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Effect of vitamin D and β-sitosterol on immune function of macrophages

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ABSTRACT

Among the many functions of vitamin D (VD) is its role in the immunomodulation of macrophage. As VD deficiency is a wide-spread nutritional problem, there is a tendency for the public to overdose with vitamin D supplementation which can result in hypercalcemia and several associated disorders. The present study was designed to investigate the possibility that combining low doses of vitamin D with β-sitosterol (SIT), a common phytosterol in the diet without toxicity, enhances the efficacy of the vitamin. Murine macrophages were stimulated with LPS and supplemented with VD3 (80 nM) and SIT (8 μM) for 24 hr and examined for cell proliferation, release of nitric oxide (NO) and cytokines and the activation of NFκB. SIT (8 μM) was found to reduce cell proliferation by 62% while VD3 was found to be not effective. In combination, SIT and VD3 reduced cell proliferation by 75%.The amount of NO released, as influenced by 8 μM SIT or 80 nM VD3 treatments, was not significantly different from control. Combining SIT and VD3, resulted in a 220% greater increase in NO release compared to control. The SIT + VD3 treatment brought about significant increase in all the cytokine release, regardless of whether they were pro- or anti-inflammatory. The effects were either additive or synergistic. We conclude that SIT enhances the action of VD3 on the immune function of macrophages which could be beneficial to vitamin D deficient individuals and to those with autoimmune diseases such as multiple sclerosis.

1. Introduction

The most well established role of vitamin D (VD) is its role in calcium absorption. It is essential for preserving the calcium-phosphate balance in the body and thus it is vital for bone health [1–3]. Besides calcium homeostasis, VD has other functions in the human body, including its role in immune [4] and muscle function [5]. In addition, many regulatory proteins involved in cell proliferation, cell differentiation, and apoptosis are modulated by VD [1,6,7].

Nutrient deficiency can result from inadequate intake, poor absorption, added need, or increased excretion [8]. Usually vitamin D deficiency can occur when there is lower than recommended intake levels for prolonged time, limited sunlight exposure, inability of the kidneys to produce the active form of VD, or impaired absorption of VD from the digestive tract[8]. Consumption of natural food sources alone may not be enough to meet the daily dietary requirements of VD. Including VD fortified foods in their diet and exposure to sunlight are effective ways to get sufficient VD [8]. However, some people like those with disabilities, lactose intolerance, vegetarians, breast feeding mothers, or the elderly have to resort to dietary supplements to meet daily requirements [8].

Vitamin D toxicity is not likely to result from too much sunlight exposure or consumption of fortified foods [8], but excessive intake of supplements can present with many health problems [8,9]. Excessive intake of vitamin D supplements can increase serum calcium levels resulting in kidney stones, heart diseases or impaired mental status [10–12].

Although enough scientific evidence is not available to recommend the exact upper level of toxicity for vitamin D, there is no difference in opinion about the toxicity of vitamin D in excess amounts. Efforts are focused on finding a safe alternative that can augment the effects of VD. A lot of interest in this approach is being currently investigated by several drug manufacturers. Alternatively, the combination of low level of vitamin with other dietary molecules to increase its efficacy without the hypercalcemic side-effect offers an attractive solution.

One such molecule tested here is the β-sitosterol (SIT), the most common plant sterol or phytosterol, in the diet. Beside structural similarities between VD and SIT they each play role in immunomodulation [13,14]. Phytosterols are naturally found in a wide variety of plants and in good amounts in legumes such as peanuts [15] and oil seeds such sesame seeds and soybeans [16]. Consumption of gram quantities of phytosterols has no side-effects [17]. Hence, it would be of great interest to see if SIT would act additively or synergistically with vitamin D to enhance its effect in lower doses on macrophage immune function. Macrophages produce nitric oxide (NO) and cytokines that play major roles in inflammation.

