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Journal of Cellular Biochemistry 116:711–720 (2015)

Journal of Cellular Biochemistry

Vitamin D_3 Metabolites Enhance the NLRP3-Dependent Secretion of IL-1β From Human THP-1 Monocytic Cells

Sarah E. Tulk,[1] Kuo-Chieh Liao,[1] Daniel A Muruve,[2] Yan Li,[2] Paul L. Beck,[2] and Justin A. MacDonald[1]*

[1]*Department of Biochemistry & Molecular Biology, University of Calgary, 3280 Hospital Drive NW, Calgary, AB, T2N 4Z6, Canada*

[2]*Department of Medicine, University of Calgary, 3280 Hospital Drive NW, Calgary, AB, T2N 4Z6, Canada*

ABSTRACT

Vitamin D_3 has emerged as an important regulator of the immune system. With metabolic enzymes for vitamin D_3 activation and vitamin D receptors (VDR) now identified in a variety of immune cells, the active vitamin D_3 metabolite $1,25(OH)_2D_3$, is thought to possess immunomodulatory properties. We examined whether $1,25(OH)_2D_3$ might also enhance the NLRP3-dependent release of mature IL-1β from macrophages. PMA-differentiated THP-1 cells were stimulated with vitamin D_3 metabolites and assessed for CYP27, CYP24, NLRP3, ASC, pro-caspase-1 expression by western blot and real-time qPCR as well as inflammasome activation with pro-inflammatory cytokine IL-1β release measured by ELISA. Exposure to $1,25(OH)_2D_3$ had no effect on the basal expression levels of VDR; however, CYP27A1 transcript was suppressed and CYP24A1 transcript was substantively elevated. Both $1,25(OH)_2D_3$- and $25(OH)D_3$ induced IL-1β release from THP-1 cells, and these effects were blocked with application of the caspase-1 inhibitor YVAD and the NLRP3 inhibitors glyburide and Bay 11–7082. Interestingly, $1,25\ (OH)_2D_3$ exposure reduced NLRP3 protein expression but had no effect on ASC or pro-caspase-1 protein levels. The increase in mature IL-1β elicited by $1,25(OH)_2D_3$ was modest compared to that found for ATP or *C. difficile* toxins. However, co-treatment of THP-1 cells with ATP and $1,25(OH)_2D_3$ resulted in more IL-1β secretion than ATP or $1,25(OH)_2D_3$ alone. J. Cell. Biochem. 116: 711–720, 2015.

KEY WORDS: NOD-LIKE RECEPTOR; NLR; MACROPHAGE; INNATE IMMUNITY; VDR; INTERLEUKIN-1β; 1; 25-DIHYDROXY-VITAMIN D_3

Vitamin D_3 or 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$), is most well known for its role in calcium and phosphate metabolism [Norman, 2006] but has gained recent attention both in the scientific literature and popular media for its emerging role in immunity and inflammatory diseases [Prietl et al., 2013; Djukic et al., 2014; Wang et al., 2014]. Active vitamin D_3, (i.e, $1,25(OH)_2D_3$) is formed by a 3-step process [Wikvall, 2001; Henry, 2011]. First, 7-dehydocholesterol (7DHC) is converted to cholecalciferol (vitamin D_3) by UVB irradiation of the skin. Next, vitamin D_3 is hydroxylated to form 25-dihydroxyvitamin D_3 ($25(OH)D_3$) in the liver. Finally, a second hydroxylation occurs in the kidney to form the biologically active metabolite, $1,25(OH)_2D_3$. Active vitamin D_3 was recently shown to affect the expression of innate immune sensors in macrophages [Wang et al., 2010] and to induce the expression of important antimicrobial factors [Wang et al., 2004; Gombart et al., 2005]. Further emphasizing the emerging importance of $1,25(OH)_2D_3$ in innate immune function, peripheral blood macrophages can produce local supplies of $1,25(OH)_2D_3$ from $25(OH)D_3$ [Gottfried et al., 2006]. Thus, there is an emerging role for vitamin D_3 in the innate immune system wherein it may influence cellular sensing and

Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; 7DHC, 7-dehydocholesterol; 1, 25 (OH)2D3, 1,25-dihydroxy-vitamin D3; $25(OH)D_3$, 25-dihydroxyvitamin D3; DAMPs, danger-associated molecular patterns; IL, interleukin; MDP, muramyl dipeptide; NLRP, NACHT, LRR and PYD domain-containing protein; NOD, nucleotide-binding oligomerization domain-containing protein; PAMP, pathogen-associated molecular patterns; PMA, phorbol-12-myristate-13-acetate; PRR, pattern recognition receptor; VDR, calcitriol or vitamin D3 receptor; VDRE, vitamin D response element.

Grant sponsor: Canadian Institutes of Health Research (MOP-98004).

*Correspondence to: Justin A. MacDonald, Libin Cardiovascular Institute of Alberta, Department of Biochemistry and Molecular Biology, University of Calgary, Health Research Innovation Center, 3280 Hospital Drive NW, Calgary, AB, T2N 4Z6, Canada. E-mail: jmacdo@ucalgary.ca

Manuscript Received: 20 May 2014; Manuscript Accepted: 22 September 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 5 January 2015

DOI 10.1002/jcb.24985 • © 2015 Wiley Periodicals, Inc.

responses of macrophages [Prietl et al., 2013; Ryynänen and Carlberg, 2013; Verma et al., 2014] and dendritic cells [Karthaus et al., 2013; Kundu et al., 2014]. $1,25(OH)_2D_3$ was recently shown to induce gene and protein expression of the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) in both monocytes and epithelial cells [Wang et al., 2010]. This intracellular pattern recognition receptor (PRR) stimulates inflammatory signaling pathways following activation by pathogen-associated molecular patterns (PAMPs). Specifically, NOD2 is responsible for activating the inflammatory response following detection of muramyl dipeptide (MDP). This was the first study to show that $1,25(OH)_2D_3$ could directly modulate the expression of an innate immune receptor, and thus may be able to directly affect detection of PAMPs by the innate immune system. Further, $1,25(OH)_2D_3$ was shown to upregulate the production of pro-interleukin(IL)-1β in the differentiated human monocytic cell line U937 [Lee et al., 2011]. In this case, $1,25(OH)_2D_3$ exposure increased the expression of the pro-IL-1β gene with a concurrent increase in intracellular IL-1β, as assessed by flow cytometry. Thus, $1,25(OH)_2D_3$ is increasingly being linked to pro-inflammatory signalling of macrophages.

