



In vitro Modulation of the LPS-Induced Proinflammatory Profile of Hepatocytes and Macrophages- Approaches for Intervention in Obesity?

Ramiar K. Kheder^{1,2}, James Hobkirk³ and Cordula M. Stover^{1*}

¹ Department of Infection, Immunity, and Inflammation, University of Leicester, Leicester, UK, ² College of Nursing, University of Raparin, Ranya, Iraq, ³ Department of Sport, Health, and Exercise Science, University of Hull, Hull, UK

OPEN ACCESS

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*Correspondence:

Cordula M. Stover
cms13@le.ac.uk

Specialty section:

This article was submitted to
Lipidology,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 11 March 2016

Accepted: 07 June 2016

Published: 22 June 2016

Citation:

Kheder RK, Hobkirk J and Stover CM
(2016) *In vitro* Modulation of the
LPS-Induced Proinflammatory Profile
of Hepatocytes and Macrophages-
Approaches for Intervention in
Obesity?. *Front. Cell Dev. Biol.* 4:61.
doi: 10.3389/fcell.2016.00061

Low grade endotoxemia is a feature of obesity which is linked to development of steatohepatitis in non-alcoholic fatty liver disease. In this study, macrophages (J774) and hepatocytes (HepG2) were stimulated with lipopolysaccharide (LPS) from *E. coli* 0111: B4 and analyzed for modulation of this response when preconditioned or stimulated subsequent to LPS, with different doses of Vitamin D₃ or docosahexaenoic acid (DHA) over a time period of 1 and 5 days. Pro-inflammatory TNF α and pro-fibrotic TGF β released into the supernatants were measured by ELISA; qPCR was performed for Srebp-1c and PPAR α mRNA (genes for products involved in fatty acid synthesis and catabolism, respectively). Vitamin D₃ and DHA exerted a consistent, dose dependent anti-inflammatory effect, and increased PPAR α relative to Srebp-1c in both cell types. By contrast, addition of free fatty acids (FFA, oleic acid/palmitic acid 2:1) caused aggravation of LPS-induced inflammatory reaction and an increase of Srebp-1c relative to PPAR α . Our results argue in favor of dietary supplementation of Vitamin D₃ or DHA (and avoidance of monounsaturated/saturated fatty acids) to alleviate development of fatty liver disease.

Keywords: Vitamin D₃, docosahexaenoic acid, HepG2, J774, lipopolysaccharide

INTRODUCTION

Low grade endotoxemia has been detected in circumstances as diverse as a bolus of high fat diet (Erridge et al., 2007), hemodialysis (Terawaki et al., 2010), and surgery (Fujita et al., 2003). Drained via the portal vein, abdominally derived endotoxin, a pathogen associated molecular pattern, PAMP, directly stimulates the reticuloendothelial system of the liver but also hepatocytes. Obesity, from which insulin resistance develops, is linked to increased translocation of endotoxins from the gut (Brun et al., 2007). Steatosis is the accumulation of fat in hepatocytes which can lead to an overall increase in liver size, so-called hepatomegaly. Accumulation of lipids may lead to inflammation; this is called non-alcoholic steatohepatitis, to differentiate the disease from alcohol-induced liver injury, either of which can progress to cirrhosis. It is estimated that there is evidence of non-alcoholic steatohepatitis in up to a third of populations in the developed world (Preiss and Sattar, 2008). Because of increasing prevalence of obesity in these populations, fatty liver disease has recently become a pediatric diagnosis (Giorgio et al., 2013). Bacterial overgrowth, increased gut

permeability and intestinal dysmotility are characteristics of patients with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). Endotoxin levels were elevated in patients diagnosed with NAFLD compared to control subjects (Harte et al., 2010). One approach of modulating hepatic disease activity is to target the microbiome (Zhu et al., 2014), but another might be to modulate the effects of bacterial lipopolysaccharide (LPS) on hepatic cells, which express its receptor, TLR4 (Sharifnia et al., 2015). Of note, circulating free fatty acids may also function as endogenous ligands for TLR binding as so-called danger associated molecular patterns, DAMPs.

Vitamin D₃ and docosahexaenoic acid (an omega 3 fatty acid) offer promise for the adjuvant treatment of NAFLD (Nobili et al., 2011; Eliades and Spyrou, 2015). In fact, Vitamin D₃ deficiency has been found in patients with NAFLD, and omega 3 fatty acid deficiency is associated with the development of insulin resistance, fatty liver disease, and dyslipidemia (Targher et al., 2013).

Therefore, the aim of this study was to expose macrophages and hepatocytes to LPS and to gauge preventative or curative effects of Vitamin D₃ and docosahexaenoic acid (DHA) *in vitro* while quantifying a detrimental effect of FFA, a mixture of oleic acid and palmitic acid. Reactions were assessed by measuring TNF α and TGF β in supernatants of cells for which mRNA expressions for Srebp-1c and PPAR α were determined in parallel.

MATERIALS AND METHODS

Materials

LPS *E. coli* 0111:B4 was from Invivogen (Toulouse, France), docosahexaenoic acid, oleic acid, and palmitic acid were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK), and Vitamin D₃ was from DSM Nutritional products (Basel, Switzerland).

Cell Culture

J774, a mouse macrophage cell line, and HepG2, a human hepatocellular carcinoma cell line, were grown to semi confluence at 37°C in a humidified atmosphere (5% CO₂) in RPMI 1640 medium or DMEM, respectively, supplemented with 10% (v/v) fetal calf serum, 100 μ g/ml streptomycin, 100 IU/ml penicillin. They were passaged using trypsin EDTA. Cells were counted and adjusted to 60,000/ml for J774, 40,000/ml for HepG2 in 25 cm² flasks (5 ml) and treated when 70% confluent.

Experimental Design

The doses of Vitamin D₃ (0.4, 2, 4 μ g/ml) were chosen based on a pilot experiment that investigated induction of insulin receptor mRNA in target cells, a described, beneficial effect of Vitamin D₃ aimed at increasing insulin sensitivity (Maestro et al., 2000; Supplementary Figure 1). DHA was efficient at 50 μ M (16 μ g/ml) to dampen the proinflammatory response of J774 induced by LPS (100 ng/ml) after 5 days of stimulation (Oliver et al., 2012). Doses of 8, 16, 32 μ g/ml DHA were chosen for this work. The composition of FFA was oleic acid/palmitic acid (2:1) and was added at 15 and 30 mM (Yao et al., 2011).