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2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM) without phenol red, sulfanilamide, N-1-naphthylethylenediamine dehydrochloride, lipopolysaccharide (LPS) and β-sitosterol (SIT) were purchased from Sigma (St. Louis, MO). Vitamin D3 (VD3) or calcitriol was generously donated by SAFC Pharma, Inc. (Madison, WI); WST-8 reagent was obtained from BioVision (Mountain View, CA). ELISA ^{MAX} deluxe kits for mouse interleukin-10 (IL-10), mouse monocyte chemoattractant protein (MCP-1), mouse interleukin-6 (IL-6) and mouse tumor necrosis factor – alpha (TNF-α) were purchased from BioLegend (San Diego, CA). NFκB p65 profiler Kit was purchased from Active Motif (Carlsbad, CA). Sodium pyruvate, L-glutamine, Penicillin - Streptomycin and phosphate saline buffer (D-PBS) were from GIBCO (Carlsbad, CA). Other chemicals were obtained either from VWR or Fischer Scientific.

2.2. Methods

2.2.1. Cell culture

Murine J774A.1 macrophage cells (ATCC) were maintained in DMEM supplemented with 4 g/L glucose, 1.5 g/L sodium bicarbonate, 10 ml/L Pen-Strep, 10 ml/L sodium pyruvate and 10% fetal bovine serum.

2.2.2. Cell proliferation measurement

J774A.1 cells (5×10^5cells/250 μl) were seeded in a 96-well tissue culture plate and stimulated with LPS to a final concentration of 100 ng/ml [18]. To examine the effect of VD3 and SIT on cell proliferation, cells were incubated with different concentrations of these agents at the same time cells were stimulated with LPS. After 24 hours of incubation, the conditioned media were removed and 10 μL of WST-8 reagent in 90 μL media was added to all wells and incubated for 30 minutes to 4 hours under standard culture conditions according to the manufacture instructions. During that time the tetrazolium salt of WST-8 is cleaved to form formazan by mitochondrial dehydrogenase. The amount of the formazan produced was quantified by measuring the absorbance at 450 nm which is directly proportional to the number of viable cells. The reference wavelength used was 650 nm.

2.2.3. Nitric oxide measurement

LPS-stimulated cells were incubated with SIT, VD3 or both and incubated for at 37 °C in a 5% CO_2 - 95% air humidified atmosphere for 24 hours. Untreated cells were used as controls. Nitrite accumulation in the conditioned media was measured using the Griess assay [19].

2.2.4. Cytokine release measurement

Cells were treated either with SIT, VD3 or both and the experiment was performed as above. After 24 h incubation, conditioned media were collected from the 96-well plates and stored at -80 °C until cytokine assays were performed. ELISA^{MAX} Deluxe kits were used for measuring TNF-α, IFN-γ, IL-6, IL-10 and MCP-1 released from the treated cells. Standard curves were generated as per the instructions of the manufacturer to read the concentration of each cytokine released. The amount of cytokine released was expressed as percentage of control.

2.2.5. Measurement of phosphorylated and total NFκB

Cells were seeded in 96-well plates and grown to about 80% confluence. Then they were treated with LPS and the test agents, SIT, VD3, either alone or in combination. After 24 h of incubation, the cells were fixed by replacing the growth medium with formaldehyde in PBS. The amount of phosphorylated NFκB at Serine 468, Serine 536 and total NFκB in the experimental samples were determined using NFκB p65 profiler kit. The relative number of cells in each well was then determined according to the manufacturer's instructions.

2.2.6. Statistical analysis

The values are expressed as means ± SEM and presented as percentages of control. Analysis of variance (ANOVA) followed by the student Newman-Keuls post-hoc test were utilized to examine the significance between groups. Differences between the means were considered to be statistically significant at a p-value <0.05. ProStat software (Pearl River, NY) was used to analyze the data.

