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Vitamin D: A Negative Regulator of Triggering Receptor Expressed on Myeloid Cell-1 (TREM-1) in Hepatocellular Carcinoma Cells

By

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A THESIS

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Abstract

Hepatocellular carcinoma (HCC) accounting for more than 90% of cases of primary liver cancer, is the third most common cause of cancer-related death worldwide. Exposure to toxic substance and exposure to infectious agents like HBV (hepatitis B virus) and HCV (hepatitis C virus) are the main risk factors for development of HCC. Chronic inflammation precedes the development of cirrhosis and HCC. TREM-1 (triggering receptor expressed on myeloid cell-1) is an inflammatory marker and amplifier of inflammation. HMGB-1 secreted from necrotizing cells activates TREM-1 at cell surface. It further signals through PI3K and ERK1/2 to activate NF-κB transcription factor resulting in the production of pro-inflammatory cytokines namely IL-6 and TNF-α. These pro-inflammatory cytokines further enhance the inflammation to change the microenvironment and predisposing the liver to carcinogenesis. Low levels of vitamin D have been associated with liver diseases such as alcoholic liver disease, non-alcoholic steatohepatitis, and HBV and HCV infection. Low level of vitamin D is a poor prognostic factor and supplementation of vitamin D with antiviral therapy in HCV has proven beneficial. Vitamin D is an anti-inflammatory agent and acts through vitamin D receptor in the cells to suppress inflammation in various diseases. Vitamin D also decreases proliferation in various tumors and hence it can be used to decrease chronic inflammation in liver diseases. I found significantly increased expression of TREM-1, HMGB-1 and DAP-12 in HepG-2 cells (hepatocellular carcinoma cell line) compared to THLE-2 (epithelial cells transformed with Sv40 large T antigen) cells. Pro-inflammatory cytokines, IL-6 and TNF-α further significantly increased the TREM-1 expression in HepG-2 and THLE-2 cells. Treatment of HepG-2 cells with calcitriol attenuated the TREM-1 expression as well as the stimulatory effect of pro-inflammatory cytokines IL-6 and TNF-α on TREM-1 expression. Further, calcitriol also decreased the proliferation, invasion and migration of HepG-2 cells, suggesting that vitamin D supplementation can be used to
attenuate the ongoing chronic inflammation in hepatitis and cirrhosis, and the progression to hepatocellular carcinoma can be delayed. This study provides molecular and biochemical evidence that suppression of TREM-1 by vitamin D supplementation together with conventional treatment of hepatitis, fibrosis and chronic hepatitis may be a novel strategy and promising target for delaying the progression of liver disease and hepatocellular carcinoma.
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<tr>
<td>ALD</td>
<td>Alcoholic liver disease</td>
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<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
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<tr>
<td>DAP-12</td>
<td>DNAX activation protein of 12 KDa</td>
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<tr>
<td>HepG-2</td>
<td>Hepatocellular carcinoma cell line</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
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<td>HMGB-1</td>
<td>High mobility group box-1</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
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<td>NAFLD</td>
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<td>NF-kB</td>
<td>Nuclear factor-kappa B</td>
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<tr>
<td>PCOS</td>
<td>Polycystic ovarian syndrome</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RNA</td>
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<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>THLE-2</td>
<td>Homo sapiens-Epithelial Cells Transformed with Sv40 Large T Antigen</td>
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<tr>
<td>TLRs</td>
<td>Toll like receptors</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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<tr>
<td>TREM-1</td>
<td>Triggering receptor expressed on myeloid cell-1</td>
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<tr>
<td>TREM-2</td>
<td>Triggering receptor expressed on myeloid cell-2</td>
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<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
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Introduction

Liver cancer is the sixth most common cancer diagnosed and third most common cause of cancer-related death worldwide [1]. Hepatocellular carcinoma (HCC) accounts for more than 85% of the primary liver cancer and is a major public health problem in humans [2]. The incidence of HCC is increasing around the world including the United States[3]. Correlation of chronic inflammation and development of cancers have been suspected for a long time and hepatocellular carcinoma is one of the most studied inflammation induced cancers. Chronic inflammation in the liver tissue involves in changing the microenvironment of the liver and plays a major role in pathogenesis of development of HCC. The main triggering agents are exposure to infectious agents and toxic compounds (Fig.1). The most common risk factors for HCC are hepatitis B virus (HBV) and hepatitis C virus (HCV) infection[1]; nearly accounting for 75% of the HCC.(Fig.2) Chronic inflammation in liver due to HBV and HCV infection, alcoholic liver injury, primary sclerosing cholangitis and non-alcoholic steatohepatitis (NASH) disrupt the normal architecture and regenerative mechanism and result in fibrosis, cirrhosis and potential development of HCC [4-6] (Fig.1). In humans, HCC is usually preceded by either chronic hepatitis or cirrhosis. The death of hepatocytes during chronic inflammation, chronic hepatitis or cirrhosis is followed by secretion of pro-inflammatory cytokines by resident (Kupffer) cells and recruited (neutrophils and monocytes) inflammatory cells. High mobility group box-1 (HMGB-1) released from the necrotic cells mediate the activation of surface receptors like triggering receptor expressed on myeloid cell-1 (TREM-1) and toll like receptors (TLRs) further activating signalling pathways and secretion of pro-inflammatory cytokines [7].(Fig.3) These pro-inflammatory cytokines in general induce inflammatory response which is initially beneficial to liver and compensate for the loss of hepatocytes. But the dysregulation between innate and adaptive immunity due to chronic inflammation results in loss of hepatocyte and regeneration. This leads to over secretion of
pro-inflammatory cytokines involving signalling pathways and transcription factors mediating excessive tissue remodelling, loss of tissue architecture, apoptosis, necrosis, increasing the risk of carcinogenesis and development of HCC [8-10]. (Fig. 4) It is proposed that chronic inflammation results in induction of a high rate of cell turnover and a highly oxidative microenvironment leading to increase DNA damage, mutation and carcinogenesis [11].

**Figure 1: Alcoholic and non-alcoholic liver disease.** Increased deposition of fat occurs in liver due to alcohol consumption and causes like altered fat metabolism, metabolic disorders, visceral obesity etc... Inflammation in deposited fat results in alcoholic hepatitis and non-alcoholic steatohepatitis (NASH). Chronic inflammation in liver tissue leads to cirrhosis and likely development of hepatocellular carcinoma.
Figure 2: Pathogenesis of HBV and HCV infection and hepatocellular carcinoma. HBV infection results in integration of viral DNA with host DNA and leads to increased production of HBx protein. This results in up-regulation of JNK, p38/MAPK, PI3K, ERK1/2, Akt and NF-kB signalling pathways. Upregulation of NF-kB causes chronicity of infection and increased production of pro-inflammatory cytokines. Increase in HBx protein also results in increased reactive oxygen species, genetic mutation and instability. Association of viral and host protein after HCV infection results in activation of signalling pathways and increased production of pro-inflammatory cytokines and modulation of host immunity. Increased production of pro-inflammatory cytokines, chronic inflammation and modulation of host immunity may result in development of hepatocellular carcinoma.
Figure 3: Pathogenesis of hepatocellular carcinoma. Increased infection of inflammation results in cirrhosis and necrosis of hepatocyte. Release of HMGB-1 from necrotic cell causes activation of TREM-1 through TLRs and activation of kupffer cells. Bacterial endotoxins like LPS can also activate TREM-1 through TLRs. Association of activated TREM-1 with DAP-12 leads to signalling pathway (JNK, p38/MAPK, PI3K, ERK1/2, Akt) and transcription factor NF-kB activation and transcription of TREM-1 signalling to the nucleus. Activation of transcription factor NF-kB in the nucleus results in increased production of pro-inflammatory cytokines namely IL-6 and TNF-α. Activation of kupffer cells also leads to secretion of pro-inflammatory cytokines. These pro-inflammatory cytokines may increase tumorigenesis and the development of hepatocellular carcinoma.
**Figure 4: Stages of liver disease and treatment.** HBV, HCV, alcohol, aflatoxin exposure, autoimmune and metabolic diseases, visceral adiposity result in fat deposition and increased inflammation in liver tissue and hepatitis. Chronic hepatitis causes disruption in architecture, matrix deposition, angiogenesis, fibrosis and loss of liver function at the stage of cirrhosis. Cirrhosis is the irreversible end stage liver disease and may lead to development of liver failure and hepatocellular carcinoma. Hepatitis and fibrosis are reversible liver disease and can be treated with antiviral therapy and removal of the cause. Chronic hepatitis can be treated with antiviral therapy but for cirrhosis and some hepatocellular carcinoma the only curative treatment available is liver transplant.
TREM-1 is a trans-membrane glycoprotein belonging to the immunoglobulin superfamily and family related to the natural killer cell receptor. TREM-1 is a cell surface receptor expressed on monocytes, neutrophils, macrophages and fibroblasts [12-13]. The surface expression and signalling by TREM-1 requires adaptor DNAX activation protein of 12LDa (DAP-12), attached with the intracellular domain of TREM-1 [14]. The presence of TREM-1 on endothelial cells isolated from mice liver was reported by Chen et al [15]. Liao et al reported that TREM-1 is also expressed on the hepatic stellate cells in HCC and promotes migration of HCC cells [16]. These studies suggest that TREM-1 is not only expressed on monocytes, neutrophils and macrophages but also on some liver cells. Cell proliferation and deregulation of cell death is promoted by chronic inflammation and being a mediator of inflammation, TREM-1 may play a crucial role in pathogenesis of hepatocellular carcinoma. Further TREM-1 also induces generation of various pro-inflammatory cytokines and promotes tumor progression and invasion by generating a pro-tumor microenvironment. Activation of TREM-1 results in increased secretion of TNF-α, GM-CSF, MCP-1, IL-1β, IL-6, IL-8 and IL-12 from monocytes, neutrophils and macrophages thus acts as amplifier of inflammation. Secretion of the pro-inflammatory cytokines from these cells on activation indicates a role of TREM-1 in modulation of inflammation [12, 17]. (Fig.5) TREM-1 mediated activation of Kupffer cells and hepatic stellate and its upregulation in HCC has been shown by previous studies suggesting a direct crucial role of TREM-1 in development, progression and invasion of HCC [16, 18-19].
Figure 5: Schematic presentation for molecular pathogenesis of hepatocellular carcinoma. Exposure of normal liver tissue to HBV, HCV, alcohol and aflatoxin induce inflammation and results in induction of surface receptors like TREMs and TLRs. Association of TREM-1 with DAP-12 in turn activate JNK, p38/MAPK, PI3K, ERK1/2, IRAK, STAT3, PPAR-γ and Akt signalling pathways. Further these signalling pathways stimulate the transcription factors like NF-kB, PU.1, AP-1 and others. Activation of transcription factors results in increased secretion of pro-inflammatory cytokines namely IL-6, TNF-α, IL-1β, IL-2, IL-8, IL-12, IL-17, IL-23, IL-32, MCP-1, MCP-3, GM-CSF and anti-inflammatory cytokines namely IL-10 and TGF-β. Pro-inflammatory cytokines induce tumorigenesis and the development of hepatocellular carcinoma. Vitamin D is an anti-inflammatory agent and modulates the immune response of body hence can be used to subdue inflammation, decrease secretion of pro-inflammatory cytokines and suppress activation of signalling pathways.
TREM-2 is a cell surface receptor protein belonging to immunoglobulin and lectin like superfamily and is expressed on macrophages, dendritic cells and microglial cells. TREM-2 suppresses inflammation in hepatic, lung and bone-marrow derived macrophages, osteoclasts and dendritic cells. Thus it functions as a negative regulator of the inflammatory response [20-23]. TREM-2 is also associated with cytosolic adaptor DAP-12 [24]. TREM-1 and TREM-2 glycoprotein have a single extracellular immune-globulin like domain, a transmembrane region with lysine residue and a short intracellular region [25]. DAP-12 is a common ligand for both TREM-1 and TREM-2, but the downstream signalling is different. TLRs signalling is amplified by TREM-1 in macrophage/ monocyte and neutrophils, but suppressed by TREM-2 in macrophage and dendritic cells [26].

