TET-2 up-regulation is associated with the anti-inflammatory action of Vicenin-2

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Abstract

Vicenin-2, a C-glycoside flavone that is present in many plant sources, exerts potent anti-inflammatory effects in a number of cell and animal models of inflammation. Ten-eleven translocation (TET)-2 has recently gained considerable attention due to the role it plays in regulating the inflammasome. We studied the ability of Vicenin-2 (V-2) to regulate a range of lipopolysaccharide (LPS) stimulated inflammatory activities in PMA-differentiated THP-1 cells and human primary mononuclear cells. We also investigated the action of V-2 on the secretion of NLRP3 inflammasome regulated cytokines (IL-1β and IL-18) by ELISA, and determined if V-2 can regulate the expression of *NLRP3, IL-10, IL-1Ra and TET-2*. The effect of V-2 on NF-κB signalling was investigated by fluorescent microscopy and gene reporter assay. Additionally, the effect of V-2 on LPS-induced phosphorylation of IKB-α was also investigated by Western blot analysis.

V-2 down-regulated LPS-induced secretion of proinflammatory cytokines (TNF-α and IL-1β), in both THP-1 and primary mononuclear cells. V-2 also decreased the LPS-stimulated

secretion of IL-18 in THP-1 cells. V-2 significantly down-regulated TNF-α induced NF-κB reporter activity in HEK293T transfected cells and attenuated IKB-α phosphorylation in THP-1 cells. V-2 treatment also induced enhanced nuclear staining of the p50 subunit and reduced p65 subunit of NF-κB.

V-2 treatment alone increased the expression of anti-inflammatory cytokine IL-10 expression and the regulator of the inflammasome; IL-1Ra, in the presence of LPS. V-2 significantly decreased LPS-induced NLRP3 expression while concomitantly increasing TET-2 expression.

This study demonstrates that the anti-inflammatory actions of V-2 are associated not only with increased IL-10 and IL-1Ra expression but also with TET-2 expression. Further work is required to establish if the effects of V-2 can be definitively linked to TET-2 activity and that these actions are mirrored in a range of relevant cell types.

1. Introduction

Flavone-C-glycosides are a class of naturally-occurring compounds known to have diverse anti-inflammatory actions [1]. Vicenin-2, a C-glycoside that is present in many plant sources, has been shown to exert potent anti-inflammatory effects both *in vitro* [2] and in an experimental colitis model [3]. Macrophages are immune cells that are ubiquitously expressed across tissues and play an important role in the development and resolution of the inflammatory response. For instance, exposure to bacterial lipopolysaccharide (LPS) induces an inflammatory phenotype in macrophages that is characterised by the expression of many proinflammatory cytokines including TNF-α and IL-1β [4]. The proinflammatory activities of macrophages is also associated with activation of NLR Family Pyrin Domain Containing 3 (NLRP3)-dependent inflammasome [5] and the nuclear transcription factor, NF-κB [6]. During the resolution of inflammation, macrophages produce mediators such as

IL-10 and Interleukin-1 receptor antagonist (IL-Ra), that in turn, down-regulate inflammatory processes [7]. THP-1 is a human monocytic leukaemia derived cell line that is one of the most widely used cell models for investigating monocytic differentiation and subsequent biological functions of differentiated cells [8]. THP-1 cells differentiated with Phorbol-myristate-acetate (PMA) have been shown to possess functional characteristics of mature macrophages [9, 10]. TET (ten-eleven translocation)-2 is an epigenetic modifier that has recently gained considerable attention in the pathophysiology of atherosclerosis and cardiovascular disease [11, 12]. TET-2 also plays an important role in the inflammatory response of macrophages [13], and loss of this regulator results in the up-regulation of many inflammatory mediators following LPS stimulation [14]. To date, the ability of natural products to modulate TET-2 in human macrophages has not been reported and this study determined if the anti-inflammatory actions of V-2 was associated with changes in expression of TET-2 in THP-1 cells.

