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Vitamin D Mitigates Inflammation and Downregulates Importin α3 in Non-Alcoholic Fatty Liver Disease (NAFLD)

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Abstract

Background: *Pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, seem to play a crucial role in the progression of NAFLD as they activate the transcription factor NF-κB. The activated NF-κBp50/RelA subunits are translocated to the nucleus by Importin α3 and Importin α4. Numerous studies have indicated a negative association between NAFLD and vitamin D levels. Low vitamin D levels have been correlated with histological severity, necro-inflammation, and fibrosis in NAFLD. Non-alcoholic fatty liver disease (NAFLD) affects about one-third of the population in the United States and Western countries, reaching up to 90% among obese individuals and those undergoing bariatric surgery. Characterized by hepatic fat accumulation, NAFLD progresses from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, and, in rare cases, hepatocellular carcinoma. Linked intricately with metabolic syndrome, including diabetes and obesity, NAFLD development involves insulin resistance and hyperinsulinemia. Experimental evidence consistently associates low vitamin D levels with NAFLD severity, independent of age, gender, and BMI. Pro-inflammatory cytokines, such as TNF-α and IL-6, play critical roles in NAFLD, with positive correlations between liver fibrosis and TNF-α levels in NASH patients. The inflammatory cascade involves NF-κB activation, Importin α3, and Importin α4. Vitamin D emerges as a key player, up-regulating the NF-κB inhibitor IκBα and reducing pro-inflammatory activity. Studies in NASH models highlight vitamin D's positive impact on inflammation, fibrosis, and insulin resistance. Recent research underscores calcitriol's role in decreasing Importin α3 expression, influencing NF-κB translocation. This study provides novel insights into calcitriol's effects on Importin α3 and IκBα in HepG-2 cells, unraveling cellular and molecular mechanisms in the intricate interplay between vitamin D and NAFLD pathology.* ***Objective:*** *Vitamin D may resolve the ongoing inflammation observed in NAFLD by reducing the effects of pro-inflammatory cytokines that are extensively produced in NAFLD.* ***Methods:*** *"HepG-2 cell cultures were subjected to calcitriol stimulation, both in the presence and absence of pro-inflammatory cytokines alongside the anti-inflammatory cytokine IL-10. Quantitative polymerase chain reaction (qPCR) was employed to assess mRNA expression levels of Importin α3 and Importin α3. Additionally, immunofluorescence was utilized for antibody detection."* ***Results:*** *Pro-inflammatory cytokines significantly increase the mRNA and protein expression of Importin-α3 in HepG2 cells. Treatment of HepG-2s with calcitriol significantly decreases the mRNA and protein expression of Importin-α3 and attenuates the effects of pro-inflammatory cytokines.* ***Conclusion:*** *Our data suggest that, under inflammatory conditions, calcitriol plays an inhibitory role in the regulation of NF-κB by decreasing the expression of Importin α3. Thus, calcitriol reduces the translocation of NF-κB and thereby lowers its transcriptional activities.*

Keywords: Calcitriol, importin α3, inflammation, NAFLD, pro-inflammatory cytokines, vitamin D

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) remains the predominant chronic liver disorder in

the United States and other Western countries, impacting roughly one-third of the population [1]. The surge in obesity rates has intensified the prevalence of NAFLD, particularly in obese individuals, reaching up to 90% in subjects undergoing bariatric surgery [2].

Histologically, NAFLD is characterized by the accumulation of hepatic fat, driven by the excessive deposition of free fatty acids (FFA) and triglycerides (TG) in over 5% of the liver parenchyma, culminating in steatosis and hepatic inflammation [3]. The pathological progression often commences as simple steatosis, with 20% of cases evolving into nonalcoholic steatohepatitis (NASH), stimulating an inflammatory response. In 9% of cases, this inflammatory state progresses to fibrosis or liver cirrhosis, and in 1% of cases, hepatocellular carcinoma may manifest. NAFLD is intricately linked with metabolic syndrome (MS), encompassing diabetes, obesity, hypertension, hypercholesterolemia, and hypertriglyceridemia, solidifying its status as the hepatic manifestation of metabolic syndrome [4]. Insulin resistance and associated hyperinsulinemia play pivotal roles in the development of both NAFLD and metabolic syndrome [5].

Experimental evidence has consistently demonstrated a negative correlation between NAFLD and vitamin D levels [6]. Low vitamin D levels exhibit significant associations with histological severity, necro-inflammation, and fibrosis in NAFLD, independent of age, gender, BMI, and the presence of other metabolic syndrome features [7]. These findings extend to pediatric cases of NAFLD, emphasizing the robust association between NAFLD and chronic inflammation across all stages [8, 3]. Inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, are implicated in the pathogenesis of NAFLD, with positive associations observed between liver fibrosis amounts and circulating TNF-α levels in NASH patients [9–12].

The inflammatory cascade in NAFLD is closely linked to nuclear factor κB (NF-κB) activation, particularly its association with pro-inflammatory cytokines and the stimulation of Kupffer cells [13, 14]. The regulation of NF-κB relies on factors like IκB family proteins and Importin α3 and Importin α4 [15]. Vitamin D has emerged as a key player in mitigating inflammation, as evidenced by its ability to up-regulate IκBα, an NF-κB inhibitor, and decrease pro-inflammatory NF-κB activity [16]. Additionally, studies in NASH models have demonstrated the positive impact of vitamin D on hepatic inflammation, fibrosis, and insulin resistance [17]. A recent investigation highlighted calcitriol's role in decreasing Importin α3 expression, shedding light on its potential to influence NF-κB translocation [15–18].

In this study, we provide novel insights into the effects of calcitriol on Importin α3, in HepG-2 cells, aiming to unravel the cellular and molecular mechanisms underlying the complex interplay between vitamin D and NAFLD pathology [19, 20].

MATERIALS AND METHODS

Cell Culture

The hepatocellular carcinoma cell line (HepG-2, ATCC #: HB-8065) was acquired from the ATCC cell bank. Cells were cultured in a 25 cm² cell flask containing Eagle's Minimum Essential Medium with L-Glutamine (EMEM), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cell cultures were maintained at 37°C in a humidified incubator with a 5% CO_2 and 95% air environment.

Cell Stimulation

All experiments were conducted using three biologically independent samples. The growth of cultured HepG-2 cells (maintained at 70–80% confluency) was arrested through serum starvation for 24 hours, achieved by replacing FBS-containing EMEM with FBS-free EMEM. Subsequently, cells were stimulated with calcitriol (100 nM) (D1530 Sigma-Aldrich, St. Louis, MO) in fresh EMEM for 24 hours. Recombinant TNF-α, IL-1β, IL-6, IL-8, and IL-10 (Pepro Tech, Inc. NJ) were administered at a dose of 10 ng/ml. Cell viability and cytotoxicity were assessed using the trypan blue dye method.

HepG-2 cells were exposed to varying cytokine concentrations (5 ng/ml, 10 ng/ml, 20 ng/ml, and 50 ng/ml) with viability assessed through cell counting. Multiple readings were obtained, and 10 ng/ml was identified as the most effective dose for HepG-2 cells.

RNA Isolation, Reverse Transcription, and Real-Time PCR

RNA extraction was performed to obtain total RNA, followed by Real-Time RNA quantitation using previously established methods [21]. Relative gene expression was determined by calculating differences in threshold cycles (Ct). The obtained results were normalized against the expression of GAPDH (Table 1).

Immunofluorescence (IF)

Immunofluorescence staining was conducted to assess the expression of VDR, IκBα, and Importin α3, with a negative control that omitted the primary antibody. Briefly, cells were cultured in a T25 flask, and 30,000 cells were plated in a chamber (4 chambers per slide). Subsequently, fixation was performed using 4% formalin for 10 minutes, followed by washing and the addition of 0.1% Triton for 15 minutes. After washing, cells were subjected to a blocking solution for 30 minutes (5% fetal bovine serum in PBS). The subsequent step involved incubation with the primary antibody for 2 hours at room temperature or overnight at 4°C.

Following primary antibody incubation, cells underwent three washes with PBS, each lasting five minutes. Subsequently, cells were incubated with the corresponding secondary antibody (Alexa Fluor 594 and 488 at a 1:1000 dilution) for 30 minutes, followed by additional washes. Counterstaining with DAPI (4,6-diamidino-2 phenyl indole) was performed. Immunofluorescence microscopy was conducted using an Olympus inverted fluorescent microscope (Olympus BX51). Image J software was employed to measure immunofluorescence intensity for stained slides. Ten cells per slide were randomly selected for fluorescence intensity measurement, and the mean fluorescence intensity for each gene of interest was calculated. Each experiment was independently repeated three times, utilizing distinct batches of cells.