Interleukin-6 (IL-6) is a pro-inflammatory cytokine released from T-cells in response to acute inflammation. IL-6 has been shown to be a risk factor for HCC and high serum levels promotes HCC development. It has been reported that both autocrine and paracrine secretion of IL-6 induces HCC development. Chronic inflammation has also been associated with increased levels of IL-6. IL-6 induces B cell proliferation, differentiation and antibody production and acts as hepatocyte stimulatory factor inducing acute phase reaction by modulating transcription of liver specific genes [2, 5, 27]. HBV infection results in increased level of hepatitis B virus X (HBx) protein [28]. (Fig 2) This results in delayed proliferation of hepatocyte through over expression of IL-6 and delayed liver regeneration. Correlation of IL-6 with TREM-1 in HCC has been reported and studies has suggested that TREM-1 activation increases IL-6 secretion [19].

Tumor necrosis factor-alpha (TNF-α) is another pleotropic pro-inflammatory cytokine that induce cell growth, differentiation, proliferation and survival; cellular function and metabolism; production of inflammatory mediators and cell death by activating intracellular pathways. TNF-α is produced by macrophages, neutrophils, fibroblasts, NK cells, T and B
cells. In liver TNF-α is involved in functional restoration by liver regeneration, hepatocyte proliferation as well as in hepatotoxicity [29-31]. TNF-α polymorphism has been correlated with development of HCC and high levels of TNF-α have been reported in HCC by previous studies [9, 30, 32-36]. Higher levels of TNF-α are associated with increased secretion of other pro-inflammatory cytokines, fibrogenesis, and progression of fibrosis in chronic liver disease, and severity of chronic HCV infection, accumulation of extracellular matrix by activating hepatic stellate cells predisposing the liver to development of HCC [37-42]. It is also evident that TNF-α increases the incidence of HCC in HCV infection and HCV patients have higher level of TNF-α [43].

Cirrhosis is the irreversible end stage disease and can develop into HCC. Treatment of liver disease varies according to the stage of disease, and the only curative treatment for HCC is liver resection or transplant depending on the stage of disease, but there are reports of reactivation and recurrence of HCC post-surgery (Fig. 4). The five-year survival rate post-transplant is only 30-40% [44], and there is no improvement in overall survival rate despite the advance therapeutic strategies [45]. The five-year survival of HCC is 10% [46-47], and one year survival is less than 50% [48]. Thus, there is a need for a satisfactory treatment to reduce the inflammation to slow down or prevent the progression of various stages like hepatitis and fibrosis to cirrhosis and HCC. Being an anti-inflammatory agent, vitamin D may play a potential role in slowing down the inflammation in various progressive stages and progression of HCC. There is evidence of association between low levels of vitamin D with alcoholic liver disease (ALD) [49], non-alcoholic fatty liver disease (NAFLD) [50-54], poor prognosis in infectious liver disease, cirrhosis [49, 55-59], severe fibrosis and poor prognosis in chronic HCV patients [60]. The high prevalence of low vitamin D in patients with ALD, NAFLD, HCV infection, cirrhotic hepatitis C patients and non-cirrhotic patients [61], all leading to development of HCC raises concern and hence the role of vitamin D, to prevent
these condition need to be assessed. Additionally there is a high prevalence of hypovitaminosis D in adult US population and studies have stressed on increasing the intake of vitamin D [62]. With the increasing incidence of liver disease, including cirrhosis and HCC, and the prevalence of hypovitaminosis in the US, vitamin D may be used as a therapeutic additive option. Hence to analyse the effect of vitamin D on inflammatory markers and cytokines in HCC becomes crucial and need of the time.

Vitamin D is a potent modulator of immune response in human being and plays a protective role against infectious diseases. Downregulation of various pro-inflammatory cytokines (IL-2, IL-9, IL-13, TNF-α) and chemokines (MCP-1, CX3CL-1, CXCL-10, CXCL-11) and upregulation of anti-inflammatory cytokine (IL-10) by vitamin D has been reported by Thota et al [63]. Along with its role in calcium homeostasis and bone metabolism, vitamin D also has anti-inflammatory properties, anti-tumoral/ anti-carcinogenic and anti-fibrogenic effects [64-71]. Previous studies have reported that vitamin D inhibits proliferation in breast [72-73], prostate [74-75], colon [76-77], ovarian [78-79], melanoma and pancreatic carcinoma [80] and hepatocellular carcinoma [81]. Reduction in tumor dimensions of hepatocellular carcinoma by vitamin D analogue has also been reported [82].
TREM-1 expressed on hepatic stellate cells in hepatocellular carcinoma is an inflammatory marker and amplifier of inflammation and vitamin D is an anti-inflammatory agent. But till now there are no reports on the effect of vitamin D on TREM-1 expression in hepatocellular carcinoma. To analyse the effect of vitamin D in regulating the expression of TREM-1 in hepatocellular carcinoma cells in this study I proposed the hypothesis that

“Vitamin D attenuates the expression of TREM-1 in hepatocellular carcinoma cell line”

and tested the hypothesis with following specific aims.

**Aim 1**: To identify expression of TREM-1, TREM-2, HMGB-1, DAP-12 and VDR, IL-6 and TNF-α on HCC and THLE-2 cells (Fig.5).

**Aim 2**: To check the effect of cytokines IL-6 and TNF-α on TREM-1/TREM-2 expression in presence and absence of vitamin D and effect of vitamin D on TREM-1/ TREM-2 expression in HCC and THLE-2 cells (Fig.5).

**Aim 3**: To study the effect of Vitamin D on proliferation, migration and invasion of HepG-2 and THLE-2 cells.
Material and Methods

Reagents

Primary antibodies for TREM-1(sc-19309), TREM-2(sc-48764), VDR(sc-9164), Cyp24(sc-32166), Cyp27B1(sc-67261), HMGB-1(sc-26351), DAP-12(sc-20783) and PU.1(sc-352) were from Santa Cruz Biotechnologies (Dallas, TX, USA), and antibodies for IL-6 (ab6672) and TNF-α(ab1793) were purchased from Abcam (Cambridge, MA, USA). Alexa Fluor 594 (red) and Alexa Fluor 488 (green) secondary antibodies were from Life Technologies (Grand Island, NY, USA). Cytokines recombinant human IL-6 and TNF-α were purchased from Peprotech (NJ, USA). Calcitriol (Vitamin D3, D1530) was purchased from Sigma Aldrich (St. Louis, MO, USA). PU.1 siRNA (sc-36330, Santa Cruz Technology), TREM-1 siRNA (sc-42999, Santa Cruz Technology), recombinant HMGB-1 (H4652, Sigma Aldrich) and PU.1 plasmid (LZRS PU.1 WT #34835 from Addgene, Cambridge, MA, USA) were used. Lipofectamine 2000 Transfection Reagent and Plus Reagent were from Invitrogen (Grand Island, NY, USA). NF-κB inhibitors BAY11-7085 and PTDC; PI3K inhibitor (LY294002) were from Sigma Aldrich (St. Louis, MO, USA) and ERK1/2 inhibitor (U0126) was from Calbiochem (Darmstadt, Germany).

Cell line and culture medium

Hepatocellular carcinoma cell line (HepG-2) and epithelial cells transformed with SV40 large T antigen (THLE-2) were obtained from ATCC cell bank (Manassas, VA, USA). Hep G-2 cells were grown in complete (with 10% fetal bovine serum and 1% penicillin-streptomycin) Eagle's Minimum Essential Medium with L-Glutamine (EMEM) purchased from ATCC (Manassas, VA, USA) and THLE-2 cells were grown in complete (with 10% fetal bovine serum, 1% BEpiCGS growth factor and 1% penicillin-streptomycin) Bronchial Epithelial
Cell Medium (BEpiCM) purchased from Sciencell (Carlsbad, CA, USA) at 37°C in a 5% CO₂, 95% air environment humidified incubators.

**Cell Culture and Treatment Protocol**

HepG-2 and THLE-2 cells were grown in complete medium in T25 flasks. About 50,000 cells were seeded to each chamber in chamber slide and grown overnight for immunofluorescence studies. Around 250-300,000 cells were grown in 6-well plates till 70-80% confluence for mRNA extraction. Cells were then kept in serum free medium for 2 hours before treatments. The treatment of cells with IL-6 (10ng/ml), TNF-α (10ng/ml), and calcitriol (10nM) was given for 24 hours. Immunofluorescence staining and mRNA extraction was done in the treated as well as untreated cells. In case of inhibition studies, inhibitors were added one hour before the cytokine treatment and then cells were treated with cytokine along with inhibitor for 24 hours.

**Immunofluorescence (IF)**

Immunofluorescence staining studies were carried out for protein expression of TREM-1, TREM-2, HMGB-1, DAP-12, VDR, Cyp24, Cyp27B1, PU.1, IL-6 and TNF-α; and co-localization of TREM-1 with DAP-12 and PU.1 following the standard protocol in our laboratory. Briefly, cells were grown in T25 flask and then 50,000 cells were placed in each chamber of four chambered slide. Cells in chamber slides were fixed with 4% formalin for 10 minutes, washed and incubated with 0.1% triton for 15 minutes. Blocking of cells with 5% fetal bovine serum in PBS for half hour was followed by primary antibody incubation (goat anti-TREM-1 in 1:50 dilution, rabbit anti-TREM-2 in 1:50 dilution, goat anti-HMGB-1 in 1:50 dilution, rabbit anti-DAP-12 in 1:50 dilution, rabbit anti-VDR in 1:50 dilution, rabbit anti-Cyp24 in 1:50 dilution, goat anti-Cyp27B1 in 1:50 dilution, rabbit anti-PU.1 in 1:50 dilution, rabbit anti-IL-6 in 1:1000 dilution and mouse anti-TNF-α in 1:500 dilution) for 2
hours at room temperature. Cells were washed again with PBS three times five minutes each followed by the incubation with corresponding secondary antibody (Alexa Fluor 594 and 488 in 1:1000 dilution) for 30 minutes. After 3 washings, cells were counterstained with DAPI (4, 6-diamidino-2-phenylindole) for nuclear staining. Stained cells were scanned by using Olympus inverted fluorescent microscope (Olympus BX51). Mean immunofluorescence intensity was calculated by Image –pro software using 10 cells for each measurement. The experiment was repeated three times.

**RNA isolation, cDNA synthesis, and real-time PCR:** TRI reagent (Trizol reagent, Sigma, St Louis, MO, USA) was used to isolate the total RNA from cells according to the manufacturer’s instructions and yield of RNA quantitation was done by using Nano drop (Thermo Scientific, Rockford, IL, USA). The cDNA was prepared by using oligo (dT) primer and Improm II reverse transcription kit (Promega, Madison, WI, USA) following the manufacturer’s instructions. Real time PCR (RT-PCR) was performed in triplicate using SYBR Green using real-time PCR system (CFX96, BioRad Laboratories, and Hercules, CA, USA). The primers for different genes were obtained from Integrated DNA Technologies (Coralville, IA, USA). The PCR cycling conditions were 5 min at 95°C for initial denaturation, 40 cycles of 30s at 95°C (denaturation), 30s at 55-60°C (according to the primer annealing temperatures) and 30s at 72°C (extension) followed by melting curve analysis. Determination of fold change in expression of mRNA transcripts relative to controls was done after normalizing to GAPDH. The oligonucleotide primer sequences for TREM-1, TREM-2, PU.1, IL-6, and TNF-α, NF-kB, Ki-67 and GAPDH are shown in Table 1. The experiment was repeated three times.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>TREM-1</td>
<td>5’-AGT TAC AGC CCA AAA CAT GC-3’</td>
<td>5’-CAG CCC CCA CAA GAG AAT TA-3’</td>
</tr>
<tr>
<td>TREM-2</td>
<td>5’-ACA GAA GCC AGG GAC ACA TC-3’</td>
<td>5’-CCT CCC ATC ATC TTT CTT CA-3’</td>
</tr>
<tr>
<td>PU.1</td>
<td>5’-AAG GGC AAC CGC AAG AA-3’</td>
<td>5’-GGT AGG TGA GCT TCT TCT TCA C-3’</td>
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<tr>
<td>IL-6</td>
<td>5’-AAA TTC GGT ACA TCC TCG ACG GCA-3’</td>
<td>5’-CAG TGC CTC TTT GCT GCT TTC ACA-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’ACC CTC AAC CTC TCC TGG CTC AAA-3’</td>
<td>5’-AAT CCC AGG TTT CGA AGT GGT GGT-3’</td>
</tr>
<tr>
<td>NF-kB</td>
<td>5’-GAC TAC GAC CTG AAT GCT GTG-3’</td>
<td>5’-GTC AAA GAT GGG ATG AGG AAG G-3’</td>
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<td>Ki-67</td>
<td>5’-CTT TGG GTG CGA CTT GAC G-3’</td>
<td>5’-GTC GAC CCC GCT CCT TTT-3’</td>
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<td>GAPDH</td>
<td>5’-GGT GAA GGT CGG AGT CAA CGG ATT TGG TCG-3’</td>
<td>5’-GGA TCT CGC TCC TGG AAG ATG GTG ATG GG-3’</td>
</tr>
</tbody>
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**Table 1:** Nucleotide sequence for the primers used for mRNA analysis of TREM-1, TREM-2, PU.1, IL-6, and TNF-α, NF-kB, Ki-67 compared to GAPDH by RT-PCR.

