

## Research Article

# 25(OH)-D3 Alleviate Liver NK Cytotoxicity in Acute but not in Chronic Fibrosis Model of BALB/C Mice due to Modulations in Vitamin D Receptor

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## Abstract

**Background:** Low 25-Hydroxy-vitamin-D; “25(OH)-D3” serum and vitamin D receptor (VDR) levels were recently correlated to advanced fibrosis. However, VDR mechanism in liver fibrosis modulations is not well understood.

**Aim:** To evaluate associations of VDR with NK cell modulations during fibrogenesis.

**Methods:** Carbon-tetrachloride (CCl<sub>4</sub>) hepatic-fibrosis was induced in BALB/c mice for 1 and 4 weeks as an acute and chronic fibrosis model, respectively. Along 1<sup>st</sup> to 4<sup>th</sup> weeks; vitamin D were i.p injected/2x week. Liver were assessed histologically and for proteins quantification for VDR and fSMA expressions. *In vitro*, potential killing of NK cells were evaluated following co-culture with primary-hepatic-stellate-cells (pHSCs) obtained from BALB/c WT-mice.

**Results:** Hepatic-fibrosis increased along 4 weeks of CCl<sub>4</sub> as indicated by serum ALT, αSMA expressions (P<0.02) and histological assessments. These results were associated with increased NK1.1 activations and hypercalcemia. While vitamin D administrations delayed fibrosis of early stages, vitamin D worsen hepatic-fibrosis of late stages of CCl<sub>4</sub>. In week 4, no further activations of NK cells were seen following vitamin D injections and were associated with down-expressions of VDR (1.7 Fold, P<0.004) indicating the inability of vitamin D to ameliorate hepatic fibrosis. *In vitro*, NK cells from the chronic model of CCl<sub>4</sub> did not affect pHSCs killing and fail to reduce fibrosis.

**Conclusion:** Vitamin D alleviate liver NK cytotoxicity in acute but not in chronic fibrosis model due to modulations in vitamin D receptor and calcium. Hypercalcemia associated with late fibrosis may inhibited VDR levels, however, may not explain the profibrogenic effects of vitamin D.

**Keywords:** Hepatic fibrosis; NK cells; Vitamin D receptor; Hypercalcemia

## Abbreviations

Vitamin D receptor- VDR, Hepatic stellate cells-HSCs, Carbon tetrachloride-CCl<sub>4</sub>, 25-(OH) D3 -Vitamin D.

## Introduction

Vitamin D is an important prohormone with known effect on calcium homeostasis [1], but recently there is increasing recognition that vitamin D also is involved in cell proliferation and differentiation, it has immunomodulatory and anti-inflammatory properties [2]. The effects of vitamin D are mediated through the vitamin D receptor (VDR) [3]. VDR is a member of the nuclear receptor super-family of ligand-inducible transcription factors, which are involved in many physiological processes, including cell growth and differentiation, embryonic development and metabolic homeostasis [4]. The transcriptional activity of this receptor is modulated by several ligands, such as steroids, retinoids and other lipidsoluble compounds, and by nuclear proteins acting as co-activators and co-repressors [5]. The liganded VDR heterodimerizes with the retinoid X receptor and

binds to vitamin D response elements in the promoter of target genes, thereby affecting their transcription. The genomic organization of the VDR at locus 12q13.1 shows that the VDR gene itself is quite large (over 100kb) and has an extensive promoter region capable of generating multiple tissue-specific transcripts [6].

Clinical observations have recently demonstrated that 25-OH D serum levels were significantly lower in patients with chronic hepatitis C than in controls and that low 25-OH D serum levels were associated with more severe fibrosis and lower responsiveness to interferon-based therapy in those patients [7,8]. Other studies have shown that low 25-OH D serum levels are associated with poor liver function and more advanced stages of liver fibrosis in hepatitis C virus patients [9]. Moreover, a possible role for vitamin D in liver fibrosis has gained further support from the finding that VDR is expressed in human as well as in rat liver nonparenchymal cells, such as Hepatic stellate cells (HSCs) [10]. It has recently been suggested that VDR polymorphism is associated with primary biliary cirrhosis [11]. Vitamin D deficiency is a common phenomenon in chronic liver disease, particularly in

advanced fibrosis and cirrhosis [12]. Whether this association reflects the cause of accelerated fibrosis progression or the consequence of impaired liver function in advanced disease is still unclear.