Two basic stimulation models were performed on HepG2 and J774, namely, independently, preconditioning and subsequent stimulation with LPS as well as initial stimulation with LPS and *post-hoc* stimulation: In the preconditioning model, J774 and HepG2 were exposed to different doses of Vitamin D₃ (0.4, 2, 4 μ g/ml), docosahexaenoic acid, DHA (8, 16, 32 μ g/ml) or FFA, free fatty acids (oleic acid/palmitic acid 2:1, 15, and 30 mM) prior to stimulation with LPS *E. coli* 0111:B4 (100 ng/ml) for 24 h. In the *post-hoc* stimulation model, J774 and HepG2 were exposed to different doses of Vitamin D₃ (0.4, 2, 4 μ g/ml), docosahexaenoic acid, DHA (8, 16, 32 μ g/ml) or FFA, free fatty acids (oleic acid/palmitic acid 2:1, 15, and 30 mM) after stimulation with LPS *E. coli* 0111:B4 (100 ng/ml) for 4 h. While Vitamin D₃ and DHA are protective agents, FFA simulates an obesity relevant aggravating agent. Unstimulated controls were run in parallel.

cDNA Synthesis and qPCR

RNA was prepared using RNeasy Mini Kit (Qiagen, Manchester UK), genomic DNA was digested and cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Loughborough, UK). Primer sequences are available in Supplementary Table 1. Their efficiency was tested and sensitivity of amplification verified by linearity of product yield vs. dilutions of cDNA. Real time PCR was used to quantify the relative changes in gene expression by $2^{-\Delta\Delta C_t}$ method using SYBR Green formulation and Corbett, Rotor gene 6000 real time rotary analyser. GAPDH mRNA expression was found to be stable. Any variation was corrected by relating expression of gene of interest to GAPDH mRNA expression of the respective controls as part of the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

ELISA

ELISAs for mouse and human Transforming growth factor β , TGF β , were from R&D Systems (Abingdon, UK), ELISAs for mouse and human Tumor necrosis factor α , TNF α , were from PeproTech EC Ltd (London, UK). Supernatants were used neat and assayed in triplicate. 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System was used.

Statistics

For ELISA measurements, standard curves were constructed according to the manufacturers' instructions and levels in supernatants extrapolated using Graph Prism Pad. For qPCR analyses, the data were presented as the fold change in gene expression normalized to GAPDH and relative to unstimulated control. Data from triplicate determinations were expressed as means from averages \pm SD. Unpaired one-way ANOVA with Tukey's multiple comparisons testing was performed and adjusted *p*-values expressed.

RESULTS

J774 and HepG2 were used as model cells to investigate in parallel the modulation of their LPS induced response by the presence of Vitamin D₃ and DHA using markers of relevance in the inflammatory, profibrotic, and lipogenic response. Free

Fatty Acids, FFA (oleic acid/palmitic acid 2:1), were used as a stimulus to aggravate LPS-induced reactivity. A special focus was to capture adaptation of the cells to long term corrective exposure with Vitamin D₃ or DHA before and after LPS stimulation with a low endotoxin dose or the detrimental accumulating effect of FFA when cells were exposed before or after the LPS stimulus.

The dose of LPS *E. coli* 0111:B4 to model responses toward low grade endotoxemia was compared in a pilot experiment to a 10-fold higher dose. The dose of 1000 ng/ml was found to negatively impact TLR and VDR mRNA expression in J774, which behaved as acute sensors of the presence of this PAMP in comparison with HepG2 (Supplementary Figure 2). The dose of 100 ng/ml LPS has been used by others to study the anti-inflammatory effect of DHA on macrophages (Mullen et al., 2010). To determine that the dose of FFA was not toxic, a pilot experiment investigated the effect of FFA on a morphological feature of HepG2 cells, namely lipid accumulation. FFA was found to enhance Oil Red O positive cell inclusions, which could be reduced in the presence of Vitamin D₃, whilst retaining cell intactness (Supplementary Figure 3). DHA (50 μ M) was shown to have a similar effect on palmitate-induced lipids in HepG2 (Luo et al., 2012).

Beneficial Effect of DHA and Vitamin D₃ on LPS-Induced TNF α and TGF β Production by Macrophages and Hepatocytes

A robust induction of TNF α production by 24 h stimulation with LPS was observed in J774 and HepG2 (Figures 1A–D). There was a significant and dose dependent reduction when parallel cultures of J774 or HepG2 were preconditioned with varying concentrations of Vitamin D₃ (0.4, 2, 4 μ g/ml) or DHA (8, 16, 32 μ g/ml) for 1 or 5 days. Five days' preconditioning with Vitamin D₃ (0.4, 2, 4 μ g/ml) or DHA (8, 16, 32 μ g/ml) before stimulation with LPS was not more effective in reducing the inflammatory gene response than preconditioning for 1 day only. The TNF α response in J774 and HepG2 elicited by an acute 4 h-stimulation with LPS was significantly reduced at 1 and 5 days of subsequent incubation with Vitamin D₃ or DHA at three different doses each (Figures 1E–H). Macrophages at 5 days' stimulation with Vitamin D₃ or DHA seemed particularly depressed in their TNF α production (Figures 1E,G).

Analysis of the same supernatants for TGF β showed that Vitamin D₃ and DHA significantly reduced TGF β production of J774 and HepG2 when preconditioned with these modulators prior to LPS stimulation (Figures 2A–D). LPS led to a robust production of TGF β in macrophages and hepatocytes. It is known that LPS can trigger TGF β production in monocytes (Toossi et al., 1996). Macrophages at 5 days' stimulation with Vitamin D₃ or DHA seemed particularly depressed in their TGF β production (Figures 2A,C). Addition for 1 day and 5 days of higher doses of Vitamin D₃ (2, 4 μ g/ml) or DHA (16, 32 μ g/ml) prior to the LPS stimulation reduced the production of TGF β by HepG2 (Figures 2B,D). LPS-induced TGF β production by J774 and HepG2 cells was reduced in a dose dependent manner by the subsequent addition of Vitamin D₃ or DHA for 1 and 5 days

(Figures 2E–H). HepG2 seemed more sensitive to the action of Vitamin D₃ or DHA compared to J774.