**Blocking experiments for analysis of Signalling Pathways and downstream transcription factor:**

Pre-confluent HepG-2 cells in serum free media were treated with inhibitors against ERK1/2 (U0126), PI3K (LY294002) and NF-kB inhibitors BAY11-7085 and PTDC at 10μM for 1 hour followed by treatment with IL-6, TNF-α at 10ng/ml and calcitriol at 10nM along with these inhibitors for 24 hours. The mRNA was extracted from treated cells and the fold change in mRNA expression of TREM-1 was analysed by RT-PCR. The experiment was repeated three times.

**Small interfering RNA (siRNA) Transfection Assay**

PU.1 siRNA transfection of HepG-2 cells was done according to manufactures instructions in 6-well plate using Lipofectamine 2000, Plus reagent and Opti-MEM medium (Gibco). 4μl (40nM) of siRNA, 14.4μl of Lipofectamine and 6μl of Plus Reagent was used for transfection. Transfection assay was done for 48 hours, repeated three times. LPS treatment of the transfected cells was done for 24 hours. The mRNA was extracted from the transfected
cells and subjected to RT-PCR studies for PU.1 and TREM-1 expression. The experiment was repeated three times.

**Plasmid cDNA Transfection Assay**

PU.1 plasmid transfection was carried out in HepG-2 cells according to manufactures protocol. Briefly plasmid was propagated with LB Agar (L24030-500, RPI Corp. Mount Prospect, IL, and USA). Plasmid cDNA was prepared by using QIAprep Spin Miniprep Kit (Quigen #27106) following manufactures protocol and quantitated by Nanodrop (Thermo Scientific, Rockford, IL, USA). Titration studies were done using 1μg, 2μg, and 4μg and 10μg cDNA. Maximum effect was found with 10μg and hence was used for further studies. Transfection of PU.1 plasmid cDNA was done with the same protocol as in case of siRNA transfection. The mRNA was extracted from the transfected cells and mRNA expression studies were done for PU.1 and TREM-1 by RT-PCR. The experiment was repeated three times.

**Migration and Invasion Assay**

Transwell chamber inserts (Corning Inc. Corning, NY, USA) with 8μm size were used for invasion assay. HepG-2 and THLE-2 cells in a concentration of 5x10⁴/ml in respective serum free medium were inoculated on the upper chamber of the transwell. Serum free medium with (IL-6, TNF-α, calcitriol) and without treatment was added to lower compartment. Cells were incubated in humidified incubator (5%CO₂, 37⁰C) for 24 hour. Migrated cells through the permeable membrane were fixed with 100% methanol and stained with Hem-diff stain. Cells in 10 fields (20X) were scanned. Invasion and migration assay for each treatment was repeated three times.
Scratch test for cell migration in vitro with cytokine IL-6 (10ng/ml), TNF-α (10ng/ml) and calcitriol (10nM) and without treatment was also carried out. The effect of calcitriol on migration of HepG-2 and THLE-2 cells in presence and absence of cytokines was analysed. Cells were grown in complete medium till 80% confluence and the well was divided into 8 parts by scratching a vertical line in middle and three equidistance horizontal lines. Each chamber was photographed at the 6 intersection fields along with the marked lines at 0hr, 6hr, 24hr, 48hr and 72 hr. The experiment was repeated three times.

**Proliferation Assay**

Proliferation assay to determine the number of growing cells with and without the effect of cytokines IL-6 and TNF-α, and calcitriol on HepG-2 was done using cell counting kit-8 (CCK-8, DJ689 Dojindo). RT-PCR studies were also carried out to analyze the effect of various treatments on the proliferation marker Ki-67, and fold change in mRNA expression of Ki-67 was calculated. About 1500 HepG-2 cells were seeded in the wells of 96 well plate in 100μl of serum free media. Cells were incubated for 12hr, 24hr, 48hr, 72hr and 96hr with and without treatments. 10μl of CCK-8 was added and incubated for 4 hours in dark (5%CO2, 37°C) and then OD values were calculated using the EnSpire 2300 Multilabel Reader (Perkin Elmer) at 450nm. The experiment was repeated three times.

**Statistical analysis**

All data are presented as mean ± SD from three independent experiments using HepG-2 and THLE-2 cells and were analysed using Graph Pad Prism. Statistical analysis was performed using Student’s *t* test and ANOVA. A *p* value of <0.05 was considered significant (*=*0.05, **=*0.01, ***=*0.001 and ****=*0.0001).
Results

**Increased TREM-1 expression in Hep G-2 cells:** Expression of TREM-1 and TREM-2 was examined by immunofluorescence staining studies. Interestingly IF showed higher immunoreactivity of TREM-1 in HepG-2 cells (Fig. 6 D) compared to THLE-2 cells (Fig. 6 A) while the immunoreactivity for TREM-2 was higher in THLE-2 cells (Fig. 6 G) compared to HepG-2 cells (Fig. 6 J). Immunofluorescence studies showed that both TREM-1 and TREM-2 have cytoplasmic expression in cells. Each experiment was repeated three times.

**Increased HMGB-1 and DAP-12 expression in HepG-2 cells:** To examine the expression of HMGB-1 and DAP-12 IF with corresponding antibody on HepG-2 and THLE-2 cells was carried out. Immunofluorescence staining studies showed higher immunoreactivity of HMGB-1 and DAP-12 in HepG-2 cells (Fig. 7 G and J) compared to THLE-2 cells (Fig. 7 A and D). Immunofluorescence studies showed that both HMGB1 and DAP-12 have nuclear and cytoplasmic but more nuclear expression in cells. Cytoplasmic as well as nuclear expression of HMGB-1 and DAP-12 shows the cytoplasmic and nuclear presence of HMGB-1 and is in accordance to the fact that HMGB-1 is secreted from nucleus into the cytoplasm from a necrotic cell. Further cytoplasmic expression of DAP-12 suggests that TREM-1 and TREM-2 binds to DAP-12 on cytoplasmic membrane after activation (Fig. 3). Each experiment was repeated three times.
Figure 6: Immunofluorescence staining for expression of TREMs in HepG-2 and THLE-2 cells. Immunofluorescence staining of THLE-2 (A-C and G-I) and HepG-2 (D-F and J-L) cells for TREM-1 and TREM-2 respectively was performed with corresponding antibody using the standard protocol. Alexa Fluor 594 was used as secondary antibody for TREM-1 (A and D) and TREM-2 (G and H). DAPI (B, E, H and K) was used to counterstain the nucleus. Images C, F, I and L are the merged images for Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Figure 7: Immunofluorescence staining for expression of HMGB-1 and DAP-12 in HepG-2 and THLE-2 cells. Immunofluorescence staining of THLE-2 (A-F) and HepG-2 (G-L) cells for HMGB-1 (A-C and G-I) and DAP-12 (D-F and J-L) was performed with corresponding antibody using the standard protocol. Alexa Fluor 594 was used as secondary antibody for HMGB-1 (A and G) and DAP-12 (D and J). DAPI (B, E, H and K) was used to counterstain the nucleus. Images C, F, I and L are the merged images for Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Co-localization of TREMs and DAP-12 in HepG-2 cells: Since DAP-12 is a common ligand for both TREM-1 and TREM-2, to examine the presence of TREM-1 and TREM-2 along with DAP-12 on HepG-2 cells IF studies were conducted. The results showed the co-localization of TREM-1 with DAP-12 (Fig.8 F and G) and TREM-2 with DAP-12 (Fig.9 F and G). Each experiment was repeated three times.

Expression of vitamin D receptor (VDR), Cyp 24A1 and Cyp 27A1 in HepG-2 and THLE-2 cells: Vitamin D acts through VDR and metabolised by Cyp24A1 and Cyp27A1 enzymes. Immunofluorescence studies of HepG-2 and THLE-2 cells for VDR, Cyp24A1 and Cyp27A1 showed that VDR, Cyp24A1 and Cyp27A1 have nuclear expression in both HepG-2 (Fig. 10 J, M and P) and THLE-2 (Fig.10 A, D and G) cells. The immunoreactivity of VDR, Cyp24A1 and Cyp27A1 was higher in HepG-2 cells compared to THLE-2 cells. Each experiment was repeated three times.

Increased expression of cytokines IL-6 and TNF-α in HepG-2 cells: Immunofluorescence studies showed that IL-6 and TNF-α have cytoplasmic expression more than nuclear expression in HepG-2 and THLE-2 cells (Fig.11 A-L). Interestingly it was found that immunoreactivity of IL-6 and TNF-α was more in HepG-2 cells (Fig.11G and J) compared to THLE-2 cells (Fig.11 A and D). The increased immunoreactivity of IL-6 and TNF-α shows higher level of these cytokines in hepatocellular carcinoma cell line compared to normal cell line. Each experiment was repeated three times.
Figure 8: Dual immunofluorescence staining for co-localization of TREM-1 and DAP-12 in HepG-2 cells. Dual immunofluorescence staining was done for co-localization of TREM-1 with DAP-12 using corresponding antibody and the standard protocol. Alexa Fluor 594 was used as secondary antibody for TREM-1 (A) and Alexa Fluor 488 was used for DAP-12 (B). DAPI (C) was used to counterstain the nucleus. D-merged image for TREM-1 and DAPI; E-merged image for DAP-12 and DAPI; F-merged image for TREM-1, DAP-12 and DAPI; G-merged image for TREM-1 and DAP-12. This is a representative image of three separate experiments.
Figure 9: Dual immunofluorescence staining for co-localization of TREM-2 and DAP-12 in HepG-2 cells. Dual immunofluorescence staining was done for co-localization of TREM-2 with DAP-12 using corresponding antibody and the standard protocol. Alexa Fluor 594 was used as secondary antibody for TREM-2 (A) and Alexa Fluor 488 was used for DAP-12 (B). DAPI (C) was used to counterstain the nucleus. D-merged image for TREM-2 and DAPI; E-merged image for DAP-12 and DAPI; F-merged image for TREM-2, DAP-12 and DAPI; G-merged image for TREM-1 and DAP-12. This is a representative image of three separate experiments.
Figure 10: Immunofluorescence staining for expression of vitamin D receptor (VDR), cyp24A1 and Cyp27A1 in THLE-2 and HepG-2 cells. Immunofluorescence staining for VDR (A-C and J-L), Cyp24 (D-F and M-N) and Cyp27 (G-I and P-R) was done in THLE-2 (A-I) and HepG-2 (J-R) cells with corresponding antibody using the standard protocol. Alexa Fluor 594 was used as secondary antibody for VDR (A and J), Cyp24 (D and M) and Cyp27 (G and P). DAPI (B, E, H, K, N and Q) was used to counterstain the nucleus. Images C, F, I, L, O and R are the merged images for Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Figure 11: Immunofluorescence staining for expression of IL-6 and TNF-α in HepG-2 and THLE-2 cells. Immunofluorescence staining for IL-6 (A-C and G-I) and TNF-α (D-F and J-L) was done in THLE-2 (A-F) and HepG-2 (G-L) cells with corresponding antibody using the standard protocol. Alexa Fluor 594 was used as secondary antibody for IL-6 (A and G) and TNF-α (D and J). DAPI (B, E, H and K) was used to counterstain the nucleus. Images C, F, I and L are the merged images for Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Cytokines IL-6 and TNF-α increase while vitamin D decreases TREM-1 expression in HepG-2 cells: The RT-PCR studies showed that the mRNA expression of TREM-1 was significantly higher (p<0.05) in HepG-2 cells compared to THLE-2 cells. The mRNA expression of TREM-1 further increases significantly (p<0.05) by treating the cells with IL-6 and TNF-α, and decreases with calcitriol treatment (Fig. 12A). RT-PCR studies also showed that mRNA expression of TREM-2 is higher in THLE-2 cells compared to HepG-2 cells. Calcitriol, IL-6 and TNF-α significantly (p<0.05) enhances the mRNA expression of TREM-2 in HepG-2 cells, and the upregulation of TREM-2 expression is more with calcitriol than with IL-6 and TNF-α (Fig.12B). It is noteworthy to observe that calcitriol suppresses the effect of IL-6 and TNF-α on TREM-1 expression, while it has an additive effect on TREM-2 expression in HepG-2 cells. These findings suggest that vitamin D downregulates the TREM-1 expression as well as the effect of pro-inflammatory cytokines in HepG-2 cells. Each experiment was repeated three times.