3. Results

3.1. Effect of VD3 and SIT on cell proliferation of macrophages

The LPS-treated macrophages were incubated with respective treatments for 24 hours at 37°C and 5% CO_2. Fig. 1 demonstrates the results of the first set of experiments conducted to identify the appropriate concentrations of the sterols used for subsequent experiments. The values are expressed as percentage of control.

VD3 treatments had no pronounced effects on cell proliferation. Here also, higher concentration of VD3 (160 nM) showed decrease in cell proliferation (by 45%) although not significantly different from control. Fig. 1 illustrates that with VD3 (80 nM), there was only a smaller reduction in cell proliferation (20%). Neither of the VD3 treatments was significantly different from each other.

SIT (1 μM) treatment resulted in 59% decrease in cell proliferation while SIT (8 μM) brought it down to 63% (Fig. 1). The decrease in cell proliferation with both the SIT treatments was found to be significantly different from control. The two SIT treatments did not significantly different from each other.

Since the VD3 at two concentrations studied were not different, we decided to use VD3 (80 nM) for subsequent experiments based on published literature [13,20]. In the case of SIT, although insignificant, SIT at 8 μM was able to induce more pronounced decrease in cell proliferation compared to 1 μM. In addition, SIT (8 μM) corresponds to serum SIT level associated with the consumption of the western mixed diet. Accordingly, SIT (8 μM) was used in further experiments.

Fig. 1 demonstrates the results of a set of experiments performed to examine the effect of the combined treatment (SIT + VD3) on cell proliferation. The treatment groups included control (no SIT or VD3),

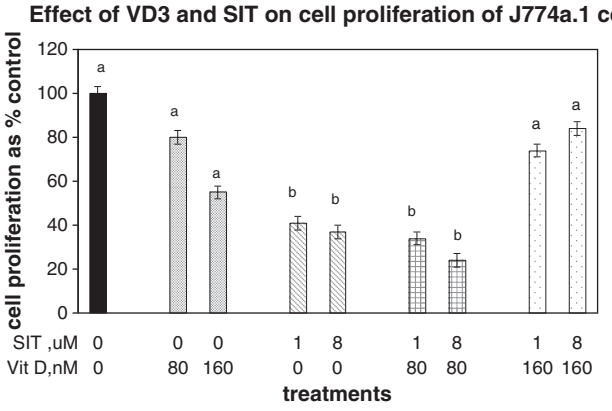

Fig. 1. Effect of VD3 and SIT treatments on cell proliferation of LPS-stimulated J774A.1 cells. J774A.1 cells (5×10^5 cells/ 250 μl) were seeded in a 96-well tissue culture plate. LPS (100 ng/ml) was added to each well and incubated with SIT, VD3, both or none (control). After 24 h incubation, conditioned media was removed from each well and cells were subjected to WST-8 cell proliferation assay. Values are expressed as percentage of control. Columns represent average of three observations and bars represent SEM. Bars with different letters indicate significant differences ($p<0.05$) as evidenced by student Newman-Keuls post-hoc test. Abbreviations: SIT = β-sitosterol, VD3 = vitamin D3.

SIT (8 μM), VD3 (80 nM) and SIT + VD3. Cell proliferation is expressed as the percentage of the control, with the control representing 100%. VD3 treatment did not reveal any significant change in proliferation from control, while SIT treatment decreased it by 63%. The SIT treated cells showed significant inhibition of cell proliferation compared to VD3 treated cells.

Fig. 1 also illustrates that the SIT + VD3 treatment exhibited the greatest effect, as it led to a significant 76% decrease in cell proliferation. SIT treated cells showed no significant difference in cell proliferation from SIT + VD3 treated cells. The reverse effect was noticed between VD3 and SIT + VD3 treated cells. There was significant difference between these two groups. Overall, the treatments with SIT, alone or in combination with VD3, proved to be effective in reducing cell proliferation.

