams KH_2PO_4

Complete into 1 lt with ddH_2O. Adjust to pH=6,8 with HCl. For 1X PBS, pH should be ≈ 7,2-7,4. After adjustment of pH, autoclave.

TAE (Tris-Acetate-EDTA) [50X]

- 242 grams Tris (C4H11NO3)
- 37,2 grams Tritiplex 3 (EDTA= $C_{10}H_{14}N_2Na_2O_2.2H_2O$)
- 57,1 ml Glacial acetic acid

Complete to 1 lt final volume with ddH_2O. Autoclave and dilute to 1X prior to use.

PBS-BSA-Na azide

- 500 ml 1x PBS
- 5g BSA (1%)
- 125mg Na-Azide (0,25%)

RPMI-1640 (Hyclone)

- 5 % : 25 ml FBS (Oligo FBS = inactivated at 65°C, Regular FBS = inactivated at 55°C)

- 5 ml Penicillin/Streptomycin (50 μg/ml final concentration from 10 mg/ml stock)
- 5 ml HEPES (Biological Industries), (10 mM final concentration from 1M stock)
- 5 ml Na Pyruvate, (0,11 mg/ml final concentration from 100mM, 11 mg/ml stock)
- 5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x concentrate stock)
- 5 ml L-Glutamine, (2 mM final concentration from 200 mM, 29.2 mg/ml stock)

Prepare in 500 ml media.

MesenCult® (STEMCELL Technologies, Canada)

- 400 ml MesenCult® Basal Medium for Mouse Mesenchymal Stem Cells
- 100 ml MesenCult® Mesenchymal Stem Cell Stimulatory Supplements
- 5 ml Penicillin/Streptomycin (50 μg/ml final concentration from 10 mg/ml stock).