Statistical Analysis

The data are presented as Mean ± Standard Deviation (SD). Statistical analyses were conducted using GraphPad Prism 4.0 biochemical statistical software (GraphPad Software, Inc., San Diego, CA) for both data analysis and graph plotting. One-way Analysis of Variance (ANOVA) was employed to identify statistically significant differences among groups. Post-hoc tests, including Dunnett or Bonferroni's test, were subsequently applied for pairwise comparisons between groups. A significance threshold of $p < 0.05$ ($p < 0.05$, $p < 0.001$, **$p < 0.0001$) was established to determine statistical significance.

Results

Modulatory Effects of Tnf-α and Calcitriol in the Regulation of Importin α3 Expression

We further tested if the Importin α3 expression was modulated by pro-inflammatory cytokines. HepG-2 cells were cultured and treated with 10 ng/ml of TNF-α for 24 hours in the presence and absence of 25 or 100 ng/ml calcitriol. After treatment, cells were washed and fixed for immunofluorescence. The immunofluorescence staining of Importin α3 expression in HepG-2 cells treated with 10 ng/ml TNF-α showed the highest immunoreactivity. HepG-2 cells treated with 100 nM calcitriol exhibited the lowest immune sensitivity for Importin α3 gene. Importin α3 showed cytoplasmic expression. *Immunofluorescence and RT-PCR analyses demonstrated that TNF-α significantly increased Importin α3 expression, whereas calcitriol treatment markedly reduced this effect (Figure 1).*

The RT-PCR analysis revealed that unstimulated HepG-2 cells express Importin α3. Treatment with 10 ng of TNF-α for 24 hours significantly increased mRNA expression of Importin α3 ($p < 0.05$). Addition of 25 and 100 nM of calcitriol showed a significant decrease ($p < 0.05$) in mRNA expression of Importin α3 in HepG-2. Calcitriol also showed a reduction in mRNA expression of Importin α3, and

concomitantly, calcitriol-treated cells exhibited higher immunoreactivity.

Immunofluorescence studies were conducted to assess the expression of Importin α3 in HepG-2 cells following treatment with TNF-α, with or without concurrent administration of calcitriol. Alexa Fluor 488 (green) served as the secondary antibody. The expression of Importin α3 is visualized in images A (untreated HepG-2), D (HepG-2 treated with TNF-α), G (HepG-2 treated with TNF-α and calcitriol), and J (HepG-2 treated with calcitriol only). Nuclei were counterstained with DAPI (B, E, H, and K). Merged images of Alexa Fluor and DAPI are presented in images C, *F*, I, and L. This figure represents a typical image from three independent experiments. M and N show RT-PCR analysis demonstrating the effects of TNF-α and Vit D on Importin α3 gene expression. HepG-2 cells underwent 24-hour serum starvation followed by a 24-hour treatment with TNF-α in the presence and absence of different doses of calcitriol (25–100 nM). The extracted RNA was analyzed via RT-PCR to evaluate Importin α3 expression. The figure illustrates the fold change in mRNA expression of Importin α3. Results are presented as mean ± SD from three individual samples, with statistical significance indicated by $p < 0.05$. *The quantitative RT-PCR data further confirmed these findings, showing a dose-dependent reduction in Importin α3 mRNA expression following calcitriol treatment (Figure 2)*.

Effect of Il-1β and Vitamin d on Importin α3

We further examined the effects of IL-1β and Vitamin D on Importin α3 gene expression. HepG-2 cells were cultured and exposed to 10 ng/ml of IL-1β for 24 hours in the presence and absence of 25 or 100 ng/ml calcitriol. Following treatment, cells were washed and fixed for immunofluorescence staining. The immunofluorescence results revealed the lowest immunoreactivity for Importin α3 expression in HepG-2 cells treated with 100 nM/ml calcitriol, while the highest immunoreactivity was observed in HepG-2 cells treated with 10 ng/ml IL-1β. Importin α3 exhibited cytoplasmic expression.

The RT-PCR analysis demonstrated that unstimulated HepG-2 cells express Importin α3. Treatment with 10 ng IL-1β for 24 hours significantly increased mRNA expression of Importin α3 ($p < 0.05$). The addition of 25 and 100 nM calcitriol significantly ($p < 0.05$) decreased mRNA expression of Importin α3 in HepG-2 cells. Calcitriol was shown to reduce mRNA expression of Importin α3 in HepG-2 cells. Our data suggest that IL-1β induces an increase, while calcitriol results in a decrease in Importin α3 expression in HepG-2 cells.

Immunofluorescence studies were conducted to assess the expression of IκBα in HepG-2 cells after treating cells with IL-1β, with or without calcitriol. Alexa Fluor 488 (green) served as the secondary antibody. The expression of IκBα is depicted in [image A (untreated HepG-2), D (HepG-2 treated with IL-1β), G (HepG-2 treated with IL-1β and calcitriol), and J (HepG-2 treated with calcitriol only)]. DAPI (B, E, H, and K) was used to counterstain the nucleus. Images C, *F*, I, and L represent the merged images of Alexa Fluor and DAPI. This is a representative image from three separate samples. RT-PCR analysis was performed to investigate the effects of IL-1β and Vitamin D on Importin α3 in HepG-2 cells (M and N). The cells were subjected to serum starvation for 24 hours, followed by treatment with IL-1β in the presence and absence of different doses of calcitriol (25–100 nM) for an additional 24 hours. The extracted RNA was then analyzed using RT-PCR to assess Importin α3 gene expression. The figure illustrates the fold change in mRNA expression of Importin α3. Data are presented as mean ± SD from three individual experimental samples; $p < 0.05$. *IL-1β treatment significantly enhanced Importin α3 expression at both protein and mRNA levels, while calcitriol markedly suppressed this response (Figure 3).*

Effect of IL-6 and Vitamin d on Importin A3

We further tested if IL-6 modulated Importin α3 gene expression. HepG-2 cells were cultured and exposed to 10 ng/ml of IL-6 for 24 hours in the presence and absence of 25 or 100 ng/ml calcitriol. Subsequently, cells were subjected to washing and fixation for immunofluorescence staining. The immunofluorescence results revealed the highest immunoreactivity for Importin α3 in HepG-2 cells

treated with 10 ng/ml IL-6, while the lowest immunoreactivity was observed in HepG-2 cells treated with 100 nM calcitriol. Importin α3 exhibited cytoplasmic expression. The RT-PCR analysis demonstrated that unstimulated HepG-2 cells express Importin α3; treatment with 10 ng IL-6 for 24 hours led to an insignificant increase in mRNA expression of Importin α3 ($p > 0.05$). The addition of 25 or 100 nM calcitriol with 10 ng IL-6 showed a statistically insignificant ($p > 0.05$) decrease in mRNA expression of Importin α3 in HepG-2 cells. Calcitriol, as indicated, reduces the mRNA expression of Importin α3, and concomitantly, calcitriol treatment resulted in higher immunoreactivity. These findings suggest that calcitriol reduces the expression of Importin α3 in HepG-2 cells. Together our data suggest that IL-6 induces an increase, while calcitriol results in a decrease in Importin α3 expression in HepG-2 cells.

Immunofluorescence studies were conducted to assess the expression of IκBα in HepG-2 cells following treatment with IL-6, with or without calcitriol. Alexa Fluor 488 (green) served as the secondary antibody. The expression of IκBα is illustrated in [image A (untreated HepG-2), D (HepG-2 treated with IL-6), G (HepG-2 treated with IL-6 and calcitriol), and J (HepG-2 treated with calcitriol

Table 1. The primer sequences utilized in this analysis are as follows:

Gene	Forward primer	Reverse primer
GAPDH	5'- GGG AAG GTG AAG GTC GGA GT-3'	5'- TTG AGG TCA ATG AAG GGG TCA-3'
VDR	5'- CTT CAG GCG AAG CAT GAA GC-3'	5'- CCT TCA TCA TGC CGA TGT CC-3'
IκBα	5-'ACA CTA GAA AAC TTC AGA TGC-3	5'- ACA CAG TCA TCA TAG GGC AG-3'
Importin α3	5'- TGT GAG CAA GCA GTG TGG GCA-3'	5'- TGG TGG TGG GTC TTT GTG GCG-3'
Cyp27B1	5'- TGG CCC AGA TCC TAA CAC ATT T-3'	5'- GTC CGG GTC TTG GGT CTA ACT-3'
Cyp24A1	5'- TGT GAG CAA GCA GTG TGG GCA-3'	5'- TGG TGG TGG GTC TTT GTG GCG-3'

