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Free versus total serum 25-hydroxyvitamin D in a murine model of colitis

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Graphical abstract

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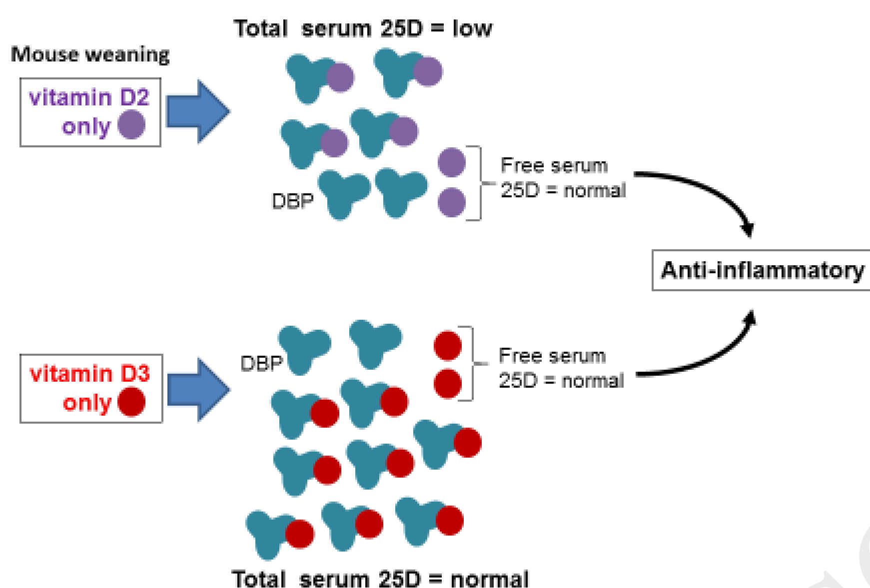

Highlights

- Mice fed a diet with vitamin D2 only from weaning showed lower levels of total serum 25OHD than mice fed a vitamin D3 diet at 8 weeks of age
- Mice fed vitamin D2 only showed higher levels of free serum 25OHD than vitamin D3 mice
- Mice fed vitamin D2 or vitamin D3 diets showed similar severity of colitis induced by dextran sodium sulphate
- Free 25OHD may be more important than total 25OHD for anti-inflammatory responses

Abstract

Inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease have been linked to vitamin D-deficiency. Using a dextran sodium sulphate (DSS)-induced model of IBD we have shown previously that mice raised on vitamin D-deficient diets from weaning have lower serum 25-hydroxyvitamin D (25OHD) levels and develop more severe colitis compared to vitamin D-sufficient counterparts. We have also shown *in vitro* that immune responses to

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25OHD may depend on 'free' rather than total serum concentrations of 25OHD. To investigate the possible effects of free versus total 25OHD on anti-inflammatory immune responses *in vivo* we have studied DSS-induced colitis in wild type C57BL/6 mice raised from weaning on diets containing vitamin D2 (D2) or vitamin D3 (D3) only (both 1000 IU/kg feed). 25OHD2 has lower binding affinity for the vitamin D binding protein than 25OHD3 which results in higher levels of free 25OHD2 relative to free 25OHD3 in mice raised on a D2-only diet. Total serum 25OHD concentrations, measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), showed that D2 mice had significantly lower levels of 25OHD than D3 mice (6.85±2.61 nmol/L vs. 49.16±13.8 nmol/L for D2 and D3 respectively). Despite this, direct ELISA measurement showed no difference in free serum 25OHD levels between D2 and D3 mice (13.62±2.26 pmol/L vs. 14.11±2.24 pmol/L for D2 and D3 respectively). Analysis of DSS-induced colitis also showed no difference in weight loss or disease progression between D2 and D3 mice. These data indicate that despite D2-fed mice being vitamin D-deficient based on serum total 25OHD concentrations, these mice showed no evidence of increased inflammatory colitis disease relative to vitamin D-sufficient D3 mice. We therefore propose that free, rather than total serum 25OHD, may be a better marker of immune responses to vitamin D *in vivo*.

1. Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), is a potent immunomodulator that stimulates both innate antimicrobial [1-3] and adaptive anti-inflammatory [4, 5] responses. These immune regulatory effects appear to be mediated via intracrine conversion of pro-hormone 25-hydroxyvitamin D (25OHD) to $1,25(OH)_2D$ by antigen presenting cells (APC) such as macrophages and dendritic cells (DC) [6, 7]. Thus,

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the delivery of 25OHD to target APC is a critical component of the mechanism by which vitamin D is able to influence immune function. As with the majority of steroid hormones, vitamin D metabolites are chaperoned in the circulation by serum globulins, of which vitamin D binding protein (DBP) accounts for 85-90% of bound 25OHD and albumin 10-15%; hence, less than 1% remains in the unbound or 'free' state [8]. Endocrine activation of vitamin D by the kidney utilises bound 25OHD for proximal tubule reabsorption via megalin/cubilin-mediated receptor endocytosis [9]. However, many other target cells do not express the megalin/tubulin complex and are thus only able to access 25OHD and other vitamin D metabolites via non-facilitated mechanisms. Prominent amongst these is the 'free-hormone hypothesis', which proposes that only unbound steroid hormones are able to enter target cells [10, 11]. In previous studies *in vitro* using megalin/tubulin-negative monocytes, we have shown that DBP impairs antibacterial responses to 25OHD and $1,25(OH)_2D$ [12, 13] suggesting that free 25OHD may be particularly important for regulation of immune responses. The aim of the current study was to assess the importance of free versus total serum 25OHD as a determinant of anti-inflammatory immune function *in vivo*.

The amount of free 25OHD is dependent on serum concentrations of DBP and albumin, and the DBP binding affinity of 25OHD [13, 14]. Knockout of the gene for DBP (*Gc*) in mice provides only a limited perspective on the relationship between total and free 25D, because total serum levels of vitamin D metabolites are substantially reduced in both $Gc^{+/-}$ and $Gc^{-/-}$ mice [15]. In recent studies we have shown that it is possible to disengage total and free serum 25OHD by exploiting the greater DBP binding affinity of 25OHD3 relative to its vitamer equivalent 25OHD2 [16-18]. Mice raised on diets containing exclusively vitamin D2 showed increased serum concentrations of free 25OHD relative to mice raised on vitamin D3 only despite having similar levels of total 25OHD2 or 25OHD3 respectively [19]. The increased free 25OHD in vitamin D2 mice was associated with significant changes in bone and immune cell function, suggesting that total serum 25OHD measurements alone did not

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reflect the biological potential of this vitamin D metabolite [19]. To determine the relative impact of total versus free 25OHD on immune responses *in vivo* we carried out further studies using mice raised on D2 and D3 diets and then treated to induce inflammatory disease. The over-arching objective in this case was to determine whether free 25OHD rather than total 25OHD is a better determinant of the anti-inflammatory activity of 25OHD.

2. Materials and Methods

2.1 *Animals*

Male C57BL/6 mice (Charles River UK) aged three weeks were fed diets that contained either vitamin D2 (D2, n=16) or vitamin D3 (D3, n=16), at concentrations of 1000 IU/kg (Research Diets Inc. New Brunswick, NJ 08901 USA), for five weeks. Mice were housed four per cage, had *ad libitum* access to food and water throughout the study and were maintained on a 12:12 hour light-dark cycle at a temperature of 21-22 °C. All procedures were in accordance with the Animals (Scientific Procedures) Act 1986, regulated by the UK Home Office and were conducted at the Biomedical Services Unit, University of Birmingham, UK.

2.2 *Induction of colitis in mice using dextran sodium sulphate*

At eight weeks of age, 2.5% dextran sodium sulphate (DSS, MP pharmaceuticals, Santa Ana, USA) was administered to eight mice per group via drinking water for seven days, leaving the remaining eight per group as water treated controls. The treatment period was followed by a three day resolution phase, whereby DSS was withdrawn from drinking water to allow mice a period of recovery.

2.3 *Mouse health Score*

To monitor health during experiments, mice were scored daily upon starting DSS treatment. By calculating percentage weight loss and assigning faecal consistency and anal bleeding scores, colitis disease progression could be quantified. The scoring system used was as

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follows. A calculated weight loss of less than 1% was scored as 0, a 1-5% loss as 1, a 5-10% reduction was given a 2, a 10-20% weight loss as 3, and if a mouse lost more than 20% it would be euthanised as this indicated an end point. Faecal consistency was scored thus; well-formed pellets were scored 0, pasty stools were given a 2, and watery stools scored as 4. Finally, no anal bleeding was scored as 0, if a spot of blood was observed in the anus a score of 2 was assigned, whilst gross bleeding was given a score of 4. Scores were added together and mice with combined scores greater than 9 were euthanised (**Table 1**).