3.2. Effect of VD3 and SIT on NO release from J774A.1 macrophages

Using similar design to that used in cell proliferation experiments, NO release from treated macrophages was examined (Fig. 2). VD3 (80 nM) treatment had an effect on NO released from macrophages. The amount of NO released, as influenced by SIT treatment, was significantly different from control. Combining these two treatments also yielded significant results. SIT + VD3 treatment resulted in more than 220% increase in NO release above the control. This effect noticed with the combined treatment was the summation of the individual effects i.e. the effect was additive.

3.3. Effect of VD3 and SIT on cytokine release

The analyzed cytokines included IL-10, IL-6, TNF-α, MCP-1 and IFN-γ in the conditioned media of treated cells:

IL-10

IL-10 is an anti-inflammatory cytokine. Fig. 2 illustrates the release of IL-10 from the macrophages as influenced by treatments. VD3 treatment increased IL-10 production from J774A.1 cells by 32%. SIT treatment was found to significantly increase the IL-10 release by 114% from macrophages. The SIT + VD3 treatment resulted in a 230% increase in IL-10 production compared to control. The amount of IL-10 produced on treating with SIT + VD3 was also significantly higher than the summation of individual treatment groups suggesting a synergistic effect.

IL-6

With regards to IL-6 (Fig. 2), VD3 treatment did not produce any noticeable change in IL-6 release from macrophages. On the other hand, SIT treatment produced significantly higher amounts of this Th2 cytokine compared to the control. The SIT + VD3 treatment significantly increase IL-6 production compared to control and other treatment groups. Compared to the control, the increase in IL-6 for SIT + VD3 group was almost 3 times higher, suggesting a synergistic effect.

TNF-α

TNF-α is a pro-inflammatory cytokine produced by macrophages upon stimulation by foreign substances. TNF-α released from macrophages treated with VD3 was not significantly different from control (Fig. 2). It can be observed that the treatment with SIT produced significant increase (by 150%) in TNF-α production from J774A.1 cells. The SIT + VD3 treatment led to a 4 times increase in TNF-α production compared to control. With SIT + VD3 treatment the increase in TNF-α production was 2.5 times higher than SIT alone and 4 times more than VD3 alone.

MCP-1

MCP-1 is a chemokine that recruits monocytes to the site of injury or infection. Fig. 2 illustrates that the effect of SIT and VD3 on MCP-1 release followed the same pattern as in the case of TNF-α. VD3 produced significant rise in MCP- 1 release from the macrophages. Treatment with SIT led to a noticeable increase in MCP-1 production. The combination treatment produced a substantial increase in MCP-1 release. Like TNF-α, the SIT + VD3 treatment produced a 4-fold increase in MCP-1 production compared to control, suggesting synergistic effect.

IFN-γ

IFN-γ is a cytokine needed for innate and adaptive immunity. Fig. 2 shows that IFN-γ release was markedly different for all the treatments. This followed the same pattern as that for MCP-1 and TNF-α. The VD3 treatment had no effect on IFN-γ release from the macrophages. IFN-γ was increased significantly with SIT treatment by 80%. The SIT + VD3 produced a sizeable increase (4 times) in IFN-γ release from macrophages with respect to control. The combined effect was also significantly different from those of SIT and VD3 treatment cells.

