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Concise report

Vitamin D_3 down-regulates intracellular Toll-like receptor 9 expression and Toll-like receptor 9-induced IL-6 production in human monocytes

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Abstract

Objective. To determine whether vitamin D_3 modulates monocytic expression of intracellular Toll-like receptors (TLRs) 3, 7 and 9.

Methods. Human monocytes were isolated from peripheral blood and cultured with 100 nM vitamin D_3 for 24, 48 and 72 h. Expression of CD14 and TLR2, TLR3, TLR4, TLR7 and TLR9 were examined by flow cytometry. Monocytes exposed to vitamin D_3 for 48 h were then stimulated with a TLR9 agonist for a further 24 h. The level of IL-6 secretion was measured by ELISA.

Results. CD14 was up-regulated, whereas TLR2, TLR4 and TLR9 expression was down-regulated by vitamin D_3 exposure in a time-dependent manner. TLR3 expression was unaffected by vitamin D_3 and there was no measurable expression of TLR7 on the monocytes. TLR9-induced IL-6 production was impaired in monocytes treated with vitamin D_3 compared with untreated cells.

Conclusion. The intracellular TLRs are differentially regulated by vitamin D_3, with TLR9 being down-regulated by vitamin D_3 exposure whereas TLR3 was unaffected. This decreased TLR9 expression in monocytes had a downstream functional effect as these cells subsequently secreted less IL-6 in response to TLR9 challenge. This may have significant biological relevance and may be a factor in the association of vitamin D deficiency with susceptibility to autoimmune disease.

Key words: Vitamin D_3, Toll-like receptors, TLR9, Systemic lupus erythematosus, IL-6.

BASIC SCIENCE

Introduction

Activation of Toll-like receptors (TLRs) results in initiation of innate and adaptive immune responses [1]. Recognition of pathogen-encoded TLR ligands activates intracellular signalling pathways that culminate in rapid induction of pro-inflammatory cytokines and chemokines [1]. Manipulation of TLR activity has now been recognized as a potential therapeutic tool for boosting immune responses [2]. However, the beneficial effects of TLRs can also be viewed as a double-edged sword in light of accumulating evidence that TLR recognition of self-nucleic acids, specifically by TLR7 and TLR9, can play an important role in the pathogenesis of autoimmune diseases, such as SLE [3]. In SLE, it is thought that self-reactive nucleic acids or DNA fragments, produced through apoptosing cells, can bind to both TLR9 and the B-cell receptor simultaneously. This interaction is hypothesized to break B-cell tolerance and cause generation of DNA autoantibodies, which are characteristically seen in this disease [4]. In such a situation, suppression of TLR responses is the desired clinical goal.

Vitamin D has been demonstrated as having potential immunomodulatory activity; vitamin D analogues are effective in the treatment of psoriasis [5] and have shown promising results in clinical trials of a number of conditions, including prostate cancer [6]. Most circulating

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vitamin D is in the storage form ($25OHD_3$) [7] but it is the active form, $1,25(OH)_2D_3$ (vitamin D_3) which has been shown to have an immunomodulatory role. Vitamin D_3 has been shown to down-modulate T-, B- and dendritic-cell responses [8] and also to down-regulate monocyte TLR2 and TLR4 expression [9]. However, the effects of vitamin D_3 on intracellular TLR expression have not yet been reported. Other effects of this vitamin on monocytes include reduced MHC and co-stimulatory molecule expression, which causes a reduction in the capacity of these cells to activate T cells [10]. However, antimicrobial defence mechanisms, such as phagocytosis and autophagy, are increased after vitamin D_3 treatment [11, 12].

Epidemiological studies suggest that there is an association between vitamin D deficiency and susceptibility to autoimmune disease, such as RA [13] and SLE [14]. Increased intake of vitamin D has been associated with a reduced risk of developing RA [13], and animal studies have shown vitamin D_3 to interfere with the course of experimental SLE [15]. Conflicting data have been published on serum levels of $25OHD_3$ in SLE patients; however, in most reports, levels show an inverse correlation with disease activity [16]. The association of vitamin D deficiency with SLE and the observed immunosuppressive effects of vitamin D_3 on TLR activity prompted us to examine whether intracellularly expressed TLRs, particularly TLR7 and TLR9, shown to play a role in SLE [3], are modulated by exposure to vitamin D_3, similar to that observed for surface expressed TLR2 and TLR4 [9].