Score	**0**	**1**	**2**	**3**	**4**
Weight loss	None	1-5%	5-10%	10-20%	≥20%
Stool consistency	Well formed	-	Pasty	-	Watery
Anal bleeding	None	-	Spot	-	Gross

Table 1. Disease scoring of a mouse model of colitis. Mice were monitored daily for weight loss, stool consistency and anal bleeding. These parameters were assigned scores dependent on presence and/or severity. Any mouse with a combined health score of nine or greater was humanely sacrificed; as were mice with a weight loss of ≥20%.

2.4 *Tissue collection*

At the conclusion of the study, cardiac bleeds were performed under terminal anesthesia and bloods collected into micro centrifuge tubes, left at room temperature to allow coagulation, were centrifuged and resultant sera stored at -80 °C. Tissues were excised, dissected and promptly snap-frozen in LN_2 and fixed in 4% formaldehyde for later analyses.

2.5 *Quantification of serum 25OHD*

Both total and free 25OHD were quantified from collected mouse sera. Free 25OHD analysis was performed using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Diasource, cat no: KAPF1991). This assay has 77% cross-reactivity with 25D2. Total serum 25OHD was analysed using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) as described previously, with slight modifications

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[20, 21]. In brief, samples were prepared for analysis by protein precipitation and supported liquid-liquid extraction (SLE). Samples were derivatized with 4-(2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl)-1,2,4-triazoline-3,5-dione (DMEQ-TAD) as previously described [22]. Analysis of serum was performed on a Waters ACQUITY ultra performance liquid chromatography (UPLC) coupled to a Waters Xevo TQ-XS mass spectrometer. The LC-MS/MS method has been validated on US Food and Drug Administration guidelines for analysis of these metabolites as previously described (32).

2.6 *Histological analysis of colon*

Excised colons were washed with PBS before being coiled, placed into processing cassettes and fixed in 10% neutral-buffered formalin (4% formaldehyde) prior to embedding in paraffin wax. Sections were cut to 5 μm and haematoxylin and eosin staining performed using standard procedures. Briefly, sections were de-waxed and re-hydrated through serial dilutions of industrial methylated spirits, stained in haematoxylin and washed in Scott's water. This was followed by staining in eosin, washing in water and dehydration through serial dilutions of industrial methylated spirits before clearing and mounting. Sections were viewed using light microscopy and images taken using a Leica imaging system.

Histological scoring of haematoxylin and eosin stained colonic tissue sections was performed blinded as described previously [23] for: 1) inflammatory cell infiltration. The presence of occasional inflammatory cells in the lamina propria=0; Increased numbers of inflammatory cells in the lamina propria=1; Confluence of inflammatory cells, extending into the submucosa=2; Transmural extension of the infiltrate=3. 2) ulceration, and tissue damage**.** No mucosal damage was scored=0; Discrete lymphoepithelial lesions=1; Surface mucosal erosion or focal ulceration=2; Extensive mucosal damage and extension into deeper structures of the bowel wall=3. Values for n=4 fields of vision from each of the mouse colons for vitamin D2 and vitamin D3 treated mice were obtained. Values were totalled and

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divided by the total number of fields of vision. The combined histological score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage).

2.7 *Statistics*

Data for body weight changes and scoring were compared using student's *t*-test. Total and free 25OHD values were compared using ANOVA, with Tukey's multiple comparisons test; with significance assigned where P<0.05.

3. Results

3.1 Vitamin D2 is associated with deficient levels of total but *not* free serum 25OHD

At the completion of the study, control (water only) D3-fed mice had higher serum concentrations of 25OHD3 (42.61±3.40 nmol/L) than D2-fed mice (12.41±0.85 nmol/L, p<0.0001). DSS-treated D3-fed mice also had higher serum concentrations of 25OHD3 (49.16±5.21 nmol/L) than D2-fed mice (6.85±0.92 nmol/L, P<0.0001) mice (**Fig. 1A)**.