Similar to NOD2, the NACHT, LRR and PYD domain-containing protein 3 (NLRP3) is a PRR that responds to PAMPs and danger-associated molecular patterns (DAMPs) with the activation of the pro-inflammatory IL-1β cascade. Unlike NOD2, which responds solely to MDP, NLRP3 detects a wide range of stimuli (reviewed in: Schroder and Tschopp [2010]; Latz et al. [2013]). In response to these stimuli, NLRP3 oligermerizes with pro-caspase-1 via an adaptor protein, the apoptosis-associated speck-like protein containing a CARD, ASC [Agostini et al., 2004; Dowds et al., 2004; Yu et al., 2006]. This in turn converts pro-caspase-1 to the active caspase-1, which can then cleave pro-IL-1β to the pro-inflammatory cytokine IL-1β. Numerous intrinsic and extrinsic activators of NLRP3 inflammasomes have been described, including crystalline material, mitochondrial DNA, intracellular $[K^+]$, reactive oxygen species (ROS), peptide aggregates and bacterial toxins [Schroder and Tschopp, 2010; Horvath et al., 2011; Latz et al., 2013]. NLRP3 activation can proceed via a canonical two-checkpoint mechanism that involves (i) an initial priming of pro-IL-1β and NLRP3 expression by transcriptionally active PRRs, and (ii) an activation step that triggers NLRP3 inflammasome stimulation and IL-1β maturation [Netea et al., 2009]. An additional non-canonical three-checkpoint activation scheme for NLRP3 activation has been reported to involve caspase-11 induction in response to Gram-negative bacteria [Kayagaki et al., 2011]. The diversity of NLRP3 agonists suggests that direct interactions between NLRP3 and all of its activators are unlikely. Currently, it is thought that extrinsic triggers of the NLRP3 inflammasome act by influencing intrinsic regulatory mechanisms of inflammasome activation (i.e., ROS and redox status, osmotic pressure, K+ ion gradients, mitochondrial and/or lysosomal contents; Schroder and Tschopp, 2010; Latz et al., 2013).

Given the results of Wang et al. [2010] which demonstrated $1,25(OH)_2D_3$ could stimulate NOD2 signaling in macrophages, we hypothesized that $1,25(OH)_2D_3$ may also modulate the activity of other innate immune signalling receptors, namely the NLRP3 inflammasome. Herein we show that both $1,25(OH)_2D_3$ and its precursor $25(OH)_2D_3$ are able to stimulate the maturation of IL-1β from PMA-differentiated THP-1 cells via an NLRP-dependent process.

METHODS

REAGENTS

7DHC, vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ were obtained from Sigma-Aldrich (Oakville, ON) and dissolved in 100% molecular grade ethanol. Z-VAD-FMK (ZVAD, Enzo Life Sciences; Farmingdale, NY), Z-YVAD-FMK (YVAD, Enzo Life Sciences) and glyburide (Sigma-Aldrich) were prepared in 100% DMSO. All reagents for cell treatments were sterile filtered through a 0.22 μM filter prior to use. *Clostridium difficile* toxin (containing TcdA and TcdB) was prepared as described previously [Lee et al., 2013].

CELL CULTURE

THP-1 cells (ATCC; #TIB-202) were maintained at 37°C and 5% CO_2 in RPMI (Invitrogen/Life Technologies; Burlington, ON) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% penicillin-streptomycin and β-mercaptoethanol. To limit inter-experiment variability due to differences in cell passage, cells were used for experiments between the passages of 10 and 25, after which they were discarded and new cells were initiated from frozen liquid N_2 stocks. For all experiments, THP-1 cells were plated at a density of 500,000 cells/well in 24-well plates in the aforementioned media preparation with 100 nM of PMA and incubated overnight to allow for differentiation into a macrophage-like phenotype. Following differentiation, PMA-containing media was replaced with serum-free optiMEM for all treatments.

ANALYSIS OF MRNA TRANSCRIPT LEVELS

To quantitatively assess gene expression levels, mRNA was isolated using a standard TRIzol-chloroform extraction protocol and purified using the RNeasy kit (Qiagen, Toronto, ON, Canada). First strand cDNA synthesis was performed using the RT^2 First Strand Kit (SABiosciences/Qiagen) and RT-qPCR with the RT^2 SYBR Green Master qPCR Mix (SABiosciences) paired with the appropriate RT^2 qPCR Primer Assay Kit (all from SABiosciences/Qiagen: pro-caspase-1 #PPH00105B, Pro-IL-1β #PPH00171B, NLRP3 #PPH13170A, ASC #PPH00907A, CYP24A1 #PPH01279A, CYP27A1 #PPH01277A, CYP27B1 #PPH01242B, GAPDH PPH00150E) using an ABI Prism 7000 Sequence Detection System. GAPDH was used as an endogenous control, and all samples were normalized to an internal calibrator.

WESTERN BLOTTING AND ELISA

Pro-caspase-1, caspase-1, pro-IL-1β, VDR, NLRP3, ASC and β-actin expression were analyzed using standard western blotting protocols with antibodies obtained from Santa Cruz (VDR #sc-1008, pro-caspase-1 / caspase-1 #sc-622), Sigma-Aldrich (β-actin #A5441), Cell Signaling (cleaved IL-1β D116 #2021), Millipore (pro-IL-1β C-terminal #MABF22), R & D Systems (ASC #AF3805) and Adipogen (NLRP3 mAb #AG-20B-0014). THP-1 cell lysates were separated via SDS-PAGE electrophoresis and transferred to 0.2 μm nitrocellulose

or modified-PVDF at 100 V for 1 h at 4°C. VDR, NLRP3 and ASC protein expressions were assessed using a standard two-step western blotting protocol. Membranes were blocked in PBS containing 0.1% (v/v) Tween-20 (PBS-T) with 5% (w/v) milk for a minimum of 1 h at room temperature with gentle shaking. Membranes were then incubated in primary antibody overnight at 4°C. All primary antibodies were diluted 1:1,000 in PBS-T with 5% (w/v) milk. Membranes were then washed five times with PBS-T with each wash lasting 5 min. Secondary antibodies were diluted 1:5,000 in PBS-T containing 5% milk. Membranes were incubated in secondary for 1 h at room temperature with gentle shaking. Pro-caspase-1, caspase-1 and pro-IL-1β protein levels were assessed using a three-step western blotting protocol. The two-step protocol was followed until secondary incubation at which time membranes were incubated at room temperature with biotin-conjugated α-rabbit IgG (Cell Signalling) which was diluted 1:10,000 in PBS-T with 5% (w/v) milk. Membranes were washed as previously described, and incubated for 30 min at room temperature with streptavidin-conjugated horseradish peroxidase diluted 1:2,000 in PBS-T (no milk). Membranes were then washed as previously described prior to developing. To detect bands, either ECL or ECL Plus was used as per the manufacturer's protocol (GE Healthcare; Mississauga, ON). Blots were then visualized using an LAS 4000 Luminescent Image Analyzer (GE Healthcare). IL-1β was measured in cell supernatants using the OptEIA IL-1β ELISA Set II kit (BD Biosciences, Mississauga, ON) according to the manufacturer's protocol.

