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Decreased sensitivity to 1,25-dihydroxyvitamin D3 in T cells from the rheumatoid joint

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ABSTRACT

1,25-dihydroxyvitaminD$_3$ (1,25(OH)$_2$D$_3$), has potent anti-inflammatory effects, including suppression of IL-17 + and IFNγ+ T cells implicated in rheumatoid arthritis (RA), but efficacy at the site of active disease is unclear. To investigate this, T cells from synovial fluid (SF) and paired blood of patients with active RA were studied. 1,25(OH)$_2$D$_3$ had significantly less suppressive effect on Th17 cells (IL-17+IFNγ-) and Th17.1 cells (IL-17+IFNγ+) from SF compared to those from blood, and had no effect on SF CD4$^+$ or CD8$^+$ IFNγ+ T cell frequencies. Memory T cells (CD45RO+) predominate in SF, and 1,25(OH)$_2$D$_3$ had less effect on memory T cells relative to naïve (CD45RA+) T cells. RT-PCR and flow cytometry showed that this was not due to decreased expression of the vitamin D receptor or its transcription partners in memory T cells. Further studies using stimulated CD4$^+$ T cells sorted according to IL-17 and IFNγ expression confirmed the ability of 1,25(OH)$_2$D$_3$ to suppress pre-existing cytokines. However, 1,25(OH)$_2$D$_3$ was most effective at suppressing *de novo* IL-17 and IFNγ induction. Correspondingly, T cell responses to 1,25(OH)$_2$D$_3$ correlated directly with capacity for phenotype change, which was lower in cells from SF compared to blood. These findings indicate that anti-inflammatory effects of 1,25(OH)$_2$D$_3$ in active RA are impaired because of reduced effects on phenotype-committed, inflammatory memory T cells that are enriched in SF. Restoration of 1,25(OH)$_2$D$_3$ responses in memory T cells may provide a new strategy for treatment of inflammatory diseases such as RA.

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1. Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) promotes anti-inflammatory responses in a diverse array of cell types, supporting the potential use of vitamin D in the prevention and/or treatment of inflammatory disorders [1,2]. In previous studies we have shown that anti-inflammatory actions of vitamin D may occur indirectly through localized synthesis of 1,25(OH)$_2$D$_3$, and reduced expression of major histocompatibility and co-stimulatory molecules by antigen-presenting dendritic cells (DCs), monocytes and macrophages [3–6]. However, 1,25(OH)$_2$D$_3$ can also act directly on T-lymphocytes (T cells), inhibiting their proliferation [7], especially under conditions of weak co-stimulation [8], and suppressing their production of pro-inflammatory cytokines such as IFNγ, IL-17 and IL-21 [9–13], whilst promoting their expression of regulatory markers including CTLA-4, FoxP3, and IL-10 [13] even in the presence of pro-inflammatory cytokines [14]. Crucially almost all of these observations have stemmed from experiments using T cells from the blood of healthy donors and much less is known about the effects of 1,25(OH)$_2$D$_3$ in established inflammatory disease, especially its effects upon T cells from inflamed compartments such as the joint of a patient with rheumatoid arthritis (RA).

Epidemiology suggests that many autoimmune diseases and

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common chronic inflammatory diseases such as RA are associated with vitamin D-deficiency [15–17]. Vitamin D metabolites such as $1,25(OH)_2D_3$ may therefore provide an alternative strategy for the prevention and/or treatment of RA [18], possibly as an adjunct to existing RA therapies [8]. Previous studies have highlighted aberrant metabolism of vitamin D in disease-affected synovial fluid (SF) from RA patients [19], but the impact of $1,25(OH)_2D_3$ on T cells from the site of inflammation, the inflamed joint, has yet to be studied.

IFNγ+ Th1 cells and IL-17 + Th17 cells are regarded as important mediators of chronic synovial inflammation. Increased levels of their hallmark and differentiating cytokines are found in the serum and synovial fluid of patients relative to controls [20], and elevated frequencies of both have been detected in the blood and joints of RA patients [21]. IFNγ produced by Th1 cells promotes APC maturation whilst IL-17 has pleiotropic effects, driving fibroblast-like synoviocytes (FLS) to release pro-inflammatory cytokines and chemokines [22], that further amplify the inflammation by facilitating the recruitment and retention of immune cells, including CCR5+CXCR3+ Th1 cells [23,24] and CCR6+ Th17 [25,26] cells into the joint. IL-17 from Th17 cells also promotes cartilage and bone resorption by stimulating MMP release from FLS [22] and the induction of RANKL on FLS and osteoblasts leading to activation of RANK + osteoclasts [27]. The ability of $1,25(OH)_2D_3$ to affect T cell function is important in RA, as T cells accumulate in the RA synovium and genetic risk factors for RA are largely related to T cell activation [20–25]. The aim of the current study was to investigate further the potential use of vitamin D as a therapy for RA, by determining whether anti-inflammatory effects of $1,25(OH)_2D_3$ are achievable on T cells from the site of inflammation.

2. Materials and methods

Patients were recruited for the study if they fulfilled 1987 ACR criteria for RA [28]. All patients and age and gender-matched healthy controls gave full written consent. Patient demographics are summarized in Table 1. Ethical approval for the work was granted by Solihull Research Ethics Committee (REC reference number 07/Q2706/2) and the University of Birmingham Ethics Committee (ERN_14-0446). For naïve and memory T cell comparison studies, as well for as cytokine-expression cell capture experiments, cells were isolated from fully anonymysed leukocyte cones obtained from the National Blood Service, Birmingham, UK.

