Extended report

Vitamin D receptor regulates TNF-mediated arthritis

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ABSTRACT

Objective Reduced vitamin D intake has been linked to increased susceptibility to develop rheumatoid arthritis (RA) and vitamin D deficiency is associated with increased disease activity in RA patients. The pathophysiological role of vitamin D in joint inflammation is, however, unclear.

Methods To determine the influence of absent vitamin D signalling in chronic arthritis, vitamin D receptor (VDR)-deficient mice were crossed with human tumour necrosis factor (TNF) transgenic mice (hTNFtg), which spontaneously develop chronic arthritis.

Results Clinical signs and symptoms of chronic arthritis were aggravated in hTNFtg mice lacking functional VDR signalling. Moreover, synovial inflammation was clearly increased in VDR −/− hTNFtg mice as compared to hTNFtg mice and was associated with an increased macrophage influx in inflamed joints. In vitro, VDR-deficient monocytes were proinflammatory and hyper-responsive to TNF stimulation associated with prolonged mitogen-activated protein kinase activation and cytokine secretion. Also, VDR −/− monocytes showed enhanced potential to differentiate into bone resorbing osteoclasts in vitro. In line, VDR −/− hTNFtg mice had significantly increased cartilage damage and synovial bone erosions.

Conclusions VDR plays an important role in limiting the inflammatory phenotype in a mouse model of RA. Absent VDR signalling causes a proinflammatory monocyte phenotype associated with increased inflammation, cartilage damage and bone erosion.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease presenting as a symmetric and chronic polyarthritis. The typical trias synovial inflammation, cartilage damage and bone resorption causes structural damage leading to significant morbidity and mortality. 1 The pathogenesis is only partially understood but may include genetic susceptibility factors such as major histocompatibility class antigens, environmental triggers and the activation of the innate and adaptive immune system. 2 The successful introduction of diverse therapeutic strategies treatments such as cytokine blockers, B cell depletion and inhibition of co-stimulation has indeed made clear that distinct pathways are involved in driving RA. 3

Vitamin D is a steroid hormone playing a major role in the maintenance of proper bone metabolism: profound vitamin D deficiency causes rickets in children and osteomalacia in adults. 4 The active form of vitamin D, 1,25(OH) 2 D 3 is required for its physiological function: regulation of calcium and phosphorus uptake from the gut and kidney, thus providing the substrate for proper bone mineralisation. However, the discovery of the vitamin D receptor (VDR) in 1974 in non-skeletal tissue, particularly in immune cells, such as lymphocytes and monocytes indicated a possible role for vitamin D in the immune system. 5 Depending on the experimental conditions, vitamin D has indeed been shown to be necessary for T cell proliferation and activation but also monocyte differentiation, dendritic cell maturation and many other functions. Importantly, vitamin D and vitamin D analogues show a suppressive effect on disease development and disease severity in animal models of autoimmune diseases. 6 7

Interestingly, vitamin D deficiency or reduced intake has been linked to an increased susceptibility to develop autoimmune diseases such as multiple sclerosis (MS) and type 1 diabetes in large population studies. 8 9 Low vitamin D levels are also linked to increased disease activity in RA. 10 The exact mechanism for the inverse relationship between low vitamin D level and increased inflammatory disease activity is, however, unclear. Importantly, it may merely be an association rather than a pathogenetic link. Thus, the effects of absent vitamin D signalling receptor were investigated in human TNF transgenic mice, a mouse model of inflammatory arthritis, by interbreeding with VDR-deficient mice.

MATERIAL AND METHODS

Animals

Heterozygous human tumour necrosis factor α transgenic mice (hTNFtg mice, strain tg197), 11 and VDR knock-out mice (VDR −/− , both: genetic background C57/BL6) 12 were interbred to receive the following genotypes at generation F2: wildtype (wt), VDR −/− , hTNFtg and VDR −/− hTNFtg mice. A total of 80 mice were used in this study (n=20/group) and only littermates were used. All four genotypes were assessed weekly (starting at week 3) for arthritis and weight as described previously. 26 All animal studies were approved by the local ethics committee.