2. Materials and Methods

2.1. Cell culture

Ethical approval was obtained from the Cardiff School of Health Sciences Research Ethics Committee (reference: 7758). All compounds and chemicals were sourced commercially: V-2 and LPS (*E.coli*) were purchased from Sigma Aldrich (UK) and reconstituted in dimethyl sulfoxide (DMSO). TNF-α was obtained from Peprotech (UK). THP-1 cells (ATCC, UK) were cultured in supplemented RPMI 1640 (Gibco, UK). To differentiate cells, THP-1 (6 x 10^5 cells/ml) were treated with 5ng/ml PMA (Sigma Aldrich, UK) for 48 hours then rested in PMA-free media for a further 48 hours before experiments. HEK293T (ATCC, UK) cells were cultured in supplemented DMEM (Gibco, UK). Human peripheral blood mononuclear cells (PBMCs) were obtained from a pooled buffy coat sample of five healthy donors. PBMCs were isolated from buffy coat using Lymphoprep™ (Stemcell

Technologies, UK) according to manufacturer's instructions. PBMCs were cultured in supplemented RPMI-1640 and stimulated with PMA (5ng/ml) for 48 hours. The cells were further cultured in fresh medium for 48 hours before experiments.

2.2. Cytokine Analysis

The level of cytokines in cell-free supernatants was measured using Enzyme-linked immunosorbent assay (ELISA). All ELISA kits (TNF-α (Cat. #DY210); IL-1β (Cat. #DY401) and IL-18 (Cat. # DY318-05)) were obtained from R & D systems (Oxford, UK) and undertaken using protocols outlined by manufacturer.

2.3. RNA extraction and quantitative RT-PCR

RNA was isolated from cell monolayer using TRIzol® reagent (Life Technologies, UK) according to manufacturer's guidelines. RNA was converted to cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). Gene expression was analysed using Applied Biosystems Fast 7500 Real-Time PCR System (Applied Biosystems, UK). The comparative C_T method was used to calculate relative gene expression. The SYBR® Green methodology was employed to measure the expression of *IL-10 and IL-1Ra* using the primer sequences published in [15]. *TET-2 and NLRP3* gene expression was determined using Taqman methodology. Pre-designed probes for *GAPDH* (Hs99999905_m1), *GUSB* (Hs00939627_m1), *TET-2* (Hs00325999_m1) and *NLRP3* (Hs00918082_m1) were obtained from Thermofisher (UK). C_T values of target genes were normalized against the housekeeping genes; *GAPDH* and *GUSB*. Untreated samples were used as experimental controls.

2.4. NF-κB gene reporter assay

HEK 293T cells were co-transfected with 50ng of NF-κB and 5ng of the *Renilla* luciferase reporter plasmid DNA (Pr6tk; Promega, UK) using Lipofectamine® for 24 hours before experiment. Cells were lysed 24 hours after stimulation and analysed using Dual-glo luciferase assay (Promega, UK), in line with manufacturer's guidelines.

2.5 Western Blotting

To extract total proteins, the cell culture media was removed from wells and Radio-Immunoprecipitation Assay (RIPA) Buffer was added to wells and placed on ice for 5 minutes. The cell lysate was centrifuged at 14,000 x g for 15 minutes at 4˚C. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermofisher, UK). A total of 25μg total protein was separated on 10% Bis-Tris NuPAGE® gels (Invitrogen Ltd, UK) and transferred to a nitrocellulose membrane using the iBlot® dry blotting system (Invitrogen Ltd., UK). The membrane was blocked with 5% Marvel milk powder for 1 hour then incubated with the primary antibody overnight at the dilution of 1:300. The membrane was washed and incubated with HRP-conjugated secondary antibody for 1 hour. Subsequently, the membrane was visualised with ECL HRP-substrate (Amersham™, UK) and exposed to Hyperfilm™ ECL film (Sigma Aldrich, UK).

2.6 Immunofluorescence.

THP-1 cells were seeded at a density of 6 x 10^5 cells/ml in a multi-well plate containing sterile 1.6mm coverslips (EMS, Hatfield, UK). Cells were differentiated with PMA as described in 2.1. Differentiated THP-1 cells were stimulated with V-2 for 1 hour before stimulation with LPS for 30 minutes. The cells were then fixed with 4% paraformaldehyde for 10 minutes then incubated with 0.25% Triton X-100 (Sigma, Aldrich, UK) for a further 15 minutes. The cells were incubated with primary monoclonal antibodies for p50 (Abcam, UK) or p65 (Invitrogen, UK) for 1 hour then incubated with fluorescently-labelled

secondary antibodies (p65: Dylight 488 (Abcam, UK); p50: CFL-555 (Santa Cruz Biotechnology)) for 1 hour. Cells were counterstained with 150nM of DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride; Sigma Aldrich, UK) and visualized using Nikon Eclipse 80i (Nikon UK Ltd., Surrey, UK). Images were captured using Volocity 5.5 (Perkin Elmer, UK).