8.2. Appendix B-1

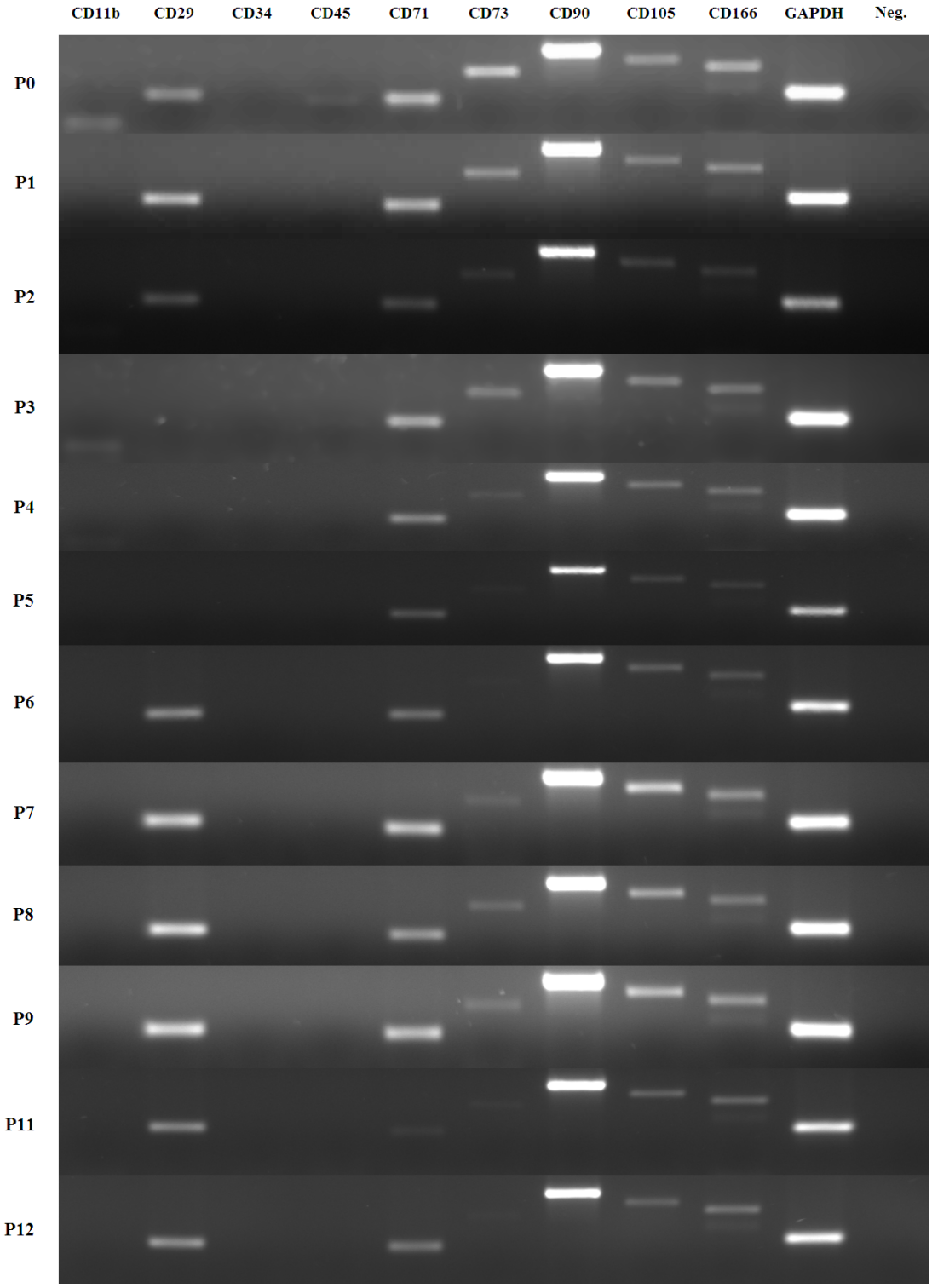


Figure 8.1 - CD marker expression panels over passages for characterization