Methods

All reagents were from Sigma-Aldrich (Gillingham, Dorset, UK) unless otherwise stated.

Cell isolation and culture

Heparinized blood was collected from healthy volunteers, having obtained approval from the Leeds (Central) Research Ethics Committee, and informed consent was given by participants. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation using Lymphoprep (Axis-Shield UK, Kimbolton, Cambridgeshire, UK). Monocytes were isolated by magnetic cell sorting using the Monocyte Isolation Kit II (Miltenyi Biotec, Bisley, Surrey, UK). Isolated monocytes were cultured at 1×10^6 cells/ml in Roswell Park Memorial Institute medium 1640 supplemented with 10% heat-inactivated fetal calf serum (Autogen Bioclear, Calne, Wiltshire, UK), 1% Hepes buffer, 2 nM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in the presence or absence of vitamin D_3 at indicated concentrations. Cells were incubated at 37°C supplemented with 5% CO_2.

Vitamin D_3

A stock solution of 5 μM vitamin D_3 [$1,25(OH)_2D_3$] (Sigma-Aldrich) was prepared in 75% ethanol and stored in sterile aliquots at −20°C.

Flow cytometry analysis

Human monocytes were cultured at 1×10^6 cells/ml and exposed to 100 nm vitamin D_3 in a time course stimulation of 0, 24, 48 and 72 h, to examine the expression of TLR2, TLR3, TLR4, TLR7 and TLR9 by flow cytometry. FITC-conjugated antibodies against CD14 (AbD Serotec, Kidlington, UK), TLR3 and TLR9 (Alexis Biochemicals, Enzo Life Sciences Exeter, UK) were used, with FITC-conjugated mouse IgG1 isotype control (BD Pharmingen, San Jose, CA, USA) as a negative control. Unconjugated monoclonal IgG_1 antibodies against TLR2 and TLR4 (Santa Cruz, CA, USA) were used and labelled with a goat anti-mouse IgG $F(ab')_2$-FITC secondary antibody. An unconjugated goat polyclonal TLR7 antibody (Santa Cruz), labelled with donkey anti-goat IgG-PE secondary antibody (Research Diagnostic, Inc, Fitzgerald Industries International, North Acton, MA, USA), was used with goat serum as the negative control. For staining intracellular TLRs (TLR3, TLR7 and TLR9), cells were fixed and permeabilized using Leucoperm (AbD Serotec) according to the manufacturer's instructions. Analysis was carried out using a FACScan (BD Pharmingen).

IL-6 ELISA assay

Human monocytes were cultured at 1×10^6 cells/ml in media alone, or with 100 nM vitamin D_3 for 48 h prior to stimulation for a further 24 h with the TLR9 agonist, ODN2006 at 1 μg/ml (Autogen Bioclear). The supernatant from cultured cells was stored at −80°C until analysed. Levels of secreted IL-6 in the supernatant were measured using a BD OptEIA Human IL-6 ELISA set (BD Biosciences, Oxford, UK) according to the manufacturer's instructions.