Incubation with FFA elicited a dose dependent TNF α release from J774 and HepG2 compared to basal levels (Figures 3A–D). When preconditioned with FFA, macrophages showed a TNF α response to subsequent LPS stimulation which was of the same magnitude as LPS alone (Figure 3A). Stimulation with FFA subsequent to LPS, however, led to an impaired TNF α response (Figure 3C). In HepG2, stimulation with 30 mM FFA for 5 days prior to LPS exposure, produced TNF α levels which were reduced compared to those generated by LPS alone (Figure 3B), while subsequent exposure with FFA after LPS stimulation further increased TNF α secreted by HepG2 compared to LPS stimulated cells (Figure 3D). Regarding TGF β , the levels produced by J774 were highest after 5 days of either preconditioning of cells with 30 mM FFA before LPS stimulation, or *post-hoc* treatment of cells with 30 mM FFA after LPS stimulation (Figures 3E,G). The same observation was made for HepG2 cells: stimulation with 30 mM FFA for 5 days prior to LPS exposure, produced TGF β levels which exceeded those generated by LPS alone (Figure 3F), as did *post-hoc* exposure with 30 mM FFA after LPS (Figure 3H).

Effect of DHA and Vitamin D₃ on LPS-Induced mRNA Expression of Srebp-1c and PPAR α by Macrophages and Hepatocytes

LPS exposure of J774 and HepG2 stimulated Srebp-1c mRNA expression (Figure 4) as expected (Costales et al., 2013). Preconditioning of J774 for 1 day with Vitamin D₃ led to a dose dependent reduction of Srebp-1c mRNA, which was further reduced after 5 days' preconditioning. This means that 5 days of incubation with a minimum dose of Vitamin D₃ (0.4 μ g/ml) prevented the induction of Srebp-1c observed after addition of 100 ng/ml LPS for 24 h (Figure 4A). The same pattern was observed for HepG2 cells, although higher doses of Vitamin D₃ (2, 4 μ g/ml) were efficient after just 1 day of preconditioning (Figure 4B). Preconditioning of J774 and HepG2 with DHA for 5 days efficiently downregulated Srebp-1c mRNA with a minimum dose studied of 8 μ g/ml (Figures 4C,D). One day prestimulation with 8 μ g/ml DHA significantly reduced Srebp-1c mRNA in HepG2, while higher doses were necessary to achieve this effect in J774 (Figures 4C,D). Exposure of LPS stimulated J774 with Vitamin D₃ or DHA yielded a dose dependent reduction in Srebp-1c mRNA for both durations of incubation, 1 and 5 days (Figures 4E,G). Higher doses of Vitamin D₃ (2, 4 μ g/ml) were needed to maintain the reduction in Srebp-1c mRNA observed at 1 day *post-hoc* stimulation compared to 5 days (Figure 4E). DHA, by contrast, significantly downregulated Srebp-1c mRNA at 5 days' incubation after LPS stimulation compared to 1 day incubation (Figure 4G). In the presence of Vitamin D₃, LPS-induced Srebp-1c mRNA expression was effectively downregulated at 1 and 5 days' stimulation in HepG2 (Figure 4F), whereas higher doses of DHA had this effect at 5 days (Figure 4H).

An opposing pattern of stimulation is obtained when studying PPAR α mRNA expression (Figures 5A–H), whose translation

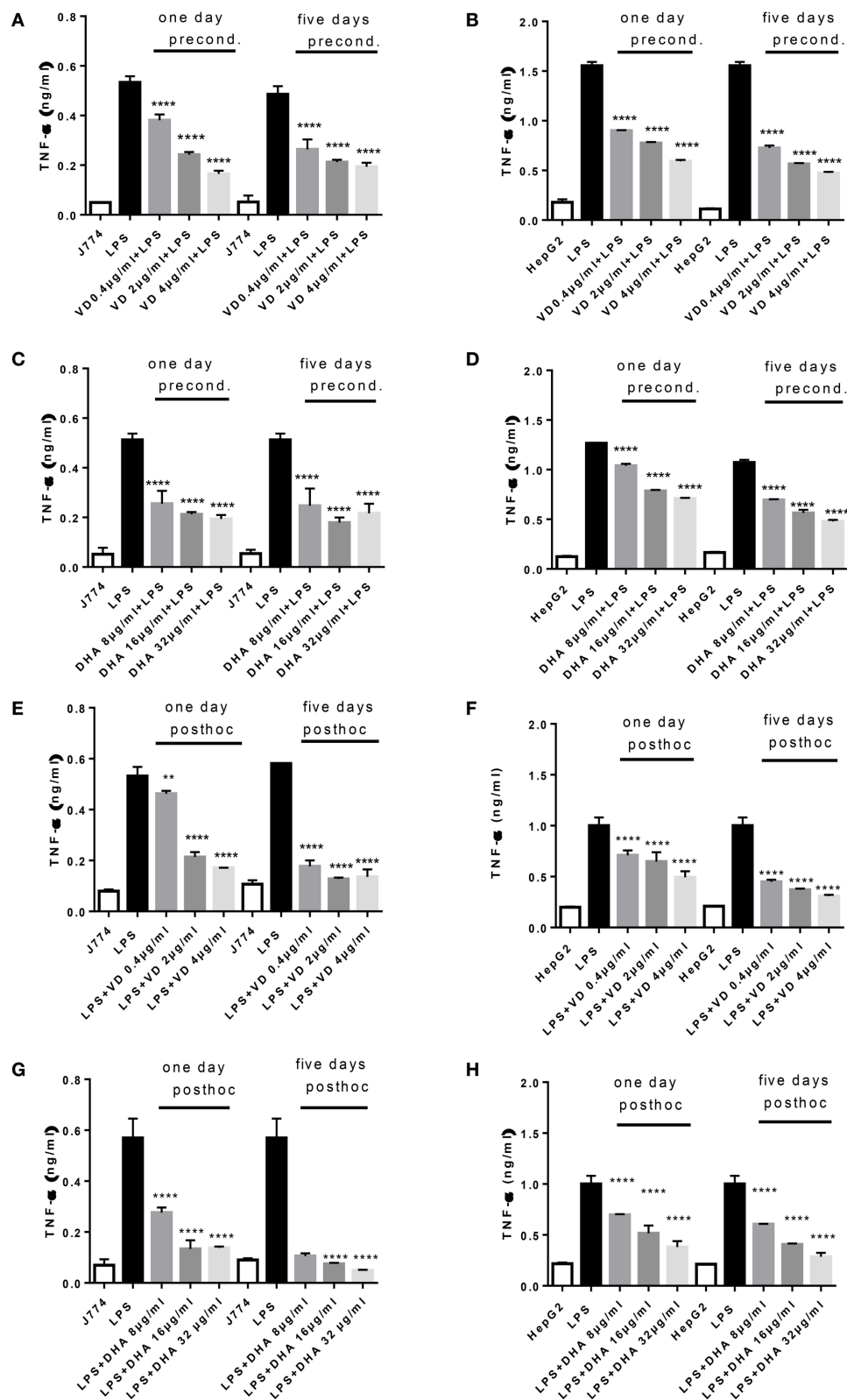


FIGURE 1 | (A–H) TNFα response of J774 (A,C,E,G) and HepG2 (B,D,F,H) toward LPS and its modulation by preconditioning with Vitamin D₃ (A,B) or DHA (C,D) or *post-hoc* (subsequent to LPS) stimulation with Vitamin D₃ (E,F) or DHA (G,H). Asterisks indicate significant differences to the LPS induced response. Results are presented as averages ±SD from triplicate determinations. ***p* < 0.01, *****p* < 0.0001 (adjusted *p*-values).