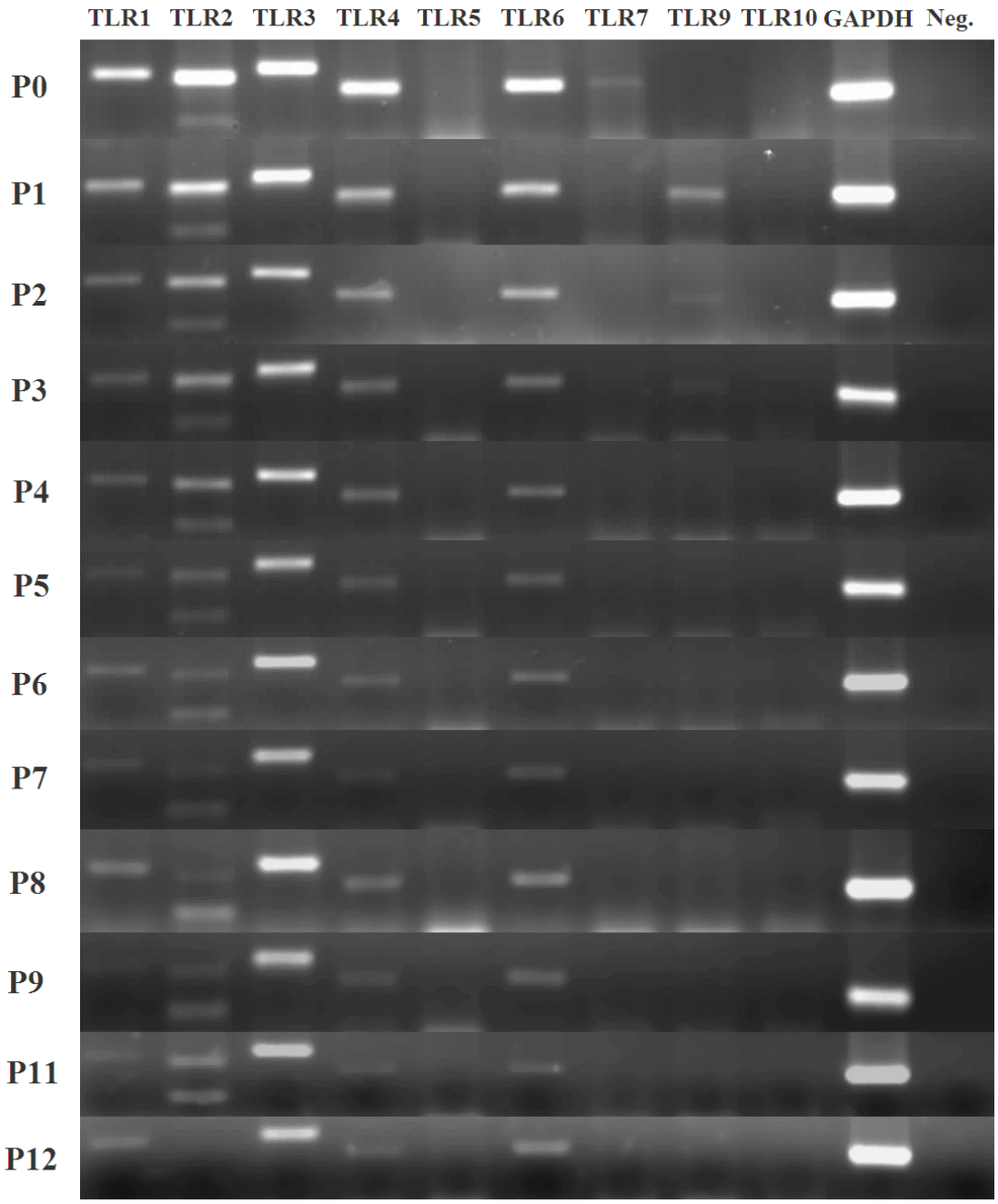
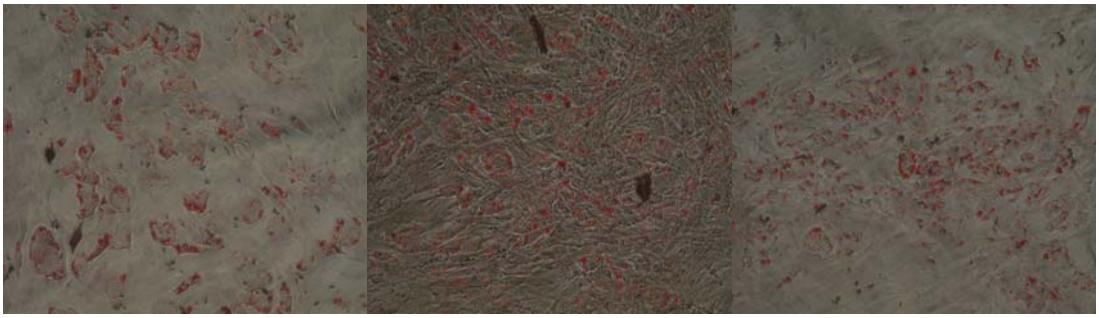
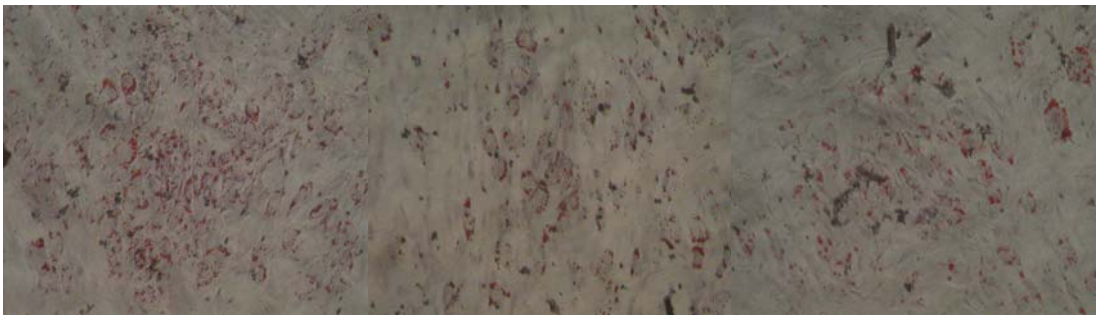