2.7. Statistical analysis

All data are presented as mean ± standard deviation (SD). One-way or Two-way analysis of Variance (ANOVA) was conducted for within group comparisons. The statistical package, Graphpad Prism 7.0 was used for statistical analyses. Statistically significant differences between treatments are denoted as * for $p < 0.05$; $p < 0.01$ (**) or $p < 0.001$(***). Error bars represent standard deviation of at least 3 replicates.

3. Results

3.1. V-2 down-regulates proinflammatory cytokines in THP-1 cells and primary mononuclear cells.

We initially determined if pre-treatment of dTHP-1 cells with V-2 can regulate LPS-induced inflammatory cytokine secretion. As shown in Fig 1A and 1B, V-2 pre-treatment over a concentration range of 16-160nM significantly down-regulated LPS-induced TNF-α and IL-1β secretion with no significant effect on cell-viability. On the basis of the data in Figs. 1A and 1B, further studies on the modulatory actions of V-2 were restricted to using the 80nM concentration. Given that the assembly of NLRP3 inflammasome mediates the processing and release of both IL-1β and IL-18 [5, 16] we also explored the effect of V-2 on the release of IL-18. Fig. 1C confirms that like IL-1β, a significant reduction in LPS-induced IL-18 was observed following V-2 pre-treatment (LPS: 117.4 ± 9.3 vs V-2 + LPS: 66.6 ± 6.3 pg/ml). We also explored if the inflammation modulatory ability of V-2 that we observed in dTHP-1 cells was also present in primary mononuclear cells. As was seen with

dTHP-1 cells, LPS-induced TNF-α and IL-1β secretion were significantly reduced by V-2 pre-treatment (fig. 1D), supporting the view that the modulatory effects of V-2 was not unique to dTHP-1cells.
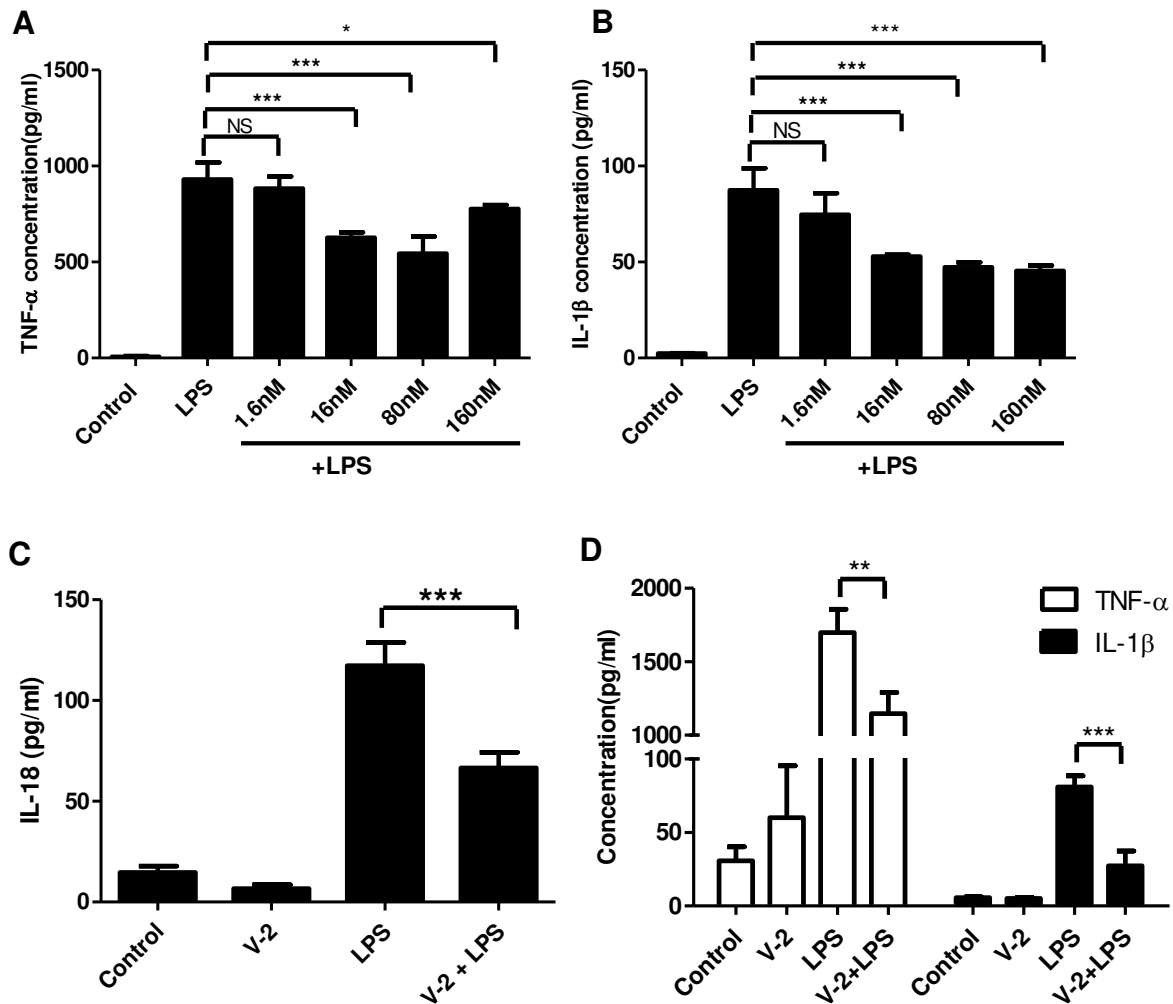