RESULTS

$1,25(OH)_2D_3$ TREATMENT OF PMA-DIFFERENTIATED THP-1 CELLS ALTERS CYTOCHROME P450 CYP27A1 AND CYP24A1 TRANSCRIPT LEVELS BUT NOT VITAMIN D RECEPTOR (VDR)

PMA-differentiated THP-1 cells exhibited a basal expression of VDR mRNA and protein that was not affected by 24 h treatment with 500 nM of $1,25(OH)_2D_3$ (Fig. 1A and B). The formation of $1,25(OH)_2D_3$ is catalyzed by cytochrome P450 (CYP27) enzymes [Henry,

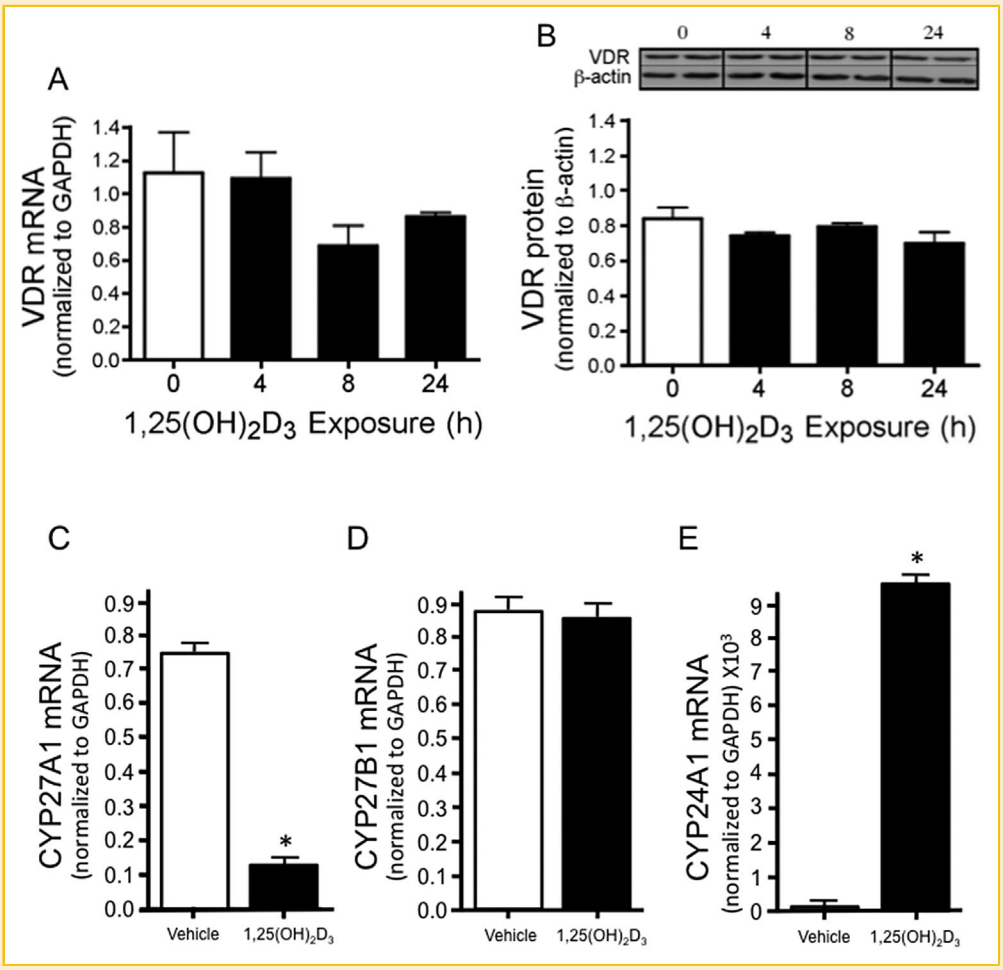

Fig. 1. Effect of $1,25(OH)_2D_3$ exposure on the expression of CYP27A1, CYP27B1, CYP24A1 hydroxylases and the vitamin D receptor (VDR) in PMA-differentiated THP-1 cells. In (A), the vitamin D receptor (VDR) transcript was not affected by up to 24 h exposure to 100 nM $1,25(OH)_2D_3$. PMA-differentiated THP-1 cells express the VDR protein as assessed using western blot (B), and VDR protein levels were unaffected by up to 24 h of exposure to 500 nM $1,25(OH)_2D_3$. Data represent the mean ± S.E.M. for n = 12. The transcript levels of vitamin D hydroxylase CYP27A1 (C), CYP27B1 (D) and CYP24A1 (E) are differentially affected by 24 h exposure to 100 nM $1,25(OH)_2D_3$. Data represent the mean ± S.E.M. for n = 4. *Significantly different from vehicle-treated (Student's *t*-test; $P < 0.05$).

2011; Jones et al., 2012]. PMA-differentiated THP-1 cells do exhibit basal expression of CYP27 family members (Supplemental Fig. 1). The PCR produced a single product of the expected size (~2000 bp) with exon-flanking primers, and the transcript was sequenced to confirm its identity. One of the most important regulators of vitamin D_3 abundance is actually its active hormonal form (i.e., $1,25(OH)_2D_3$ itself), which can act by direct negative feedback mechanisms to regulate cytochrome P450 transcription [Henry, 2011]. When PMA-differentiated THP-1 cells were treated with $1,25(OH)_2D_3$ (100 nM, 24 h), the transcript level of CYP27A1 was suppressed (~80% decrease; Fig. 1C) whereas that of CYP27B1 remained unchanged (Fig. 1D). As the cytochrome P450 component that catalyzes the conversion of $25(OH)D_3$ and $1,25(OH)_2D_3$ into 24-hydroxylated products, CYP24A1 constitutes the main degradation pathway for the vitamin D_3 molecule [Jones et al., 2012]. In this case, we observed a substantive increase in CYP24A1 transcript (~2,800-fold) with exposure of THP-1 cells to $1,25(OH)_2D_3$ (Fig. 1E).

IL-1β RELEASE FROM PMA-DIFFERENTIATED THP-1 CELLS IS INDUCED BY EXPOSURE TO $25(OH)D_3$ AND $1,25(OH)_2D_3$

To assess whether $1,25(OH)_2D_3$ or any of its precursors could stimulate PMA-differentiated THP-1 cells to release IL-1β, cells were treated with 7DHC, vitamin D_3, $25(OH)D_3$ or $1,25(OH)_2D_3$ (100 nM, 24 h). Subsequently, the IL-1β secretion into the culture supernatant was measured via ELISA (Fig. 2A). While neither 7DHC nor vitamin D_3 caused a significant increase in IL-1β release as compared to the vehicle-treated control, both $25(OH)D_3$ and $1,25(OH)_2D_3$ were able to stimulate IL-1β secretion. To further characterize $25(OH)D_3$- and $1,25(OH)_2D_3$-induced IL-1β release, PMA-differentiated THP-1 cells were treated with each agent over a concentration range (i.e, 1 pM - 500 nM) for 24 h and then assessed for IL-1β release. Exposure to 25 $(OH)D_3$ caused a significant increase in IL-1β release at a minimum concentration of 100 nM (Fig. 2B), while $1,25(OH)_2D_3$ caused a significant increase in IL-1β secretion at a minimum concentration of 1 nM (Fig. 2C). Thus, the release of IL-1β from THP-1 cells appeared to be more sensitive to $1,25(OH)_2D_3$ exposure.