## Materials and Methods

### Animals

Male mice on the BALB/c background, 12 weeks of age, weighing  $22 \pm 0.5$  g, received care according to NIH guidelines. Mice were purchased commercially from Harlan Laboratories, Jerusalem-Israel. All animal protocols were approved by the institutional animal care ethical committee of the Hebrew University and housed in a barrier facility.

### CCl<sub>4</sub> Model of Liver Injury and Fibrosis

BALB/c mice were IP injected with 0.5ml/kg body weight CCl<sub>4</sub> (1:10 v/v in corn oil from Sigma) or vehicle (corn oil) twice a week for 4 weeks. 25(OH) D3 (Biogems, Cat# 3220632-10mg) at the dose of 0.5 microgram/100g body weight was IP injected twice a week, commencing one day after the first dose of CCl<sub>4</sub>. The animals were terminated 72hr after the final CCl<sub>4</sub> injection, and whole livers and serum were collected for histological, cytological and biochemical analyses.

### Alanine aminotransferase (ALT)

Blood samples were collected from BALB/c male hearts at the volume of 1ml blood; the samples were centrifuged for 5min at 4000rpm. Serum samples were drawn to an Eppendorf tubes, after blood centrifuge. Serum samples of 32 $\mu$ l were dropped onto the strip of the ALT (Reflotron Company) and analyzed by Reflovet<sup>®</sup> plus Roche.

### Liver NK cells isolation

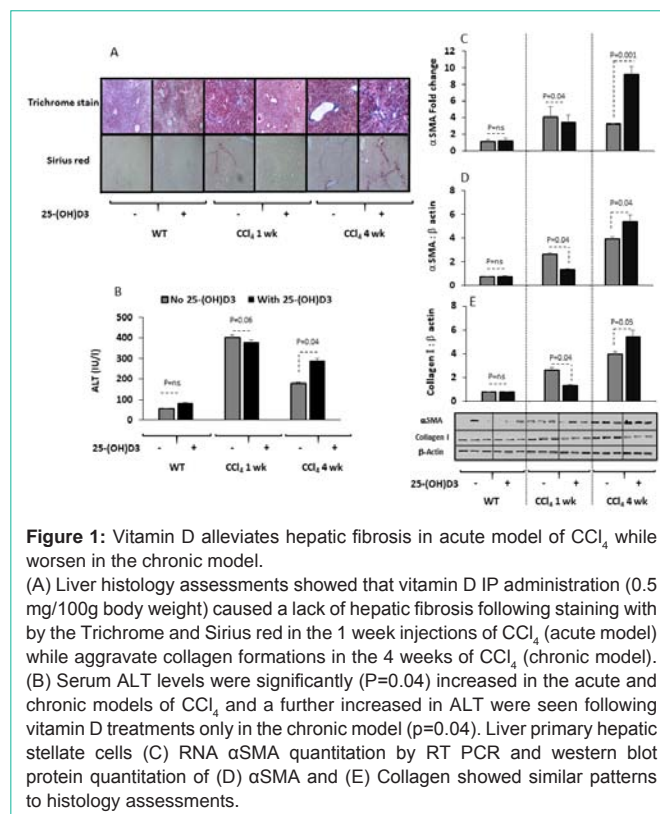
Under deep ether anesthesia, mice were euthanized by isoflurane, USP 100% (INH), then the liver was removed and a part of the liver was transferred to Petri dish that contains 5ml DMEM medium (Biological industries; Cat# 01-055-1A). The liver tissue was thoroughly dissected by stainless steel mesh, the cells were harvested with the medium and added to 50ml tubes that contain 10ml DMEM, and then carefully cells were transferred to new tubes that contain Ficoll (Abcam; Cat# AB18115269). Tubes were centrifuged for 20 minutes, at 1,600rpm at 20°C. The supernatant in each tube was transferred to a new tube, for another centrifuge for 10 min, at 1,600 rpm at 4°C. After the second centrifuge, the pellet in each tube was resuspended in 1ml of DMEM for NK isolation kit (Stem Cells; Cat# 19665).

### Primary HSCs isolation

HSCs were isolated from 12-week-old male BALB/c mice by in situ pronase, collagenase perfusion, and single-step Histogenz gradient as previously reported (Hendriks et al., 1985; Knook et al., 1982). Isolated HSCs were cultured in Dulbecco's modified Eagle's medium (Mediatech) containing 20% fetal bovine serum (Hyclone) on six-well plates for 40hr prior to end-point assays.