Joint histology

Hind paws were fixed overnight in 4% formalin and then decalcified in EDTA (Sigma-Aldrich, St. Louis, Missouri, USA) until bones were pliable. Serial paraffin sections (2 μm) of hind paws were stained with H&E, toluidine blue and tartrate-resistant acid phosphatase (TRAP). Area of inflammation, area of bone erosions and the extent of proteoglycan loss was determined as described previously. 13

Immunohistochemical analysis

To analyse different cell populations in the inflamed joints of hind paws, immune-phenotyping for...
B cells (anti-CD45R; BD Bioscience Pharmlingen, San Jose, California, USA), T cells (anti-CD3; Serotec, Oxford, UK), neutrophil granulocytes (Serotec, Oxford, UK) and macrophages (F4/80; Serotec, Oxford, UK) was performed as described previously. Quantitative analysis of positively stained cells in the inflamed synovium was performed using a microscope (Nikon, Düsseldorf, Germany) and the Osteomasure System (OsteoMetrics, Atlanta, Georgia, USA).

Micro CT imaging studies
MicroCT (μCT) images of tibial bones were acquired on a vivaCT40 system (Scanco Medical, Bassersdorf, Switzerland). The scanner generates a cone beam at 5 mm spot size and operates at 50 keV. A region of 402 slices was imaged at 10 μm isotropic resolution, starting from the proximal end of the tibia.

Bone histomorphometry
Formalin-fixed tibiae were embedded undecalcified in methacrylate (Heraeus Kulzer, Wehrheim, Germany). After polymerisation, 3 μm sections were prepared with a Jung microtome (Jung, Heidelberg, Germany), deplastinated in methoxymethylmethacrylate (Merck, Darmstadt, Germany) and stained using von Kossa’s stain, Goldner trichrome and toluidine blue. The following histomorphometric parameters were measured according to international standards.

Dynamic labelling of bone
All mice at 9 weeks of age were given injections of calcine green (Sigma-Aldrich) at a dose of 30 mg/kg body weight 7 days apart, and killed 2 days after the final injection. Mineral apposition rate (μm/day) was calculated as described previously.

Bone marrow monocyte cultures
Monocytes of VDR−/− and wildtype mice were isolated from bone marrow and cultured in α-MEM with 10 mg/ml macrophage colony stimulating factor (M-CSF) for 3 days. Adherent macrophages were starved for 6 h and afterwards stimulated with 10 ng/ml mTNF (R&D System, Minneapolis, Minnesota, USA) or without stimulation for 5 min, 15 min, 30 min, 1 h and 2 h. Cells were lysed and western blotting was performed with polyclonal antibodies against the phosphorylated and non-phosphorylated forms of p38MAPK, ERK and JNK (all antibodies from Cell Signalling) as well as β-actin (Sigma-Aldrich). For determination of spontaneous cytokine production, freshly isolated monocytes were cultured for 24 h in α-MEM supplemented with FCS. After 24 h, RNA and supernatant were collected for further analysis. All experiments were performed with three different donors and representative blots are shown.

Ex vivo osteoclastogenesis assay and RNA analysis
Osteoclasts were generated as described previously. Briefly, non-adherent bone marrow cells were stimulated with 30 ng/ml M-CSF and 30 ng/ml RANKL (R&D Systems) for 3 days. Osteoclasts were determined as TRAP-positive cells with three or more nuclei and counted. Additionally, RNA was isolated from osteoclast cultures by Trizol reagent (Invitrogen, Carlsbad, California, USA) and reverse transcribed with murine leukaemia virus reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems, Foster City, California, USA) and oligo(dT) primers. Quantitative real-time RT-PCR was performed using LightCycler technology (Roche Diagnostics, Indianapolis, Indiana, USA) and the Fast Start SYBR Green I kit for amplification and detection. In all assays, cDNA was amplified using a standardised program. The expression of the target molecule was normalised to the expression of β-actin. All experiments were performed with three different donors.

Enzyme-linked immunosorbent assays
Serum and cell culture supernatant levels of TNF, interleukin (IL)-1, osteoprotegerin (OPG) and RANKL were measured using ELISAs according to the manufacturer’s recommendations (all R&D systems).

Statistical analysis
Data are presented as the mean ± SEM. For group comparisons, a non-parametric Mann–Whitney U test was used. A p value <0.05 was considered significant.