$25(OH)D_3$- AND $1,25(OH)_2D_3$-INDUCED IL-1β RELEASE IS ATTENUATED BY TREATMENT WITH CASPASE-1 AND NLRP3 ANTAGONISTS

Mature IL-1β is produced by the cleavage of pro-IL-1β by caspase-1, which is in turn can be produced by the activated NLRP3 inflammasome. To assess whether $25(OH)D_3$- and $1,25(OH)_2D_3$-induced IL-1β release was dependent on this processing pathway,

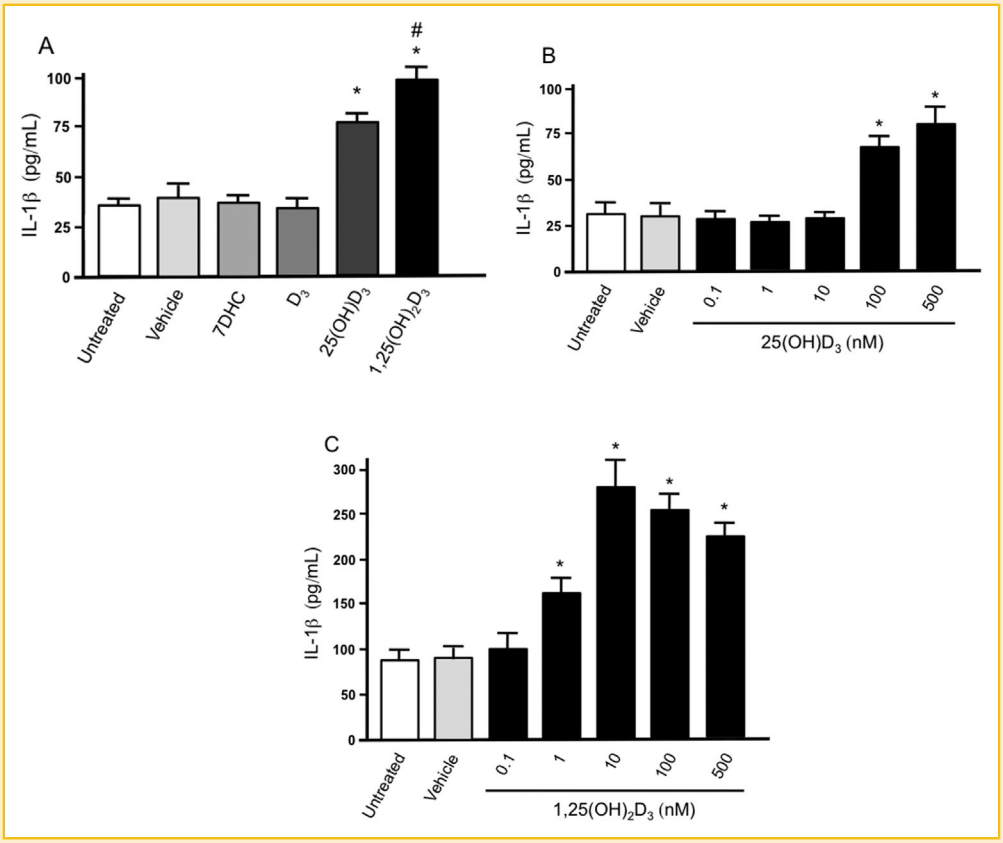

Fig. 2. Treatment with $25(OH)D_3$ or $1,25(OH)_2D_3$ increased IL-1β secretion from PMA- differentiated THP-1 cells. In (A), PMA-differentiated THP-1 cells were treated (100 nM, 24 h) with vehicle, 7 dehydrocholesterol (7DHC), vitamin D_3 (D_3), $25(OH)D_3$ or $1,25(OH)_2D_3$. IL-1β released into cell culture supernatants was assessed via ELISA. Exposure to $25(OH)D_3$ was found to cause a significant increase in IL-1β release at 100 nM (B), while $1,25(OH)_2D_3$ elicited a significant increase in IL-1β release at 1 nM (C). *Significantly different from vehicle-treated; #significantly different from $25(OH)D_3$-treated (ANOVA with Dunnett's *post hoc* test; $n = 6$; $P < 0.05$).

PMA-differentiated THP-1 cells were exposed to 100 nM $25(OH)D_3$ or $1,25(OH)_2D_3$ in the presence of a variety of inhibitors of NLRP3 inflammasome signaling. The pan-caspase inhibitor ZVAD (100 μM) and the caspase-1 specific inhibitor YVAD (100 μM) were applied 30 min prior to and throughout 24 h exposures to 100 nM $25(OH)D_3$ (Fig. 3A) or $1,25(OH)_2D_3$ (Fig. 3B). When treated with ZVAD, $1,25(OH)_2D_3$-induced IL-1β production was abolished. This finding suggests that $1,25(OH)_2D_3$-dependent IL-1β production was dependent on a member of the caspase family. More specifically, it suggests a dependency on caspase-1 activity, as YVAD treatment also significantly reduced $25(OH)_2D_3$- and $1,25(OH)_2D_3$-dependent IL-1β production. The requirement for NLRP3 was assessed by treatment with the NLRP3 antagonist glyburide [Lamkanfi et al., 2009] a known NLRP3 inflammasome antagonist. In this case, IL-1β secretions were shown to be linked with NLRP3 inflammasome signaling, as treatment with glyburide inhibited IL-1β production following stimulation with both $25(OH)D_3$ and $1,25(OH)_2D_3$ (Fig. 3A and B, respectively). Glyburide, which blocked the effects of $1,25(OH)_2D_3$ on IL-1β secretion, is thought to act indirectly on the NLRP3 inflammasome. The compound does not affect the ATPase activity of NLRP3 and may act upstream of NLRP3 and downstream of the P_2X_7 receptor to block NLRP3 inflammasome activation [Juliana et al., 2010]. We also completed additional studies with the Bay 11–7082 compound, which has been shown to inhibit the ATPase activity of NLRP3 as well as block the IKKβ kinase activity responsible for NF-kB pathway activation [Juliana et al., 2010]. In this case, a significant inhibition of IL-1β secretion in response to $1,25(OH)_2D_3$ exposure (100 nM, 24 h) was observed when Bay 11–7082 concentrations above 2 μM were used (Fig. 3C).