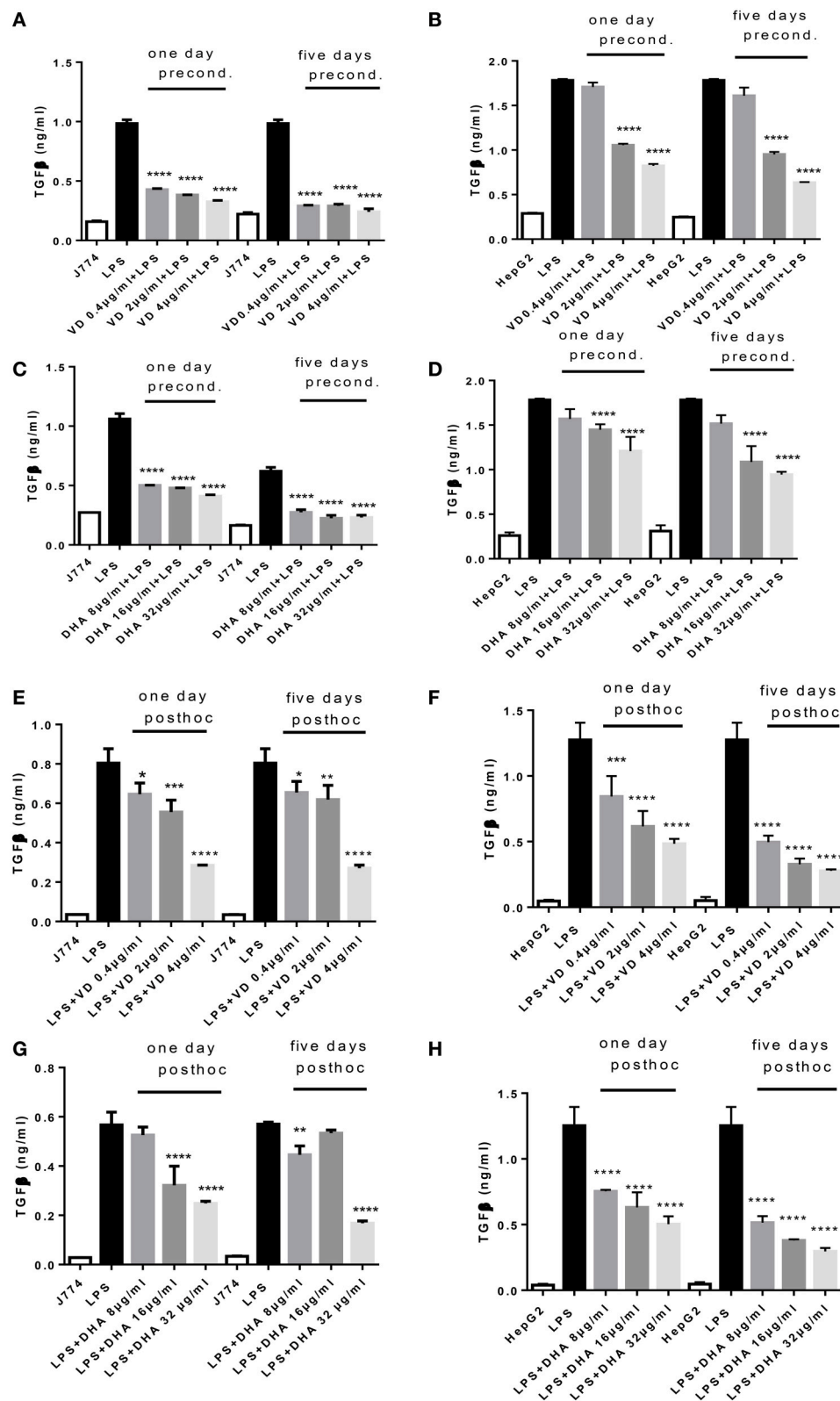


FIGURE 2 | (A–H) TGFβ response of J774 (A,C,E,G) and HepG2 (B,D,F,H) toward LPS and its modulation by preconditioning with Vitamin D₃ (A,B) or DHA (C,D) or *post-hoc* (subsequent to LPS) stimulation with Vitamin D₃ (E,F) or DHA (G,H). Asterisks indicate significant differences to the LPS induced response. Results are presented as averages ±SD from triplicate determinations. **p* < 0.05, ***p* < 0.01, ****p* < 0.005, *****p* < 0.0001 (adjusted *p*-values).