Figure 1. V-2 down-regulates proinflammatory cytokine secretion. (A, B) dTHP-1 cells were pre-treated with a range of concentration of V-2 (1.6-160nM) for 1 hour then stimulated with 100 ng/ml LPS. The level of TNF-α and IL-1β was measured by ELISA in cell-free media at 6 and 24 hours, respectively. Treatment of cells with 1.6-160nM of V-2 alone did not change cytokine expression (Data not shown). (C) dTHP-1 cells were pre-treated with 80nM V-2 for 1 hour and then stimulated with 100ng/ml LPS for 24 hours and the level of IL-18 was determined in cell culture supernatants. (D) Effect of V-2 on primary mononuclear cells. Primary mononuclear cells were pre-treated with 80nM of V-2 for 1 hour then stimulated with 100 ng/ml LPS. The level of TNF-α and IL-1β was measured in cell-free media by ELISA at 6 and 24 hours, respectively. Data represents mean ± S.D (n=3).

3.2. V-2 attenuates NF-κB reporter activity and phosphorylation of IKB-α.

To elucidate the mechanism by which V-2 regulates inflammatory cytokine release, we hypothesized that the major regulator of inflammatory cytokine release, NF-κB [6], is affected by V-2 pre-treatment. We investigated the ability of V-2 to regulate nuclear translocation of NF-κB p50 and p65 subunits using immunofluorescence. Fig. 2A demonstrates that V-2 pre-treatment alone induces enhanced nuclear translocation of the p50 subunit, while LPS treatment induced co-localization of both p50 and p65. Pre-treatment of cells with V-2 followed by LPS challenge resulted in reduced nuclear staining of p65 and enhanced p50. We would contend that these data is supportive of V-2 inducing enhanced anti-inflammatory activity through p50 homodimerization. To further explore whether V-2 is capable of selectively targeting NF-κB activity, mediated through the activation of p50/p65 heterodimers, we employed a reporter assay with a specific p50/p65 construct as originally described by [17]. The transfection