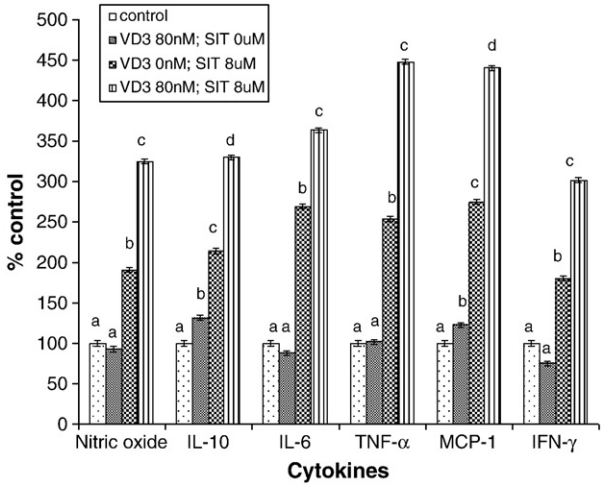

Fig. 2. Effect of VD3 and SIT treatments on nitric oxide and cytokine release from LPS-stimulated J774A.1 cells. J774A.1 cells (5×10^5 cells/ 250 μl) were seeded in a 96-well tissue culture plate. LPS (100 ng/ml) was added to each well and incubated with SIT, VD3, both or none (control). After 24 h incubation, conditioned media was collected from each well and quantified for NO by Griess assay. The conditioned media collected after 24 h incubation was also quantified for the respective cytokines by ELISA kit. Values are expressed as percentage of control. Columns represent average of three observations and bars represent SEM. Bars with different letters within an inflammatory marker indicate significant differences ($p<0.05$) as evidenced by student Newman-Keuls post-hoc test. Abbreviations: SIT = β-sitosterol, VD3 = vitamin D3.

3.4. Effect of VD3 and SIT on total and phosphorylated NFκB

By determining the phosphorylation of NFκB on Ser536, one would be able to examine the role of NFκB pathway in the observed results. Since the kit we used can also measure Ser 468 phosphorylation by activating NFκB by another pathway with cytokines we decided to examine Ser 468 phosphorylation, as well. The experiment was repeated with similar results. Fig. 3 demonstrates the results in one of these experiments. There was a decrease in Ser 536 phosphoylation with both SIT and VD3 + SIT treatments. VD3 treatment also showed a slight decrease in Ser 536 phosphorylation. On the other hand, a noticeable increase in Ser 468 phosphorylation was found with SIT and SIT + VD3 treatments, while VD3 decreased it (Fig. 4). In Fig. 5, the total cellular NFκB remained relatively unchanged except for SIT + VD3 treatment where a slight increase was noticed. Similar results were obtained when the experiment was repeated.

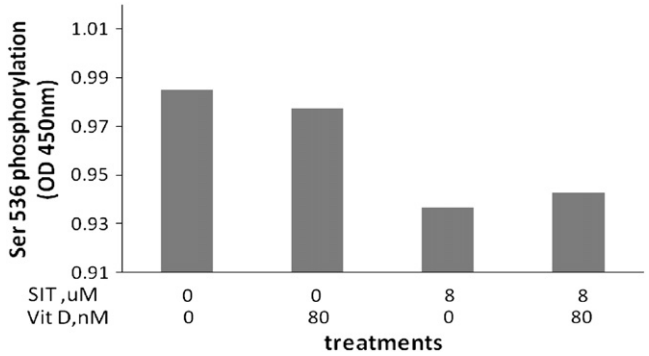

Fig. 3. Effect of VD3 and SIT treatments on Ser 536 phosphorylation. J774A.1 cells (3×10^5 cells/ 100 μl) were seeded in a 96-well tissue culture plate. LPS (100 ng/ml) was added to each well and incubated with SIT, VD3, both or none (control). After 24 h incubation, the cells were subjected to FACE assay and values shown have been corrected for cell number. Abbreviations: SIT = β-sitosterol, VD3 = vitamin D3.

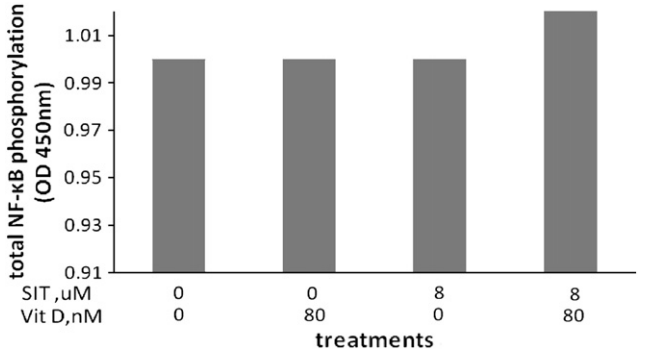

Fig. 5. Effect of VD3 and SIT treatments on total NFκB. J774A.1 cells (3×10^5 cells/ 100 μl) were seeded in a 96-well tissue culture plate. LPS (100 ng/ml) was added to each well and incubated with SIT, VD3, both or none (control). After 24 h incubation, the cells were subjected to FACE assay and values shown have been corrected for cell number. Abbreviations: SIT = β-sitosterol, VD3 = vitaminD3.