### Flow cytometry

Harvested mice liver NK cells were adjusted to 10<sup>6</sup>/ml in buffer saline containing 1% bovine albumin (Biological Industries; Cat# 02-023-5A), and were stained for the following antibodies: Anti-mice NK1.1 (murine NK cell marker) (Biogems; Cat# 83712-70), Anti CD49a (MACS; Lot# 5150716246), and anti-mice Lysosomal-



**Figure 1:** Vitamin D alleviates hepatic fibrosis in acute model of CCl<sub>4</sub> while worsen in the chronic model.

(A) Liver histology assessments showed that vitamin D IP administration (0.5 mg/100g body weight) caused a lack of hepatic fibrosis following staining with by the Trichrome and Sirius red in the 1 week injections of CCl<sub>4</sub> (acute model) while aggravate collagen formations in the 4 weeks of CCl<sub>4</sub> (chronic model). (B) Serum ALT levels were significantly (P=0.04) increased in the acute and chronic models of CCl<sub>4</sub> and a further increased in ALT were seen following vitamin D treatments only in the chronic model (p=0.04). Liver primary hepatic stellate cells (C) RNA αSMA quantitation by RT PCR and western blot protein quantitation of (D) αSMA and (E) Collagen showed similar patterns to histology assessments.

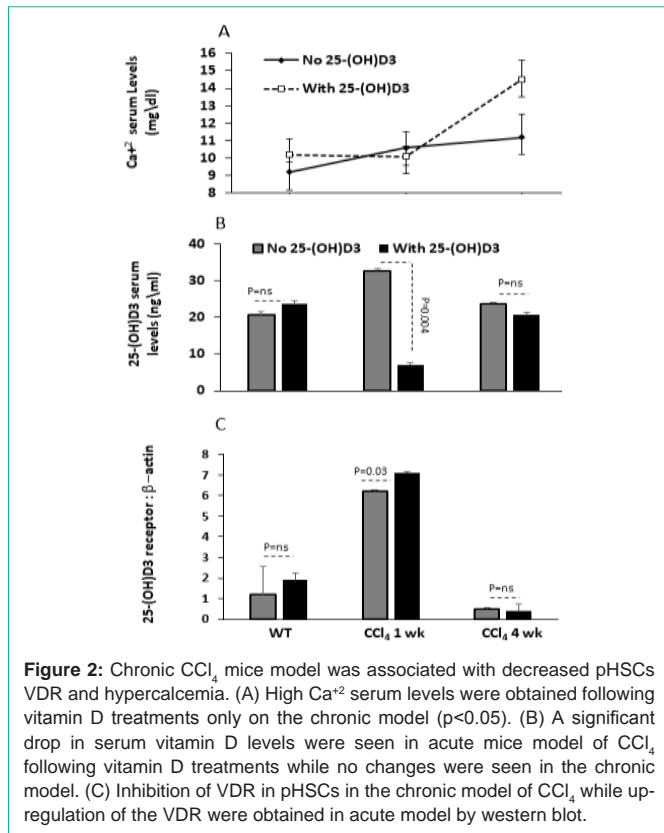
associated membrane protein-1 (CD107a; NK1.1 cells cytotoxicity marker) (eBioscience; Cat# 48-1071). All antibodies were incubated for 45min at 4°C. The cells were washed with 0.5ml of staining buffer and fixed with 20ml of 2% paraformaldehyde. All stained cells analyzed with a flow cytometer (The BD LSRFortessa™, Becton Dickinson, Immunofluorometry systems, Mountain View, CA).

### Real Time PCR

**Tissue RNA isolation:** Total cellular RNA was isolated from liver tissue, using 2ml TRI Reagent (Bio LAB; Cat# 90102331) for each cm<sup>3</sup> of tissue. The samples were homogenized for 5min at room temperature of 25°C. Chloroform at volume of 0.2ml (Bio LAB; Cat# 03080521) were added to each sample, incubated for 15min at room temperature and centrifuged (1,400rpm) for 15min at 4°C. RNA precipitation: The supernatant in each sample was transferred to new Eppendorf, 0.5ml of isopropanol (Bio LAB; Cat# 16260521) left for 10min at 25°C and centrifuged (12,000rpm) for 10min at 4°C. The supernatants were removed and one ml of ethanol 75% were added to the pellet and centrifuged (7,500rpm) for 5min. The pellets were dried in air at room temperature for 15min, 50 $\mu$ l of DEPC were added, and the samples were heated for 10min at 55°C.