To further validate my results I conducted the immunofluorescence studies to observe the TREM-1 expression and effect of cytokines at protein level in HepG-2 cells. Immunofluorescence studies showed higher immunoreactivity of TREM-1 in HepG-2 cells compared to THLE-2 cells (Fig.13 D and A). It was observed that treatment of the HepG-2 cells with calcitriol downregulates (Fig.13 G) and with IL-6 and TNF-α upregulates (Fig. 13 J and P) the TREM-1 expression. Further the treatment of IL-6 and TNF-α treated HepG-2 cells with calcitriol suppresses the effect of IL-6 and TNF-α on TREM-1 expression (Fig. 13 M and S). IF studies for TREM-2 expression showed that TREM-2 immunoreactivity is higher in THLE-2 cells compared to HepG-2 cells (Fig.14 A). The immunoreactivity of TREM-2 enhances with calcitriol (Fig.14 G), IL-6 (Fig.14 J) and TNF-α (Fig. 14 P) in HepG-2 cells. Further upregulation of TREM-2 immunoreactivity was observed when IL-6 and TNF-α treated HepG-2 cells were treated with calcitriol (Fig.14 M and S). Mean fluorescence
intensity showed higher TREM-1 intensity for TREM-1 in HepG-2 cells compared to THLE-2 cells. The TREM-1 intensity increases by IL-6 and TNF-α and decreases by calcitriol, and the effect of IL-6 and TNF-α on TREM-1 expression decreases by calcitriol (Fig.17 A- B). These observations of immunofluorescence further support the results of RT-PCR that TREM-1 expression and stimulatory effect of IL-6 and TNF-α on TREM-1 is attenuated by calcitriol.

Figure 12: RT-PCR analysis for effect of vitamin D on TREMs expression. HepG-2 and THLE-2 cells were treated with IL-6 and TNF-α with and without calcitriol and calcitriol alone. The extracted RNA was subjected to RT-PCR for TREM-1 (A and C) and TREM-2 (B and D) expression. Figure shows fold change in the mRNA expression of TREM-1 (A) and TREM-2 (B) in untreated and treated HepG-2 cells compared to untreated control THLE-2 cells. Figure C and D are the mRNA expression of TREM-1 and TREM-2 respectively in untreated and treated THLE-2 cells. Values are shown as mean ± SD. Data are representation of three individual experiments. Each test was run in triplicate, p<0.05 compared to control THLE-2 cells, N=3.
Figure 13: Immunofluorescence staining for effect of vitamin D on TREM-1 expression in HepG-2 cells. Immunofluorescence staining was done for TREM-1 expression in HepG-2 cells treated with calcitriol (G-I), IL-6 (J-L), IL-6+ calcitriol (M-O), TNF-α (P-R) and TNF-α + calcitriol (S-U). TREM-1 (Alexa Fluor 594-red) expression of HepG-2 (D) was compared to TREM-1 expression in THLE-2 (A) control cells and with TREM-1 expression in different treatments (G, J, M, P and S). DAPI (B, E, H, K, N, Q and T) was used to counterstain nucleus. Images C, F, I, L, O, R and U are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Figure 14: Immunofluorescence staining for effect of vitamin D on TREM-2 expression in HepG-2 cells. Immunofluorescence staining was done for TREM-2 expression in HepG-2 cells treated with calcitriol (G-I), IL-6 (J-L), IL-6+ calcitriol (M-O), TNF-α (P-R) and TNF-α + calcitriol (S-U). TREM-2 (Alexa Fluor 594-red) expression of HepG-2 (D) was compared to TREM-2 expression in THLE-2 (A) control cells and with TREM-2 expression in different treatments (G, J, M, P and S). DAPI (B, E, H, K, N, Q and T) was used to counterstain nucleus. Images C, F, I, L, O, R and U are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Increased TREM-1 expression by cytokines IL-6 and TNF-α and vitamin D in THLE-2 cells:

The RT-PCR studies showed upregulated mRNA expression of TREM-1 in THLE-2 cells with calcitriol, IL-6 and TNF-α and the enhancing effect of IL-6 and TNF-α is suppressed by calcitriol (Fig. 12C). RT-PCR studies also showed that mRNA expression of TREM-2 is significantly (p<0.05) enhanced by calcitriol and attenuated by IL-6 and TNF-α. Thus, the suppressing effect of IL-6 and TNF-α on TREM-2 expression is diminished by calcitriol and compared to IL-6 and TNF-α treated cells, cells treated with IL-6 and TNF-α along with calcitriol have higher immunofluorescence (Fig. 12 D). These results suggest that calcitriol downregulates the TREM-1 expression and upregulates the TREM-2 expression in THLE-2 cells.

To further validate the RT-PCR results, immunofluorescence studies were conducted to observe the TREM-1 expression and effect of cytokines at protein level in THLE-2 cells. Immunofluorescence studies showed upregulation of TREM-1 immunoreactivity with IL-6 (Fig. 15 G) and TNF-α (Fig. 15 M) treatment and attenuation with calcitriol (Fig. 15 D). Further the effect of IL-6 and TNF-α on TREM-1 immunoreactivity was suppressed by calcitriol (Fig. 15 J and P).

The immunoreactivity of TREM-2 enhances with calcitriol (Fig.16 D) and attenuates with IL-6 (Fig.16 G) and TNF-α (Fig. 16 M) in THLE-2 cells. Further upregulation of TREM-2 immunoreactivity was observed when IL-6 and TNF-α treated THLE-2 cells were treated with calcitriol (Fig.16 J and P) suggesting suppression of the effect of IL-6 and TNF-α by calcitriol. Mean fluorescence intensity showed downregulation of TREM-1 immunoreactivity with calcitriol and upregulation with IL-6 and TNF-α (Fig. 17 C). Further TREM-2 intensity increases with calcitriol and attenuates with IL-6 and TNF-α (Fig. 17 D). The results of
immunofluorescence and IF quantitation further support our results of RT-PCR that TREM-1 expression and stimulatory effect of IL-6 and TNF-α on TREM-1 is attenuated by calcitriol in THLE-2 cells.

Figure 15: Immunofluorescence staining for effect of vitamin D on TREM-1 expression in THLE-2 cells. Immunofluorescence staining was done for TREM-1 expression in THLE-2 cells treated with calcitriol (D-F), IL-6 (G-I), IL-6+ calcitriol (J-L), TNF-α (M-O) and TNF-α + calcitriol (P-R). TREM-1 (Alexa Fluor 594-red) expression in THLE-2 (A) was compared with TREM-1 expression of different treatments (D, G, J, M and P). DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Figure 16: Immunofluorescence staining for effect of vitamin D on TREM-2 expression in THLE-2 cells. Immunofluorescence staining was done for TREM-2 expression in THLE-2 cells treated with calcitriol (D-F), IL-6 (G-I), IL-6+ calcitriol (J-L), TNF-α (M-O) and TNF-α + calcitriol (P-R). TREM-2 (Alexa Fluor 594-red) expression in THLE-2 (A) was compared to TREM-2 expression in different treatments (D, G, J, M and P). DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Figure 17: Mean immunofluorescence intensity (MFI) quantification of TREMs in HepG-2 and THLE-2 cells. Mean immunofluorescence intensity quantification for TREM-1 and TREM-2 in HepG-2 (A and B) and THLE-2 (C and D) was done using Image-pro software. Ten individual cells were arbitrarily taken and mean intensity was calculated. MFI quantification was done for control as well as treated cells. All data are shown as mean ± SD. p<0.05; N=10.
Increased TREM-1 expression with rHMGB-1 protein: HMGB-1 secreted from necrotic cells binds to TREM-1 and activates TREM-1 resulting in activation of signalling pathways and transcription factors (Fig. 3). To analyse the effect of recombinant HMGB-1 protein on TREM-1 I treated the HepG-2 cells with rHMGB-1 and mRNA was subjected to RT-PCR. The RT-PCR studies showed that the mRNA expression of TREM-1 significantly (p<0.05) upregulates with rHMGB-1 indicating the role of HMGB-1 in TREM-1 activation (Fig. 18 A). Additionally the mRNA expression of IL-6 (Fig. 18 B) and TNF-α (Fig. 18 C) also upregulate with rHMGB-1 treatment. To further confirm the activation of TREM-1 with rHMGB-1 at protein level, IF studies were performed with rHMGB-1 treated HepG-2 cells. Immunofluorescence study showed that treatment with rHMGB-1 upregulates immunoreactivity of TREM-1 in HepG-2 cells (Fig.19 G) and THLE-2 cells (Fig.19 J). Further the immunofluorescence studies of rHMGB-1 treated HepG-2 and THLE-2 cells for IL-6 and TNF-α immunoreactivity showed that rHMGB-1 upregulates the immunoreactivity for IL-6 and TNF-α in HepG-2 (Fig. 19 S and AE) and THLE-2 cells (Fig. 19 V and AH).
Figure 18: RT-PCR analysis for the effect of rHMGB-1 on TREM-1, IL-6 and TNF-α expression in HepG-2 cells. HepG-2 cells were treated with rHMGB-1 and after 24 hours incubation RNA was extracted. The extracted RNA was subjected to RT-PCR for TREM-1 (A), IL-6 (B) and TNF-α (C) expression. Figure shows fold change in mRNA expression of TREM-1 (A) IL-6 (B) and TNF-α (C) compared to untreated cells. Values are shown as mean ± SD. Data are representation of three individual experiments. Each test was run in triplicate, p<0.05, N=3.
Figure 19: Immunofluorescence staining for the effect of rHMGB-1 on TREM-1, IL-6 and TNF-α expression in HepG-2 and THLE-2 cells. Immunofluorescence staining was done for TREM-1, IL-6 and TNF-α expression using corresponding primary antibody in THLE-2 and HepG-2 cells without and with rHMGB-1 treatment. Alexa Fluor 594 was used as secondary antibody for TREM-1, IL-6 and TNF-α. Image A-C TREM-1 expression in untreated and image G-I TREM-1 expression in rHMGB-1 treated HepG-2 cells; image D-F TREM-1 expression in untreated and image J-L TREM-1 expression in rHMGB-1 treated THLE-2 cells. Image M-O IL-6 expression in untreated and image S-U IL-6 expression in rHMGB-1 treated HepG-2 cells; image P-R IL-6 expression in untreated and image V-X IL-6 expression in rHMGB-1 treated THLE-2 cells. Image Y-AA TNF-α expression in untreated and image AE-AG TNF-α expression in rHMGB-1 treated HepG-2 cells; image AB-AD TNF-α expression in untreated and image AH-AJ TNF-α expression in rHMGB-1 treated THLE-2 cells. DAPI (B, E, H, K, N, Q, T, W, Z, AC, AF and AI) was used to counterstain nucleus. Images C, F, I, L, O, R, U, X, AA, AD, AG and AJ are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Decreased TREM-1 expression with PI3K and ERK1/2 inhibition:

Activated TREM-1 is translocated to the nucleus through the PI3K, p38MAPK, JNK and ERK1/2 signalling pathways (Fig. 3). To analyse the effect of inhibition of these pathways on TREM-1 and TREM-2 in HepG-2 cells, and effect of vitamin D on the inhibitors of PI3K and ERK1/2, I treated the HepG-2 cells with inhibitors of PI3K (LY294002) and ERK1/2 (U0126) signalling pathways in presence and absence of vitamin D. The mRNA from the treated cells was subjected to RT-PCR analysis. The results of RT-PCT studies showed that inhibition of PI3K and ERK1/2 with LY294002 and U0126 respectively significantly (p<0.05) attenuate the mRNA expression of TREM-1 and TREM-2 (Fig. 20 A-B). Interestingly it was observed that subsequent treatment of HepG-2 cells with IL-6 and TNF-α in presence of inhibitors decreases the effect of inhibitors while calcitriol addition has minimal effect in case of TREM-1(Fig.20 A). Though the inhibitors of PI3K and ERK1/2 suppresses the TREM-2 expression in HepG-2 cells, addition of IL-6, TNF-α and calcitriol decreases the effect of inhibitors and increases the mRNA expression of TREM-2 compared to inhibitor treatment alone (Fig.20 B). To validate the results of RT-PCR, I carried out the IF studies to analyse the effect of these inhibitors at protein level. Immunofluorescence staining for TREM-1 and TREM-2 expression after treating the HepG-2 cells with PI3K (LY294002) and ERK1/2 (U0126) inhibitors along with IL-6 and TNF-α showed significantly decreased immune-reactivity of TREM-1 with PI3K inhibition (Fig. 21 G and J and Fig. 23 G and J) and ERK1/2 inhibition (Fig. 21 M and P and Fig. 23 M and P). Immunofluorescence studies for expression of TREM-2 showed significantly decreased immune-reactivity of TREM-2 on treating the HepG-2 cells with PI3K inhibitor (Fig. 22 G and J and Fig. 24 G and J) and ERK1/2 inhibitor (Fig. 22 M and P and Fig. 24 M and P). These results suggest that PI3k and ERK1/2 signalling pathways are involved in signal transduction of activated TREM-1 from cytoplasmic membrane to nucleus and also involves inTREM-2 expression.
Figure 20: RT-PCR analysis for effect of vitamin D and signalling pathway inhibitors on TREM-1 expression in HepG-2 cells. HepG-2 cells were treated with calcitriol, IL-6 and TNF-α without and with inhibitors of signalling pathways PI3K (LY294002) and ERK1/2(U0126). The mRNA extracted from treated cells was subjected to RT-PCR for the mRNA expression of TREM-1 and TREM-2 in HepG-2 cells. Figure shows fold change in mRNA expression of TREM-1 (A) and TREM-2 (B) compared to untreated HepG-2 cells. Values are shown as mean ± SD. Data are representation of three individual experiments. Each test was run in triplicate, p<0.05, N=3.
Figure 21: Immunofluorescence for TREM-1 expression in HepG-2 cells after signalling pathway inhibitor treatment. Immunofluorescence studies were done for the expression of TREM-1 in HepG-2 cells after treating cells with calcitriol and IL-6 with and without PI3K (LY294002) and ERK1/2 (U0126) inhibitors. Alexa Fluor 594 (red) was used as secondary antibody. Expression of TREM-1 [image A (untreated HepG-2 cells), D (HepG-2 cells treated with IL-6), G (cells treated with IL-6 and PI3K inhibitor), J (cells treated with IL-6, PI3K inhibitor and calcitriol), M (cells treated with IL-6 and ERK1/2 inhibitor) and P (cells treated with IL-6, ERK1/2 inhibitor and calcitriol)]. DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Figure 22: Immunofluorescence for TREM-2 expression in HepG-2 cells after signalling pathway inhibitor treatment. Immunofluorescence studies were done for the expression of TREM-2 in HepG-2 cells after treating cells with calcitriol and IL-6 with and without PI3K (LY294002) and ERK1/2 (U0126) inhibitors. Alexa Fluor 594 (red) was used as secondary antibody for TREM-1. Expression of TREM-2 [image A (untreated HepG-2 cells), D (HepG-2 cells treated with IL-6), G (cells treated with IL-6 and PI3K inhibitor), J (cells treated with IL-6, PI3K inhibitor and calcitriol), M (cells treated with IL-6 and ERK1/2 inhibitor) and P (cells treated with IL-6, ERK1/2 inhibitor and calcitriol)]. DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Figure 23: Immunofluorescence for TREM-1 expression in HepG-2 cells after signalling pathway inhibitor treatment. Immunofluorescence studies were done for the expression of TREM-1 in HepG-2 cells after treating cells with calcitriol and TNF-α with and without PI3K (LY294002) and ERK1/2 (U0126) inhibitors. Alexa Fluor 594 (red) was used as secondary antibody. Expression of TREM-1 [image A (untreated HepG-2 cells), D (HepG-2 cells treated with TNF-α), G (cells treated with TNF-α and PI3K inhibitor), J (cells treated with TNF-α, PI3K inhibitor and calcitriol), M (cells treated with TNF-α and ERK1/2 inhibitor) and P (cells treated with TNF-α, ERK1/2 inhibitor and calcitriol)]. DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Figure 24: Immunofluorescence for TREM-2 expression after signalling pathway inhibitor treatment. Immunofluorescence studies were done for the expression of TREM-2 in HepG-2 cells after treating cells with calcitriol and TNF-α with and without PI3K (LY294002) and ERK1/2 (U0126) inhibitors. Alexa Fluor 594 (red) was used as secondary antibody. Expression of TREM-2 [image A (untreated HepG-2 cells), D (HepG-2 cells treated with TNF-α), G (cells treated with TNF-α and PI3K inhibitor), J (cells treated with TNF-α, PI3K inhibitor and calcitriol), M (cells treated with TNF-α and ERK1/2 inhibitor) and P (cells treated with TNF-α, ERK1/2 inhibitor and calcitriol)]. DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Co-localization of TREM-1 and TREM-2 with PU.1: PU.1 is a transcription factor binding to promoter region of TREM-1 gene and regulates expression of TREM-1, so to show the presence of PU.1 in HepG-2 and THLE-2 cells, IF studies were carried out and it was found that PU.1 has nuclear expression (Fig. 25 B, F, J and N) and PU.1 co-localizes with TREM-1 (Fig. 25 D and L) in THLE-2 and HepG-2 cells. It was also observed that the immunoreactivity for PU.1 was more in HepG-2 cells (Fig. 25 J) compared to THLE-2 cells (Fig. 25 B).

IL-6 and TNF-α attenuate and vitamin D enhance PU.1 mRNA expression in HepG-2 cells: PU.1 is a downstream signalling molecule regulating the expression of TREM-1. To analyse the effect of IL-6, TNF-α and calcitriol on PU.1, the mRNA of the HepG-2 cell treated with IL-6, TNF-α and calcitriol was subjected to RT-PCR study. The results of RT-PCR study showed significant (p<0.05) attenuation of mRNA expression of PU.1 transcription factor on treatment of HepG-2 cells with pro-inflammatory cytokines like IL-6 and TNF-α and significant (p<0.05) enhancement with calcitriol (Fig. 26 A). These results indicate the inhibitory effect of IL-6 and TNF-α and stimulatory effect of vitamin D on PU.1.

Increased TREM-1 mRNA expression by PU.1 siRNA in HepG-2 cells: Since PU.1 regulates the expression of TREM-1, I transfected the HepG-2 cells with PU.1 siRNA to suppress the mRNA expression of PU.1 transcription factor. The titration with 40nM and 80nM dosages of PU.1 siRNA was done and the higher inhibitory effect was found with 80nM (Fig. 26 B). It was found that TREM-1 expression gets upregulated on transfecting the cells with PU.1 siRNA (Fig. 26 C).

Decreased TREM-1 mRNA expression by PU.1 plasmid in HepG-2 cells: Transfection of HepG-2 cells with PU.1 plasmid enhances the PU.1 mRNA expression. Titration of the plasmid cDNA at concentration of 1μg, 2μg, 4μg was carried out. The maximum effect on the
mRNA expression enhancement of PU.1 was found with 10μg (Fig. 26 D). Further RT-PCR studies showed that transfection with PU.1 plasmid cDNA downregulates the TREM-1 expression in HepG-2 cells (Fig. 26 E). These results suggest that suppression of PU.1 results in upregulation and over expression of PU.1 downregulates the TREM-1 mRNA expression.

**Figure 25: Immunofluorescence staining for co-localization of TREMs and PU.1 in HepG-2 and THLE-2 cells.** Immunofluorescence for TREM-1 and TREM-2 co-localization with PU.1 was done using TREM-1, TREM-2 and PU.1 primary antibody. Alexa Fluor 594 (red) was used for TREM-1 (A and I) and TREM-2 (E and M), and Alexa Fluor 488 (green) was used for PU.1 (B, F, J and N) as secondary antibody. Images A-D (TREM-1 and PU.1) and images E-H (TREM-2 and PU.1) are THLE-2 cells. Images I-L (TREM-1 and PU.1) and images M-P (TREM-2 and PU.1) are HepG-2 cells. DAPI (B, F, J and N) was used to counterstain nucleus. The merged image with DAPI, D and L are co-localization of TREM-1 and PU.1, images H and P are co-localization of TREM-2 with PU.1 in THLE-2 and HepG-2 cells respectively. This is a representative image of three separate experiments.
Figure 26: RT-PCR studies for PU.1 expression and TREM-1 expression after treating HepG-2 cells with PU. siRNA and plasmid. The RNA extracted from HepG-2 cells without and with treatment of calcitriol, IL-6 and TNF-α was subjected to RT-PCR for mRNA expression of PU.1 (A). HepG-2 cells were treated with siRNA and the mRNA expression of PU.1 (B) and TREM-1 (C) was analysed with RT-PCR. HepG-2 cells were also treated with PU.1 plasmid and the mRNA expression of PU.1 (D) and TREM-1 (E) was analysed with RT-PCR. Figure shows fold change in mRNA expression of PU.1 (A) in calcitriol, IL-6 and TNF-α treated cells; PU.1 (B) in PU.1 siRNA treated cells; TREM-1 (C) PU.1 siRNA treated cells; PU.1 (D) in PU.1 plasmid treated cells and TREM-1 (E) in PU.1 plasmid treated cells. Values are shown as mean ± SD. Data are representation of three individual experiments. Each test was run in triplicate, p<0.05, N=3.
**Vitamin D downregulates NF-kB expression in HepG-2 cells:** Treatment of HepG-2 cells with calcitriol downregulates the mRNA expression of NF-kB. Though cytokines IL-6 and TNF-α upregulates the mRNA expression of NF-kB but calcitriol suppresses the effect of pro-inflammatory cytokines and decreases the mRNA expression of NF-kB (Fig. 27 A).