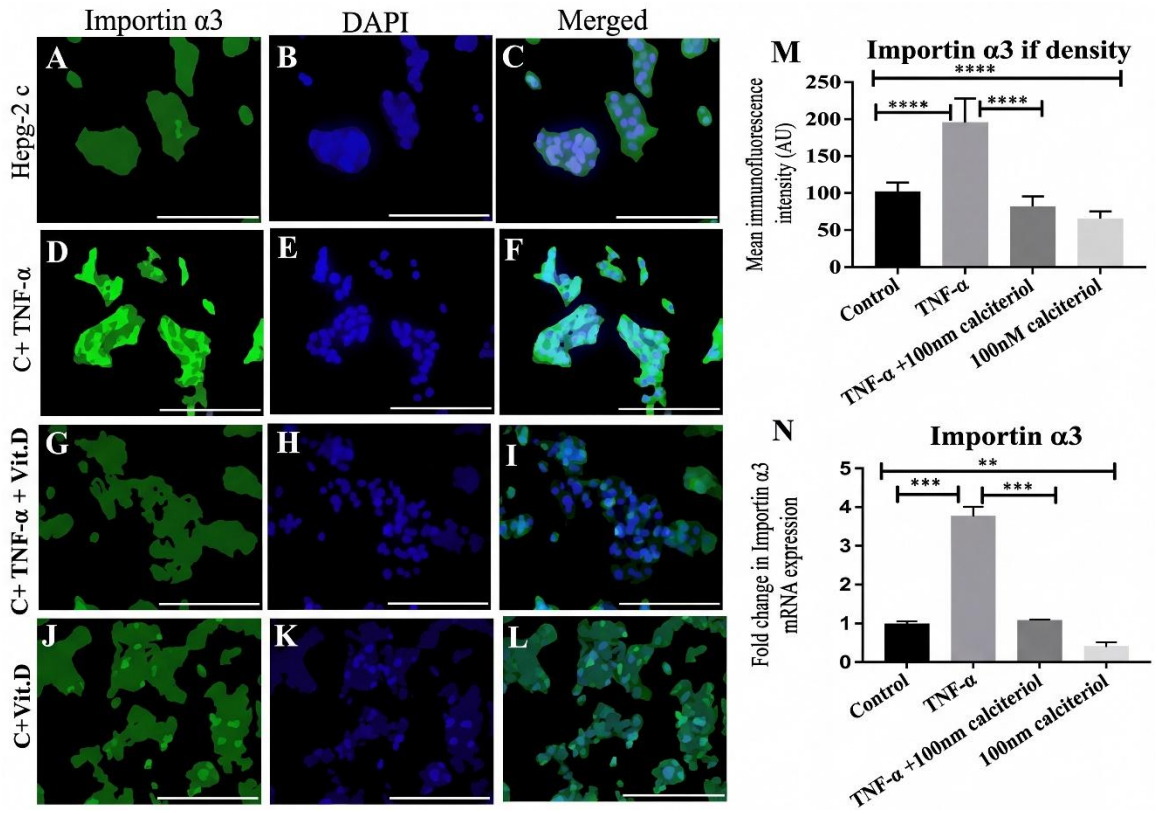

Figure 1. Immunofluorescence staining for the effects of TNF-α on Importin α3 in HepG-2.

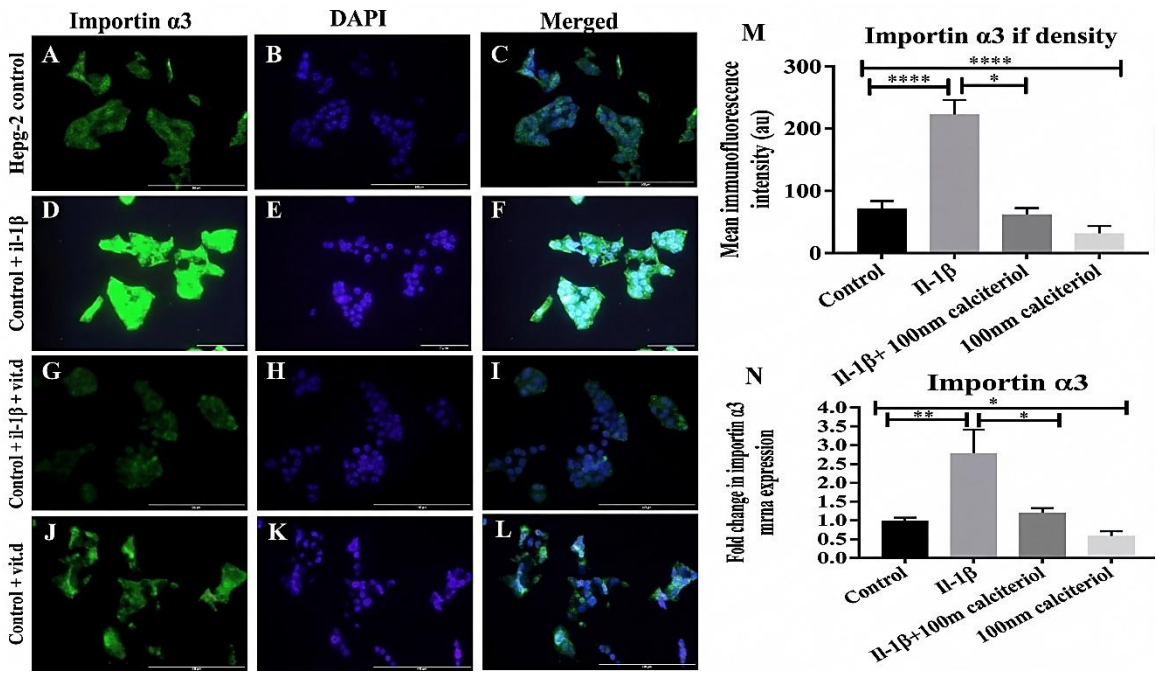

Figure 2. Immunofluorescence staining for effect of IL-1β and vitamin D on Importin α3 in HepG-2 cells.

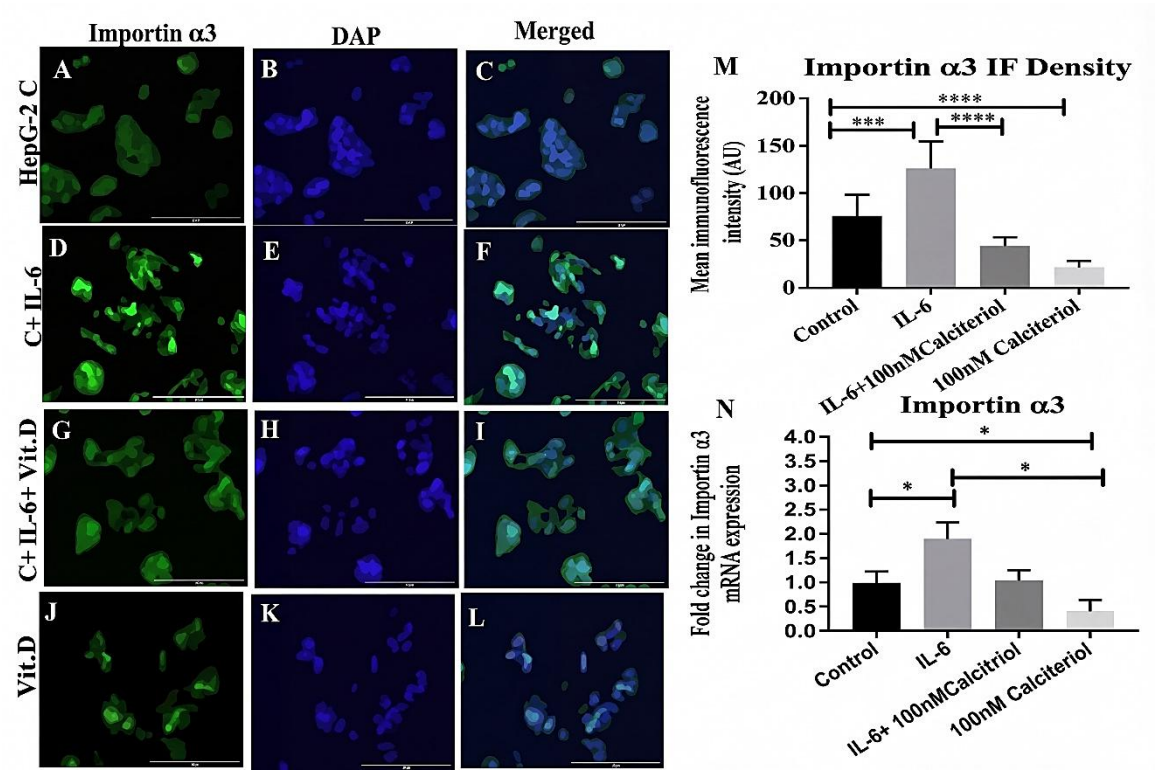

Figure 3. Immunofluorescence staining for the effects of IL-6 on Importin α3 in HepG-2.

only)]. DAPI (B, E, H, and K) was used to counterstain the nucleus. Images C, *F*, I, and L represent the merged images of Alexa Fluor and DAPI. This serves as a representative image from three separate experiments. RT-PCR analysis was conducted to investigate the effects of IL-6 and Vitamin D on Importin α3 (M and N). HepG-2 cells were serum-starved for 24 hours, followed by treatment with IL-6 in the presence and absence of different doses of calcitriol (25–100 nM) for an additional 24 hours.