efficiency of dTHP cells with NF-κB was very low; therefore we used HEK293T cells to determine the role of V-2 in regulating NF-κB activity. As shown in Fig. 2B, pre-treatment of HEK293T cells with V-2 significantly decreased TNF-α induced NF-κB activity (TNF-α: 32.7 ± 0.5-fold vs V-2 + TNF-α: 24.8 ± 1.5-fold). IKB-α regulates the nuclear translocation of the p50/p65 heterodimers by sequestering the complex in the cytoplasm [18]. Upon stimulation of cells IKB-α is phosphorylated and degraded [6], allowing the nuclear translocation of the p50/p65 heterodimer and activation of NF-κB. Accordingly, we explored whether V-2 could attenuate LPS induced phosphorylation of IKB-α in dTHP-1 cells. Fig. 2C demonstrates that V-2 pre-treatment markedly inhibited LPS-induced phosphorylation of IKB-α.
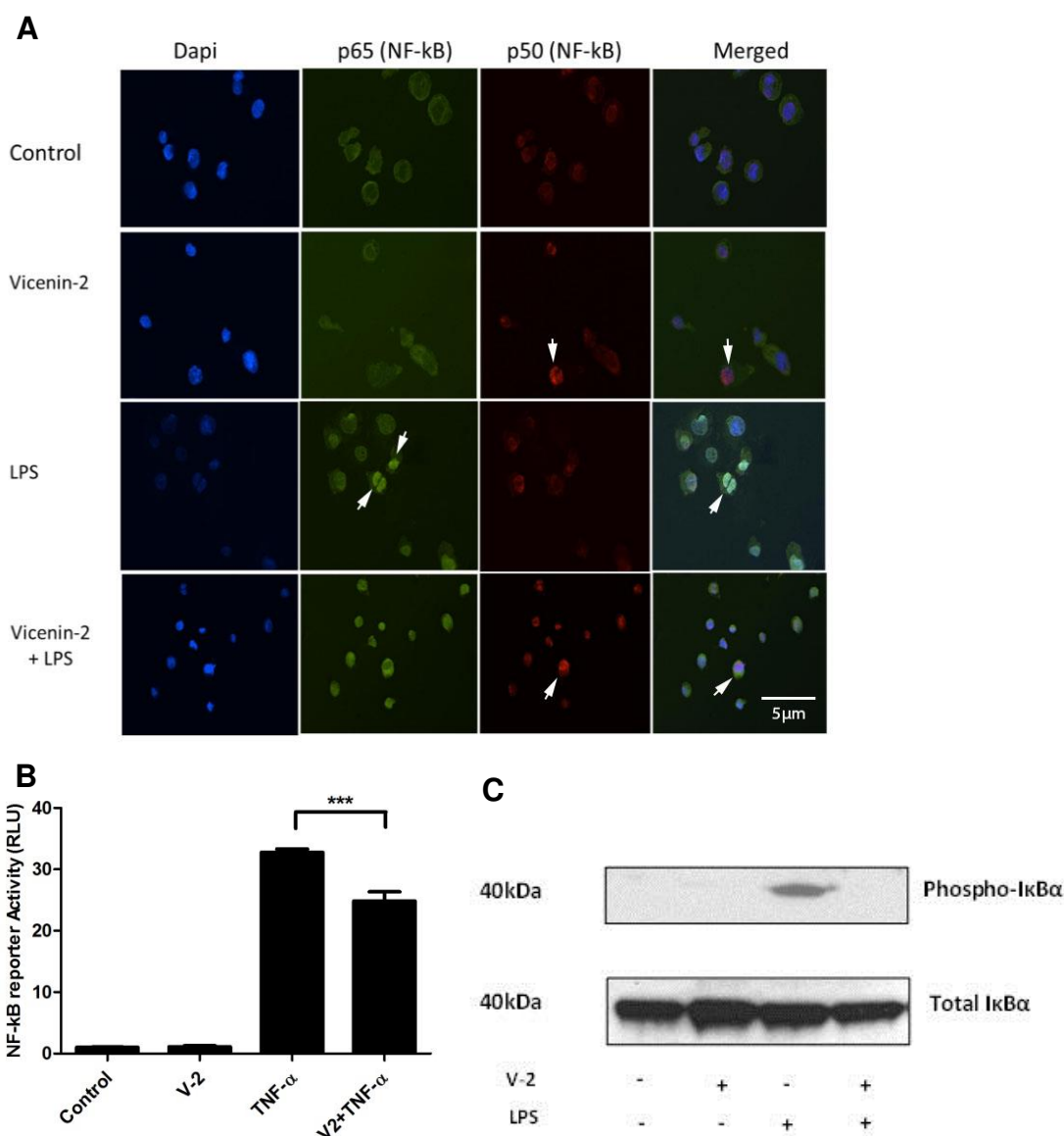
Figure 2. Effect of V-2 on NF-κB signalling. (A) Confocal microscopy analysis of the location of p50 and p65 subunits of NF-κB in dTHP-1 cells. Cells were pre-treated with V-2 for 1 hour before stimulation with LPS for 30 minutes. Cells were fixed, permeabilized and immunolabelled with primary antibodies for p50 and p65. Secondary antibodies conjugated to Dylight 488 (green) and CFL 555 (red) was raised against p65 and p50 antibodies, respectively. DAPI (blue) was used to counterstain cell nuclei. The scale bar represents 5 µm. (B) Effect of V-2 on NF-κB reporter activity. HEK-293T cells cultured to 80% confluence then pre-treated with V-2 for 1 hour before stimulation with 10 ng/ml of TNF-α. Cells were lysed after 24 hours and gene-reporter activity was determined using the Dual-glo luciferase assay. (C) Effect of V-2 on LPS-induced phosphorylation of IKB-α. Differentiated THP-1 cells were pre-treated with or without V-2 for 1 hour and then stimulated with 100 ng/ml of LPS for 30 minutes. The protein expression of IKB-α and Phospo-IKBα was analysed by immunoblotting. Data represents mean ± S.D (n=3).