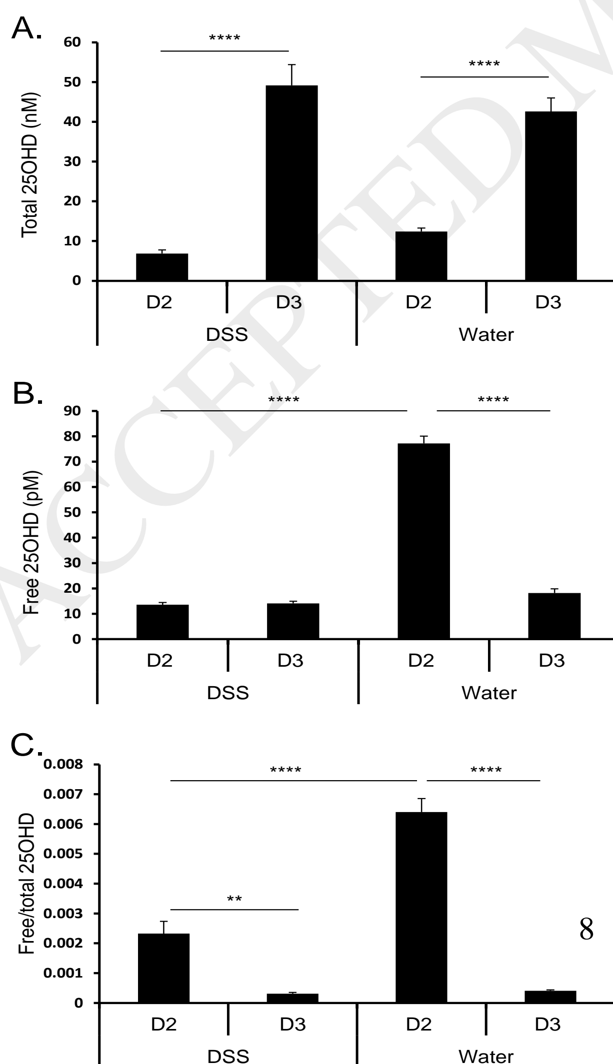

Figure 1. Total and free 25OHD in vitamin D2 and vitamin D3 fed mice. In DSS- or water-treated D2 and D3 mice: (A) Total serum 25OHD2 and 25OHD3 determined by LC-MS/MS (B) Free serum 25OHD determined by ELISA (C) Ratios of free to total 25OHD. Data are shown as mean values ± SEM; statistical analyses performed using ANOVA; **P<0.01, ****P<0.0001; n=8 mice/treatment group.

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In contrast to the total 25OHD data, analysis of free serum 25OHD by ELISA showed that in DSS treated mice, levels were similar in both groups (13.62±0.80 pmol/L vs. 14.11±0.85 pmol/L for D2/D3 respectively). However, in water control mice, free 25OHD levels were four-fold higher in D2 mice compared to D3 mice (77.2±2.83 pmol/L vs. 18.21±1.60 pmol/L, P<0.0001 for D2/D3 respectively)**(Fig. 1B)**. The ratio of free to total 25OHD was 7.5 and 16 times higher in D2 fed mice relative to D3 fed mice (1:430 vs. 1:3239 and 1:156 vs. 1:2472 for DSS-treated and water controls respectively) **(Fig. 1C)**. Additional serum metabolites measured for the D3 fed mice showed significantly decreased levels of 1,25$(OH)_2$D3 (157.50±19.20 pmol/L vs. 239.57±28.82 pmol/L, P<0.05), 3-Epi-25OHD3 (1.17±0.26 nmol/L vs. 4.78±0.61 nmol/L, $p<0.001$) and 24,25$(OH)_2$D3 (32.83±4.69 nmol/L vs. 92.13±12.03 nmol/L, P<0.01) in DSS-treated mice versus water controls.

3.2 *Effects of D2 and D3 on DSS-induced colitis.*

Daily weighing of mice at the commencement of DSS treatment showed there was no significant difference between body weight changes at the conclusion of the study between D2 vs. D3 fed mice cohorts, although D3 fed mice did gain significantly more weight in the first four days than their D2 fed counterparts (**Fig. 2 and Fig 3A**). Moreover, both D2 and D3 groups lost weight at similar rates between days 5 and 7 of DSS treatment: -0.3/-0.7%, -4.5/-4.7%, -9.5/-10.7% on days 5, 6 and 7 for D2/D3 treated mice respectively. In contrast to DSS-treated mice, control water-only mice showed steady increases in body weight throughout the 10 day treatment period with D2 mice showing statistically higher weight gain on days 6 and 8 (**Fig. 3B**).