Statistical analysis

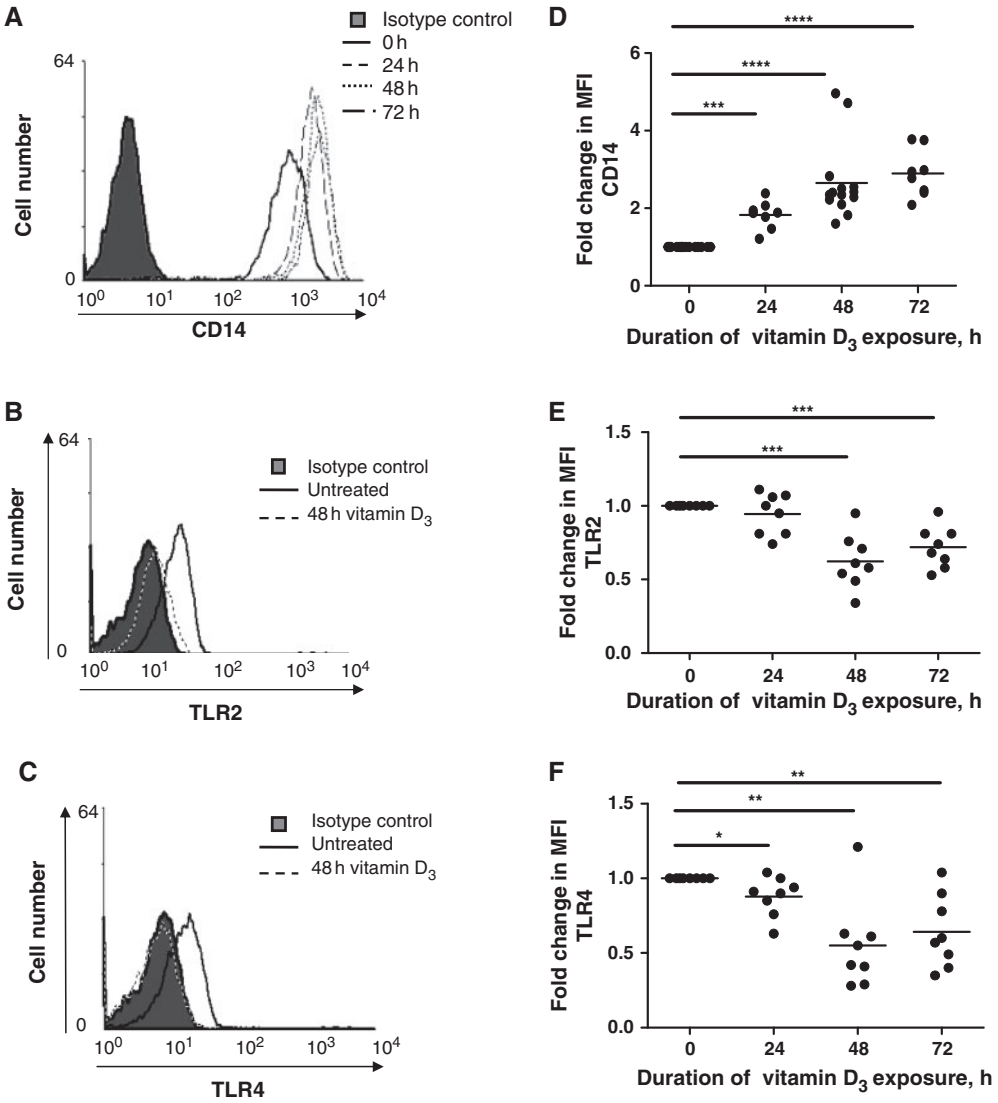
A normality test and paired *t*-test were used to examine the statistical significance. $P < 0.05$ was deemed to be statistically significant.

Results

CD14 is up-regulated in response to vitamin D_3 exposure, while TLR2 and TLR4 expression is down-regulated

Due to the previous findings that vitamin D_3 up-regulated CD14 expression on human monocytes [9], we repeated this study to confirm the findings and test the robustness of this model to investigate vitamin D_3 exposure on intracellularly expressed TLR. Justification for the dose of vitamin D_3 used here was based on dose titration studies on CD14 expression (data not shown). Exposure of monocytes to 100 nM vitamin D_3 for 24, 48 and 72 h demonstrated a time-dependent increase in surface expression of CD14 compared with untreated cells (Fig. 1A); this was statistically significant at all time points (24 h, $P = 0.0003$; 48 h, $P = 0.0001$; and 72 h, $P = 0.0001$), with the greatest increase observed at 72 h (Fig. 1D). Conversely, surface expression of both TLR2 (Fig. 1B) and TLR4 (Fig. 1C) was decreased following exposure over the same time course

Fig. 1 Vitamin D_3 up-regulates CD14 expression and down-regulates TLR2 and TLR4 expression on human monocytes in a time-dependent manner. CD14 and extracellular TLR2 and TLR4 expression as determined by flow cytometry. MFI changes were compared with expression on untreated cells, which were normalized to one. Significance was calculated using paired *t*-test. Monocytes cultured in presence and absence of 100 nM vitamin D_3 for 24 h ($n = 8$), 48 h ($n = 14$) or 72 h ($n = 8$). Histograms to demonstrate: (**A**) CD14 expression; (**B**) TLR2; and (**C**) TLR4 expression in response to vitamin D_3 treatment. Statistically significant changes of vitamin D_3 dose on: (**D**) CD14 expression; (**E**) TLR2 expression; and (**F**) TLR4 expression $^*P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.001$, $^{****}P = 0.0001$. Data represent individual responses and the mean fold change.