Figure 8.2 - TLR expression panels over rMSC passages

8.3. Appendix B-2

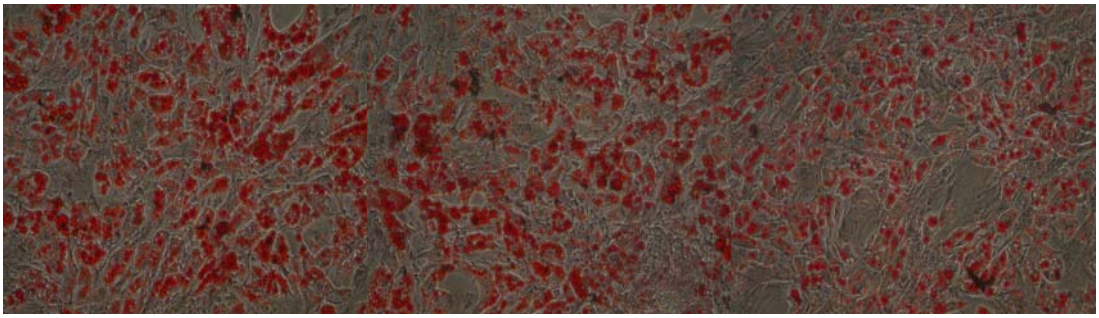


TLR9L = CpG > Adipogenic medium only > TLR2L = PGN

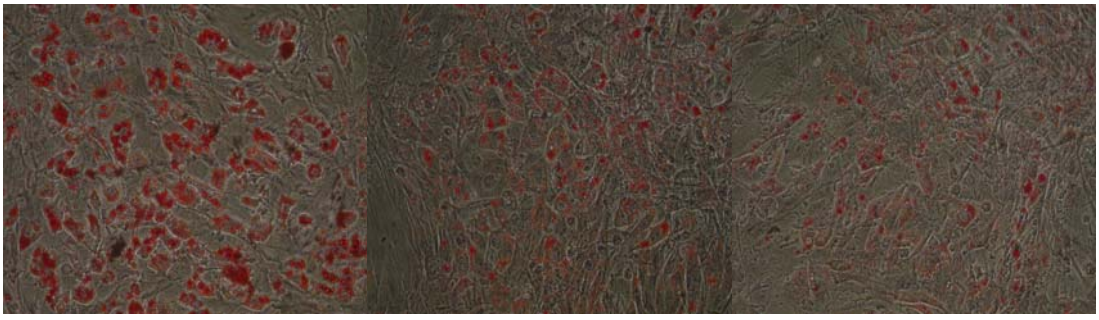


Control ODN > TLR3L = p(I:C) > TLR7L = R848

Figure 8.3 - Photomicrographs of rMSC (@P3) differentiated to adipocytes at D5 (Mag = 20X)