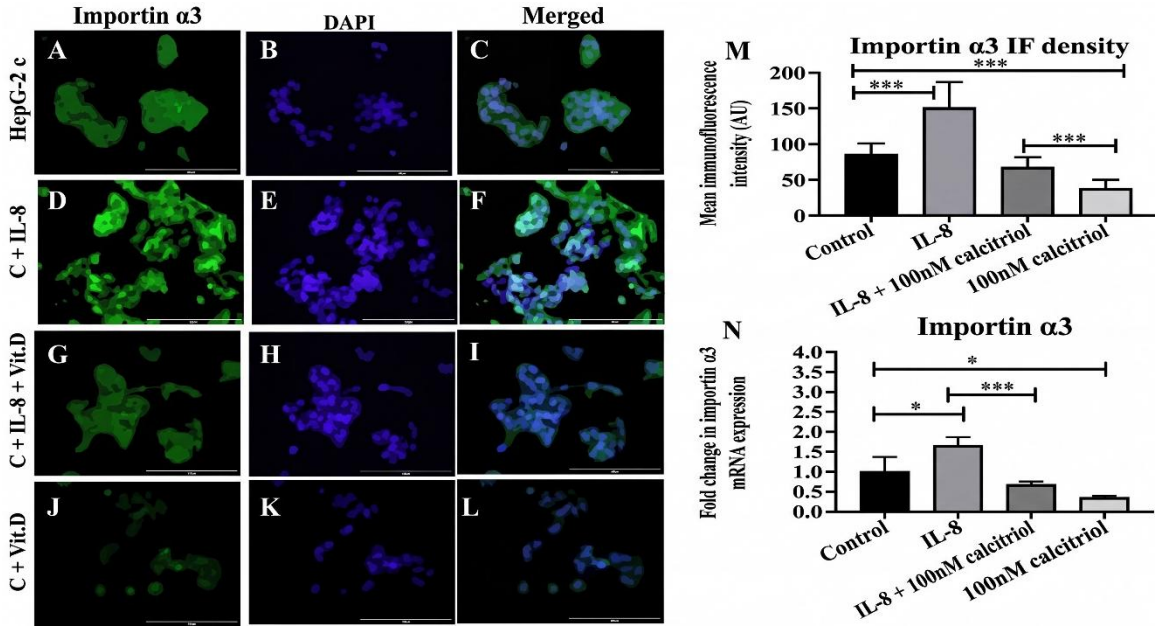

Figure 4. Immunofluorescence staining for the effects of IL-8 on Importin α3 in HepG-2.

The extracted RNA was subjected to RT-PCR for Importin α3 expression. The figure illustrates the fold change in mRNA expression of Importin α3. Data are presented as mean ± SD from three individual samples; $p < 0.05$. *IL-6 moderately increased Importin α3 expression, which was attenuated by calcitriol treatment, as demonstrated by immunofluorescence and RT-PCR analyses (Figure 4).*

EFFECT OF IL-8 AND VITAMIN D ON IMPORTIN α3

HepG-2 cells were cultured and treated with 10 ng/ml of IL-8 for 24 hours in the presence and absence of 25 or 100 ng/ml calcitriol. After treatment, cells were washed and fixed for immunofluorescence. Immunofluorescence staining showed the highest immunoreactivity for Importin α3 in HepG-2 cells treated with 10 ng/ml IL-8, with the lowest observed in HepG-2 cells treated with 100 nM calcitriol. Importin α3 exhibited cytoplasmic expression.

The RT-PCR analysis demonstrated that unstimulated HepG-2 cells express Importin α3. Treatment with 10 ng IL-18 for 24 hours significantly increased the mRNA expression of Importin α3 ($p < 0.05$). The addition of 25 or 100 nM of calcitriol significantly ($p < 0.05$) decreased the mRNA expression of Importin α3 in HepG-2. Calcitriol has shown the capability to reduce the mRNA expression of Importin α3 in HepG-2 cells. These data suggest that calcitriol, by reducing the expression of Importin α3, diminishes the pro-inflammatory effects of IL-8 in HepG-2. Our data show that IL-8 increases Importin α3 expression whereas calcitriol decreases.

Immunofluorescence studies were conducted to assess the expression of Importin α3 in HepG-2 cells following treatment with IL-8, with or without calcitriol. Alexa Fluor 488 (green) served as the secondary antibody. The expression of Importin α3 is depicted in Image A (untreated HepG-2), D (HepG-2 treated with IL-8), G (HepG-2 treated with IL-8 and calcitriol), and J (HepG-2 treated with calcitriol only). DAPI (B, E H, and K) was used to counterstain the nucleus. Images C, *F*, I, and L represent the merged images of Alexa Fluor and DAPI. This image is a representative snapshot of three distinct experiments. RT-PCR analysis was performed to investigate the effects of IL-8 and Vitamin D on Importin α3 (M and N). HepG-2 cells were serum-starved for 24 hours, followed by treatment with IL-8 in the presence and absence of different doses of calcitriol (25–100 nM) for 24 hours. The extracted RNA was subjected to RT-PCR to assess the expression of Importin α3. The figure illustrates the fold change in mRNA expression of Importin α3. Data are presented as mean ± SD from three individual experimental samples; $p < 0.05$. *IL-8 significantly upregulated Importin α3 expression, whereas calcitriol treatment reversed this effect at both transcriptional and protein levels (Figure 5).*

Effect of IL-10 and Vitamin d on Importin α3

IL-10, an anti-inflammatory cytokine mainly produced by the liver, regulates inflammation in various organs [19]. In the liver, IL-10 eases hepatocellular injury by inhibiting T-cells, monocytes, and macrophages [20]. Its role in NAFLD's pathogenesis, insulin resistance, and progression is unclear. Research on mice lacking IL-10, fed a high-fat diet, suggested that IL-10 prevents hepatic steatosis but is not linked to insulin resistance [21]. Another study using IL-10 interventions in an NAFLD mouse model increased pro-inflammatory cytokines and reduced insulin signal transduction and steatosis [22]. The role of IL-10 in preventing metabolic syndromes, including NAFLD, shows mixed results across studies, raising critical questions about its effectiveness in different populations.

Further investigations were conducted to examine if IL-10 and calcitriol had modulated Importin α3 expression. HepG-2 cells were cultured and subjected to a 24-hour treatment with 10 ng/ml of IL-10 in the presence and absence of 25 or 100 ng/ml calcitriol. Following treatment, cells were washed and fixed for immunofluorescence staining. HepG-2 cells treated with 10 ng/ml IL-10 and 100 nM calcitriol exhibited the lowest immunoreactivity for Importin α3 protein expression compared to the control. Importin α3 demonstrated a cytoplasmic expression pattern.

RT-PCR analysis of unstimulated HepG-2 cells revealed the expression of Importin α3 mRNA. Treatment with IL-10 (10 ng/ml) significantly decreased the mRNA expression of Importin α3 ($p < 0.05$). Similarly, calcitriol significantly ($p < 0.05$) reduced the mRNA expression of Importin α3 in HepG-2 cells. Notably, this study highlights how calcitriol augments the anti-inflammatory effects of IL-10 by reducing the mRNA expression of Importin α3. Our data indicate that IL-10 and calcitriol collectively diminish Importin α3 mRNA expression in HepG-2 cells.