RESULTS
VDR deficiency aggravates clinical signs of TNF-mediated arthritis
To determine the effect of vitamin D deficiency on experimental arthritis, mice lacking the VDR (VDR−/−) were interbred with human TNF transgenic (hTNFtg) mice, which spontaneously develop a chronic erosive arthritis. Wildtype and VDR−/− mice did not develop clinical signs of arthritis. Interestingly, the absence of VDR had a significant impact on the clinical course of arthritis in hTNFtg mice: arthritis occurred earlier and was more severe in VDR−/−hTNFtg mice as evident by significantly earlier onset of disease and worsening clinical scores. At week 9, VDR−/−hTNFtg mice showed drastically increased grip strength loss (mean score ± SEM −2.8 ± 0.1 vs −1.9 ± 0.1, p<0.01) and paw swelling (mean score 2.1 ± 0.3 vs 1.1 ± 0.4, p<0.01) as compared to hTNFtg mice (figure 1A–B).

Figure 1 Clinical assessment of arthritis. hTNFtg and VDR−/−hTNFtg mice (n=8/group) were clinically assessed from week 4 until week 9. Clinical signs of arthritis were determined by assessment of grip strength loss (A) and paw swelling (B). Data are the mean ± SEM. Asterisks mark statistically significant difference (p < 0.05).
VDR modulates TNF-induced synovial inflammation and macrophage influx into the joint
To further investigate the effects of vitamin D on arthritis, a comparative histological analysis of synovial inflammation in hTNFtg and VDR<sup>−/−</sup>hTNFtg mice was performed. hTNFtg mice developed severe, erosive arthritis (mean inflammation 0.94 ± 0.18 mm<sup>2</sup>). In line with the clinical findings, arthritis was even more severe in VDR<sup>−/−</sup>hTNFtg mice causing a highly destructive phenotype (+48%, mean area 1.40 ± 0.19 mm<sup>2</sup>, p<0.05 vs hTNFtg mice). Representative images are shown in figure 2A. To determine if the absence of functional vitamin D affects the cellular distribution of the inflammatory infiltrate, immunohistochemical analyses of synovial cells was performed. No quantitative differences in distribution of synovial T cells, B cells and neutrophils among hTNFtg and VDR<sup>−/−</sup>hTNFtg mice could be detected (figure 2B–D). However, a profound increase in synovial macrophages was found in VDR<sup>−/−</sup>hTNFtg mice as compared to hTNFtg mice (p<0.05; figure 2E).

Macrophages lacking VDR are proinflammatory and hyper-responsive to TNF
To determine the role of VDR in osteoclast precursors in vitro, first, cytokine production of isolated monocytes from wildtype and VDR<sup>−/−</sup> mice ex vivo was measured. Interestingly, VDR<sup>−/−</sup> monocytes revealed increased TNF and IL-1 mRNA production after 24 h of ex vivo culture. Moreover, TNF and IL-1 levels were increased in cell culture supernatant when VDR was lacking (figure 3A). Finally, increased serum levels of TNF (mean

![Figure 2](http://ard.bmj.com/)
death was observed in 14.8 ± 1.7% of articular chondrocytes of hTNFtg mice but in 20.0 ± 2.2% (p<0.05 vs hTNFtg mice) of VDR −/− hTNFtg mice (figure 4).

Absence of VDR enhances inflammatory osteoclastogenesis

Next, osteoclast formation and bone erosion in hTNFtg and VDR −/− hTNFtg mice was addressed. As determined by quantitative histological analysis, synovial bone erosions were significantly increased in VDR −/− hTNFtg (mean area 0.35 ± 0.07 mm²) as compared to hTNFtg mice (mean area 0.17 ± 0.04 mm², p<0.05). This was based on a strongly enhanced synovial osteoclastogenesis in arthritic mice lacking VDR signalling (figure 5A–B).