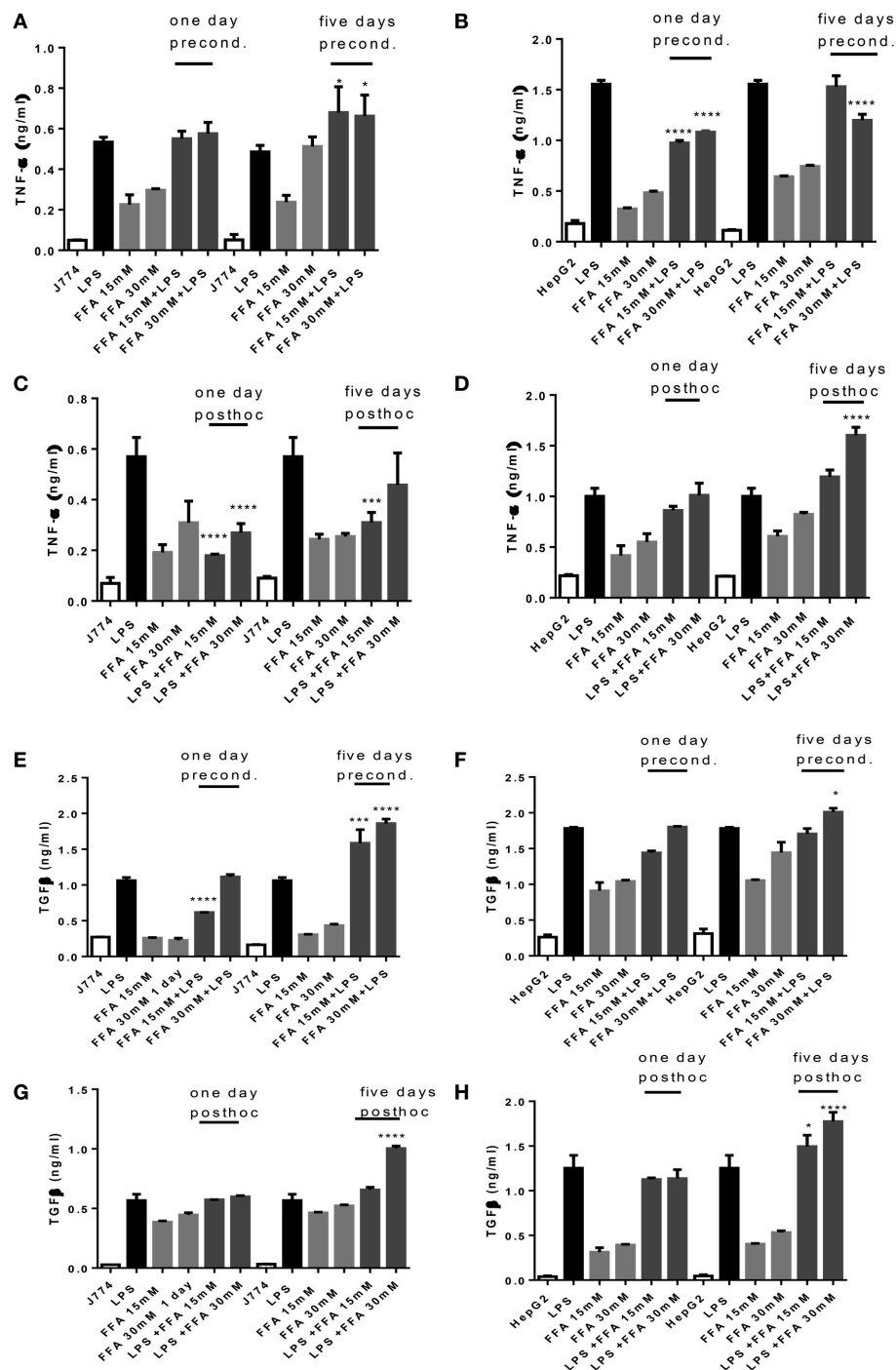


FIGURE 3 | (A–H) TNF α and TGF β response of J774 (A,C,E,G) and HepG2 (B,D,F,H) toward LPS and its modulation by preconditioning with FFA (A,B,E,F) or *post-hoc* (subsequent to LPS) stimulation with FFA (C,D,G,H). Asterisks indicate significant differences to the LPS induced response. Results are presented as averages \pm SD from triplicate determinations. * p < 0.05, *** p < 0.005, **** p < 0.0001 (adjusted p -values).

product is involved in the breakdown of fatty acids. LPS-induced stimulation of PPAR α mRNA was very low, contrasting with the robust increase of Srebp-1c mRNA. High PPAR α mRNA was achieved in conditions of preconditioning with Vitamin D₃ for 1 day prior to LPS stimulation of J774, whereas this required 5 days

preincubation for HepG2 (Figures 5A,B). There was a notable delay in PPAR α mRNA response in HepG2 cells preconditioned with Vitamin D₃ (Figure 5B). A dose dependent response in PPAR α mRNA expression is recorded when stimulating J774 or HepG2 with LPS and subsequently with Vitamin D₃ or