Immunofluorescence studies were conducted to evaluate the expression of Importin α3 in HepG-2 cells following treatment with IL-10, with or without calcitriol. A secondary antibody, Alexa Fluor 488 (green), was utilized. The expression of Importin α3 is illustrated in Image A (untreated HepG-2), D (HepG-2 treated with IL-10), G (HepG-2 treated with IL-10 and calcitriol), and J (HepG-2 treated with calcitriol only). DAPI (B, E, H, and K) was employed to counterstain the nucleus. Images C, *F*, I, and L depict the merged images of Alexa Fluor and DAPI. This representation is derived from three distinct samples. RT-PCR analysis was conducted to investigate the impact of IL-10 and Vitamin D on Importin α3 (M and N). HepG-2 cells were subjected to a 24-hour serum starvation period, followed by treatment with IL-10 in the presence and absence of different doses of calcitriol (25–100 nM) for an additional 24 hours. Subsequently, RNA was extracted and subjected to RT-PCR to assess the gene expression of Importin α3. The figure illustrates the fold change in mRNA expression of Importin α3. The data are presented as mean ± SD from three individual experimental samples; $p < 0.05$. *IL-10 alone and in combination with calcitriol significantly reduced Importin α3 expression in HepG-2 cells (Figure 6).*

Effect of Tnf-α and Vitamin d on iκbα Expression

We have further assessed the effects of pro-inflammatory cytokine TNF-α on IκBα expression. HepG-2 cells were cultured and treated with 10 ng/ml of TNF-α for 24 hours in the presence and absence of 25 or 100 ng/ml calcitriol. After treatment, cells were washed and fixed for immunofluorescence. Accordingly, immunofluorescence staining showed the highest immunoreactivity of IκBα in calcitriol-treated HepG-2 cells. HepG-2 cells treated with 10 ng/ml TNF-α showed the lowest IκBα expression. IκBα exhibited cytoplasmic expression in HepG-2 cells.

To further explore the role of calcitriol in the regulation of the effects of TNF-α on IκBα, RT-PCR was conducted. Unstimulated HepG-2 cells exhibited mRNA expression of IκBα. Following TNF-α (10 ng/L) treatment, there was a significant decrease in mRNA expression of IκBα in HepG-2 cells. The addition of calcitriol at concentrations of 25–100 nM significantly ($p < 0.05$) increased mRNA expression of IκBα. These results suggest that calcitriol is capable of up-regulating the transcription of IκBα in HepG-2 cells and attenuating the effects of TNF-α on IκBα gene expression in HepG-2 cells.

Immunofluorescence studies were conducted to examine the expression of IκBα in HepG-2 cells after

treating the cells with TNF-α with or without calcitriol. Alexa Fluor 594 (red) was used as the secondary antibody. The expression of IκBα is depicted in [image A (untreated HepG-2), D (HepG-2 treated with TNF-α), G (HepG-2 treated with TNF-α and calcitriol), and J (HepG-2 treated with calcitriol only)]. DAPI (B, E, H, and K) was used to counterstain the nucleus. Images C, *F*, I, and L represent the merged images of Alexa Fluor and DAPI. This is a representative image from three separate experiments. RT-PCR analysis for the effects of TNF-α and Vitamin D on IκBα expression (M and N): HepG-2 cells were serum starved for 24 hours followed by treatment with TNF-α in the presence and absence of different doses of calcitriol (25–100 nM) for 24 hours. The extracted RNA was subjected to RT-PCR for IκBα expression. Figure shows fold change in mRNA expression of IκBα. Data are shown as mean ± SD from three individual samples; $p < 0.05$. *TNF-α markedly suppressed IκBα expression, while calcitriol significantly restored its expression, as shown by immunofluorescence and RT-PCR analyses (Figure 7).*

Effects of Il-1β and Vitamin d on Iκb

We further investigated the effects of IL-1β and Vitamin D on IκBα. HepG-2 cells were cultured and treated with 10 ng/ml of IL-1β for 24 hours in the presence and absence of 25 or 100 ng/ml calcitriol. After treatment, cells were washed and fixed for immunofluorescence staining. Accordingly, immunofluorescence staining for IκBα expression in HepG-2 cells treated with 10 ng/ml IL-1β showed the lowest immunoreactivity compared to other treatments. IκBα showed cytoplasmic expression in HepG-2.

The unstimulated HepG-2s expressed mRNA of IκBα. Following IL-1β (10 ng/L) treatment, there was significant decrease in mRNA expression of IκBα in HepG-2s ($p < 0.05$); addition of calcitriol (100 nM) significantly ($p < 0.05$) increased mRNA expression of IκBα in HepG-2. Our data suggest that IL-1β decreases IκBα expression while calcitriol increases in HepG-2 cells. Immunofluorescence studies were completed for the expression of IκBα in HepG-2 cells after treating cells with IL-1β with or without calcitriol. Alexa Fluor 594 (red) was used as secondary antibody. Expression of IκBα [image A (untreated HepG-2), D (HepG-2 treated with IL-1β), G (HepG-2 treated with IL-1β and calcitriol) and J (HepG-2 treated with calcitriol only)]. DAPI (B, E, H, and K) was used to counterstain nucleus. Images C, *F*, I and L are the merged images of images of Alexa Fluor and DAPI. This is a representative image of three separate samples. RT-PCR analysis for the effects of IL-1β and Vitamin D on IκBα (M and N). HepG-2 cells were starved for 24 hours followed by treatment with IL-1β in the presence and absence of different doses of calcitriol (25–100 nM) for 24 hours. The extracted RNA was subjected to RT-PCR for IκBα gene expression. Figure shows fold change in mRNA expression of IκBα. Data are shown as mean ± SD from three individual experimental samples; $p < 0.05$. *IL-1β treatment resulted in a significant reduction of IκBα expression, which was reversed upon calcitriol treatment (Figure 8).*

Effect of IL-6 and Vitamin d on Importin α3

Interleukin-6 (IL-6), a versatile cytokine, is released by various cells, such as fibroblasts, endothelial cells, adipocytes, monocytes, and T-cells in response to acute inflammation [23]. Its intricate role in liver pathogenesis and its contribution to NAFLD development are not fully elucidated. Nevertheless, IL-6 has systemic effects, including inflammation stimulation, immune defense regulation (B- and T-cell functions), support of hematopoiesis, and oncogenesis [24].

Elevated IL-6 levels in the serum of obese individuals serve as a robust predictor for the onset of Type 2 diabetes, metabolic syndrome, and cardiovascular diseases [25, 26]. Moreover, IL-6 exhibits slightly inhibitory effects on adipogenesis, impacting adiponectin production and lipolysis. In the context of NAFLD, IL-6 from visceral fat enters the liver, activating signaling transducer protein JAK/STAT3 in hepatocytes. This cascade triggers the transcription of various genes, including suppressor cytokine signaling 3 (SOCS-3), affecting insulin signaling and contributing to hepatic insulin resistance, thereby fostering NAFLD development [27]. While initial studies suggested a protective mechanism of IL-6 in liver steatosis by promoting hepatocyte proliferation and mitigating

oxidative stress, more recent research demonstrates a dual role.

Here we have assessed the effects of IL-6 and Vitamin D on Importin α3 expression. HepG-2 cells were cultured and treated with 10 ng/ml of IL-6 for 24 hours in the presence and absence of 25 or 100 ng/ml calcitriol. After treatment, cells were washed and fixed for immunofluorescence staining. Immunofluorescence staining showed the highest immunoreactivity for Importin α3 in HepG-2 cells treated with 10 ng/ml IL-6, with the lowest in HepG-2 cells treated with 100 nM calcitriol. Importin α3 exhibited cytoplasmic expression.

The RT-PCR analysis showed that unstimulated HepG-2 cells express Importin α3. Treatment with 10 ng IL-6 for 24 hours insignificantly increased mRNA expression of Importin α3 ($p > 0.05$). The addition of 25 or 100 nM calcitriol with 10 ng IL-6 showed insignificantly ($p > 0.05$) decreased mRNA expression of Importin α3 in HepG-2 cells. Calcitriol, as shown, reduces mRNA expression of Importin α3, along with having higher immunoreactivity. These data suggest that IL-6 upregulates Importin α3 expression whereas calcitriol downregulates in HepG-2 cells.