To find out whether this osteoclastogenic effect is intrinsic due to the absence of VDR on osteoclast precursors, osteoclast differentiation and functional assays were performed. Interestingly, increased in vitro osteoclastogenesis in VDR −/− hTNFtg cells as compared to hTNFtg cells could not be detected (figure 4C). However, osteoclasts generated from VDR −/− hTNFtg cells were more active than wildtype osteoclasts as determined by the ability to resorb an osteogenic matrix in vitro (mean resorption area: VDR −/− hTNFtg 38.3% vs hTNFtg 24.8%, p<0.05; figure 5D). These findings were underlined by transcriptional analysis of osteoclasts: osteoclasts generated from VDR −/− hTNFtg mice showed enhanced mRNA levels of osteoclast differentiation markers TRAP, cathepsin K and c-fos as compared to hTNFtg

Cartilage proteoglycan loss is enhanced in hTNFtg mice lacking VDR

Next, whether or not VDR is involved in cartilage damage during TNF-mediated arthritis was examined. hTNFtg mice showed a pronounced loss of cartilage proteoglycans (mean area 15.3 ± 3.4%). Again, the absence of VDR signalling led to a more severe phenotype: cartilage proteoglycan loss was more pronounced (mean area 24.3 ± 4.4%, p<0.05 vs hTNFtg mice). Chondrocyte death was also compared, with similar results: chondrocyte death was observed in 14.8 ± 1.7% of articular chondrocytes of hTNFtg mice but in 20.0 ± 2.2% (p<0.05 vs hTNFtg mice) of VDR −/− hTNFtg mice (figure 4).

Absence of VDR enhances inflammatory osteoclastogenesis in hTNFtg mice

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performed. VDR−/− mice did exhibit severe osteopenia as compared to wildtype mice. Bone volume (BV) was significantly reduced in VDR−/− mice (BV/tissue volume (TV) 20.3 ± 0.6% vs wildtype 33.9 ± 0.4%, p<0.05) as evident by μCT (figure 6A).

hTNFtg mice revealed osteopenia as well (BV/TV 20.6 ± 6.2%, p<0.05), comparable to VDR−/− mice. VDR−/− hTNFtg mice showed a further, but non-significant, reduction of bone mineral density (BV/TV 12.2 ± 9.3%). These findings were also reflected, when trabecular numbers, thickness and separation were analysed: deficiency of VDR and TNF overexpression led to a loss of trabecular structures with a lower number and thinner trabeculae. VDR−/− hTNFtg mice again showed again a trend for a more severe phenotype. To further investigate this bone phenotype, static and dynamic bone histomorphometry was performed.

Enhanced systemic bone resorption in VDR−/− hTNFtg mice

Next, systemic bone architecture distant from the inflamed joints was investigated and μCT analysis and bone histomorphometry performed. VDR−/− mice showed highly increased osteoblasts and osteoid surface as compared to wildtype mice.
Figure 6  Systemic bone mineral density analysis. Micro-CT (µCT) based reconstruction of vertebral bones: wildtype, VDR^{-/−} hTNFtg and VDR^{-/−} hTNFtg mice (n=6/group) A. µCT based analysis of structural bone parameters: bone volume per tissue volume (BV/TV), trabecular number (Tb.N.), trabecular thickness (Tb.Th.) and trabecular separation (Tb.Sp.). Bone histomorphometry analysis: Goldner staining of tibial bones from 9-week-old female wildtype, VDR^{-/−}, hTNFtg and VDR^{-/−} hTNFtg mice (n=6–8/group). B. Osteoid surface per bone surface (OS/BS), number of osteoblasts per bone perimeter (No.Ob/B.Pm.) and number of osteoclasts per bone perimeter (No.Oc/B.Pm.) were determined. Mineral apposition rate (µm/day) was evaluated using calcein green in hTNFtg and VDR^{-/−} hTNFtg mice (C). Data are the mean ± SEM. Asterisks mark statistically significant difference (*p < 0.05; **p < 0.01).

In contrast, hTNFtg mice did not show any significant alterations of osteoblast activity. Interestingly, VDR^{-/−} hTNFtg mice had an osteoblast phenotype comparable to VDR^{-/−} mice with increased osteoblast numbers and osteoid surface (figure 6B). These findings were confirmed by dynamic histomorphometry: mineral apposition rate was significantly higher in VDR^{-/−} mice regardless of whether TNF was overexpressed or not (figure 6C). Thus, the phenotype of the VDR^{-/−} hTNFtg mouse is the result of a combination of osteomalacia and inflammation-induced osteopenia.

DISCUSSION
Vitamin D is a key hormone involved in the regulation of calcium and phosphorus metabolism. Vitamin D deficiency leads to a counter-regulatory secondary hyperparathyroidism and defective bone mineralisation causing rickets in children and
osteoanomalacia in adults. Besides these well-established functions in general bone health, vitamin D deficiency possibly is a susceptibility factor for autoimmune diseases: reduced vitamin D intake is associated with an increased risk of developing MS, and vitamin D deficiency has also been found in inflammatory bowel disease and systemic lupus erythematosus. Moreover, the Iowa Women’s Health Study showed that subjects with low vitamin D intake had a higher risk of developing RA during follow-up.