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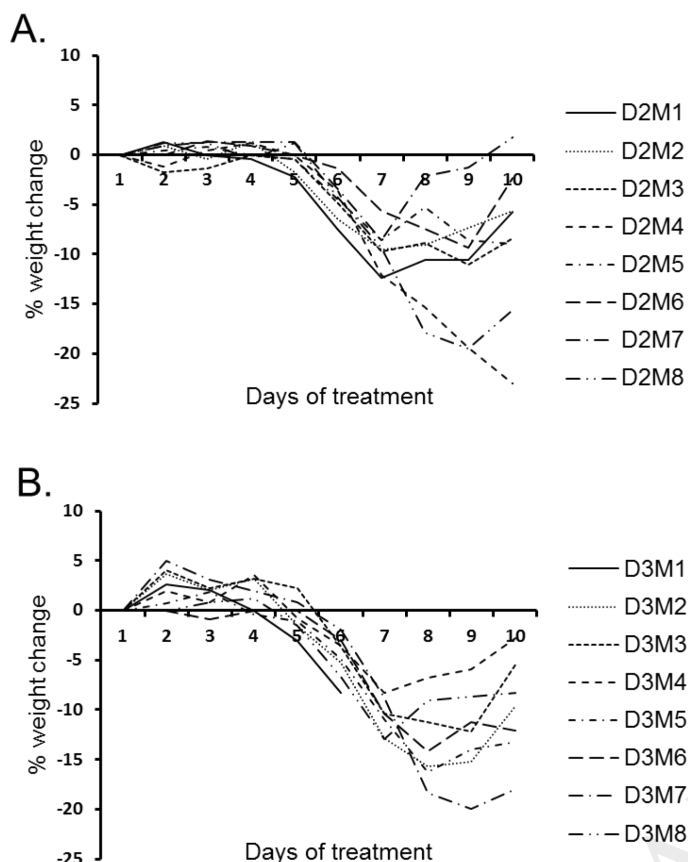

Figure 2. Individual weight changes for DSS-treated D2 and D3 mice. % change in body weight for individual mice during (day 0-7) and after (day 8-10) treatment with DSS (2.5% in water). A. Individual D2 mice (D2M1-D2M8). B Individual D3 mice (D3M1-D3M8).

Mouse health and colitis disease progression was also monitored by observing faecal consistency and the presence or extent of anal bleeding. D2 fed mice appeared to show earlier effects of DSS treatment, manifesting in aberrant stool formation approximately a day earlier than in D3 fed mice **(Fig. 3C)**. Consequently, D2 mice reached peak colitis disease severity at day 7 and D3 mice at day 8 (scores of 6±0.5 and 3.57±0.62, $P<0.01$ for D2/D3 respectively) of DSS treatment. D3 fed mice also appeared to recover more quickly than D2 fed mice (scores of 3.57±0.37 and 2.29±0.29, $P<0.05$) after removal of DSS treatment. H&E staining of paraffin embedded colonic sections for D2 and D3 fed mice showed variable, but comparable pathology of the colon, with similar levels of inflammatory cell tissue invasion and mucosal ulceration damage for D2 and D3 mice **(Fig. 4)**.