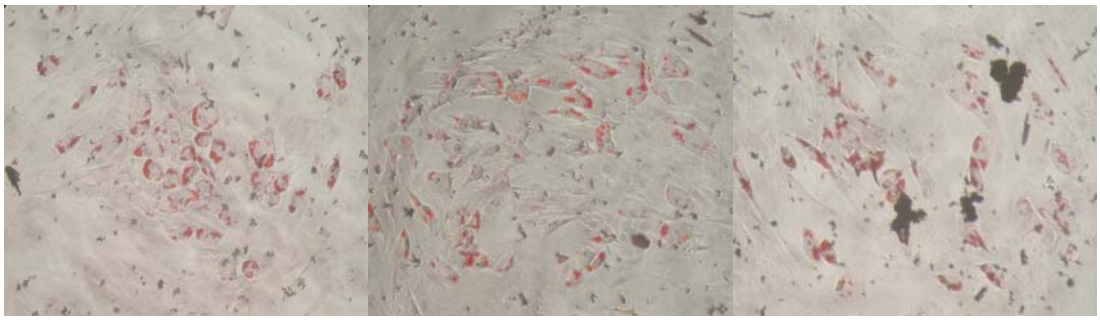


TLR9L = CpG TLR7L = R848 TLR2L = PGN

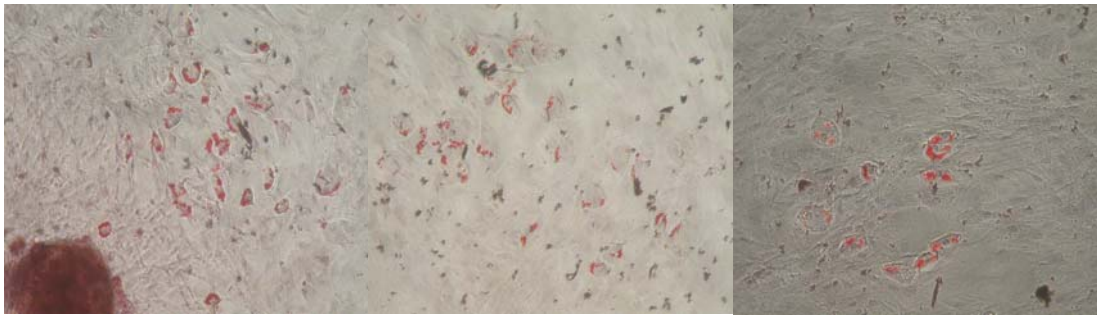


TLR3L = p(I:C) > Control ODN > Adipogenic medium only

Figure 8.4 - Photomicrographs of rMSC (@P3) differentiated to adipocytes at D11 (Mag = 20X)