**Decreased TREM-1 expression with inhibitors of NF-kB:**

The inhibition of NF-kB transcription factor by NF-kB inhibitors BAY 11-7085 and PTDC significantly (p<0.05) downregulates the mRNA expression of TREM-1 in HepG-2 cells. Treatment of HepG-2 cells with calcitriol along with NF-kB inhibitors further downregulate the mRNA expression of TREM-1. The results of PCR studies suggest that the effect of cytokines (IL-6 and TNF-α) decreases with NF-kB inhibitors (Fig. 27 B). It was also observed that NF-kB inhibition does not affect the mRNA expression of TREM-2 in HepG-2 cells (Fig. 27 C). To further validate our results of PCR studies I did immunofluorescence studies and results showed that treatment of IL-6 and TNF-α treated HepG-2 cells with NF-kB inhibitors (BAY 11-7085 and PTDC) significantly (p<0.05) downregulates the TREM-1 immunoreactivity (Fig. 28 G, J, M and P and Fig.29 G, J, M and P) but does not affect the immunoreactivity of TREM-2 (Fig. 30 G, J, M and P and Fig. 31 G, J, M and P). The findings of IF further supported the results of PCR studies.
Figure 27: RT-PCR analysis for NF-kB mRNA expression and TREM-1 expression in HepG-2 cells treated with signalling pathway PI3K and ERK1/2 inhibitors. The RNA extracted from HepG-2 cells treated with calcitriol, IL-6 and TNF-α was subjected to RT-PCR for the mRNA expression of NF-kB (A). Also RNA extracted from NF-kB inhibitors (BAY 11-7085 and PTDC), calcitriol, IL-6, and TNF-α treated HepG-2 cells was subjected to RT-PCR for the mRNA expression of TREM-1 (B) and TREM-2 (C). Figure shows fold change in the mRNA expression of TREM-1 (A) and TREM-2 (B) compared to untreated HepG-2 cells. Values are shown as mean ± SD. Data are representation of three individual experiments. Each test was run in triplicate, p<0.05, N=3.
**Figure 28: Immunofluorescence for TREM-1 expression in HepG-2 cells after NF-kB inhibitor treatment.** Immunofluorescence studies were done for the expression of TREM-1 in HepG-2 cells after treating cells with calcitriol and IL-6 with and without NF-kB (PTDC and BAY 11-7085) inhibitors. Alexa Fluor 594 (red) was used as secondary antibody. Expression of TREM-1 [image A (untreated HepG-2 cells), D (HepG-2 cells treated with IL-6), G (cells treated with IL-6 and PTDC), J (cells treated with IL-6, PTDC and calcitriol), M (cells treated with IL-6 and BAY11-7085) and P (cells treated with IL-6, BAY11-7085 and calcitriol)]. DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
**Figure 29: Immunofluorescence for TREM-2 expression in HepG-2 cells after signalling pathway inhibitor treatment.** Immunofluorescence studies were done for the expression of TREM-2 in HepG-2 cells after treating cells with calcitriol and IL-6 with and without NF-kB (PTDC and BAY 11-7085) inhibitors. Alexa Fluor 594 (red) was used as secondary antibody for TREM-1. Expression of TREM-2 [image A (untreated HepG-2 cells), D (HepG-2 cells treated with IL-6), G (cells treated with IL-6 and PTDC), J (cells treated with IL-6, PTDC and calcitriol), M (cells treated with IL-6 and BAY11-7085) and P (cells treated with IL-6, BAY11-7085 and calcitriol)]. DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Figure 30: Immunofluorescence for TREM-1 expression in HepG-2 cells after signalling pathway inhibitor treatment. Immunofluorescence studies were done for the expression of TREM-1 in HepG-2 cells after treating cells with calcitriol and TNF-α with and without NF-κB (PTDC and BAY 11-7085) inhibitors. Alexa Fluor 594 (red) was used as secondary antibody. Expression of TREM-1 [image A (untreated HepG-2 cells), D (HepG-2 cells treated with TNF-α), G (cells treated with TNF-α and PTDC), J (cells treated with TNF-α, PTDC and calcitriol), M (cells treated with TNF-α and BAY11-7085) and P (cells treated with TNF-α, BAY11-7085 and calcitriol)]. DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
Immunofluorescence studies were done for the expression of TREM-2 in HepG-2 cells after treating cells with calcitriol and TNF-α with and without NF-kB (PTDC and BAY 11-7085) inhibitors. Alexa Fluor 594 (red) was used as secondary antibody. Expression of TREM-2 [image A (untreated HepG-2 cells), D (HepG-2 cells treated with TNF-α), G (cells treated with TNF-α and PTDC), J (cells treated with TNF-α, PTDC and calcitriol), M (cells treated with TNF-α and BAY11-7085) and P (cells treated with TNF-α, BAY11-7085 and calcitriol)]. DAPI (B, E, H, K, N and Q) was used to counterstain nucleus. Images C, F, I, L, O and R are the merged images of Alexa Fluor 594 and DAPI. This is a representative image of three separate experiments.
**Vitamin D decreases the proliferation, migration and invasion of HepG-2 cells:**

The scratch test for migration assay of HepG-2 cells with and without calcitriol treatment was performed at 5 time points (0hr, 6hr, 24hr, 48hr and 72 hr) and it shows that calcitriol decreases the migration of HepG-2 cells (Fig. 32 G, H and I) and increases the migration of THLE-2 cells (Fig. 32 J, K and L) at 24hr, 48hr and 72 hr. It was observed that cell migration of HepG-2 cells increases with IL-6 (Fig. 32 M, N and O) and TNF-α (Fig. 32 Y, Z and AA). The migration of THLE-2 cells decreases with IL-6 (Fig. 32 P, Q and R) and TNF-α (Fig. 32 AB, AC and AD). Additionally it was observed that calcitriol decreases the effect of IL-6 and TNF-α on migration of HepG-2 (Fig. 32 S, T, U and Fig. 32 AE, AF, AG) and THLE-2 cells (Fig. 32 V, W, X and Fig. 32 AH, AI, AJ). Transwell invasion assay of HepG-2 and THLE-2 cells shows that IL-6 and TNF-α increase invasion of HepG-2 cells (Fig. 33 H and I) and decrease invasion of THLE-2 cells (Fig. 33 B and C) while calcitriol increase invasion of THLE-2 cells (Fig. 33 D) and decrease invasion of HepG-2 cells (Fig. 33 J). Additionally it was also found that calcitriol decreases the effect of IL-6 and TNF-α on migration of HepG-2 (Fig. 33 K and L) and THLE-2 cells (Fig. 33 E and F).

To analyse the effect of vitamin D on proliferation and cytokine I performed the proliferation assay for Ki-67 and with CCK-8. The RT-PCR studies for expression of proliferation marker Ki-67 were carried out at two time points (24hr and 72hr). At 24 hr it was observed that IL-6 and TNF-α increase mRNA expression in HepG-2 cells (Fig. 34 A) and in THLE-2 cells (Fig. 34 B). It was seen that calcitriol decreases the mRNA expression HepG-2 cells (Fig. 34 A) and increase the mRNA expression in THLE-2 cells (Fig. 34 B). It was noteworthy to observe that mRNA expression of Ki-67 for HepG-2 cells remains upregulated (Fig.34 C) but get downregulated for THLE-2 cells (Fig.34 D) at 72 hours. Further the proliferation assay with CCK-8 kit showed increase in proliferation of HepG-2 cells till a time point of 48 hour with IL-6 and TNF-α (Fig. 35 A, D, E, F and G) and decrease in proliferation with calcitriol.
(Fig. 35 A and C) supporting the results of RT-PCR studies. Further the effect of these cytokines and calcitriol decreases after 48 hour.

**Figure 32: Scratch test for migration of HepG-2 and THLE-2 cells.** HepG-2 and THLE-2 cells were grown in 6 well plate. Untreated HepG-2 and THLE-2 cells and after treatment with calcitriol, and IL-6 and TNF-α with without calcitriol were subjected to scratch test at three time points 0 hour, 24 hours and 48 hours. Migration of the cells was analysed. Images A-C (HepG-2) and D-F (THLE-2) untreated cells; images G-I (HepG-2) and J-L (THLE-2) treated with calcitriol; Images M-O (HepG-2) and P-R (THLE-2) cells treated with IL-6; images S-U (HepG-2) and V-X (THLE-2) cells treated with IL-6 + calcitriol; images Y-AA (HepG-2) and AB-AD (THLE-2) cells treated with TNF-α and images AE-AG (HepG-2) and AH-AJ (THLE-2) cells treated with TNF-α + calcitriol. Images A, G, M, S, Y and AE (HepG-2) and images D, J, P, V, AB and AH (thle-2) are 0 hour; images B, H, N, T, Z and AF (HepG-2) and images E, K, Q, W, AC and Al (THLE-2) are at 24 hours; images C, I, O, U, AA and AG (HepG-2) and images F, L, R, X, AD and AJ (THLE-2) are at 48 hours. This is a representative image of three separate experiments.
Figure 33: Transwell invasion assay of HepG-2 and THLE-2 cells. Transwell invasion assay of HepG-2 and THLE-2 cells was performed to see the effect of IL-6, TNF-α and calcitriol on invasion. Images A-F are THLE-2 cells and G-L are HepG-2 cells. A (control THLE-2); B (THLE-2 treated with IL-6); C (THLE-2 treated with TNF-α); D (THLE-2 + calcitriol); E (THLE-2 treated with IL-6 + calcitriol); F (THLE-2 treated with TNF-α + calcitriol); G (control HepG-2); H (HepG-2 treated with IL-6); I (HepG-2 treated with TNF-α); J (HepG-2 + calcitriol); k (HepG-2 treated with IL-6 + calcitriol); K (HepG-2 treated with TNF-α + calcitriol). This is a representative image of three separate experiments.
Figure 34: RT-PCR analysis for the mRNA expression of proliferation marker Ki-67 in HepG-2 and THLE-2 cells. The RNA extracted from untreated and treated with calcitriol, IL-6 and TNF-α HepG-2 and THLE-2 cells was subjected to RT-PCR for the mRNA expression of Ki-67 at 24 and 72 hours. Figure shows fold change in the mRNA expression of ki-67 (A- HepG-2 cells at 24 hours, B- THLE-2 cells at 24 hours, C- HepG-2 cells at 72 hours and D- THLE-2 cells at 72 hours). Values are shown as mean ± SD. Data are representation of three individual experiments. Each test was run in triplicate, p<0.05, N=3.
Figure 35: Proliferation assay of HepG-2 cells using CCK-8 kit. CCK-8 kit was used to analyse the proliferation of HepG-2 cells at 12 hour, 24 hours, 48 hours, 72 hours and 96 hours. OD values were plotted at various time points in relation to treatments given. A-All time points with treatments, B- control cells, C- cells treated with calcitriol, D-cells treated with IL-6, E-cells treated with IL-6 + calcitriol, F-cells treated with TNF-α and G-cells treated with TNF-α + calcitriol. Each experiment was run three times.
Discussion

HCC is one of the most extensively studied inflammation-related carcinomas. The chronic inflammation in liver triggers compensatory proliferation of hepatocyte initiating development and progression of HCC. Apoptosis and necrosis occurring in chronic hepatitis and cirrhosis triggers and activates kupffer cells and initiates inflammatory response[8]. Recent reports indicate increasing incidence of liver disease and hepatocellular carcinoma due to hepatitis (HBV, HCV and alcoholic). Some patients suffering from acute hepatitis develop chronic hepatitis, and of these patients, some develop HCC. Chronic inflammation is involved in the development of these diseases as well as HCC, and patients with higher levels of inflammatory markers may be identified as patients at risk for HCC. There is a need for novel early markers of inflammation that can serve as both a tool for early diagnosis as well as a therapeutic target for the treatment of HCC. Recently discovered inflammatory marker TREM-1 may serve as an early marker of inflammation in at risk patients. TREM-1 is an amplifier of inflammation [83] and plays a role in pathogenesis of development of HCC; suppression or inhibition of TREM-1 activity may serve as a tool to decrease the ongoing inflammation in liver. Vitamin D is a well-known anti-inflammatory agent and has been used to suppress inflammation [84]. Hence vitamin D may be used to suppress TREM-1 activity. Up to now, no study has reported the role of vitamin D on TREM-1 activity, so I analyzed the effect of vitamin D on TREM-1 expression in normal liver and hepatocellular carcinoma cells.