3.2. V-2 down-regulates the expression of NLRP3 and increases the expression of the regulators of inflammation, IL-10 and IL-1 receptor antagonist and TET-2 in the presence of LPS

After demonstrating the effect of V-2 on NF-κB signalling cascade, the effect of V-2 on the expression of anti-inflammatory cytokine (IL-10), NLRP3 inflammasome and its regulators (IL-1Ra and TET-2) was determined. IL-10 is produced by macrophages to down-regulate LPS-stimulated TNF-α expression [19]. Fig. 3A demonstrates that V-2 treatment alone significantly up-regulated IL-10 expression at 6 and 24 hours (6.9 ± 0.2 and 5.9 ± 0.9-fold, respectively). The pattern of IL-10 expression with LPS treatment was similar to that of V-2 at both 6 and 24 hour time points, however, co-stimulation of dTHP-1 cells with V-2 and LPS significantly increased IL-10 expression at 24 hours compared to V-2 or LPS treatments alone. We further explored whether the significant reductions in LPS-induced IL-1β and IL-18 secretion (Figs. 1B and 1C) were associated with changes in *NLRP3* expression by qRT-PCR. Fig. 3B demonstrates that gene expression of *NLRP3* was itself significantly down regulated by V-2 in dTHP-1 cells stimulated by LPS.

IL-1Ra negatively regulates NLRP3 inflammasome activation [20], consequently, we hypothesized that V-2 can modulate IL-1Ra expression. Fig. 3C demonstrates that although V-2 treatment alone did not increase IL-1Ra expression, pre-treatment of cells with V-2 before LPS treatment significantly increased IL-1Ra expression at 6 hours (LPS: 69.6 ± 0.5 vs V-2 + LPS: 76.8 ± 0.2) and 24 hours (LPS: 55.9 ± 0.3 vs V-2 + LPS: 62.8 ± 0.2), respectively. Given the interest in the role of TET-2 in the regulation of the NLRP3 inflammasome [21], we also explored if V-2 was capable of modulating TET-2 expression both in the presence and absence of LPS. Fig. 3D demonstrates that V-2 treatment alone significantly increased TET-2 expression at 6 hours post-stimulation (V-2: 2.4 ± 0.1-fold increase). LPS treatment induced a significant increase in TET-2 expression and a further