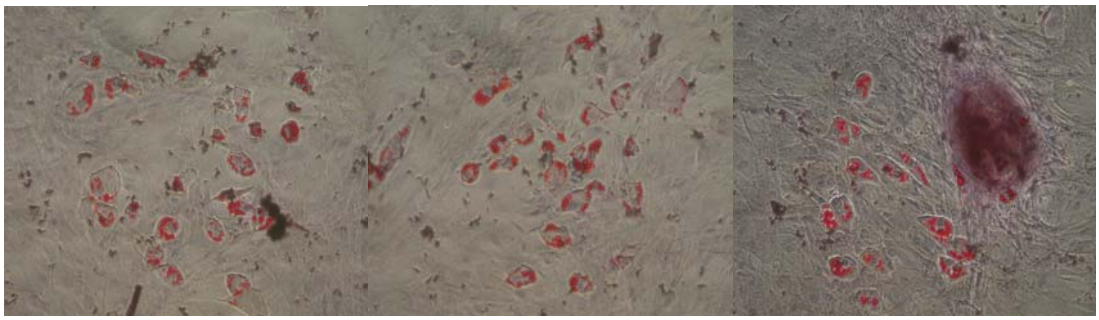


Control ODN > TLR7L = R848 > TLR2L = PGN

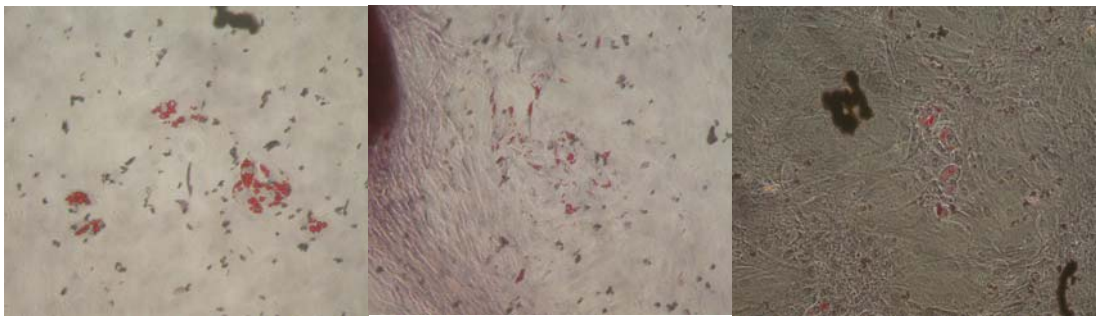


TLR3L = p(I:C) > TLR9L = CpG > Adipogenic medium only

Figure 8.5 – Photomicrographs of rMSC (@P6) differentiated to adipocytes at D5 (Mag = 20X)

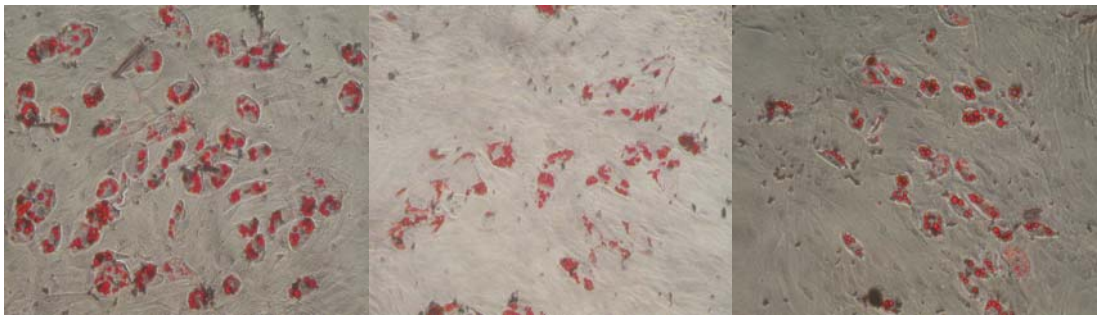


TLR9L = CpG > TLR3L = p(I:C) > Adipogenic medium only

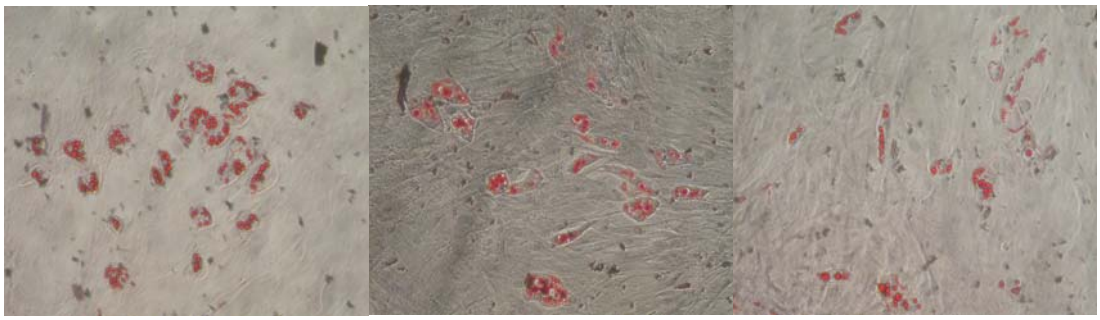


Control ODN > TLR2L = PGN > TLR7L = R848

Figure 8.6 – Photomicrographs of rMSC (@P6) differentiated to adipocytes at D8 (Mag = 20X)