Hepatocellular carcinoma cell line (HepG-2) showed increased expression of TREM-1 compared to THLE-2 cells (Fig. 6) indicating increased association of higher level of TREM-1 with HCC[62]. Previous studies has also described about the association of TREM-1 with lung and hepatic cancer [16, 85]. Duan et al reported the increased expression of TREM-1 in HCC carcinoma [19]. The expression of TREM-2 was higher in THLE-2 cells compared to
HepG-2 cell (Fig. 6) suggesting that TREM-2 expression decreases during development of carcinoma. TREM-2 is an anti-inflammatory marker and negatively regulates the immune response; hence the decreased expression of TREM-2 in HepG-2 cells and increased expression in THLE-2 cells in our study are in accordance with the previous reports that TREM-2 reduces the inflammation [22]. Chen et al reported the enhanced clearance of bacteria and improvement in organ injury by TREM-2 overexpression, suggesting its anti-inflammatory role [24]. Increased expression of TREM-1 and decreased expression of TREM-2 by immunofluorescence (Fig.6) and RT-PCR (Fig. 12 A and B) in HepG-2 cells compared to THLE-2 cells indicate that HepG-2 cells has higher level of inflammation compared to THLE-2 cells. So to study the effect of vitamin D on TREM-1 and TREM-2 expression, first it is necessary to analyse the presence of vitamin D receptor (VDR) in HepG-2 and THLE-2 cell lines, as vitamin D have its biological effect through VDR. Immunofluorescence staining of HepG-2 and THLE-2 cells show the presence of VDR in HepG-2 and THLE-2 cells (Fig.10).

1,25-Dihydroxyvitamin D (calcitriol-vitamin D3) is the hormonally active form of vitamin D maintaining the calcium homeostasis in human body [86]. Main sources of vitamin D to humans are sunlight and dietary supplements like egg, fish, milk and mushrooms. Dietary form of vitamin D and vitamin D obtained from sunlight get converted in to active form of calcitriol involving CYP2R1 and Cyp27A1 in liver and Cyp24A1 and Cyp27B1 in kidney [60] (Fig. 36). The presence of these proteins in HepG-2 cell line was determined by immunofluorescence staining (Fig. 10). It has been reported that vitamin D enhances the expression of these protein in HCC [87]. Calcitriol mediates its biological activity and anti-proliferative effect through vitamin D receptor (VDR). VDR is a nuclear hormone receptor expressed in most human cells including cells involving in innate immunity. It has been reported that VDR also regulates the activation of NF-kB and mediate a balance between
autophagy and apoptosis [88-90]. I found increased immunoreactivity for VDR in HepG-2 cells (Fig. 10) in accordance with the previous reports that VDR is expressed on HepG-2 cells and plays a role in development of HCC [91-92]. It is noteworthy that VDR polymorphism has been associated with chronic liver disease and development of HCC in chronic HCV. Vitamin D receptor polymorphism also plays a role in susceptibility and in the clinico-pathological status of HCC and may affect the inhibitory role of vitamin D on tumor cells [93-94]. Decreased expression of VDR in hepatocyte in HCV infection has also been reported suggesting the diminished protective role of vitamin D [50]. It also indicate that in such conditions increase amount of vitamin D will be needed to perform its action up to normal level and hence it is important to analyse the expression of VDR, Cyp24 and Cyp27 in hepatocytes. Reduced expression of VDR may indicate severity and consequence of underlying liver disease. These studies suggest that vitamin D signalling pathway can be a potential target for treatment of HCC by decreasing and delaying the tumor growth.
Figure 36: Schematic diagram showing metabolism of vitamin D and its role in regulation of TREM-1. Ergocalciferol and cholecalciferol from sunlight, milk, egg, fish and mushroom gets converted to calcidiol in liver with the help of enzyme cyp27A1. Further cyp27B1 converts calcidiol to calcitriol in kidney and this calcitriol (vitamin D3) is the biologically active form of vitamin D. Vitamin D acts through vitamin D receptor (VDR) and has an inhibitory effect on NF-kB and stimulatory effect on PU.1. Inhibition of NF-kB results in decreased secretion of IL-6 and TNF-α further decreasing TREM-1 activation. Stimulation of PU.1 results in decreased translation of TREM-1. Both these effects will result in decreased inflammation and tumorigenesis.

Chronic inflammation in HBV and HCV results in chronic hepatitis in some patients. Among patients with chronic inflammation and suppressed immune response, few develop HCC (Fig. 3). IL-6 upregulate the expression of hepatitis B virus X-protein (HBx) and high levels of IL-6 has been reported in liver cirrhosis patients. Engagement of IL-6 with its receptor (IL-6R) in hepatocyte results in increased inflammatory activity and activation of JAK-STAT pathway, ERK pathway, p38MAPK pathway and PI3K pathway [95-97]. IL-6 induces the expression of mitogenic and pro-neoangiogenic and hepatocyte growth factors and decreases the HCC cell apoptosis. Further the role of IL-6 in stimulating hepatic DNA synthesis and
natural killer cell dysfunction has been suggested. This enhances tumor development and progression by escape mechanism from immune response of body [96]. Recently many studies have suggested IL-6 as a tumor marker and prognostic factor for HCC [11, 96, 98] so it became important to study the effect of vitamin D on IL-6 and its effect on TREM-1. To modulate the immune response in human body supra-physiological concentrations of calcitriol is needed, so I treated the Hep G-2 cells with calcitriol at 10nM concentration and found that treatment with calcitriol decreases the mRNA expression of TREM-1 (Fig. 12) as well as immunoreactivity of TREM-1 (Fig. 13). It was observed that IL-6 and TNF-α increases the TREM-1 expression in both HepG-2 and THLE-2 cells (Fig.12-16), but treatment of IL-6 and TNF-α treated HepG-2 cell with calcitriol attenuated the effect of IL-6 and TNF-α on mRNA expression of TREM-1(Fig.12). Similar results were found with immunofluorescence studies showing attenuated immunoreactivity of TREM-1 in HepG-2 cells (Fig.13) after treating the cells with calcitriol. These results suggest that calcitriol suppresses the stimulatory effect of pro-inflammatory cytokines on TREM-1 and suppresses the expression of TREM-1.

Chronic inflammation is involved in carcinogenesis of HCC and TNF-α is the most crucial pro-inflammatory cytokine involved in HCC development. The role of TNF-α in liver disease and its complication has been discussed by previous studies [36]. Higher levels of TNF-α mediate hepatic inflammation, fibrosis, cirrhosis and tissue damage in liver, and induce pro-malignant chemokines, metalloproteinases, angiogenic mediators, cell adhesion molecules resulting in pro-carcinogenic microenvironment. Regulation of TNF-α expression and secretion is regulated at transcriptional level (via AP-1, PU.1 and NF-κB) as well as post-transcriptional level [9, 30, 32, 35, 99]. It has been reported that vitamin D can decrease the production of TNF-α [59] and can improve the deleterious effect of pro-inflammatory cytokines in liver disease. Suppression of the effect of IL-6 and TNF-α on TREM-1
expression by calcitriol and downregulation of TREM-1 expression in our study suggests that vitamin D supplementation can be used to suppress ongoing inflammation in progressing stages of HCC. Reduction in level of IL-6 with vitamin D supplementation in chronic HCV infection [100] and reduction in levels of TNF-α and IL-6 in monocyte with vitamin D [101] has been reported.

To my surprise I found that calcitriol increases the expression of TREM-1 and TREM-2 in THLE-2 cells (Fig.12 C-D). This shows that in normal conditions calcitriol upregulates the immune response by enhancing the expression of TREM-1 and increased production of pro-inflammatory cytokines to initiate the immune response against inflammation. When the THLE-2 cells were treated with IL-6 or TNF-α to induce inflammation, it resulted in increased TREM-1 and decreased TREM-2 expression, but addition of calcitriol to these IL-6 and TNF-α treated cells results in decreased effect of IL-6 and TNF-α on TREM-1 and TREM-2 expression (Fig.12 C-D) suggesting that calcitriol suppresses the inflammation in diseased state and increases production of pro-inflammatory cytokines in non-diseased state. Being an anti-inflammatory agent vitamin D can play a role in upregulation of innate immunity. Treatment with calcitriol up regulates TREM-1 expression in differentiating and mature monocytes/macrophages and human myometrial cells [102] and downregulates TREM-2 expression in human myometrial cells [63]. The upregulation of TREM-1 expression involves NF-kB signalling pathway. It was suggested that enhanced TREM-1 expression is due to increased synthesis of the protein (increased TREM-1 transcription via functional vitamin D response element-VDRE) and not due to endotoxin contamination. Upregulation of TREM-1 by calcitriol suggests the role of vitamin D in enhancing innate immunity.

High mobility group box 1 (HMGB-1) is a non-histone nuclear binding protein promoting the maintenance of nucleosomal structure, and regulating the gene transcription, DNA
recombination, DNA repair and replication. HMGB-1, a TREM-1 ligand, gets secreted from the necrotic hepatocyte due to DNA damage, macrophage after inflammation and parenchymal cells after necrosis, and elicits an inflammatory response [7]. IF staining of HepG-2 and THLE-2 cells showed the expression of HMGB-1 and the immunoreactivity of HMGB-1 was more in HepG-2 cells compared to THLE-2 cells suggesting its higher activity in HCC. The cytoplasmic as well as nuclear staining of HMGB-1 indicates its cytoplasmic as well as nuclear location in the cell. It acts as damage associated molecular pattern and can activate the pro-inflammatory pathways. HMGB-1 association with TREM-1 receptor on hepatocyte results in activation of pro-inflammatory pathways [8]. To further analyse the role of HMGB-1 in activating the TREM-1 in HCC cells, I treated the HepG-2 cells with recombinant HMGB-1 protein (rHMGB-1) and found that there is an increase in immunoreactivity of TREM-1 after rHMGB-1 treatment (Fig.18-19). Similar results were found in RT-PCR studies. HMGB-1 binding to the receptor for advanced glycation end product (RAGE) results in activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPK). Further it result in increased production of various pro-inflammatory cytokines namely TNF-α, IL-6, IL-1β and IL-1α. The secreted HMGB-1 is also involved in cellular movements, chemotaxis, regeneration and cell repair [7]. Since HMGB-1 stimulate the secretion of IL-6 and TNF-α, to analyse it in HepG-2 cells I treated the hepG-2 cells with rHMGB-1 and did the RT-PCR analysis of the extracted RNA. There was an increase in expression of both IL-6 and TNF-α (Fig.18). Immunofluorescence studies further supported the results of RT-PCR studies and I found increased immunoreactivity for IL-6 and TNF-α after treating the cells with rHMGB-1 (Fig. 19).

Further I examined the downstream signalling pathways and transcription factors involved in production of IL-6 and TNF-α in HepG-2 cells and the effect of calcitriol on these signalling pathways. Involvement of p65MAPK, ERK1/2, Akt, and STAT3 molecules in TREM-1
signalling pathways as downstream effectors for the TREM-1 transduction in HCC has been reported [103-104]. The secretion of pro-inflammatory cytokines and chemokines by TREM-1 is modulated by various signalling pathways. The important contributory role of TREM-1/DAP-12 signalling receptor in Kupffer cells in regulation of inflammation has been known. Further activation of TREM-1/DAP-12 signalling receptor leads to activation/phosphorylation of signalling pathways like AKT and ERK1/2 resulting in increased secretion of pro-inflammatory cytokines [8, 105-106]. It has also been reported that TREM-2/DAP-12 association leads to phosphorylation of PI3K/Akt and ERK1/2 [107], but the TREM-2 signalling is cell type specific [20]. I treated the HepG-2 cells with the inhibitors of PI3K (LY294002) and ERK1/2 (U0126) and subjected the extracted RNA to RT-PCR. I found that TREM-1 and TREM-2 expression gets attenuated (Fig.20). Further simultaneous treatment of HepG-2 cells with calcitriol and inhibitors further decreases the TREM-1 expression but the inhibitory effect of the inhibitors on TREM-2 suppresses. Addition of pro-inflammatory cytokines to the treatment attenuates the inhibitory effect of inhibitors in both TREM-1 and TREM-2 (Fig.20). Immunofluorescence studies with the inhibitors, calcitriol and pro-inflammatory cytokines gave the similar results confirming the results of RT-PCR (Fig.21-24). These results suggest that PI3K and ERK1/2 are involved in expression of TREM-1 and TREM-2 and calcitriol has an inhibitory effect on these signalling pathways. PI3K/ Akt signalling is also involved in TREM-2 mediated immune response and TREM-2 suppresses the inflammation via PI3K/Akt activation. Furthermore it has been reported that silencing of TREM-2 inhibits the PI3K/Akt phosphorylation, upregulates the production of pro-inflammatory cytokines like TNF-α, IL-1β and MIP-2[26], and over-expression of TREM-2 attenuates the production of IL-6 and TNF-α [20, 22], but has no effect on ERK, JNK and p38MAPK. The results of rHMGB-1 treatment increasing TREM-1, IL-6 and TNF-α expression and signalling inhibitors provides another potential target, by suppression of
HMGB-1 and inhibition of signalling pathways, to decrease the secretion of pro-inflammatory cytokines in diseased condition and resulting to delay in disease progression.