**cDNA preparation:** Liver RNA was extracted as described above. Preparing of c-DNA was performed using High Capacity cDNA Isolation Kit (R&D; Cat # 1406197).

**Real time PCR:** Real-time PCR is performed for the quantification of the expression of the gene that encoded alpha Smooth Muscle Actin (αSMA) and Vitamin D Receptot (VDR), compared to GAPDH as a housekeeping gene by using Taqman Fast Advanced Master Mix (Applied Biosystem; Cat # 4371130).

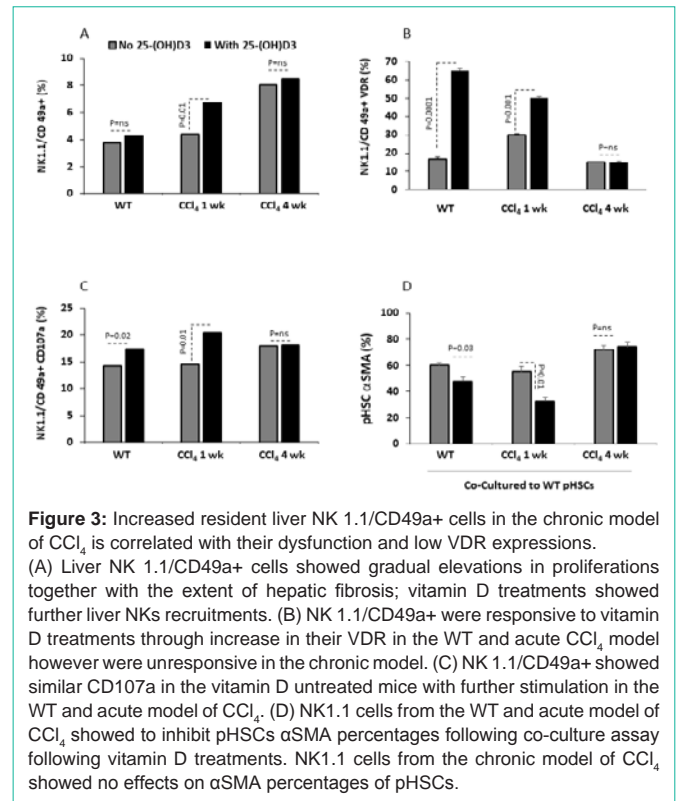


**Western blot analysis**

Immunoblot analysis of αSMA in liver extracts performed. Following isolation, whole protein extracts were prepared in liver homogenization RIPA buffer (Sigma; R0278-50ML) with protease phosphatase inhibitor cocktail (Roche; 1183617011). Next, proteins (30µg per lane) were resolved on a 10% (wt/vol) SDS-polyacrylamide gel (Novex, Groningen, The Netherlands) under reducing conditions. For immunoblotting, proteins transferred to a PVDF membrane. Blots incubated 1h at 4°C temperature at in a blocking buffer containing 5% skim milk and then incubated with either mouse Mouse Anti Human/Mice α-SMA (Novusbio; NB600-531), Rabbit Anti-Collagen I (ab34710), Rabbit Anti-VDR (ab109234) and Mouse Anti Human/Mice β-Actin (R&D System; 937215), diluted 1/1000, overnight in 4°C, and subsequently, with peroxidase-conjugated goat anti-mouse and Rabbit IgG (PARIS, Compiègne, France) diluted 1/5,000, for 1.5h at room temperature. Immunoreactivity revealed by enhanced chemiluminescence using an ECL Kit (Abcam; ab133406).

**Histological assessments of liver injury**

The posterior one third of the liver was fixed in 10% formalin for 24 hours and then paraffin-embedded in an automated tissue processor. Seven-millimeter liver sections were cut from each animal. Sections (15mm) were then stained in 0.1% sirius red F3B in saturated picric acid as well as Masson trichrome stain for connective tissue stain (both from Sigma). Sirius red staining assessed using the modified Histological Activity Index (HAI) criteria, incorporating semi quantitative assessment of periportal/periseptal interface hepatitis (0-4), confluent necrosis (0-6), focal lytic necrosis/apoptosis and focal inflammation (0-4), portal inflammation (0-4), and architectural



changes/fibrosis and cirrhosis (0-6).

**Statistical analysis**

The results were evaluated using the Student’s t-test, with statistical significance set at P<0.05. Comparison between the mean values of different experiments was carried out. All data were reported as mean ± SE.