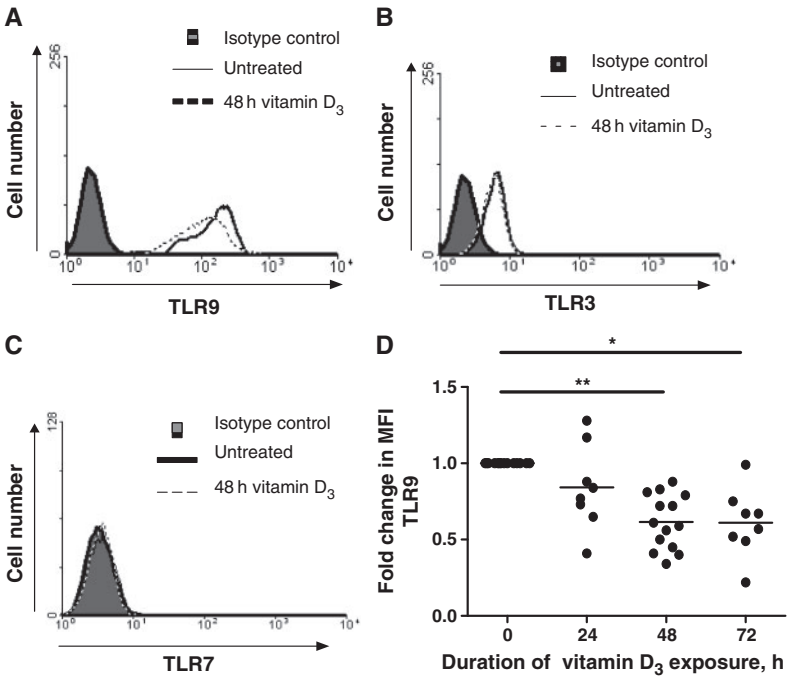


(Fig. 1E and F, respectively). This decrease was maximal at the 48 h time point for both receptors, and was statistically significant at a number of time points: TLR2 ($P = 0.0007$ at 48 h and $P = 0.0008$ at 72 h); and TLR4 ($P = 0.028$ at 24 h and $P = 0.017$ at both 48 and 72 h).

Monocyte exposure to vitamin D_3 causes down-regulation of TLR9 expression

The effect of vitamin D_3 has not been investigated with regard to expression of intracellular TLRs (TLR3, TLR7 and TLR9). TLR9 expression was down-regulated in a time-dependent manner with exposure to 100 nM vitamin D_3 (Fig. 2A and D), which was evident at 24 h and reached statistical significance at both 48 h ($P = 0.0001$) and 72 h ($P = 0.0017$). The expression of TLR3 was unaffected by exposure to vitamin D_3 for 48 h (Fig. 2B), or at any other time point (data not shown). TLR7 expression was undetectable and exposure to 100 nM vitamin D_3 failed to induce any TLR7 expression at any time point (Fig. 2C).

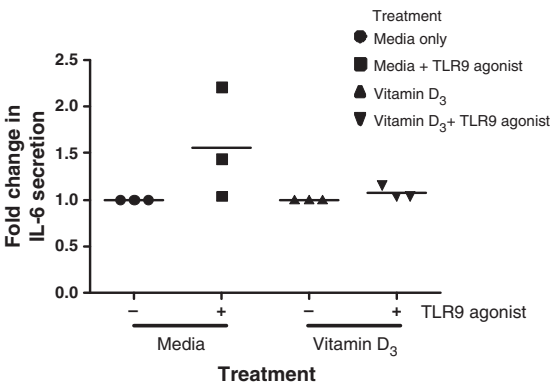
FIG. 2 Vitamin D_3 down-regulates TLR9 expression in a time-dependent manner whereas TLR3 expression is unchanged. Monocytes were cultured in presence/absence of 100 nM vitamin D_3 for 48 h. Intracellular expression of: (**A**) TLR9 ($n = 14$); (**B**) TLR3 ($n = 8$); and (**C**) TLR7 ($n = 8$) was determined by flow cytometry. (**D**) Changes in TLR9 MFI in a time course from 24 h ($n = 8$), 48 h ($n = 14$) to 72 h ($n = 8$) were calculated and compared with TLR9 expression of untreated cells, which was normalized to one. Data represent means; $^*P < 0.002$, $^{**}P = 0.0001$.