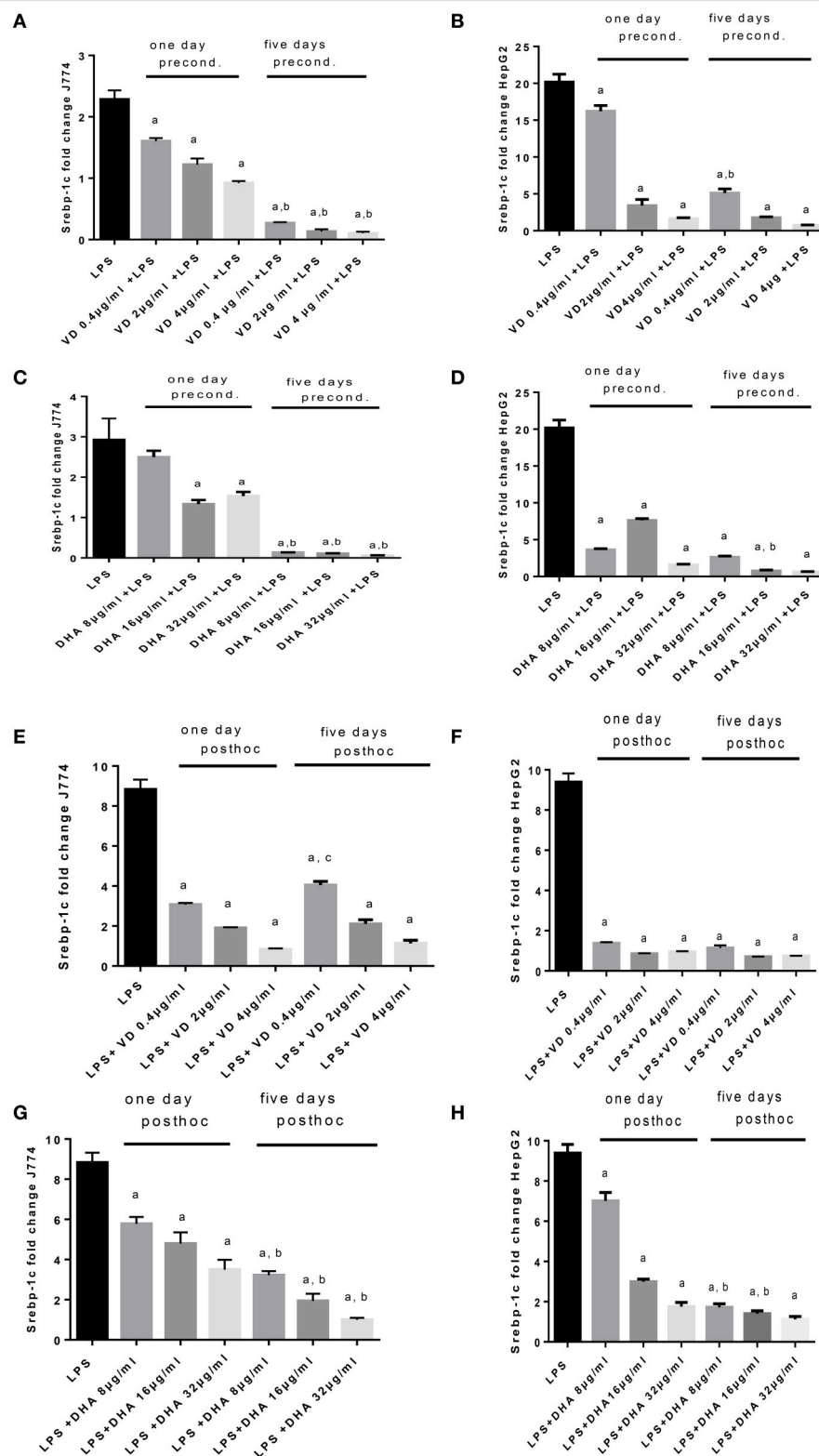


FIGURE 4 | (A–H) qPCR analysis of J774 (**A,C,E,G**) and HepG2 (**B,D,F,H**) for Srebp-1c mRNA expression induced by LPS and modulated by preconditioning with Vitamin D₃ (**A,B**) or DHA (**C,D**) or *post-hoc* (subsequent to LPS) stimulation with Vitamin D₃ (**E,F**) or DHA (**G,H**). Triplicate data were averaged, normalized to GAPDH mRNA abundance and expressed in relation to baseline Srebp-1c mRNA expression. ^a*p* < 0.0001 compared to LPS, ^b*p* < 0.0001 compared to 1 day precond, ^c*p* < 0.002 compared to 1 day *post-hoc* stimulation (adjusted *p*-values).

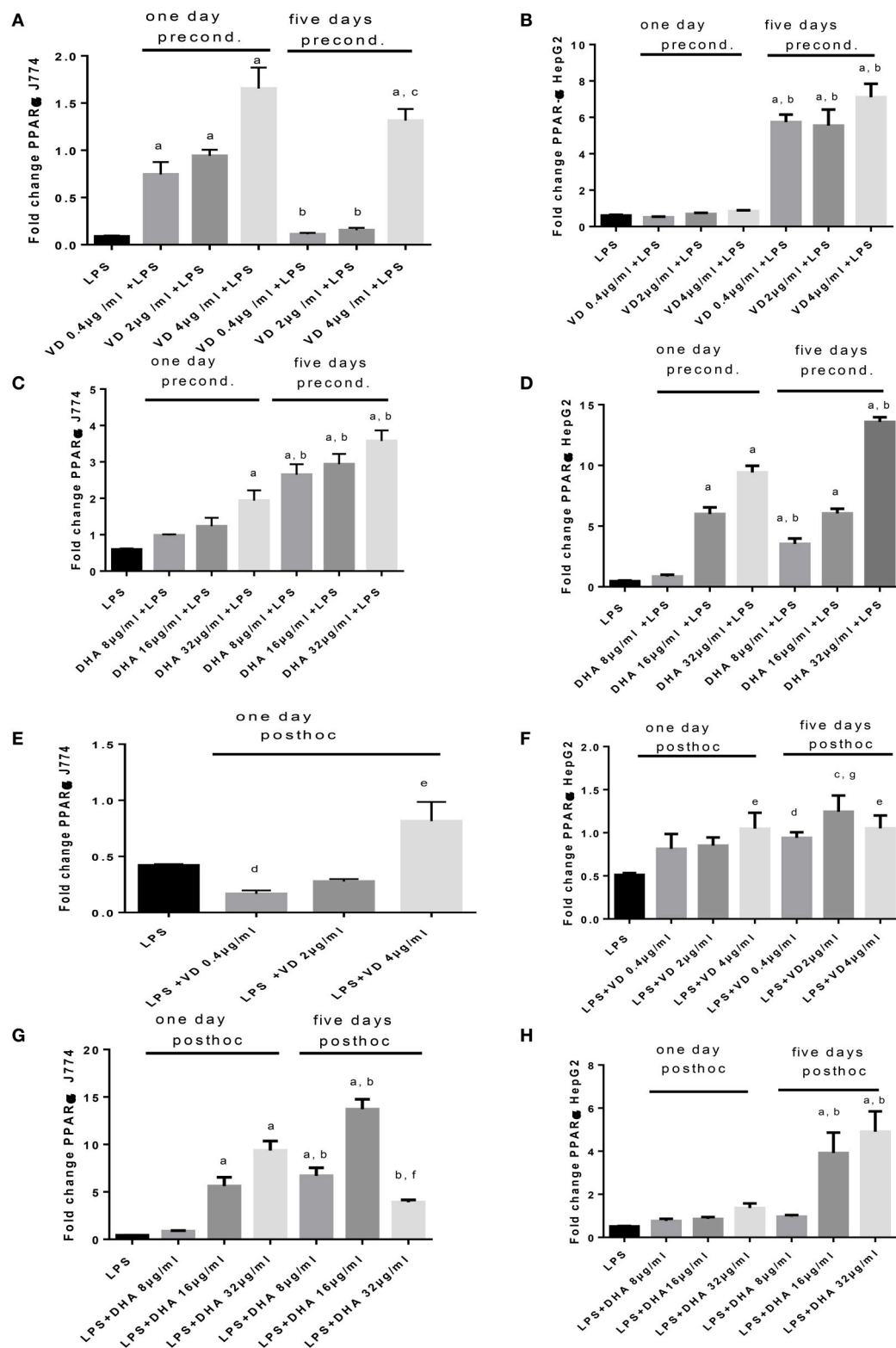


FIGURE 5 | (A–H) qPCR analysis of J774 (**A,C,E,G**) and HepG2 (**B,D,F,H**) for PPARα mRNA expression induced by LPS and modulated by preconditioning with Vitamin D (**A,B**) or DHA (**C,D**) or *post-hoc* (subsequent to LPS) stimulation with Vitamin D₃ (**E,F**) or DHA (**G,H**). Triplicate data were averaged, normalized to GAPDH mRNA abundance and expressed in relation to baseline PPARα mRNA expression. ^a $p < 0.0001$ compared to LPS, ^b $p < 0.0001$ compared to day 1, ^c $p < 0.05$ compared to day 1, ^d $p < 0.05$ compared to LPS, ^e $p < 0.005$ compared to LPS, ^f $p < 0.001$ compared to LPS, ^g $p < 0.0005$ compared to LPS (adjusted p -values).