**Results and Discussion**

**25-(OH) D3 (vitamin D) reduced liver fibrosis and inflammation in acute while worsen fibrosis in chronic model of CCl<sub>4</sub>**

Hepatic fibrosis was induced in BALB/c mice by biweekly IP CCl<sub>4</sub> injections for 1 week (acute model) and 4 weeks (chronic model) and was compared with naive vehicle-treated mice (WT). Histological staining, ALT levels and αSMA expressions were performed for fibrosis assessments. Figure 1A shows staining of Trichrome and Sirius red for the three major animal groups with and without IP administration of vitamin D as described in materials and methods. Acute and chronic model of CCl<sub>4</sub> showed gradual extent of collagenous connective tissue fibers of red fibrosis septae with both Trichrome and Sirius red stains in consistent with CCl<sub>4</sub> exposure time.

Fibrotic mice of WT and acute model of CCl<sub>4</sub> treated with vitamin D showed attenuation in their fibrosis histology that lack red fibrosis septae. On the other hand, vitamin D treatments in the chronic CCl<sub>4</sub> model, in the contrary, had propagate liver fibrosis as seen in liver histological samples (Figure 1A).

To further evaluate effects of vitamin D on liver injury, serum

ALT levels and liver samples for quantitation of fibrosis were performed. Figure 1B shows serum ALT levels were significantly ( $P=0.05$ ) increased from  $33.1\pm 7.5$  in naïve WT mice to  $401.5\pm 45$  IU/L and  $178\pm 21$  in acute and chronic model of  $\text{CCl}_4$ , respectively. While vitamin D treatments had no effects on WT mice, it decreased ALT levels in acute model of  $\text{CCl}_4$  to  $380\pm 29.4$  IU/L ( $p=0.04$ ) and caused elevations in ALT levels in the chronic model of  $\text{CCl}_4$  up to  $288.5\pm 30.5$  IU/L ( $P<0.05$ ). Gene expressions of liver  $\alpha$ SMA fibrotic marker by RT PCR (Figure 1C),  $\alpha$ SMA liver portion expressions by western blot analysis (Figure 1D) and liver collagen quantitation (Figure 1E) showed similar patterns to ALT. The above results showed dual anti- and pro- fibrotic effects of vitamin D could be related to extent of fibrosis stage.

### Liver vitamin D receptor (VDR) expressions were inhibited with fibrosis progressions and were unresponsive to vitamin D treatments

The vitamin D hormone, 1,25-dihydroxyvitamin D(3) [1,25(OH)(2)D(3)], binds with high affinity to the nuclear vitamin D receptor (VDR), and is considered as primary regulator of calcium ( $\text{Ca}^{+2}$ ). We aimed to evaluate  $\text{Ca}^{+2}$ , vitamin D and VDR changes in our mice model with and without vitamin D treatments. Figure 2A shows similar  $\text{Ca}^{+2}$  serum levels in our mice model prior to vitamin D treatments. Vitamin D treatments did not change  $\text{Ca}^{+2}$  serum levels in WT and acute model of  $\text{CCl}_4$ , however, it did significantly elevated  $\text{Ca}^{+2}$  in the chronic model of  $\text{CCl}_4$ . In Figure 2B, vitamin D serum levels were significantly elevated in the acute model of  $\text{CCl}_4$  and were inhibited in the chronic model to levels similar to WT mice. Vitamin D treatments while did not alter vitamin D serum levels in the WT and chronic model of  $\text{CCl}_4$ , it significantly dropped vitamin D serum levels 4.6 fold ( $P=0.004$ ) in the acute model.

Therefore, our next aim were whether changes in serum vitamin D are related to modulations in the VDR. For this purpose, primary hepatic stellate cells (pHSCs) obtained from livers of mice were quantitated for VDR by the western blot analysis. Figure 2C shows upregulation in the expressions of VDR in acute model of  $\text{CCl}_4$  as compared to the WT, while total loss of VDR expressions were seen in the chronic model of  $\text{CCl}_4$  and indicate that VDR expressions were modulated along the course of the liver injury. Vitamin D treatments significantly caused increased in VDR expressions in acute model of  $\text{CCl}_4$  ( $P=0.03$ ) while had no effects in VDR expressions in the WT and the chronic model of  $\text{CCl}_4$ .