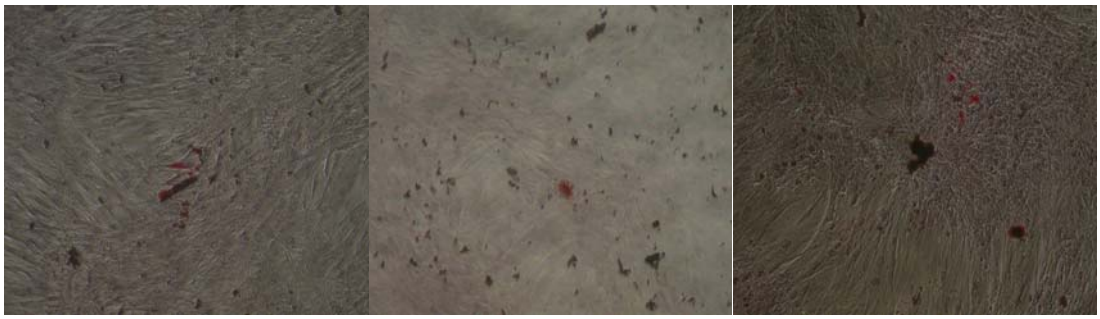


TLR3L = p(I:C) > TLR2L = PGN => TLR7L = R848

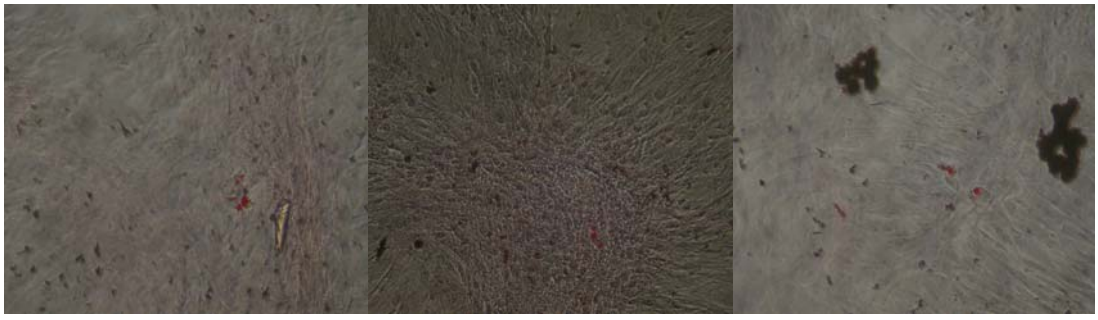


TLR9L = CpG > Control ODN > Adipogenic medium only

Figure 8.7 – Photomicrographs of rMSC (@P6) differentiated to adipocytes at D10 (Mag = 20X)