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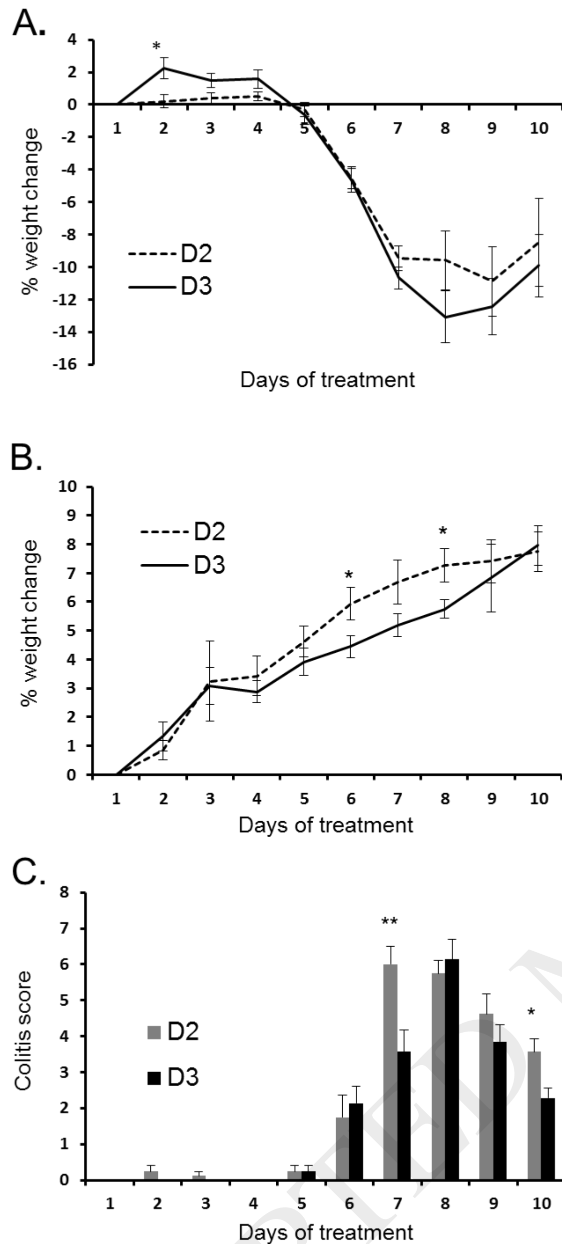

Figure 3. Effect of DSS on body weight and colitis disease score in D2 and D3 mice. A. % change in body weight for D2 and D3 mice during (day 0-7) and after (day 8-10) treatment with DSS (2.5% in water). B. In water only control mice. C. Colitis disease scores for D2 and D3 mice: during (day 0-7) and after (day 8-10) treatment with DSS (2.5% in water). Data are shown as mean ± SEM; statistical analyses performed using Student's *t*-test; *P<0.05; n=8 mice/treatment group.

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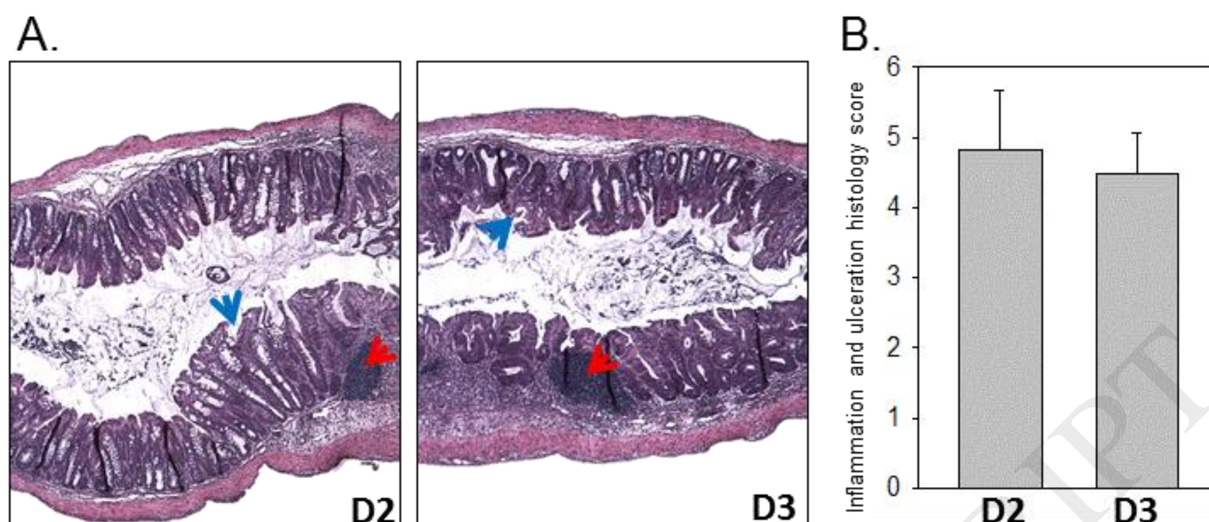

Figure 4. Histopathological analysis of excised and dissected colons for DSS-treated D2 and D3 fed mice. Colons were fixed in formalin and embedded in paraffin wax before being sectioned at 5 µm. A. Both D2 and D3 mice showed signs of inflammation (red arrows), determined by H&E staining. B. Quantification of histological scoring for inflammatory infiltration (0 – 3, red arrows) combined with scoring for mucosal ulceration damage (0 – 3, blue arrows) in D2 and D3 fed mice exposed to DSS. Data are mean ± SD for n=7 mice .