The above results indicate increased  $\text{Ca}^{+2}$  serum levels in chronic model of  $\text{CCl}_4$  treated with vitamin D could be as a result of liver injury and in particular due to degradation of vitamin D in these mice. Consequently, these effects were associated with inhibited VDR and therefore, unlike the acute model, chronic mice model of  $\text{CCl}_4$  were unresponsive to vitamin D treatments (Figure 2B and 2C). Moreover, in the acute model of  $\text{CCl}_4$  due to the upregulation of VDR, vitamin D treatments were up taken/consumed explaining their low vitamin D levels in their serum and calcium serum levels that were unchanged.

In consistent to our data, vitamin D deficiency has been frequently reported in many causes of chronic liver disease and has been associated with the development and evolution of non-alcoholic fatty liver disease (NAFLD) [12] and chronic hepatitis C (CHC) virus infection [9].

### NK cells have VDR and it is crucial for their stimulations and modulation of liver fibrosis

We next sought to determine effects of vitamin D on immune alterations in liver fibrosis. We previously showed that NK cells exert anti-fibrotic effects through killing of activated HSCs. Liver resident NK cells (NK1.1/CD49+) cellular infiltrates as well as their expressions of VDR and their activity (CD107a) were determined by the flow cytometry. Figure 3A shows an linear correlation between NK cells infiltrates with the progression of liver fibrosis. Vitamin D caused an additional increase in the NK1.1 cells infiltrates ( $p=0.01$ ) only in the acute model of  $\text{CCl}_4$  that was not seen in the WT and the chronic model of  $\text{CCl}_4$  indicating a possible fibrosis inhibition in this model that were also emphasized in our precious data summarized in Figure 1 and Figure 2. VDR expressions on liver NK1.1 increased in the acute while inhibited in the chronic model of  $\text{CCl}_4$  to levels similar to WT. The VDR were upregulated following treatments with vitamin D in the WT and acute model of  $\text{CCl}_4$ . No changes in the VDR were noticed in the NK1.1 cell from chronic model of  $\text{CCl}_4$  following the vitamin D treatments (Figure 3B). These data suggest inverse relation between NK1.1 cell proliferation (Figure 3A) and their VDR expressions (Figure 3B) prior and following vitamin D treatments.

To further correlate whether changes in the VDR on NK1.1 cells is associated with their phenotype alterations; CD107a (activity marker) was determined by the flow cytometry as described in materials and methods. CD107a NK1.1 percentages were high in both  $\text{CCl}_4$  models to similar levels of the WT. Vitamin D treatments further elevated NK1.1 cells activity in the WT and the acute model of  $\text{CCl}_4$  ( $P<0.05$ ) while did not alter NK1.1 activities in the chronic model most probably because the VDR is low in the chronic model of  $\text{CCl}_4$ . In order to determine whether changes in CD107a and VDR on NK1.1 cells could modulate liver fibrosis; we co-cultured liver NK1.1 cells with pHSCs through an *in vitro* setting assay to assess cytotoxicity/killing potential. Figure 3D shows NK1.1 cells obtained from the acute model of  $\text{CCl}_4$  reduced  $\alpha$ SMA percentages of pHSCs as compared to their WT counterparts, a result that indicate killing of pHSCs. NK1.1 cells from the chronic model of  $\text{CCl}_4$  did not change  $\alpha$ SMA of the pHSCs indicating inability of these cells to reduce fibrosis. Vitamin D treatments further exacerbate NK1.1 killing potentials in both the WT and the acute model and inhibited fibrosis while did not cause any changes in  $\alpha$ SMA of the pHSCs in the chronic model of  $\text{CCl}_4$ .

The above results are associated with VDR lost in NK1.1 cells from the chronic model of  $\text{CCl}_4$  that attenuated their potentials to kill. HSCs play a critical role in apoptosis and inflammation [10]. Activated HSCs secrete pro-fibrogenic cytokines, produce  $\alpha$ SMA and pro-collagen I, and are known to be a major source of collagens and inhibitors of matrix-degrading enzymes (tissue inhibitor of matrix metalloproteinase, TIMP) that are secreted during fibrosis [10].

Studies show that hepatocytes isolated from humans, rats, and mice express little or no VDR mRNA and protein [13,14]. On the other hand, non-parenchymal cells, including HSCs, sinusoidal endothelial cells, and Kupffer cells (KCs), strongly express VDR [15], and these probably mediate vitamin D effects in the non-diseased state. However, levels of VDR in hepatocytes increase with inflammation, giving a broader site for potential targets. Levels also increase in HSCs and KCs with inflammation, and several immune

functions of VDR described in other tissues may be involved in liver disease.

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