4. Discussion

The aim of the present study was conducted to examine the potential modulation of vitamin D3 action on macrophages with phytosterols. Phytosterols and VD3 are both sterols and thus they have structural similarity. We have demonstrated that the SIT, the main phytosterol in our diet, augments the action of VD3 on the immune function of macrophages. The markers of immune function we used in the study were cell proliferation, cytokine and nitric oxide released from LPS-challenged macrophages. To the best of our knowledge this is the first study that has examined the combined effect of SIT and VD3 on the immune function of macrophages.

The present study used a different protocol to activate the cells with LPS. Most studies pre-incubate the macrophages with the test agents and then treat them with LPS [13,21]. In this study, we incubated cells simultaneously with LPS and sterols for 24 hours to simulate the *in vivo* setting.

VD3 is an immuno-modulator [22,23] and could be produced by peritoneal macrophages during inflammation [13,24]. VD3 has been shown to down regulate the production of pro-inflammatory cytokines and up-regulate the anti-inflammatory ones [23,25–33]. VD3 exerts its effects on immune function by targeting NFκB [32,34]. VD3 has been shown to decrease the expression of TNF-α mRNA and cytokine levels in LPS-stimulated murine macrophages [35,36]. The underlying mechanism behind this down regulation is thought to be due to the stabilization of IκB mRNA and reducing the IκB phosphorylation. This reduces the nuclear NFκB-p65 complex content and increases the cytoplasmic faction.

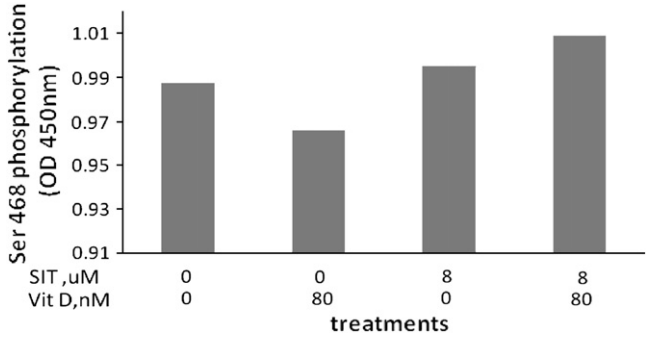

Fig. 4. Effect of VD3 and SIT treatments on Ser 468 phosphorylation. J774A.1 cells (3×10^5 cells/ 100 μl) were seeded in a 96-well tissue culture plate. LPS (100 ng/ml) was added to each well and incubated with SIT, VD3, both or none (control). After 24 h incubation, the cells were subjected to FACE assay and values shown have been corrected for cell number. Abbreviations: SIT = β-sitosterol, VD3 = vitamin D3.