DHA. The highest levels of PPAR α mRNA were achieved at 5 days' preincubation of J774 or HepG2 with 32 μ g/ml DHA (Figures 5C,D). After LPS stimulation, Vitamin D₃ (4 μ g/ml) was able to increase PPAR α mRNA expression significantly after 5 days' treatment in J774 and HepG2 cells (Figures 5E,F). One day's *post-hoc* stimulation with DHA (after LPS stimulation) at doses of 16 and 32 μ g/ml was more efficient at increasing PPAR α mRNA in J774 than in HepG2 (Figures 5G,H).

The sole addition of FFA without LPS led to significantly increased levels of Srebp-1c mRNA expression at 5 days' stimulation compared to 1 day stimulation of J774 cells. FFA at 30 mM increased Srebp-1c mRNA compared to LPS-induced expression significantly when added for 5 days to J774 culture prior to LPS stimulation (Figure 6A). While FFA at 30 mM significantly increased Srebp-1c mRNA at 5 days' stimulation compared to the shorter incubation period of 1 day, additional stimulation with LPS did not further increase Srebp-1c mRNA in HepG2 cells (Figure 6B). Prior stimulation with LPS of J774 and addition of FFA for 1 and 5 days significantly increased Srebp-1c mRNA (Figure 6C). HepG2 cells showed an inconsistent pattern of changes in Srebp-1c mRNA in response to LPS and subsequent stimulation with FFA at 15 and 30 mM (Figure 6D). FFA given for 1 and 5 days had a significant role in increasing PPAR α mRNA levels. Subsequent LPS stimulation decreased this effect (Figure 6E). HepG2 cells showed an inconsistent pattern of changes in PPAR α mRNA in response to FFA and subsequent stimulation with LPS (Figure 6F). Subsequent stimulation of J774 with FFA after 4 h LPS led to modulation of PPAR α mRNA (Figure 6G); a clear reduction in PPAR α mRNA was observed in HepG2 cells exposed to 15 mM FFA after LPS stimulation (Figure 6H).

DISCUSSION

Endotoxemia is an important factor in the progression from uncomplicated NAFLD to NASH (Finelli and Tarantino, 2014), which carries the risk of developing cirrhosis. This study analyses in parallel two TLR4 positive cell types of relevance to the liver, the macrophage and hepatocyte, for their regulation of inflammatory, fibrotic, and steatotic reactants when stimulated with Vitamin D₃ or DHA. These are dietary immune modulators (Simopoulos, 2002; Prietl et al., 2013) whose adjuvant clinical utility is beginning to be widely studied in preclinical and clinical studies. The interest of this study lay in investigating a qualitative difference in ameliorating the effect of endotoxin if Vitamin D₃ or DHA were present prior to this stimulus or available shortly after. This might give an indication whether constant dietary admixture is recommendable or whether doses of treatment after LPS stimulus were effective. TNF α is a key factor in the development of NAFLD and NASH in both humans and animals (Braunersreuther et al., 2012). Hasegawa et al. showed that TGF β 1 levels were increased in patients with NASH as compared to patients with hepatic steatosis (Hasegawa et al., 2001). Macrophages determine progression of inflammation. This is because activation of macrophages in liver leads to production of inflammatory mediators such

as TNF, Interleukin-1 and reactive oxygen species in NASH. These inflammatory mediators further stimulate hepatocytes and hepatic satellite cells to induce hepatocyte steatosis and fibrosis. Macrophages encourage development of steatohepatitis because of the interaction of chemokine-chemokine receptors on liver cells and Kupffer cells (Miura et al., 2012). Steatosis, liver injury and proinflammatory monocyte infiltration was reduced *in vivo* as a result of depletion of Kupffer cells (Tosello-Tramont et al., 2012).

This study finds that preconditioning with Vitamin D₃ or DHA prior to LPS stimulation or incubation following LPS stimulation ("*post-hoc*") decreased TNF α and TGF β production of J774 and of HepG2 significantly. Vitamin D₃ and DHA signal via distinct receptors (VDR and GPR120) but affect TLR4 mediated signaling effects in a similar manner, whether allowing cells to adapt to the presence of Vitamin D₃ or DHA prior to LPS stimulation or treating the inflammatory state of HepG2 or J774 induced by LPS with Vitamin D₃ or DHA subsequently.

Stimulation with obesity relevant FFA elicits elevated levels of inflammatory cytokines in HepG2 and J774. The use of FFA allowed consideration of development of tolerance in our system; because FFA bind to TLR4 as endogenous DAMP, it is possible that there is dampening of LPS mediated effects on subsequent challenge or, vice versa, dampening of FFA effect because of prior TLR4 engagement with LPS. In our study, there might be evidence of tolerance development when stimulating J774 with LPS, then subsequently with FFA for 1 day, because the elicited TNF α response compared to that of FFA stimulation alone and was half that achieved by LPS stimulation alone (Figure 3C). This was not observed for HepG2.

Vitamin D₃ and DHA efficiently downregulated LPS-induced Srebp-1c mRNA expression in J774 and HepG2 cells regardless of the timing of their addition, whether Vitamin D₃ and DHA were present before (preconditioning) or after (*post-hoc*) LPS stimulation. The translation product of Srebp-1c mRNA is involved in increasing cholesterol biosynthesis as part of an acute phase reaction (Diomedea et al., 2000). PPAR α mRNA expression showed an inverse pattern to that observed for Srebp-1c mRNA, with regard to LPS-induced regulation and modulation by Vitamin D₃ and DHA. Our data suggest that qPCR can detect a high ratio of Srebp-1c/PPAR α in LPS-stimulated HepG2 and J774 which is viewed as unfavorable in the obese (Pettinelli et al., 2009) and is provoked by the addition of FFA in our study. Hepatic Srebp-1c mRNA levels were significantly elevated, while hepatic PPAR α mRNA levels were significantly lower in obese patients undergoing bariatric surgery compared to non-obese patients undergoing cholecystectomy (Pettinelli et al., 2009). Relevant to our study, these obese patients with NAFLD had significantly lower DHA in liver total lipids compared to their study controls (Pettinelli et al., 2009).