Another study suggested that vitamin D is not only involved in the susceptibility of disease; patients with early inflammatory polyarthritis and low 25(OH)D levels had the highest disease activity. Finally, the VDR is expressed in the inflamed synovium of RA patients, mainly confined to monocytes, chondrocytes and synoviocytes, suggesting a possible role for vitamin D in RA pathogenesis. Serum 25(OH)D levels in the sera of 60 RA patients were measured and a strong inverse correlation to serum TNF levels was found, further indicating an anti-inflammatory role of Vitamin D (data not shown). Still, these clinical findings could merely be an association rather than a causal relationship.

Vitamin D, however, has direct effects on immune cells and could, therefore, play a role in the pathogenesis of RA. Thus, 1,25(OH)2D3 inhibits T cell proliferation, differentiation of T cells towards a Th1 response and secretion of proinflammatory cytokines such as IL-2 and interferon γ. Also, inhibition of Th17 polarisation by active vitamin D metabolites has recently been described. In line with this, treatment of T cell-dependent arthritis models such as collagen-induced arthritis with 1,25(OH)2D3 abrogates disease. Vitamin D can also inhibit autoantibody production and secretion by B cells and has profound effects on monocytes. 1,25(OH)2D3 effectively blocks the differentiation of monocytes to dendritic cells and IL-12 secretion, impairs antigen presentation and enhances phagocytosis. Pretreatment of LPS-stimulated monocytes with 1,25(OH)2D3 dramatically reduces production of proinflammatory cytokines.

Interestingly, macrophages themselves possess 1,25(OH)2D3 hydroxylase, which is the decisive enzyme responsible for the production of the active metabolite of vitamin D, 1,25(OH)2D3. Thus, immunomodulatory active vitamin D metabolites could principally be generated in the inflamed synovium itself, if sufficient amounts of 25(OH)D3 are present in the circulation and could regulate disease activity.

Here, it is demonstrated that severity of arthritis is worse when VDR is lacking. Which of the aforementioned mechanisms of action of vitamin D could be responsible for these findings? When the synovial infiltrate was analysed for the presence of immune cells, no overt difference in the frequency of T cells and B cells and neutrophil granulocytes was found. However, a dramatic increase of synovial monocytes in the synovium of VDR−/− hTNFtg mice was found. Synovial monocytes are key players in the pathogenesis of inflammatory cartilage and bone destruction: They are able to produce significant amounts of proinflammatory cytokines such as TNF, IL-1 and IL-6, thereby driving cartilage proteoglycan loss via upregulation of matrix metalloproteinases and aggrecanases in chondrocytes. In addition, these cells serve as precursors for osteoclasts.

Indeed, monocytes from mice lacking VDR display an altered phenotype ex vivo and produce more TNF and IL-1, indicating a proinflammatory phenotype. This finding was confirmed in the serum of VDR−/− mice and increased TNF and IL-1 levels were found also. Furthermore, VDR−/− monocytes are hyper-responsive to TNF stimulation, showing a modest but significantly prolonged activation of proinflammatory MAPK signalling. Furthermore, VDR−/− monocytes stimulated with TNF produce higher amounts of proinflammatory cytokines such as TNF and IL-1. Also, VDR−/− monocytes showed an increased responsiveness in ex vivo osteoclastogenesis assays showing an increased resorptive activity, whereas differentiation into the osteoclast lineage was not dramatically enhanced. This was accompanied by an altered transcriptional profile with enhanced mRNA expression of cathepsin K, TRAP and c-fos. Interestingly, the effects of VDR deficiency were more pronounced locally in the inflamed joints than in systemic bone.

In summary, evidence has been provided for a central role of a functional VDR in chronic arthritis. VDR regulates synovial inflammation, cartilage damage and bone erosion in arthritis by modulating monocyte function. Monocytes incapable of reacting to vitamin D display a proinflammatory phenotype and are effective bone resorbers once differentiated into osteoclasts.

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Competing interests None.

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REFERENCES


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