On the other hand, Zemel *et al* showed that calcitriol up-regulated inflammatory cytokine expression in RAW 264.7 cells cultured alone and co-cultured with differentiated adipocytes [37]. The cytokines so up-regulated include TNF-α, IL-6 and MCP-1 and is thought to be mediated by calcitriol stimulated Ca^{2+} signaling and diminished mitochondrial uncoupling [37]. Hakim *et al* also showed that VD3 treatment up regulated TNF- α and cytokine response both *in vitro* and *in vivo* [38]. Results from another study showed that VD3 stimulation did not affect the TNF-α production while increased IFN-γ, IL-10 and IL-6 production from PBMC of Crohn's disease patients [39]. Numerous studies have shown VD3 to be a potential anti-inflammatory agent by suppressing NFκB activity and other transcription factors [28,40–42]. Contrary to these findings, our study did not find a change in cytokine release from the macrophages with the VD3 treatment. Both the pro- and anti-inflammatory cytokines were found to be unchanged compared to vehicle control. Only MCP-1 and IL-10 were found to be significantly increased. Such a sustained response is especially useful in bacterial and viral infections where a prolonged Th1 cell response is needed to destroy the pathogens. Increased MCP-1 response is ideal for mass recruitment of monocytes to the area of inflammation. Increased IL-10 is beneficial for multiple sclerosis patients where they experience a decreased IL-10 production[43]. Our data demonstrate that calcitriol sustains the cytokine response as opposed to down regulation. The unaltered response we noticed might be due to the different cell type[37], incubation period [39], VD3 dosage [13,21,37] and differentiation status of cells [38] used in our experiment.

NO is an important defense mechanism in the body against the invading micro-organisms [44,45]. Its synthesis is mediated by nitric oxide synthases (NOS). Among the NOS, inducible NOS (iNOS) can be induced in macrophages by stimulation with LPS resulting in large amounts of NO being produced [46,47]. The NO released can decrease cell proliferation and affect cytokine release [46,47].

Calcitriol has been shown to inhibit the iNOS mRNA expression and thereby decrease NO release [21,48,49]. On the contrary, our study did not show the VD3 treatment to decrease the NO release. The sustained NO release as reported in our study is thought to be due to prolonged iNOS expression and resultant NO buildup. The unaltered NO response goes hand in hand with the pattern of IFN-γ and TNF-α release seen with VD3 treatment. Cytokines such as IFN-γ and TNF-α have been shown to activate the macrophages to produce reactive nitrogen species to combat bacterial infection [50] and our study further corroborate this evidence. Another study by Rockett et *al*so showed that VD3 could enhance iNOS mRNA expression leading to restrained bacterial growth [51].

SIT also has immuno-modulatory properties [52–56]. Several *in vitro* studies have shown SIT to reduce TNF-α and IL-6 production

[52,57,58]. On the other hand, one study has shown SIT to have no effect on IL-10, IL-4 and IFN-γ levels [59]. SIT has been shown to preferentially induce Th1 cytokines like IFN-γ compared to Th2 cytokines [60]. Th1-type cytokines mediated cellular response is important for the host to defend against viral and bacterial pathogens and curbing infection [52]. The Th2-type cytokines (IL-4, IL-6, and IL-10) are engaged in B-lymphocyte differentiation limits the damage caused by micro-organisms [52]. The inflammatory response is mediated by the intrinsic balance between Th1 and Th2 cytokines. The increased response of Th1 cytokines is responsible in eliminating pathogens [52] while increased Th2 response is beneficial in auto immune diseases like multiple sclerosis where there is a reduced IL-10 production [43]. SIT and its glycoside have been shown to increase IFN-γ secretion in allergic individuals [61]. Our data also show SIT to significantly increase the Th1 cytokine release from macrophages. SIT is shown to have no effect or to inhibit the release of Th2 cytokines [52,61]. We found SIT to increase Th2 cytokines contrary to what has been reported. Although not tested, preliminary analysis showed SIT to have a no preference for Th1 or Th2 cytokine stimulation as earlier reported [52].

SIT has been shown to decrease iNOS levels in PMA-stimulated RAW 264.7 cells [62]. However, the same study also pointed out that NO_2^- production increased from 6–24 h after PMA stimulation and a decrease in the NO production with SIT was noticed after 24 hours [62]. However the decrease in NO production was noticed only with unphysiological levels of SIT. Our study also found SIT to significantly increase NO production within 24 h of incubation with LPS. This increase in NO might be part of the initial immune response to combat the inflammation. More studies are needed to understand how physiological levels of SIT might affect the NO production after 24 h of incubation.