4. Discussion

Several lines of evidence have highlighted a role for vitamin D in anti-inflammatory responses in the gastrointestinal tract. Firstly, epidemiology has shown that patients with inflammatory bowel disease (IBD) have decreased serum levels of 25OHD [24-26]. Secondly, in humans, expression of the vitamin D-activating enzyme CYP27B1 has been detected in colonic epithelial cells [27], with CYP27B1 expression being altered in affected tissue from patients with Crohn's disease [28]. Finally, in studies using various animal models, active $1,25(OH)_2D$ has been shown to play a crucial role in the pathophysiology of

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experimental colitis [29-33]. These models include mice with knockout of the *Vdr* gene [29, 31], and knockout of the *Cyp27b1* gene [32], both of which showed increased severity of inflammatory colonic disease with different models of colitis. In previous studies we have also shown that wild type mice raised from weaning on a vitamin D-deficient diet with low serum concentrations of 25OHD are also more susceptible to experimental colitis [33]. This latter observation confirmed similar observations in humans with IBD [24-26], but also suggested that 25OHD, rather than active 1,25$(OH)_2$D, is a key determinant of immune regulation. The likely mechanism for 25OHD as an attenuator of inflammatory disease is via intracrine or paracrine synthesis of 1,25$(OH)_2$D from 25OHD [5]. However, an important additional consideration for 25OHD is that it is the vitamin D metabolite with the highest binding affinity for serum DBP. As such, the relationship between 25OHD and DBP may be a key factor in mediating both skeletal and extra-skeletal actions of vitamin D. The aim of the current study was to determine how DBP binding of 25OHD, and free 25OHD, impacts the anti-inflammatory effects of 25OHD.

The first notable observation from this study is that mice fed a D2 only diet had lower circulating levels of 25OHD than mice fed D3, in either the water control mice or with DSS-induced colitis. In a previous report by our group the serum levels of 25OHD in mice on D2- and D3-only diets were not significantly different after a similar time period (8 weeks) [19]. The most likely explanation for this is that the two different studies were carried out in different animal facilities and with wild type C57/BL6 mice from different sources. However, lower serum levels of 25OHD with a mouse vitamin D2 diet are consistent with previous analysis of non-human primates, where 2-3-fold higher serum 25OHD concentrations were observed with D3 diet relative to D2 [34]. Likewise, human studies have shown that D2 supplementation resulted in lower increases in serum 25OHD levels [35]. The proposed explanation for this is that the lower binding affinity of 25OHD2 for DBP results in less renal reabsorption of 25OHD2 via megalin-mediated endocytosis in the proximal tubule. This may,

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in turn, lead to lower levels of 1,25$(OH)_2$D2, but unfortunately we were only able to quantify 1,25$(OH)_2$D3 in the LC-MS/MS assay used in this study. It was also interesting to note that in D2 mice the low serum 25OHD2 levels in control mice decreased still further with DSS treatment. By contrast there was no effect of DSS on serum 25OHD3 levels. The reason for this is unclear, and is unlikely to be linked to DSS-induced inflammation as both sets of mice showed equivalent severity of colitis.

Although total 25OHD2 levels were much lower than total 25OHD3 in the two sets of mice, ELISA-measured free 25OHD was significantly higher in D2 control mice and no different from D3 in D2 DSS mice. As outlined above, this can be explained by the lower binding affinity of 25OHD2 for DBP relative to 25OHD3 leading to increased free 25D2, and the increased ratio of free/total 25OHD in D2 mice is consistent with our previous studies of D2 versus D3 mice [19]. Interestingly, the 16-fold higher ratio of free/total 25OHD in D2 versus D3 water control mice, was decreased to 7.5-fold in DSS-treated mice. These data suggest that the equilibrium between total and free 25OHD2 may be influenced by the inflammatory disease itself. The explanation for this is unclear, but could involve increased circulating levels of DBP and/or other serum binding proteins such as albumin. These proteins were not measured in the current set of experiments but should be an important feature of future studies. It was also interesting to note that in D3 mice serum levels of 1,25$(OH)_2$D3, 3-epi-25OHD3 and 24,25$(OH)_2$D3 were significantly lower in DSS-treated mice compared to regular water D3 controls. In previous studies of humans serum 1,25$(OH)_2$D3 levels have been reported to be elevated in some patients with Crohn's disease, but not patients with ulcerative colitis [28]. This was attributed to increased extra-renal synthesis of 1,25$(OH)_2$D3 by macrophages in disease-affected tissues, similar to granulomatous disorders such as sarcoidosis. However, studies of other inflammatory diseases such as rheumatoid arthritis an inverse correlation between disease activity and serum levels of 1,25$(OH)_2$D3 have been observed [36]. One possible explanation for this is that the systemic inflammation that is