EXPOSURE TO $1,25(OH)_2D_3$ REDUCES NLRP3 PROTEIN EXPRESSION BUT HAS NO EFFECT ON ASC OR PRO-CASPASE-1 PROTEIN LEVELS

We assessed the potential effects of $1,25(OH)_2D_3$ exposure on the expression of various NLRP3 inflammasome components. PMA-differentiated THP-1 cells were treated with $1,25(OH)_2D_3$ (100 nM) for up to 24 h, and NLRP3, ASC and pro-caspase-1 transcription (RT-qPCR) and protein production (western blot) were examined. Interestingly, we determined that $1,25(OH)_2D_3$ exposure was associated with a significant decrease in NLRP3 mRNA (Fig. 4A) and corresponding decrease in protein levels (Fig. 4C), both at the 24 h time point. Exposure to $1,25(OH)_2D_3$ did not elicit any changes in expression of ASC mRNA (Fig. 4B) or protein (Fig. 4D) when compared to untreated controls. We also examined whether $1,25(OH)_2D_3$ could independently affect pro-caspase-1 expression and/or processing. While $1,25(OH)_2D_3$ exposure elicited a 1.8-fold increase in pro-caspase-1 transcript levels at the 4 h time point compared to

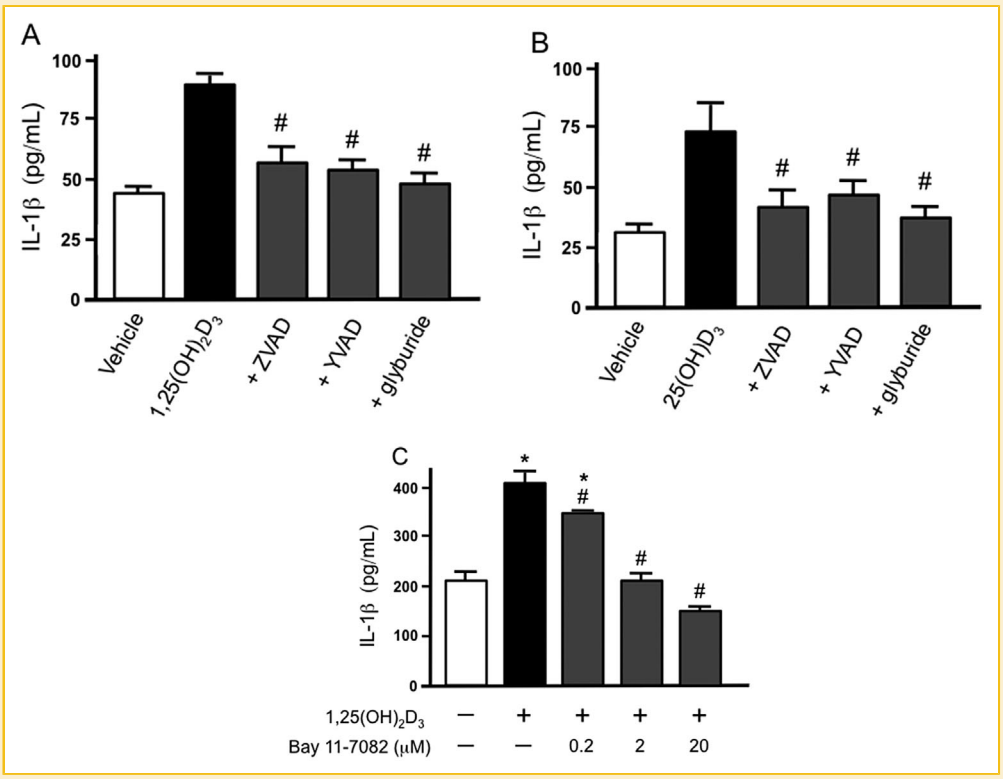

Fig. 3. Increased $25(OH)D_3$- and $1,25(OH)_2D_3$-induced IL-1β secretion from PMA- differentiated THP-1 cells is dependent on caspase-1 and NLRP3. PMA-differentiated THP-1 cells were pretreated with either vehicle (DMSO), ZVAD (100 μM), YVAD (100 μM) or glyburide (100 μM) for 30 min prior to exposure with (A) $25(OH)D_3$ (100 nM) or (B) $1,25(OH)_2D3$ (100 nM) for 24 h. Control cells were incubated for 24 h in the absence of vitamin D_3. In (C), THP-1 cells were pretreated with the NLRP3 inhibitor Bay 11–7082 prior to 24 h exposure with $1,25(OH)_2D_3$ (100 nM). Cell culture supernatants were subsequently assessed for IL-1β via ELISA. *Significantly different from control treatment; #significantly different from $25(OH)D_3$ or $1,25(OH)_2D_3$-treatment (ANOVA with Dunnett's *post hoc* test; n = 5–9; $P < 0.05$).