Immunofluorescence studies were conducted to analyze the expression of IκBα in HepG-2 cells after treating the cells with IL-6 with or without calcitriol. Alexa Fluor 488 (green) was used as the secondary antibody. The expression of IκBα is depicted in [image A (untreated HepG-2), D (HepG-2 treated with IL-6), G (HepG-2 treated with IL-6 and calcitriol), and J (HepG-2 treated with calcitriol only)]. DAPI (B, E, H, and K) was used to counterstain the nucleus. Images C, *F*, I, and L represent the merged images of Alexa Fluor and DAPI. This is a representative image from three separate experiments. RT-PCR analysis for the effects of IL-6 and Vitamin D on Importin α3 (M and N) was done. HepG-2 cells were serum-starved for 24 hours, followed by treatment with IL-6 in the presence and absence of different doses of calcitriol (25–100 nM) for 24 hours. The extracted RNA was subjected to RT-PCR for Importin α3 expression. The figure shows the fold change in mRNA expression of Importin α3. Data are presented as mean ± SD from three individual samples; $p < 0.05$. *Calcitriol counteracted IL-6-mediated modulation of IκBα expression in HepG-2 cells (Figure 9).*

Effect of IL-8 and Vitamin d on Iκbα

IL-8, a chemokine secreted by various cells, including inflammatory and endothelial cells, primarily functions to coordinate the attraction of neutrophils within inflamed tissue [28]. Despite limited data supporting its involvement in NAFLD development, recent research indicated elevated circulating levels of IL-8 in NAFLD patients, particularly in the NASH group, compared to obese or lean individuals [29]. Furthermore, serum IL-8 levels were significantly higher in the NASH group compared to those with hepatic steatosis or in a healthy control group [30, 31]. However, studies reported no discernible association between circulating IL-8 levels and NAFLD [32].

To assess the effects of IL-8 and Vitamin D on IκBα, HepG-2 cells were cultured and treated with 10 ng/ml of IL-8 for 24 hours in the presence and absence of 25 or 100 ng/ml calcitriol. After treatment, cells were washed and fixed for immunofluorescence. Accordingly, HepG-2 cells treated with calcitriol shown the highest immunoreactivity. HepG-2 cells treated with 10 ng/ml IL-8, the control, and 10 ng/ml IL-8+100 nM shown the similar immunoreactivity. IκBα showed cytoplasmic expression in HepG-2.

To further explore the role of calcitriol in the regulation of the effects of IL-8 on IκBα gene expression, RT-PCR was done, and the analysis shown unstimulated HepG-2s expressed mRNA of IκBα. Following IL-8 (10 ng/L) treatment, there was insignificantly decreased in mRNA expression of IκBα in HepG-2s ($p > 0.05$); adding of calcitriol (100 nM) significantly ($p < 0.05$) increased mRNA expression of IκBα. These data indicate that IL-8 has a limited effect on the suppression of IκBα gene expression in HepG-2. Together our data indicate that IL-8 decreases whereas calcitriol increases IκBα expression in HepG-2.

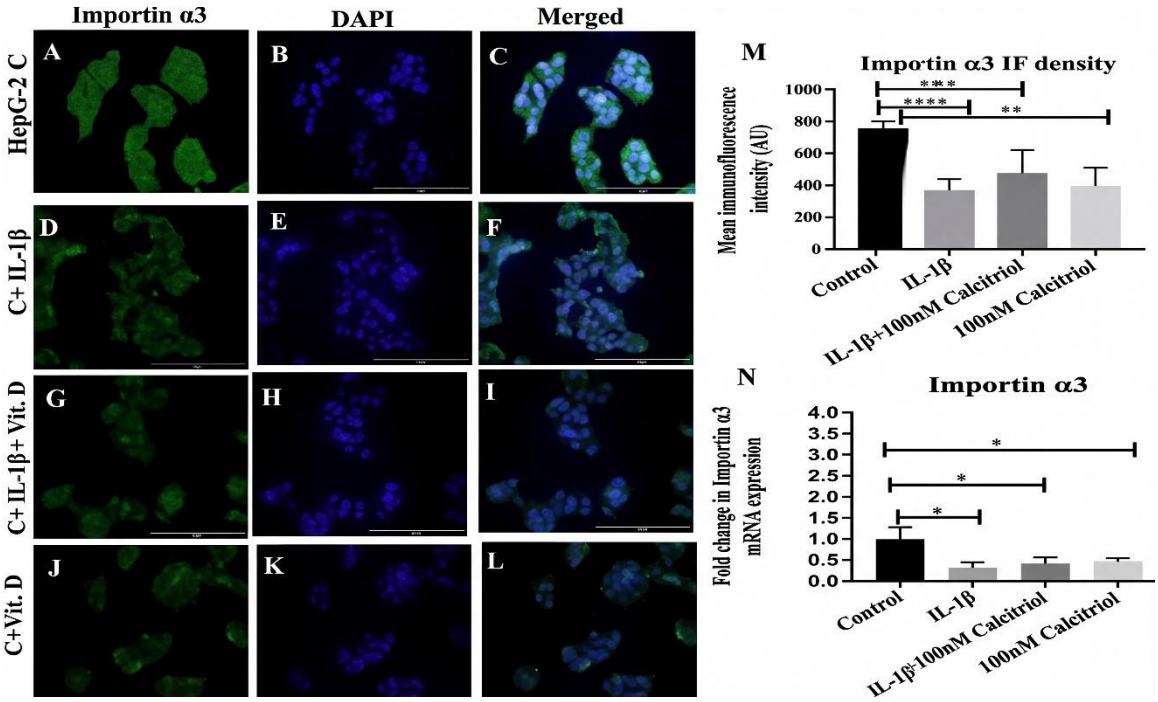

Figure 5. Immunofluorescence staining for effect of IL-10 and vitamin D on Importin α3 in HepG-2 cells.

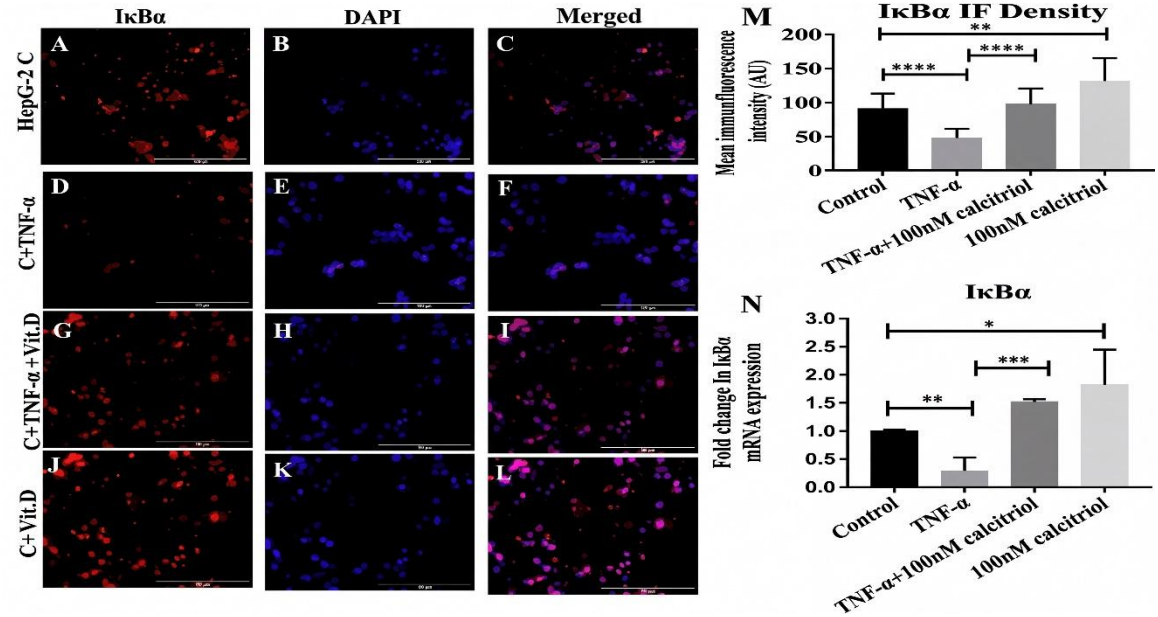

Figure 6. Immunofluorescence staining of the effects of TNF-α on IκBα in HepG-2.

Immunofluorescence studies were completed for the expression of IκBα in HepG-2 cells after treating cells with IL-8 with or without calcitriol. Alexa Fluor 594 (red) was used as secondary antibody. Expression of IκBα [image A (untreated HepG-2), D (HepG-2 treated with IL-8), G (HepG-2 treated with IL-8 and calcitriol) and J (HepG-2 treated with calcitriol only)]. DAPI (B, E, H, and K) was used to counterstain nucleus. Images C, *F*, I and L are the merged images of Alexa Fluor and DAPI. This is a representative image of three separate experiments. RT-PCR analysis for the effects of IL-8 and Vitamin D on IκBα HepG-2 cells were serum starved for 24 hours followed by treatment with IL-8 in the presence and absence of different doses of calcitriol (25–100 nM) for 24 hours. The extracted RNA was subjected to RT-PCR for IκBα expression. Figure shows fold change in mRNA expression of IκBα.