Vitamin D_3 treatment causes reduced IL-6 secretion in monocytes in response to TLR9 challenge

To test whether the down-regulation of TLR9 in response to vitamin D_3 had any functional implications, secretion of the pro-inflammatory cytokine IL-6 by human monocytes was measured in the culture supernatant by ELISA. Monocytes were cultured in media alone, or with 100 nM vitamin D_3 for 48 h, and then stimulated for a further 24 h with the CpG oligonucleotide TLR9 agonist ODN2006 (1 μg/ml). Stimulation of untreated monocytes with the TLR9 agonist induced an increase in secretion of IL-6 compared with monocytes cultured in media only, with 50% higher secretion on average, being observed. However, TLR9 stimulation after exposure to vitamin D_3 did not induce the same increased degree of secretion; levels of IL-6 were comparable to those treated only with vitamin D_3 (Fig. 3). A heterogeneous response was observed between donors reflecting donor variability, and hence the findings were not statistically significant; however, the actual IL-6 levels measured in the three controls for vitamin D_3 or vitamin D_3 plus TLR9 agonist were very tightly clustered together (Fig. 3).

FIG. 3 Vitamin D_3 decreases TLR9-induced IL-6 secretion from monocytes. Monocytes were cultured in presence/absence of 100 nM vitamin D_3 for 48 h before stimulation with the TLR9 agonist, ODN2006. Secretion of IL-6 in the culture supernatant was measured by ELISA ($n = 3$). Changes in IL-6 secretion (pg/ml) in response to TLR9 stimulation were calculated and compared with the respective unchallenged sample (normalized to one) to take into account the effect of the TLR9 agonist alone.



Discussion

The association of vitamin D deficiency with the development of autoimmunity such as Type 1 diabetes [17], RA [13], SLE [18] and IBD [19] has been known for some time. Increased vitamin D supplementation has been suggested as a way of lowering the prevalence of these diseases, particularly in the case of diabetes [20]. Vitamin D analogues have been shown to be effective in many models of autoimmune disease including SLE [15] and RA [21].

Vitamin D_3 has been shown to have a plethora of actions on monocytes, including impaired TNF and IL-1 secretion [22] and reduced NF-κB activation [9] in response to bacterial challenge, such as lipopolysaccharide. Other effects reported include decreased MHC expression and reduced ability to activate T cells due to down-regulated co-stimulatory molecule expression [23]. Conversely, vitamin D_3 has been reported to enhance monocyte phagocytosis and autophagy [11, 12], thus increasing microbial cell killing. These findings all confirm that vitamin D_3 can modulate both innate and adaptive immune responses. The reported down-regulation of TLR2 and TLR4 in response to vitamin D_3 was an interesting addition to its autoimmune regulatory role [9]. This prompted our investigations, expanding the repertoire of TLRs studied to include intracellular receptors TLR3, TLR7 and TLR9. Our data corroborate these previous findings in that we also demonstrated an increase in CD14 expression as well as down-regulation of TLR2 and TLR4 with vitamin D_3 treatment of human monocytes. Additionally, vitamin D_3 exposure reduced the expression of TLR9, which was significantly lowered at 48 and 72 h (Fig. 2D). Here, we demonstrate that this down-regulation has functional consequences as TLR9-induced IL-6 secretion was reduced after vitamin D_3 treatment when compared with untreated cells stimulated with the agonist (Fig. 3). We have also demonstrated an interesting differential response to vitamin D_3 as TLR3 levels did not change during treatment whereas TLR7 was not detectable in healthy donor monocytes (Fig. 2B and C). These findings may have significant biological and clinical implications and lead us to speculate that the vitamin D deficiency observed in SLE patients may further potentiate autoreactivity to self-nucleic acids recognized by TLR9. This hypothesis would fit well with existing observations of vitamin D deficiency in SLE patients [18], and also provide a mechanism to explain the increased frequency of TLR9-expressing monocytes, as observed in SLE patients with active disease [3]. The knock-on effect of TLR9 down-regulation by vitamin D_3 on IL-6 expression could be particularly relevant in the clinical setting, as patients with SLE are reported to have elevated IL-6 serum levels [16, 24] and anti-IL-6 treatment has demonstrated a beneficial effect on disease activity in a murine model of SLE [25]. Further to this, SLE patients with high disease activity have lower serum levels of vitamin D [16]. This study provides evidence that vitamin D_3 down-regulates TLR9 expression on human monocytes with functional implications, and reinforces the public health recommendation that vitamin D sufficiency is an important disease prophylactic.

Rheumatology key messages

- Vitamin D_3 modulates expression of TLR9 in healthy human monocytes.
- Vitamin D_3 down-regulates TLR9 expression, which is associated with decreased IL-6 production.
- Modulation by vitamin D_3 may have clinical implications for susceptibility to autoimmune disease.

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