2.1. Cell isolation and culture

Synovial Fluid (SF) was extracted by ultrasound guidance as described previously [29] or by palpation guidance. Prior to SF Mononuclear Cell (SFMC) isolation, SF was treated with hyaluronidase (10U/ml) for 30 min at 37 °C. PBMCs and SFMCs were isolated by the Ficoll-Paque PLUS method of density gradient centrifugation (GE Healthcare). SF was layered on Ficoll-Paque PLUS undiluted, fresh blood was diluted 1:1 with PBS and leukocyte cones were diluted 1:4 with PBS before layering. Isolated SFMCs and PBMCs were cultured at 37 °C, 5% CO_2 in RPMI 1640 medium and supplemented with 1% penicillin and streptomycin, 2 mM L-glutamine, and 5% self-serum or SF that was pre-filtered through a 22 μm filter. For *ex vivo* cytokine expression analysis, cells were allowed to rest overnight at 1×10^6 cells/ml without stimulation before being stimulated for 6–7 h with phorbol myristate acetate (PMA) (50 ng/ml) and ionomycin (1 μM). Brefeldin A (10 μg/ml) was added during the last 4–5 h. For stimulation mononuclear cells were treated with anti-CD3 (0.5 μg/ml, clone OKT3) at 2.5×10^5 cells/ml. $1,25(OH)_2D_3$ was added to cultures at 100 nM and ethanol used as a vehicle control at 0.1%. At seven days, cells were restimulated with PMA/ionomycin in the presence of brefeldin A for cytokine expression analysis by flow cytometry.

For experiments using isolated CD45RA + $CD4^+$ naïve T cells, CD45RO + $CD4^+$ memory T cells and $CD14^+$ monocytes, cells were enriched by negative selection using cell separation reagents (StemCell Technologies and Biolegend). For 24 h post-stimulation analysis of gene expression, T cells were stimulated with anti-CD3/CD28 dynabeads (Life Technologies) at a ratio of 1 bead: 2 T cells in medium supplemented with 5% human AB serum (TCS Biosciences, Buckingham UK). For longer-term stimulations a ratio of 1 bead: 4 T cells was used. Where T cells were stimulated with monocytes, a ratio of 1 monocyte: 4 T cells and OKT3 0.5 μg/ml was used.

2.2. Isolation and culture of Th17, Th17.1 and Th1 cells

Expanded populations of Th17, Th17.1 and Th1 cells were generated by stimulating magnetically purified monocytes and $CD4^+$ T cells at 1:5 ratio with 0.5 μg/ml antiCD3 for seven days. IL-17-PE and IFNγ-APC cytokine secretion detection kits (Miltenyi Biotech) were used to label live Th17, Th17.1 and Th1 cells. In brief, cultures were re-stimulated with Phorbol 12,13-dibutyrate (PDBu) (10 ng/ml) and ionomycin (1 nM) for 2 h before labeling with IL-17 and IFNγ catch reagents on ice at 10×10^6 cells/80 μl MACS buffer for 5 mins. Cells were transferred to pre-warmed RPMI and incubated for 40 mins at 37 °C at 4×10^5 cells/ml under continual rotation. Cells were then diluted 1:1 with ice-cold MACS buffer and chilled on ice for 10 min before centrifuging and labelling with IL-

Table 1
Patient Demographics: Disease activity score 28 based upon C Reactive Protein (CRP) (DAS28 (CRP)); Erythrocyte sedimentation rate (ESR); Rheumatoid Factor (RF); anti-cyclic citrullinated peptide antibody (anti-CCP); conventional synthetic and biological disease modifying anti-rheumatic drugs (csDMARDs/bDMARDs).

Patient	Gender	Age (yrs)	Disease duration (yrs)	DAS28 (CRP)	CRP (mg/dl)	ESR (mm/hr)	RF (±)	Anti-CCP (±)	cs/DMARDs/bDMARDs
1	f	65	4	2.89	<5	37	+	−	methotrexate, rituximab
2	m	85	4	5.18	138	NA	−	+	Sulfasalazine, prednisolone
3	m	65	3	4.88	81	72	+	+	nil
4	f	65	4	4.84	10	41	+	−	methotrexate, rituximab
5	m	42	6	5.30	95	27	−	NA	sulfasalazine, methotrexate, prednisolone, tocilizumab
6	f	40	<1	3.18	14	26	−	−	methotrexate
7	m	47	2	5.48	34	NA	+	NA	methotrexate
8	m	42	6	4.19	23	7	+	+	methotrexate, prednisolone, rituximab
9	f	40	<1	4.73	59	57	−	−	sulfasalazine
10	m	42	6	4.82	14	2	−	NA	sulfasalazine, methotrexate, prednisolone, tocilizumab
11	m	45	4	8.21	166	130	−	−	nil
12	m	59	3	6.21	29	26	+	+	methotrexate
13	f	55	5	5.08	19	42	+	−	methotrexate, sulfasalazine
14	f	35	5	5.83	20	17	+	+	methotrexate
15	f	68	27	5.10	86	46	+	+	methotrexate, etanercept

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