The downstream transcription factors involved in the activation of TREM-1 gene are AP-1, PU.1 and NF-kB [108]. Previous studies have supported the involvement of NF-kB as a link between inflammation and carcinogenesis [109-110]. NF-kB crucially regulates inflammation, cell proliferation, differentiation and apoptosis, innate and adaptive immunity [111] and serve as downstream signal transduction molecule for TREM-1[108]. PU.1, a member of Ets domain transcriptional factor family with abundant expression in B cells and macrophage, is involved in protein-protein interaction. Activation of PU.1 by LPS has been reported in monocytes and RAW cells [108]. Role of PU.1 in macrophage maturation [112] and inflammation by regulating COX-2 gene expression [113] has been reported. I found the co-localization of TREM-1 and TREM-2 with PU.1 in THLE-2 and HepG-2 cells (Fig.25). RT-PCR studies for PU.1 expression in HepG-2 cells showed that calcitriol increases PU.1 expression while the pro-inflammatory cytokines IL-6 and TNF-α decreases the PU.1 expression (Fig.26 A). It was observed that treatment of HepG-2 cells with PU.1 siRNA decreases the PU.1 expression and increases the TREM-1 expression (Fig.26 B-C). Further I treated the HepG-2 cells with PU.1 plasmid and subjected the extracted RNA to RT-PCR for PU.1 and TREM-1 expression and found that PU.1 plasmid expression increases and TREM-1 expression decreases with PU.1 plasmid (Fig.26 D and E). These results show that overexpression of PU.1 has an inhibitory regulation on TREM-1 expression and that decreased PU.1 increases TREM-1 expression. Previously it has been shown that PU.1 and NF-kB binds to the promoter region of TREM-1 gene upon LPS stimulation and activation of NF-kB induces TREM-1 expression while PU.1 has a negative regulatory role on TREM-1 expression. It has been reported that PU.1 silencing results in enhanced expression of TREM-1 while PU.1 overexpression results in attenuation of TREM-1 expression in macrophages. It
was also reported that activation of TREM-1 by LPS involves binding of NF-kB and AP-1 very early and PU.1 binding takes place at a later time point to the promoter region of TREM-1 gene [108]. NF-kB under unstimulated condition remains coupled with IkB, the inhibitor complex of NF-kB. Phosphorylation of IkB takes place on stimulation of NF-kB, by various signal transduction cascades. LPS, TNF-α, IL-1β, IL-6 and reactive oxygen species can initiate activation of NF-kB. Stimulated NF-kB influences the gene expression of various inflammatory molecules and transcription factors involved in innate immune response [109, 114-116]. It has been reported that TREM-1 expression is regulated by NF-kB at transcriptional level and upon LPS stimulation NF-kB binds to promoter region of TREM-1 and enhances TREM-1 mRNA expression. Since NF-kB plays a role in TREM-1 expression our next interest was to analyse the effect of vitamin D on expression of NF-kB and effect of NF-kB inhibition on TREM-1 expression with and without calcitriol and pro-inflammatory cytokines. Treatment of HepG-2 cells with calcitriol resulted in attenuation of NF-kB expression. Further I found that IL-6 and TNF-α increase the expression of NF-kB in HepG-2 cells but the stimulatory effect of IL-6 and TNF-α on NF-kB get suppressed by calcitriol (Fig. 27 A). When the HepG-2 cells were treated with NF-kB inhibitors (BAY11-7085 and PTDC) I found that PTDC significantly (p<0.05) decreases the expression of TREM-1 but not of TREM-2 (Fig.27 B-C) suggesting that NF-kB signalling is involved in TREM-1 expression but not in TREM-2 [13, 107]. Immunofluorescence staining of HepG-2 cells with NF-kB inhibitors (BAY11-7085 and PTDC) also reveals the suppression of TREM-1 (Fig. 28 and 30) but not of TREM-2 (Fig. 29 and 31) and supported the results of RT-PCR (Fig.27) in our study. In summary vitamin D has a stimulatory effect of PU.1 and suppressive effect on NF-kB expression (Fig. 36).

TREM-1 plays a role in invasiveness of cancer [85] and its role in invasion of HCC has been stated in previous studies [16]. TREM-1 significantly (p<0.05) promotes the proliferation and
invasion and inhibits the apoptosis of the HCC cells. High TREM-1 is correlated with poorer survival and increased recurrence of HCC and is a prognostic factor for HCC[19]. IL-6 affects the cell proliferation, survival, differentiation, migration, invasion and proliferation [117]. The enhanced and uncontrolled proliferation of the hepatocyte results in development of HCC. On treating the HepG-2 cells with calcitriol I observed that calcitriol decreases migration, invasion and proliferation of HepG-2 cells (Fig. 32-35). On treating the HepG-2 and THLE-2 cells with IL-6 and TNF-α I found increased migration of cells (Fig. 32), but additional treatment of these cells with calcitriol along with IL-6 and TNF-α resulted in decreased migration of HepG-2 cells and decreased migration of THLE-2 cells. These findings suggest that vitamin D has an inhibitory effect on pro-inflammatory cytokines or vitamin D decreases the IL-6 effect on HepG-2 cells. The suppressive effect of vitamin D on IL-6 secretion and function has been reported by previous studies [118]. Since it has been reported that suppression of IL-6 results in growth inhibition of breast [119] and prostate cancer [120], hence calcitriol can be of therapeutic use in suppressing and delaying the progression of various stages of HCC development as well as inhibition of HCC tumor. Transwell invasion assay of HepG-2 cells also reveals decreased invasion of HepG-2 cells with calcitriol (Fig. 33). The mRNA studies of proliferation marker Ki-67 and proliferation assay with CCK-8 kit showed the inhibitory effect of calcitriol on untreated and treated (IL-6 and TNF-α) HepG-2 cells (Fig. 34-35). It was interesting to observe that the inhibitory effect of calcitriol was up to 48 hours and decline thereafter. These results shows that vitamin D inhibits proliferation, migration and invasion of HepG-2 cells and the effect of increasing proliferation, migration and invasion of HepG-2 cells by pro-inflammatory cytokines like IL-6 and TNF-α gets attenuated by calcitriol. These results are in accordance with previous studies that proliferation of hepatoma cell line including HepG2 liver cancer cells can be inhibited by vitamin D [51, 81, 91, 121-122].
There are reports of low levels of vitamin D in association with liver disease, increased liver damage and mortality in alcoholic liver disease [59], non-alcoholic steatohepatitis, non-alcoholic fatty liver disease [53], and chronic hepatitis [60, 123-124]; and in development of HCC [125-127], but no direct causal relationship between vitamin D deficiency and liver disease has been established. For the first time we are reporting the suppression of TREM-1 expression as well as the pro-inflammatory cytokines effect on HepG-2 cells. These results pave a way for the further analysis of estimation of TREM-1 level as a marker of decreasing inflammation in tissues with vitamin D supplementation. It is known that chronic liver disease and HCC are accompanied with enhanced innate immune system and inflammation and conversely related to vitamin D levels. There are evidences for biological role of vitamin D in liver diseases and it has been reported that low levels of vitamin D is a marker of severity/prognosis in alcoholic cirrhosis patients. Improved viral response with vitamin D has been reported in HCV patients [128-129]. High TREM-1 level is associated with poor overall survival and decreased rate of relapse free survival and may serve as prognostic factor for post-operative HCC recurrence[19]. Results of our study suggest that vitamin D suppresses the expression of inflammatory marker and effect of pro-inflammatory cytokines and enhances the expression of TREM-2; hence can be used as a supportive therapeutic option in various staged of liver diseases and may be a prognostic factor in liver cirrhosis patients and can acts as an anti-infective agent [130].
Future directions

Hepatitis B and hepatitis C virus infection is the main risk factor for development of HCC. The prevalence of HBV infection in the United States is approximately 0.4% [131]. Approximately 5% of the world population is chronically infected with HBV and 2% is chronically infected with HCV [132]. Some of the patients with HBV and HCV infection clear the virus infection but most remain unable to clear the virus. Some progress to cirrhosis and may subsequently develop HCC. As it is known that IL-6 and TNF-α are necessary for liver regeneration and hepatocyte growth and differentiation, it is important to know what levels of IL-6 and TNF-α are responsible for progression of chronic hepatitis liver to cirrhosis and HCC. TREM-1 may serve as a marker for this purpose as IL-6 and TNF-α production is TREM-1 dependent. So measuring TREM-1 at various stages and supplementing vitamin D may serve as an adjunct therapeutic option, but further studies are needed for estimation of serum TREM-1 in fatty liver, hepatitis, steatohepatitis and cirrhosis. Another stimulus which can activate the TREM-1 in hepatocytes is bacterial endotoxin lipopolysaccharide (LPS). TREM-1 stimulation by LPS through toll like receptors (TLRs) involving MyD88 results in activation of neutrophils and monocytes as well as secretion of the pro-inflammatory cytokines [108]. LPS induced activation of TLR-4 promotes cell survival and proliferation in hepatocellular carcinoma [133]. It has been reported that LPS treatment results in enhanced production of pro-inflammatory cytokine IL-6 [28]. The role of vitamin D in activation of TLRs can also be further studied. LPS also play a role in regulation of PU.1 and NF-kB at the transcriptional level [108]. LPS is a product of inflammation and, being an anti-inflammatory agent, vitamin D may affect the LPS mediated TREM-1 and TLR-4 activation. So the effect of vitamin D on LPS mediated TREM-1 activation through TLRs and regulation of TLRs expression might be the next step to investigate. Further TREM-2 counters the amplification of inflammatory response and acts to suppress inflammation; it will be interesting to study
that how TREM-2 can be activated in hepatic cells with inflammation. TREM-2 activation may be another effective way to prevent the ongoing inflammation in hepatocytes.

Since HCC develops through various stages from fatty liver, hepatitis, fibrosis, steatohepatitis and cirrhosis, it will be important to examine the serum TREM-1 level at these stages to evaluate the inflammation. Low levels of vitamin D have been found in HBV and HCV infection [60-61, 123, 134-135] and vitamin D has been reported to suppress HCV infection [124, 136]. Administration of vitamin D at various stages may attenuate the progression of different stages to cirrhosis and HCC and modulate the HCC development. Since the growth of a tumor is due to continuous increase in the cell mass and number by replication due to chronicity of infection, inhibition of replication of HCV and HBV by vitamin D seems to be useful. Further direct administration of vitamin D or its analogue can inhibit the tumor growth [134, 136].

The association of vitamin D deficiency and various inflammatory diseases has been discussed in animal models [137-138]. It has also been suggested that vitamin D supplementation can reduce inflammatory markers and inflammation [139]. Knowing that hypovitaminosis D is prevalent in the United States [140-141] and worldwide and low vitamin D levels has been a marker for severity and prognosis in various liver diseases like alcoholic liver disease, hepatitis and cirrhosis [59], it may be important to supplement vitamin D at various stages of liver disease to delay the development of HCC by suppressing inflammation. Monitoring the serum TREM-1 level at different stages to know the effect and outcome of vitamin D supplementation may be fruitful. Thus TREM-1 and vitamin D level monitoring can be a prognostic parameter and vitamin D supplementation can be a potential therapeutic option in liver disease patients and can delay or prevent progression of liver disease and development of HCC [125]. Vitamin D suppresses TREM-1 expression, proliferation, invasion and migration, signalling pathways and transcriptional factors, and
attenuation of effect of IL-6 and TNF-α on TREM-1 expression. This provides molecular and biochemical evidence that vitamin D supplementation in conjunction with the treatment of liver disease (at the stage of hepatitis, fibrosis and chronic hepatitis) to suppress TREM-1 may be a novel strategy and promising target for delaying the progression of liver disease and hepatocellular carcinoma.
References