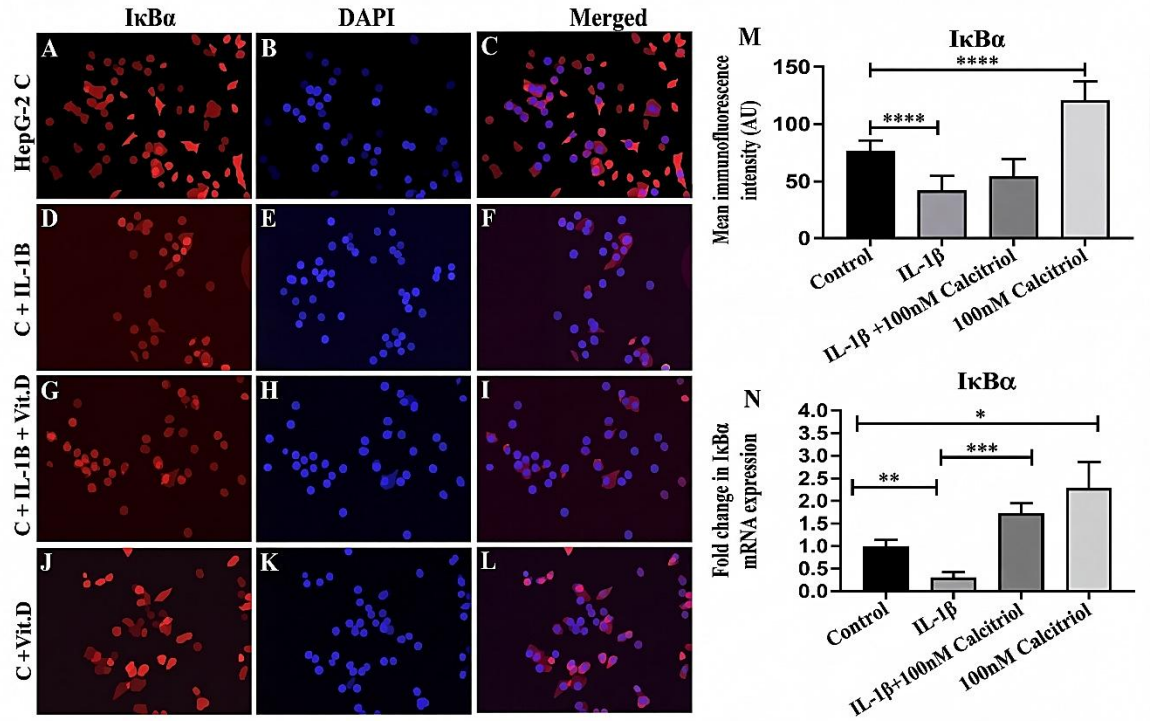

Figure 7. Immunofluorescence staining for effect of IL-1β and vitamin D on IκBα in HepG-2 cells.

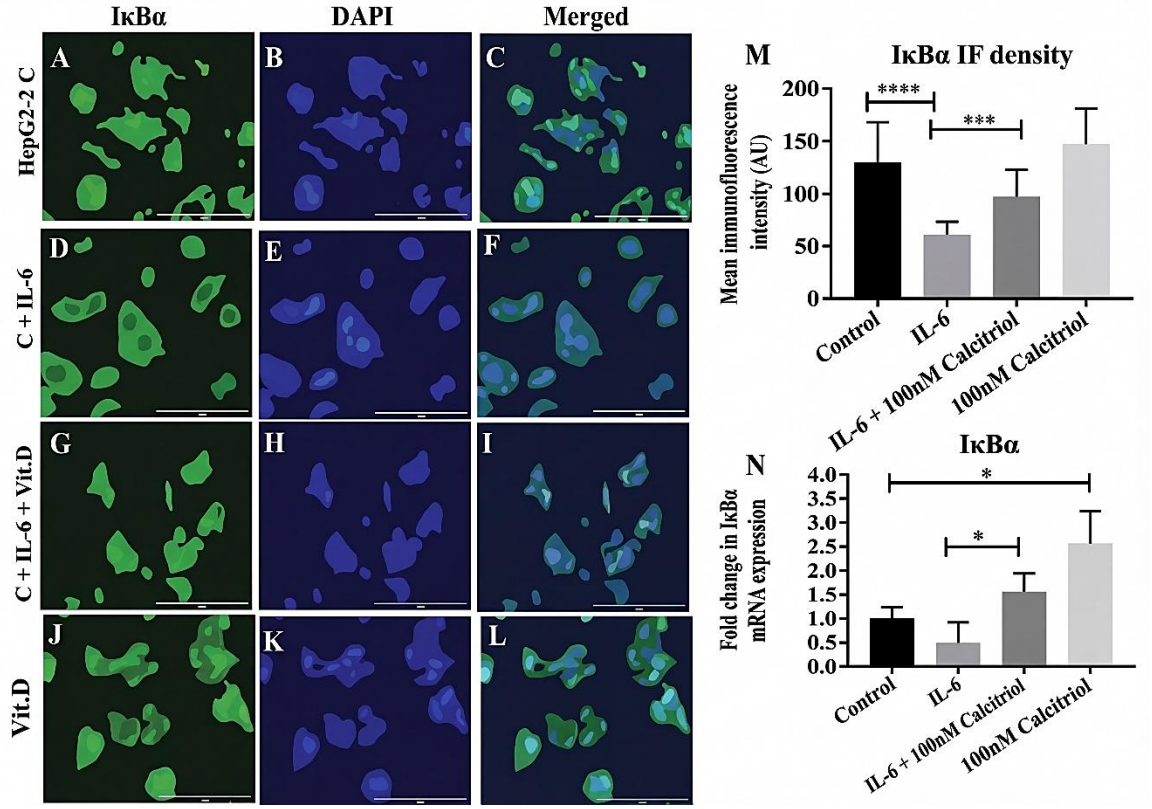

Figure 8. Immunofluorescence staining for the effects of IL-6 on IκBα in HepG-2.

Data are shown as mean ± SD from three individual experimental samples; $p < 0.05$. *IL-8 exerted minimal suppressive effects on IκBα expression, whereas calcitriol significantly enhanced IκBα levels (Figure 10).*

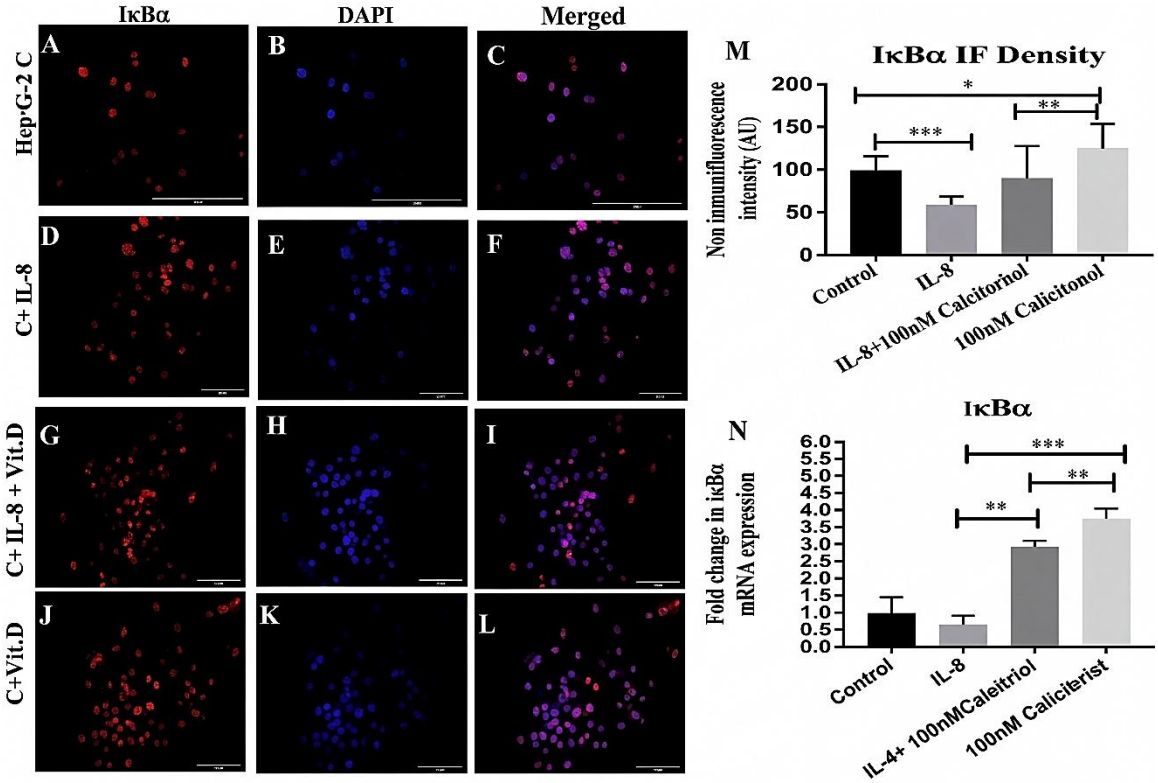

Figure 9. Immunofluorescence staining for effect of IL-8 and vitamin D on IκBα in HepG-2 cells.