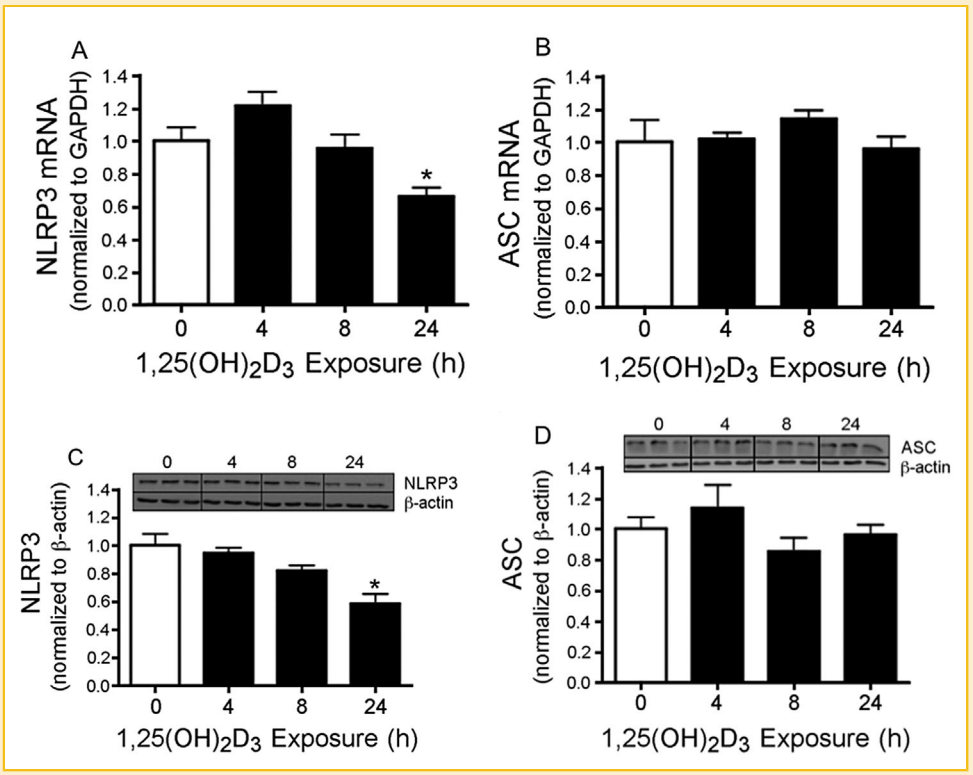

Fig. 4. Exposure to $1,25(OH)_2D_3$ decreases NLRP3 but has no effect on ASC mRNA and protein levels in PMA-differentiated THP-1 cells. Cells were treated with $1,25(OH)_2D_3$ (100 nM) for up to 24 h. The mRNA levels for NLRP3 (A) and ASC (B) were assessed with RT-qPCR (normalized to GAPDH). Protein levels for NLRP3 (C) and ASC (D) were assessed by western blot (normalized to β-actin as a loading control). Data represent the mean ± S.E.M. for n = 3–9 samples. *Significantly different from the untreated control (ANOVA with a Dunnett's *post hoc* test; n = 4–9; $P < 0.05$).

the untreated control (Fig. 5A), this did not correlate with any alteration in pro-caspase-1 protein levels (Fig. 5B). Although the release of caspase-1 p10 and p45 inflammasome components into the cell supernatant was observed over the 24 h incubation period, no changes in the abundance of active caspase-1 species (e.g., p10 or p45) was observed with exposure of 100 nM $1,25(OH)_2D_3$ (Fig. 5C). This result is consistent with the low amount of inflammasome activation observed following priming of THP-1 cells with PMA [Netea et al., 2009]. In contrast to primary monocytes, THP-1 cells do not express constitutively activated caspase-1. To release IL-1β, THP-1 cells require either double stimulation with LPS and exogenous ATP or priming with PMA, to induce constitutive release of endogenous ATP [Martinon et al., 2006; Netea et al., 2009].

$25(OH)D_3$ AND $1,25(OH)_2D_3$-INDUCED IL-1β RELEASE IS MODEST COMPARED TO DAMP AND PAMP-INDUCED IL-1β RELEASE

After determining that both $25(OH)D_3$ and $1,25(OH)_2D_3$ caused a significant increase in IL-1β release from PMA-differentiated THP-1 cells, and that this was dependent on the NLRP3 inflammasome, we examined how this increase compared with that generated by known DAMPs and PAMPs (Fig. 6A). To compare the $25(OH)D_3$- and $1,25(OH)_2D_3$-dependent stimulation of IL-1β secretion with that elicited by ATP (a DAMP) or *C. difficile* toxins A and B (TcdA/B, a PAMP), cells were treated for 24 h with either $25(OH)D_3$ (100 nM), $1,25(OH)_2D_3$ (100 nM), ATP (5 mM) or TcdA/B (10 μg/ml). While all treatments caused a significant increase in IL-1β from the baseline (untreated) value, the increase in IL-1β secretion elicited by $25(OH)D_3$ (1.6-fold increase) and $1,25(OH)_2D_3$ (2.3-fold increase) were much smaller than those elicited by ATP (5-fold and 22-fold increases at 4 and 24 h, respectively) or by TcdA/B (25-fold and 136-fold increases at 4 and 24 h, respectively). The canonical activation of the NLRP3 inflammasome is a two-step process, and vitamin D has been previously shown to stimulate priming of pro-IL-1β levels in THP-1 cells [Lee et al., 2011]. To further examine vitamin D effects on the activation step that triggers IL-1β maturation, we provided a known stimulator of the NLRP3 inflammasome (i.e., ATP) in concert with $1,25(OH)_2D_3$. In this case, the exposure of PMA-differentiated THP-1 cells to $1,25(OH)_2D_3$ amplified ATP-dependent IL-1β release (Fig. 6B). This enhancement was additive in nature with the levels of IL-1β secreted during co-treatment being approximately equivalent to the sum of the individual treatments with $1,25(OH)_2D_3$ and ATP, respectively.

DISCUSSION

Herein, we present the novel finding that both $1,25(OH)_2D_3$ and $25(OH)D_3$ can elicit significant, dose-dependent increases in the release of mature IL-1β from PMA-differentiated THP-1 cells. The $1,25(OH)_2D_3$-induction of IL-1β release was found to be dependent on caspase-1 and NLRP3, as treatments with ZVAD, YVAD and glyburide blocked the $1,25(OH)_2D_3$-induced IL-1β release. The conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ by CYP27B1 is classically