Taken together, Vitamin D₃ and DHA exerted beneficial effects in the context of endotoxin stimulation by reducing TNF α and TGF β proteins, and Srebp-1c mRNA, whilst increasing PPAR α mRNA. The translation products of Srebp-1c and PPAR α mRNA (which were not measured) are involved in cholesterol biosynthesis and triglyceride turnover, respectively. Macrophage responses are skewed toward a phenotype that

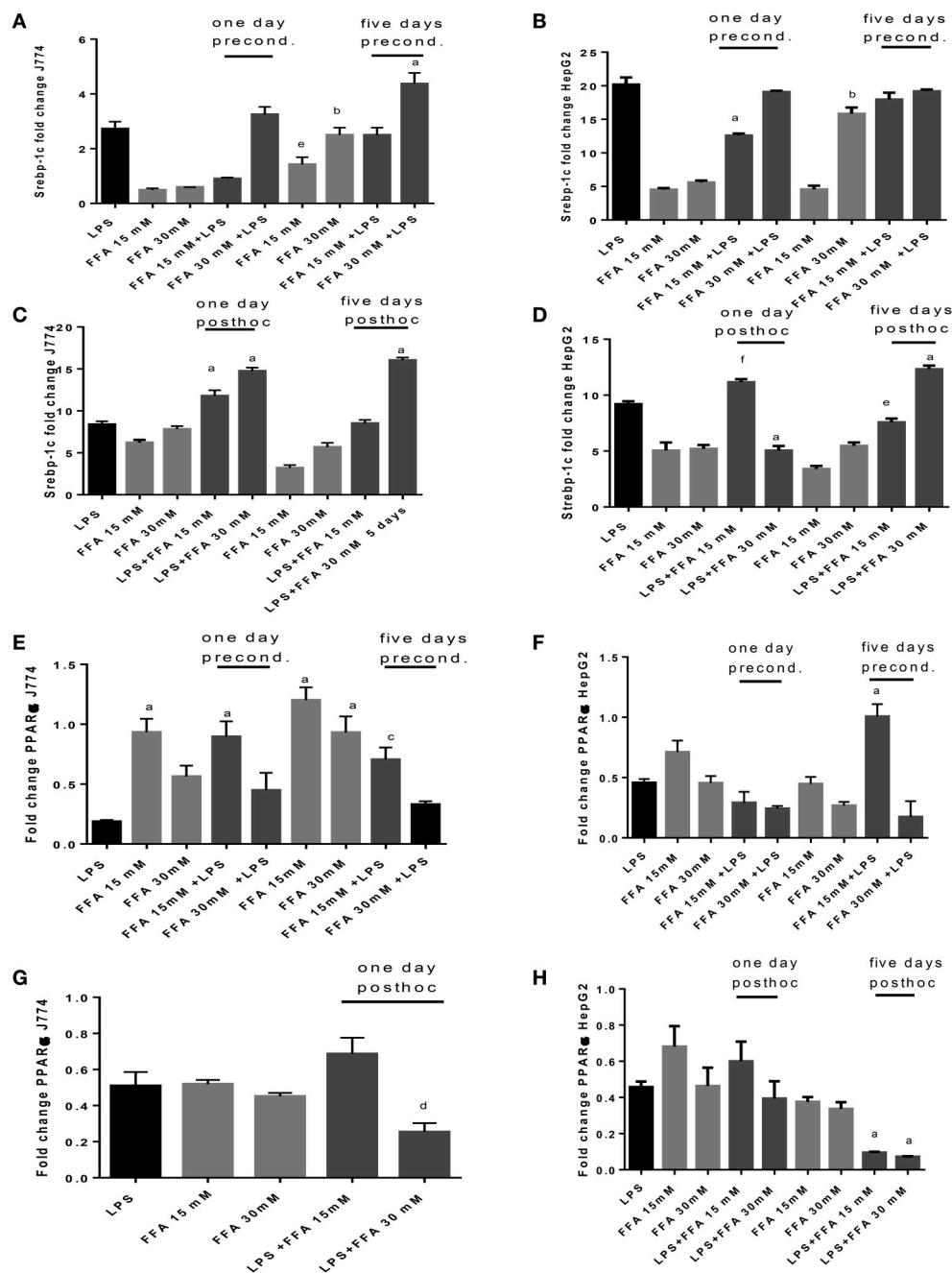


FIGURE 6 | (A–H) qPCR analysis of J774 (A,C,E,G) and HepG2 (B,D,F,H) for Srebp-1c (A–D) and PPARα (E–H) mRNA expression induced by LPS and modulated by preconditioning with FFA (A–D) or *post-hoc* (subsequent to LPS) stimulation with FFA (E–H). Triplicate data were averaged, normalized to GAPDH mRNA abundance and expressed in relation to baseline Srebp-1c or PPARα mRNA expression. ^a $p < 0.0001$ compared to LPS, ^b $p < 0.0001$ compared to day 1, ^c $p < 0.001$ compared to LPS, ^d $p < 0.005$ compared to LPS, ^e $p < 0.005$ compared to day 1, ^f $p < 0.002$ compared to LPS (adjusted p -values).

produces less proinflammatory $\text{TNF}\alpha$ and profibrotic $\text{TGF}\beta$, thereby is less able to support NAFLD development and progression to NASH (Dey et al., 2014). Both, lipid metabolism and inflammation are relevant in the development of insulin resistance (Glass and Olefsky, 2012). Our data lend support to both, curative and preventative dietary regimes involving

Vitamin D₃ or DHA to quench the detrimental effects of low grade endotoxemia. *In vivo* studies are needed next to address the efficacy of dietary supplementation with an immune modulator in enhancing the outcome from NAFLD. One recent clinical trial of supplementation with Vitamin D₃ showed significant improvement in some serum markers of inflammation, but

a greater observation time (>4 months) was likely to be necessary to observe amelioration in transaminase levels and ultrasonographic grades of NAFLD (Sharifi et al., 2014). A meta-analysis of treatments of patients with NAFLD (for a median duration of 6 months) with supplements of omega 3 fatty acids showed significant ultrasonographic improvement of steatosis, likely involving a reversal of the Srebp-1c/PPAR α ratio (Parker et al., 2012).

AUTHOR CONTRIBUTIONS

RK, Substantial contributions to the acquisition, analysis, and interpretation of data for the work, drafting the work, final approval of the version to be published, agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. JH, Substantial contributions to the conception or design of the work, final approval of the version to be published, agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. CS, Substantial contributions to the interpretation of data for the work, drafting

the work and revising it critically for important intellectual content, final approval of the version to be published, agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This study was funded by HCDP (Human Capacity Development Program) Kurdistan region government (award to RK).

ACKNOWLEDGMENTS

The study contains part of RK PhD thesis (University of Leicester). Dr S. Byrne is acknowledged for technical support, and Dr Z. Zwaini is acknowledged for critical reading of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fcell.2016.00061>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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