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characteristic of diseases such as rheumatoid arthritis acts to inhibit renal activity of the enzyme CYP27B1, possibly via NF-κB suppression of *CYP27B1* gene promoter activity [37].

The crucial observation from the current study is that although D2 mice had total serum 25OHD levels that were seven times lower than D3 mice, they showed similar responses to DSS-induced colitis. In a previous DSS-induced colitis study using mice fed a D3 diet versus a diet with no D3 (vitamin D-deficient), we observed a similar difference in total 25OHD levels to that documented in the current study [23]. In the D3-deficient mice low serum 25OHD was associated with a significant increase in colitis severity, which we were able to show was due to altered colonic expression of a range of different genes associated with immune and barrier function [23]. It is difficult to make a direct comparison between these two studies as vitamin D deficiency was not an objective of the D2 diet in the current study. Nevertheless, the current data strongly suggest that in the setting of inflammatory disease, quantification of total serum 25OHD may have only limited value. Instead the similar patterns of colitis in D2 and D3 mice would appear to be more closely linked to the similar serum concentrations of free 25OHD2 and 25OHD3 respectively in these mice. Although levels of free 25OHD are relatively small, the potential for this fraction of 25OHD to more effectively promote immune responses is consistent with our previous *ex vivo* data suggesting that free 25OHD is the key determinant of monocyte antibacterial responses to 25OHD [12]. However, it is also important to recognize that the higher levels of total 25OHD in D3 mice may also have some benefits. Notably the onset of DSS-induced disease appeared to be earlier in D2 mice (see **Figures 2** and **3**), even though the peak weight loss and colitis scores were similar for D2 and D3 treatments. It is therefore possible that early events in the onset of colitis, such as barrier integrity [38] are influenced more by total 25OHD.

Over the last 5 years there has been considerable debate concerning the relative merits of total versus free serum 25OHD [39-41]. It seems likely that in normal healthy individuals free

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serum 25OHD is very closely linked to total serum 25OHD and, as such, measurement of free 25OHD may have no significant advantage as a marker of 'vitamin D status'. However, significant variations in the ratio of free/total 25OHD have been reported in patients with diseases such as cirrhosis of the liver [42], suggesting that in some settings free 25OHD is a more accurate measure of vitamin D function. Likewise, in vitamin D-deficient patients, an early rise in free 25OHD following vitamin D supplementation was shown to be more strongly associated with changes in vitamin D biomarkers such as PTH than total 25OHD [43]. The extent to which variations in total and free 25OHD impact inflammatory disease is still unclear. In studies using elderly human subjects we were unable to determine any significant difference when using total or free serum 25OHD as correlates for circulating inflammatory cytokines [44]. However, in this case it is important to recognise that only a very limited selection of inflammatory markers were measured, and the cohort was an otherwise healthy one with no established inflammatory disease. Based on these observations, we hypothesise firstly that in the setting of inflammatory disease free 25OHD is a better indicator of the anti-inflammatory potential of vitamin D. Secondly, we propose future studies to investigate the potential benefits of supplementation with vitamin D2 versus vitamin D3 for some actions of vitamin D, notably immunomodulatory actions. As described by Bouillon *et al.* in a recent commentary [45], in this setting it may be useful to consider vitamin D2 as an 'analog' of vitamin D rather than a bioequivalent vitamer.

Author Contributions

DPL carried out animal work and tissue analyses, produced all Figures, and contributed to the design of the study and in the writing of the manuscript; CJ carried out LC-MS/MS serum analysis for Figure 1 and Table 2; CSJW performed statistical analyses on Figure 1 and Table 2; RFC contributed to experimental design; JSA and MH designed the experiments, analysed data and contributed to manuscript writing.

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Competing financial interests

The authors declare no competing financial interests.

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