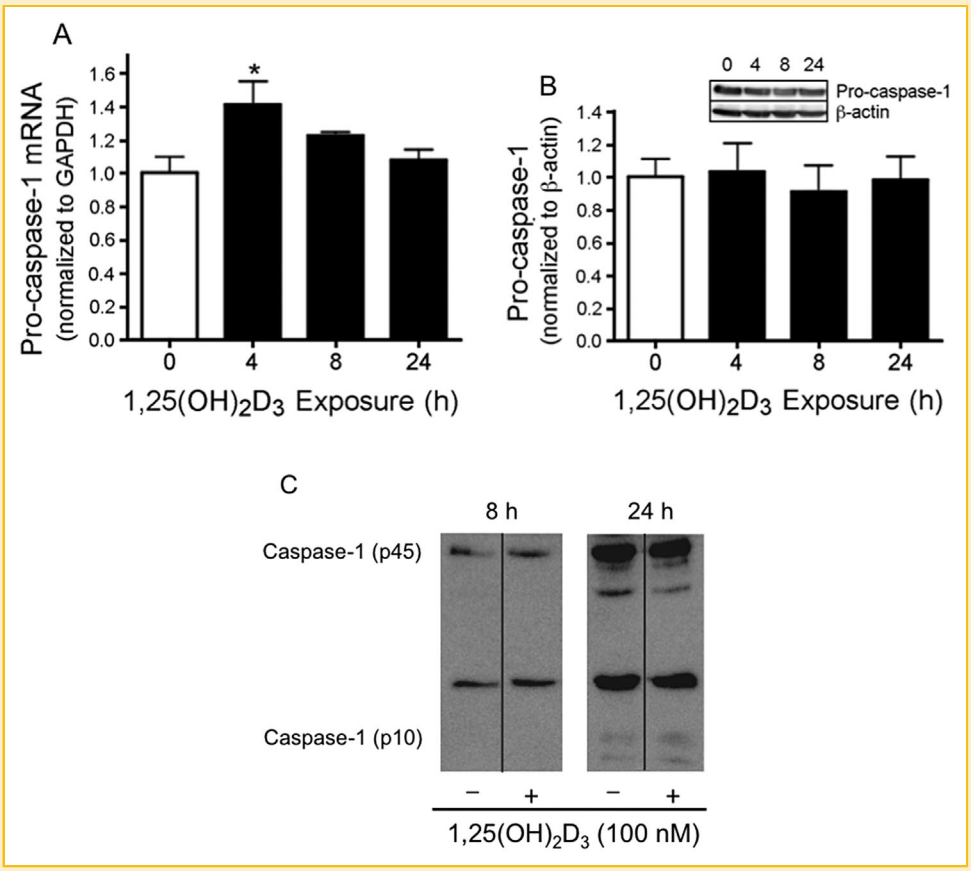

Fig. 5. Effect of $1,25(OH)_2D_3$ exposure on pro-caspase-1 and active caspase-1 in PMA-differentiated THP-1 cells. Cells were treated with $1,25(OH)_2D_3$ (100 nM) for up to 24 h. The mRNA levels (A) for pro-caspase-1 were assessed with RT-qPCR (normalized to GAPDH). Protein levels for pro-caspase-1 (B) were determined in cell lysates and normalized to β-actin as a loading control. In (C), activated caspase-1 products (p10 and p45) in the cell supernatants were assessed after 8 h and 24 h. Data represent the mean ± S.E.M. for n = 3–9 samples. *Significantly different from the untreated control (ANOVA with a Dunnett's *post hoc* test; n = 4–9; $P < 0.05$).

known to occur in the proximal tubules of the kidney [Brunette et al., 1978]. however, recent studies have determined that macrophages possess enzymes, namely CYP27B1 and CYP27A1, for the conversion of $25(OH)D_3$ to the active vitamin D_3 metabolite. The application of $25(OH)D_3$ to human monocyte-derived macrophages elicits a significant increase in $1,25(OH)_2D_3$ [Gottfried et al., 2006; Kundu et al., 2014]. The autocrine activation of $25(OH)D_3$ to $1,25(OH)_2D_3$ via CYP27B1 by macrophages is proposed to be important for host defense against infection. [Liu et al., 2006; Kundu et al., 2014]. In our study, PMA-differentiated THP-1 cells do exhibit basal expression of both CYP27A1 and CYPT27B1 hydroxylases. Moreover, while $1,25(OH)_2D_3$ exposure of THP-1 cells resulted in suppression of CYP27A1 transcription, CYPT27B1 levels were maintained. We therefore hypothesize that $25(OH)D_3$ can be converted to $1,25(OH)_2D_3$ within PMA-differentiated THP-1 cells by CYP27B1 to produce a local supply of $1,25(OH)_2D_3$ which in turn can influence IL-1β maturation. The potent up-regulation of CYP24A1 transcript during $1,25(OH)_2D_3$ exposure has been prevously reported for immune cells, including monocyte-derived dendritic cells and macrophages [Kundu et al., 2014]. This upregulation of the inactivating enzyme CYP24A1 may act to suppress the functional effects of vitamin D on inflammasome signaling and innate immune responses.

Our findings compliment the recent reports of Lee et al. [2011]; who provided evidence for $1,25(OH)_2D_3$ stimulation of pro-IL-1β transcript in both PMA-differentiated U937 and THP-1 cells as well as in human monocyte-derived macrophages, and Verway et al. [2013] who demonstrated that $1,25(OH)_2D_3$ could boost the production of mature IL-1β from macrophages during infection with *Mycobacterium tuberculosis*. Lee and colleagues proposed that $1,25(OH)_2D_3$ could enhance pro-IL-1β gene expression by increasing the expression and activation of the pro-IL-1β transcription factor CCAAT enhancer-binding protein β (C/EBPβ). One important difference between our study and that of Lee et al. [2011] is the focus on effects of $1,25(OH)_2D_3$ on either pro-IL-1β or mature IL-1β. Lee and colleagues [2011] measured intracellular IL-1β production following stimulation with $1,25(OH)_2D_3$, but this method does not discriminate between the pro- and mature forms of the cytokine. Furthermore, they did not measure the secretion of IL-1β from the macrophage, which is a key step for the initiation of inflammatory signalling. We have shown that $1,25(OH)_2D_3$ causes increased secretion of the mature form of IL-1β. Another important difference is that while Lee et al. [2011] focused on

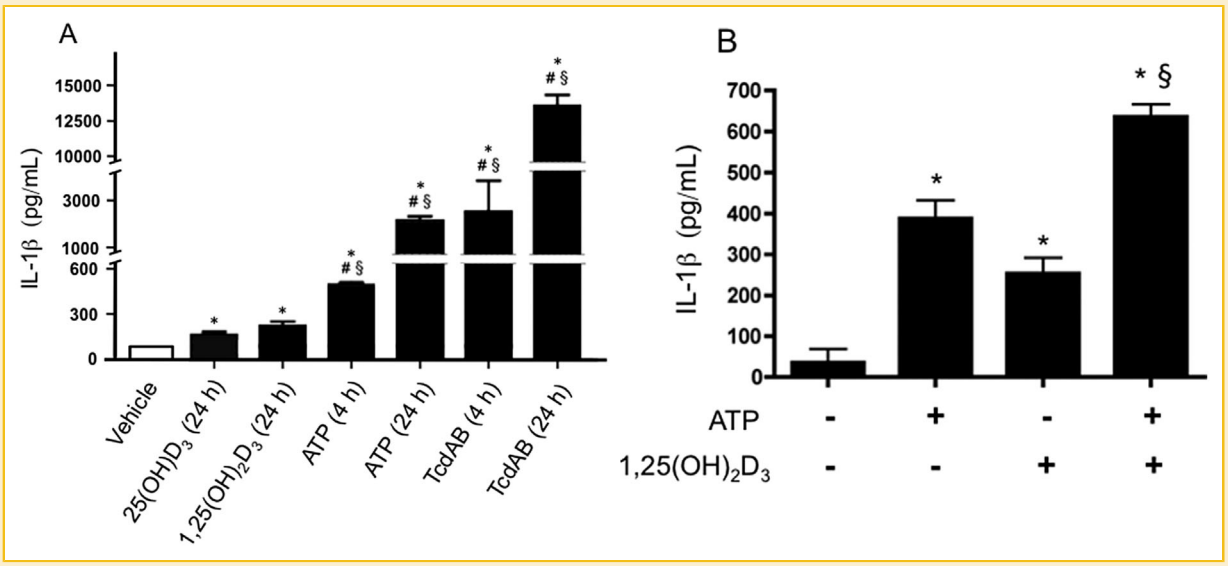

Fig. 6. Exposure to $25(OH)D_3$ or $1,25(OH)_2D_3$ elicit significantly less IL-1β release from PMA-differentiated THP-1 cells than either ATP or *C. difficile* TcdA/B. In (A), PMA-differentiated THP-1 cells were treated with either $25(OH)D_3$ (100 nM), $1,25(OH)_2D_3$ (100 nM), ATP (5 mM) or *C. difficile* TcdA/B (10 μg/ml) for 4 or 24 h. IL-1β release into the cell culture supernatants was assessed by ELISA. In (B), the ability of $1,25(OH)_2D_3$ to synergize ATP-dependent inflammasome activation was examined. PMA-differentiated THP-1 cells were treated with $1,25(OH)_2D_3$ (100 nM) and/or ATP (0.5 mM) for 24 h. *Significantly different from vehicle; #Significantly different from $25(OH)D_3$-treatment; §-significantly different from $1,25(OH)_2D_3$-treatment (ANOVA with Dunnett's *post hoc* test; $n = 4$; $P < 0.05$).