increase was observed following pre-treatment with V-2 at 6 hours (LPS: 3.6 ± 0.2 vs V-2 + LPS: 4.0 ± 0.1) and 24 hours (LPS: 4.5 ± 0.1 vs V-2 + LPS: 5.6 ± 0.1), respectively.
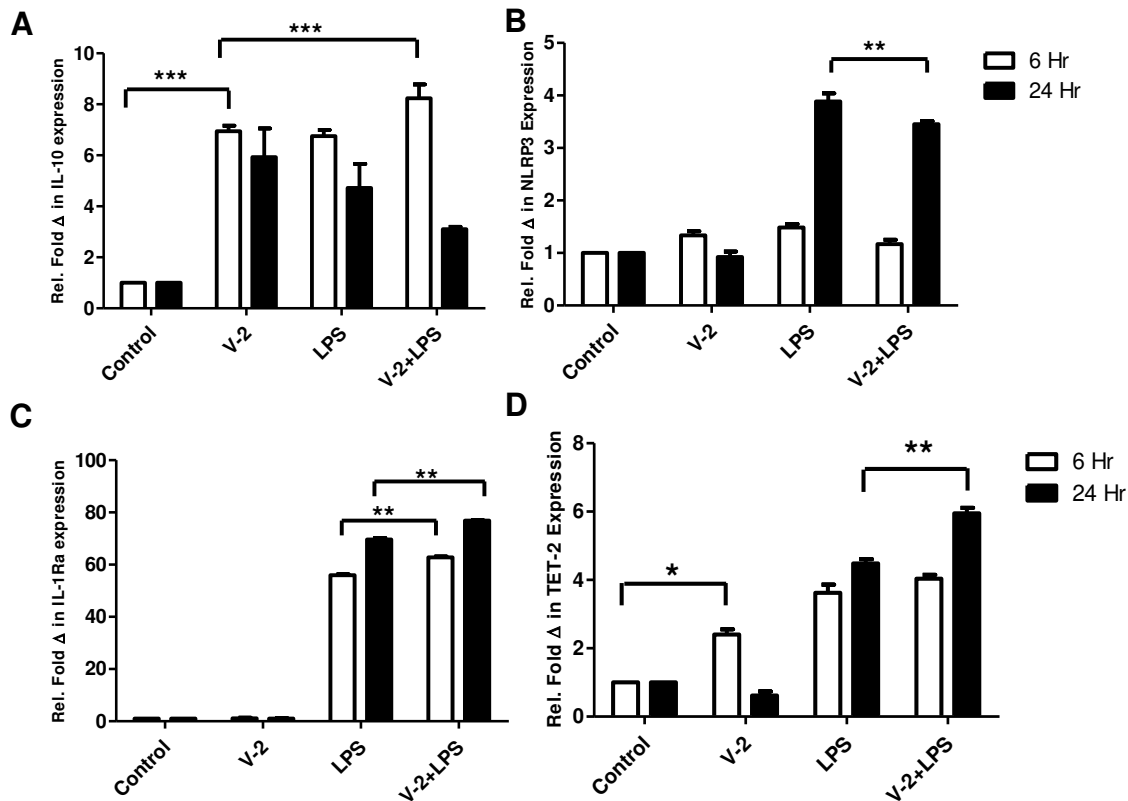

Figure 3. Effect of V-2 and/or LPS on (A) *IL-10*, (B) *NLRP3*, (C) *IL-1Ra* and (D) *TET-2* mRNA expression. dTHP-1 cells were pre-treated with 80nM V-2 (or VC) for 1 hour before stimulation with 100 ng/ml LPS for 6 and 24 hours. Gene expression at the mRNA level of *IL-10, NLRP3, IL-1Ra and TET-2* was determined by qRT-PCR. Data represents mean ± S.D (n=3).

4. Discussion

This study demonstrates that V-2 significantly down-regulates LPS induced inflammatory cytokine secretion in both dTHP-1 cells and in primary human mononuclear cells. We also present data showing that V-2 inhibits NF-κB reporter activity and increased homodimerization of the p50 subunits of the transcription factor at the expense of p50/p65 heterodimerization. Homodimerization of p50 subunit is known to induce IL-10 synthesis [22], a cytokine that is itself a regulator of inflammatory cytokine secretion. We would therefore propose that the anti-inflammatory actions of V-2 are in-part mediated by its ability to enhance p50 homodimerization and consequent IL-10 expression. The actions of