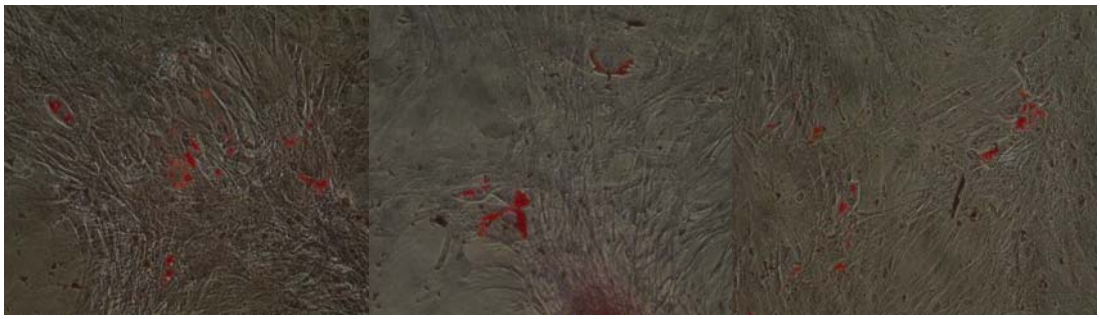


Control ODN TRL9L = CpG TLR2L = PGN

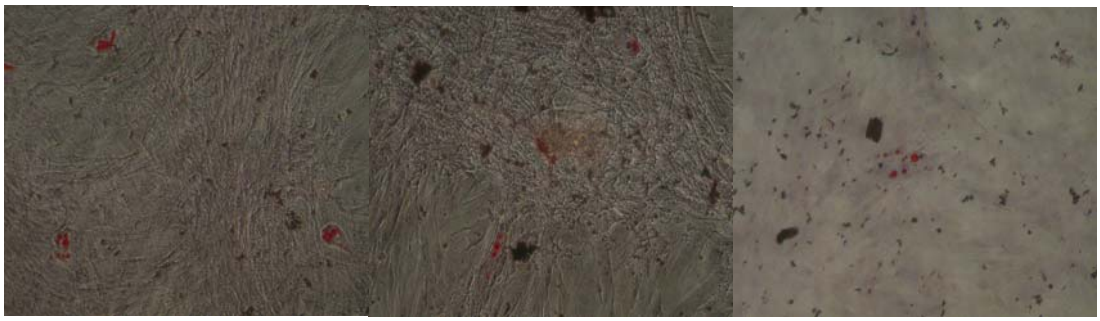


TLR3L = p(I:C) TLR7L = R848 Adipogenic medium only

Figure 8.8 – Photomicrographs of rMSC (@P9) differentiated to adipocytes at the end 1st week (Mag = 20X)



TLR2L = PGN > TLR9L = CpG > TLR7L = R848



TLR3L = p(I:C) > Control ODN > Adipogenic medium only

Figure 8.9 - Photomicrographs of rMSC (@P9) differentiated to adipocytes at the end of 2nd week (Mag = 20X)

8.4. Appendix C

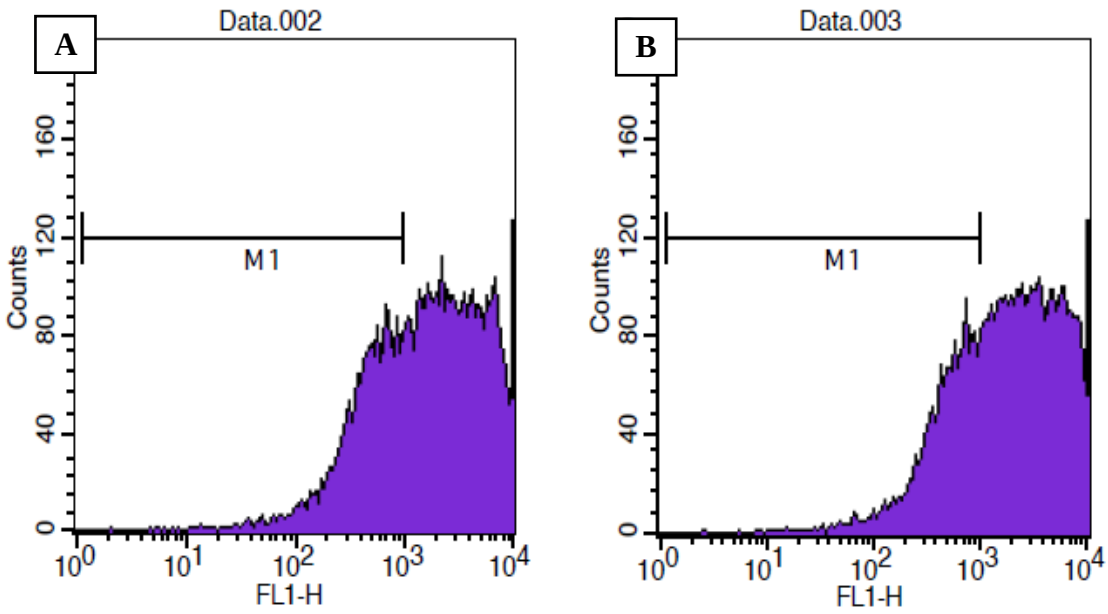


Figure 8.10 - Representative histograms and analysis of proliferation rate by CFSE assay. A) Naïve and B) p(I:C) treated MSCs

Table 8.1 - MSC proliferation rates after TLR ligand treatment expressing proliferation assessed by CFSE assay

Treatment groups	MFI analysis results (%)
MesenCult only	39.86
TLR2L = PGN	35.14
TLR3L = p(I:C)	31.91
TLR7L = R848	40.90
TLR9 = CpG	33.62
Control ODN	37.02