the mechanism for $1,25(OH)_2D_3$ boosting pro-IL-1β transcript levels, we have shown that $1,25(OH)_2D_3$ also acts downstream of the pro-IL-1β production pathway to influence IL-1β maturation by the NLRP3 inflammasome. Taken together, these results suggest that $1,25(OH)_2D_3$ can boost IL-1β production in a bi-modal fashion; first, by increasing pro-IL-1β gene transcription via C/EBPβ signalling [Lee et al., 2011], and second through increased production of mature IL-1β through the NLRP3 inflammasome (this study).

We report that $1,25(OH)_2D_3$ was able to boost the release of mature IL-1β from PMA-differentiated THP-1 cells in a NLRP3-dependent manner, yet $1,25(OH)_2D_3$ paradoxically supressed NLRP3 protein expression. NLRP3 levels were down-regulated with $1,25(OH)_2D_3$ exposure, and the treatment did not significantly alter ASC or pro-caspase-1 levels. The levels of activated caspase-1 were not increased with $1,25(OH)_2D_3$ exposure (100 nM, 24 h); this was not unexpected given that only a 2- to 3-fold increase in IL-1β secretion was observed with $1,25(OH)_2D_3$ treatment. The IL-1β ELISA is a much more sensitive method for the detection of inflammasome activation, and it would difficult to accurately quantify any enhancement in the levels of secreted p10 or p45 with western blots. The ability of YVAD to attenuate the $25(OH)D_3$ and $1,25(OH)_2D_3$–dependent increases in IL-1β secretion would suggest that caspase-1 processing was influenced by the exposure of the THP-1 cells to vitamin D_3. While $1,25(OH)_2D_3$ appears to act in "DAMP-like" manner on NLRP3 inflammasomes; there was a relatively low stimulation of mature IL-1β production when compared with other robust activators of NLRP3 signaling (e.g., a DAMP, ATP [Duncan et al., 2007] and a PAMP, *C. difficile* toxin [Ng et al., 2010]). This finding suggests that sufficient NLRP3 protein remains available for $1,25(OH)_2D_3$–dependent activation of inflammasome complex formation.

Various DAMPs have been demonstrated to activate immune cells and stimulate the formation and activation of NLRP3 inflammasomes. These DAMPs include a variety of lipids and other lipophilic molecules, such as cholesterol [Duewell et al., 2010; Rajamaki et al., 2010], ceramide [Vandanmagsar et al., 2011], palmitate [Wen etal., 2011] and other free fatty acids [Shi et al., 2006]. A number of lipophilic metabolites are suggested to enhance mitochondrial reactive oxygen species (ROS) production and provide activation of NLRP3 inflammasomes (reviewed in: De Nardo Latz [2011]). Given these reports, we speculate that $1,25(OH)_2D_3$ may act by promoting ROS production to stimulate NLRP3 activation and IL-1β maturation in the face of alterations to the core inflammasome component (i.e., NLRP3 protein availability). Indeed previous reports suggest that vitamin D metabolites can increase oxidative stress in cells and generate ROS due to increased lipid oxidation [Weitsman et al., 2003; Somjen et al., 2010]. Finally, IL-1β maturation in macrophages can be stimulated by DAMPs, such as ATP [Duncan et al., 2007; Netea et al., 2009], that are released from damaged or dying cells. LDH is present in the cytosol in healthy cells, and its release correlates with changes in cell membrane permeability and serves as a marker for cell death. The administration of $25(OH)D_3$ and $1,25(OH)_2D_3$ did not cause significant changes in LDH release throughout the experiment time-course (Supplemental Fig. 2). Thus, we propose that $1,25(OH)_2D_3$ be considered a "priming" molecule that can provide indirect NLRP3 inflammasome activation to increase IL-1β maturation.

We provide additional evidence that links vitamin D_3 and innate immune signalling. Wang and colleagues reported that exposure to $1,25(OH)_2D_3$ could induce production of both NOD2 mRNA and protein in monocytes and differentiated THP-1 cells [Wang et al.,

2010]. The study identified the effect to be dose dependent, with stronger induction of NOD2 occurring at higher $1,25(OH)_2D_3$ concentrations (i.e., 100 nM versus 10 nM). In addition, the $1,25(OH)_2D_3$-dependent induction of NOD2 expression levels was enhanced by co-treatment with LPS. Interestingly, we have identified that both NLRP3 mRNA and protein levels were reduced with 24 h of $1,25(OH)_2D_3$ exposure. This finding was distinct from results reported for $1,25(OH)_2D_3$ on NOD2 expression, where $1,25(OH)_2D_3$ caused increases in NOD2 mRNA and protein expression [Wang et al., 2010]. The divergent effects of $1,25(OH)_2D_3$ on NLRP3 and NOD2 expression could be rationalized by the potential presence of a vitamin D response element (VDRE) in the NOD2 promoter region, and a negative VDRE (nVDRE) in that of NLRP3. In a recent study, THP-1 cells were exposed for 4 h to $1,25(OH)_2D_3$, and global changes in gene expression were assessed by microarray analysis [Carlberg et al., 2012]. This analysis revealed the upregulation of NOD2 transcript in response to $1,25(OH)_2D_3$ exposure but did not identify any changes in NLRP3 expression. These data suggest that $1,25(OH)_2D_3$ did not directly affect NLRP3 gene expression through VDREs or nVDREs. However, the time point of this study was much shorter than in our study (i.e., 4 h versus 24 h) which may account for the differences observed in NLRP3 expression.

ACKNOWLEDGMENTS

This work was supported by a research grant from the Canadian Institutes of Health Research (CIHR) to J.A.M. and P.L.B. (MOP-98004). S.E.T was supported by a CIHR Canada Graduate Scholarship. D.A.M. is an Alberta Innovates Health Solutions (AIHS) Clinical Senior Scholar and holds a Canada Research Chair in Inflammation and Kidney Disease. P.L.B. is an AIHS Clinical Senior Scholar. J.A.M. is an AIHS Senior Scholar and holds a Canada Research Chair in Smooth Muscle Pathophysiology.

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