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V-2, in regulating IL-1β and IL-18 secretion and the expression of IL-1Ra and NLRP3, supports an inflammasome modulating activity in dTHP-1 cells by V-2. Recently, Fuster *et al.* [11] demonstrated that TET-2 deficient macrophages exhibited an increase in IL-1β secretion in an NLRP3-dependent manner. This suggests that TET-2 plays an important role as a 'gate-keeper' of inflammasome related activity in macrophages. Thus, a novel aspect of this study was demonstrating that V-2 was capable of increasing TET-2 expression and that this may be associated with its anti-inflammatory/inflammasome-regulatory actions. It could be argued that an increase in IL-10, IL-1Ra and TET-2 by an inflammatory mediator such as LPS is at first sight counterintuitive. However, Zhang *et al.* [14] demonstrated that LPS induces activation of TET-2 and that this event is critical to the resolution of the inflammatory response in macrophages. Jaiswal *et al.* [12] and Fuster *et al*. [11] linked the actions of TET-2 to clonal haematological malignancies and the CHIP mutation of TET-2. However, these studies confirmed a role for TET-2 in regulating the inflammasome, both in the presence and absence of the CHIP mutation. THP-1 cells are of human myelomonocytic lineage, but to best of our knowledge the CHIP mutation has not been reported in THP-1 cells.

In conclusion, we propose that V-2 has anti-inflammatory actions that are associated not only with increased IL-10 and IL-1Ra expression but also with increased TET-2 expression. We further propose that V-2's actions are mediated through its ability to regulate NF-κB activity and enhance homodimerization of the p50 subunit. Figure 4 presents a proposed mechanistic model of the actions of V-2 as it relates to NF-κB and NLRP3 inflammasome signalling in macrophages. It remains to be seen if these anti-inflammatory actions of V-2 can be definitively linked to TET-2 activity and that these actions are mirrored in a range of other relevant cell types.
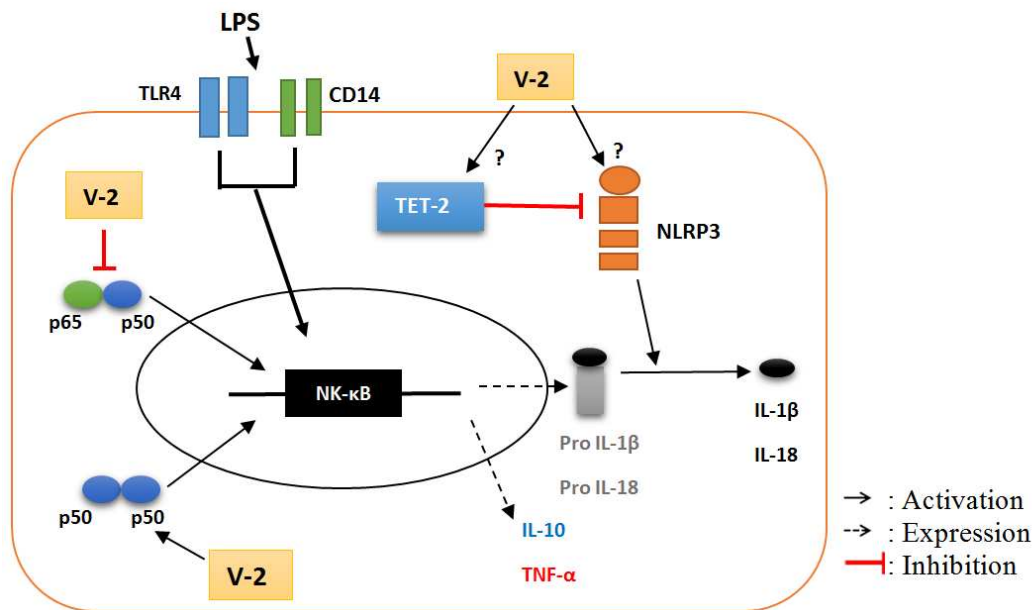
Fig 4. Mechanistic model of NF-κB/NLRP3 inflammasome-modulatory actions of Vicenin-2 (V-2) in human macrophages. NF-κB: NF-κB response element; TET-2: Ten-eleven translocation-2.

Conflict of interest declaration

The authors declare that there are no conflicts of interest.

Acknowledgement

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