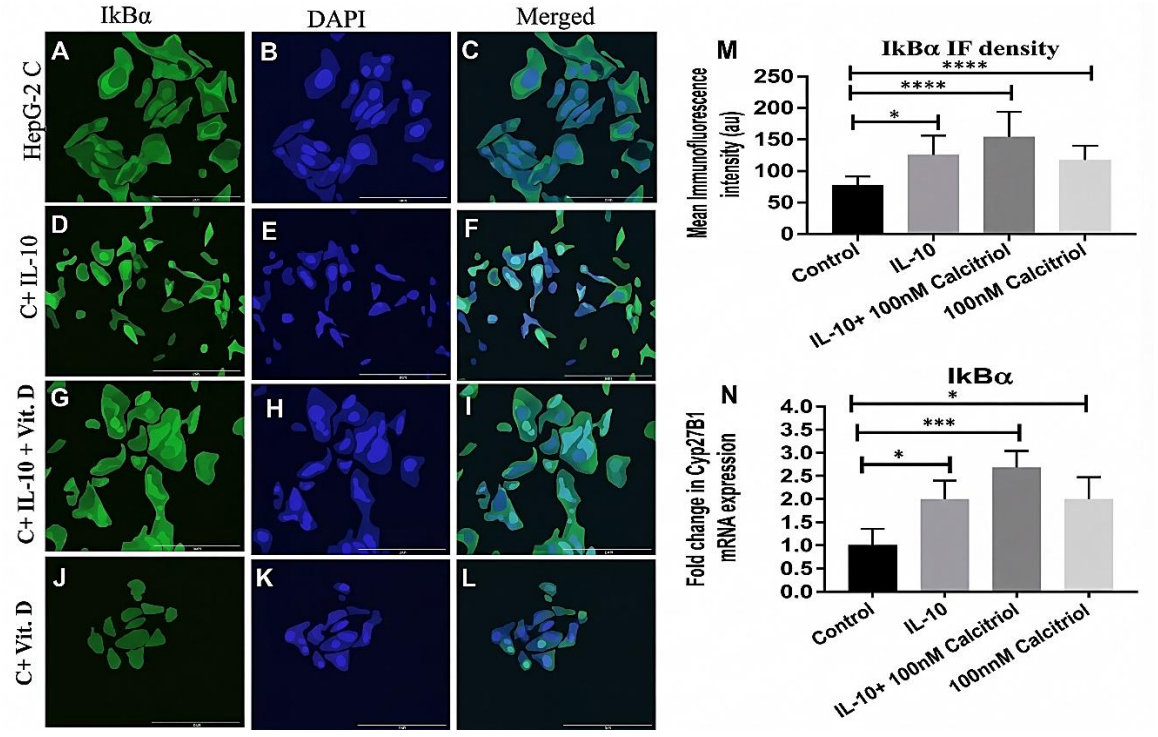

Figure 10. Immunofluorescence staining for effect of IL-10 and vitamin D on IκBα in HepG-2 cells.

Effect of IL-10 and Vitamin d on Iκbα

HepG-2 cells were cultured and treated with 10 ng/ml of IL-10 for 24 hours in the presence and absence of 25 or 100 ng/ml calcitriol. After treatment, cells were washed and fixed for immunofluorescence. Accordingly, immunofluorescence staining of HepG-2 cells treated with 10

ng/ml IL-10 showed the highest immunoreactivity expression of IκBα, followed by IL-10 + 100 nM the. IκBα showed cytoplasmic expression in HepG-2.

RT-PCR analysis of unstimulated HepG-2 cells shown expression of IκBα mRNA; treatment with IL-10 (10 ng/ml) insignificantly increased mRNA expression of IκBα; adding 25 nM calcitriol to 10 ng/ml IL-10 significantly ($p < 0.05$) increased the mRNA expression of IκBα in HepG-2. It is protrusive in this study how calcitriol enhances the anti-inflammatory effects of IL-10 on IκBα expression.

Immunofluorescence studies were completed for the expression of IκBα in HepG-2 cells, after treating cells with IL-10 with or without calcitriol. Alexa Fluor 488 (green) was used as secondary antibody. Expression of IκBα [image A (untreated HepG-2), D (HepG-2 treated with IL-10), G (HepG-2 treated with IL-10 and calcitriol) and J (HepG-2 treated with calcitriol only)]. DAPI (B, E, H, and K) was used to counterstain nucleus. Images C, *F*, I and L are the merged images of Alexa Fluor and DAPI. This image is a representative of three separate samples. RT-PCR analysis for the effects of IL-10 and Vitamin D on IκBα (M and N). HepG-2 cells were serum starved for 24 hours followed by treatment with IL-10 in the presence and absence of different doses of calcitriol (25–100 nM) for 24 hours. The extracted RNA was subjected to RT-PCR for IκBα gene expression. Figure shows fold change in mRNA expression of IκBα. *Data are shown as mean ± SD from three individual experimental samples; $p < 0.05$. Calcitriol potentiated the anti-inflammatory effects of IL-10 by significantly increasing IκBα expression.*

DISCUSSION

NF-κB, a ubiquitous cytoplasmic transcription factor, intricately governs more than 500 genes, predominantly those associated with inflammation [33]. The activation cascade involves IKK activation subsequent to IκBα degradation, culminating in NF-κB translocation to the nucleus [17]. This study unveils, for the first time, the impact of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8) on IκBα mRNA and protein expression in HepG-2 cells, revealing a significant reduction induced by these cytokines. Intriguingly, calcitriol, administered alone or concomitantly with these cytokines, markedly enhances IκBα mRNA and protein expression. Our findings align with prior research illustrating calcitriol's direct role in mitigating NF-κB activity. Specifically, in human keratinocytes, calcitriol diminished NF-κB DNA binding activity by elevating IκBα protein levels and curtailing IL-8 production [34]. Furthermore, in pancreatic islet cells, calcitriol down-regulated pro-inflammatory chemokine production while augmenting IκBα expression, ultimately attenuating NF-κB translational activity [35]. Given the limited understanding of the sustained inflammation implicated in NAFLD development, our observed mitigating effects of calcitriol on proinflammatory cytokines activating NF-κB are consequential. The observed upregulation of IκBα expression is particularly noteworthy, as it curtails the nuclear translocation of the NF-κB transcription factor [36].

In this study, we present, for the first time, the impact of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8) in the presence and absence of calcitriol on both mRNA and protein expression of Importin α3 in HepG-2 cells. Notably, these proinflammatory cytokines induced a significant increase in both mRNA and protein levels of Importin α3 [37]. Our findings reveal that calcitriol exerts a down-regulatory effect on the mRNA and protein expression of Importin α3. Importin α3, a constituent of the nuclear pore complex and harboring the nuclear localization sequence akin to ReLA and P50 subunits of NF-κB, plays a pivotal role in NF-κB stimulatory activities [15]. A previous study from our laboratory has established the role of calcitriol in diminishing the mRNA expression of Importin α3 in Primary Human Bronchial Smooth Muscle Cells [38]. Knockdown of Importin α3 in these cells attenuated TNF-α-induced expression of ReLA. Consequently, calcitriol, through the reduction of Importin α3 expression, inhibits the translocation of stimulated RelA from the cytoplasm to the nucleus in HepG-2 cells.

CONCLUSION

In conclusion, our study sheds light on the intricate regulatory mechanisms involving NF-κB, revealing its susceptibility to modulation by pro-inflammatory cytokines and the regulatory role of

calcitriol. The observed reduction in IκBα expression induced by cytokines underscores their potent inflammatory impact, while calcitriol demonstrates a mitigating effect. Additionally, our novel findings elucidate the influence of pro-inflammatory cytokines on Importin α3 in HepG-2 cells, with calcitriol exerting a down-regulatory influence. This signifies a potential therapeutic avenue for addressing NF-κB-mediated inflammation, the full spectrum of these regulatory pathways.

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