VD3 inhibits cell proliferation and promotes differentiation [29,63]. The anti-proliferative property of VD3 has been found to be beneficial in combating cancer [64–70]. VD3 stimulation of dendritic cells has been shown to induce the apoptosis in T-cells [71]. VD3 has been shown to decrease CD4+ cell proliferation in experimental autoimmune encephalitis model [72]and PBMC multiplication in inflammatory bowel disease [73]. Bartels et al also reported similar results whereby VD3 stimulation increased apoptosis and decreased cell proliferation of T-cells in Crohn's disease [39]. Our results showed a trend for VD3 to reduce cell proliferation although it did not reach significance. This could have been due to the small number of observations used in our experiment.

SIT is an anti-proliferative and apoptotic molecule to several tumor cells [74,75]. Previous studies from our lab have shown SIT to activate the sphingomyelin cycle [76,77], impede the cell cycle [78–80] and stimulate apoptotic cell death in tumor cells [81]. Others have demonstrated that SIT induces apoptosis in U937 cells by caspase -3 activation and Bax/Bcl-2 ratio up-regulation [74]. Our present results are also in accordance with previously published data and further substantiate the anti-proliferative properties of SIT in J774A.1 macrophages.

Combining SIT and VD3, an additive effect has been revealed compared to what has been noticed with their individual treatments. This suggests that the effect of these agents may be similar and in the same direction. This could also imply that both agents may influence the same signal transduction pathways. The combined treatment showed that cell proliferation decreased significantly from vehicle control. The decrease in cell proliferation, albeit insignificant, was even lower than the individual SIT treatment. The VD3, as already discussed, did not bring about much difference in the cytokine or NO release and cell proliferation. SIT, on the other hand, significantly increased the cytokine and NO response. The release of cytokines and NO from the macrophages subjected to the combined treatment was even higher. The SIT + VD3 amplified the cytokine and NO release and the effect was found to be additive and in some cases synergistic. Such an amplified Th1 and Th2 response could be beneficial in counteracting pathogens and controlling autoimmune diseases.

To understand the underlying mechanism behind this treatment response we decided to look at NF-κB phosphorylation. NFκB-p65 phosphorylation at Ser 468 is primarily induced by phorbol esters while Ser 536 phosphorylation is mediated by LPS. Although we could not apply statistical significance to our results due to limited number of observations, we found that all our treatments decreased Ser536 phophorylation. This is of importance because increased Ser536 phosphorylation is thought to inhibit the p53, mediator of apoptosis [82]. This might explain the decreased cell proliferation we observed with our treatments.

Surprisingly, increased Ser 468 phosphorylation was found to be associated with the SIT and SIT + VD3 treatments. This corresponds well with the increase in cytokine and NO release we noticed in our results. Since we observed a decrease in Ser536, this may suggest that p65 has not been activated to a greater extent. So there might be one or more alternative pathways being activated to observe such response we noticed. Since Ser 468 phosphorylation is not expected with LPS stimulation, one may consider the cross talk between the signaling pathways inside the cell a factor in this deviation. The autocrine and paracrine nature of released cytokines would make identifying the role for signal transduction pathways involved a major task.

Other mechanisms may include the possibility that SIT works as a carrier for VD3 by virtue of the similarity in structure between these molecules associated with the difference in their concentrations in the blood. SIT may also influence the binding properties of VD3 to its receptors. However, these proposed mechanisms have to wait further investigation.

In summary, although much more work is necessary to understand the underlying mechanism, we conclude that SIT promotes the immune function of VD3. This ability of SIT to enhance the immunomodulatory action of vitamin D could be beneficial to vitamin D deficient individuals. It may not be easy to overcome a vitamin D deficiency as for people living in temperate climate, with disabilities, old age or renal diseases. However, to include fresh vegetables in the diet and thus enhancing the action of vitamin D may be an option. This study has far reaching implications not only for people with vitamin D deficiency but also for people with autoimmune diseases like multiple sclerosis.

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