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Immune regulatory effects of high dose vitamin D_3 supplementation in a randomized controlled trial in relapsing remitting multiple sclerosis patients receiving IFNβ; the SOLARIUM study

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ABSTRACT

Multiple sclerosis (MS) is characterized by a disturbed immune homeostasis and low serum vitamin D levels are associated with an increased disease activity. While vitamin D has been hypothesized to promote the maintenance of immune homeostasis, vitamin D supplementation could be of benefit to patients with MS. The SOLAR study investigated the effects of high dose vitamin D_3 supplementation on clinical outcomes in a randomized controlled trial. Here we present the immune regulatory effects, investigated in the SOLARIUM sub-study.

Thirty Dutch relapsing remitting (RR) MS patients treated with IFNβ-1a received high dose vitamin D_3 supplementation and 23 patients received placebo during a period of 48 weeks. Lymphocytes were phenotypically characterized by flow cytometry and *in vitro* cytokine secretion was assessed in the presence or absence of $1,25(OH)_2D_3$ using Luminex technology. Changes in immune regulatory parameters were determined within subjects as well as between treatment groups.

The proportion of cells in the immune regulatory cell compartment (nTreg, iTreg and Breg) was not altered upon high dose vitamin D_3 supplementation. Proportions of T helper subsets were not affected by vitamin D_3, except for the proportion of $IL4^+$ Th cells, which decreased in the placebo but not in the vitamin D_3 group. T cell cytokine secretion increased, most pronounced for IL5 and latency activated protein of TGFβ, in the placebo group but not in the vitamin D_3 group. Lymphocytes remained equally reactive to *in vitro* $1,25(OH)_2D_3$.

In conclusion, high dose vitamin D_3 supplementation did not result in a relative increase in lymphocytes with a regulatory phenotype. However, this study supports the hypothesis that vitamin D contributes to the maintenance of immune homeostasis by preventing further disturbance of the T cell compartment early in the disease course of MS.

1. Introduction

Main challenges in multiple sclerosis (MS) research are to further unravel the underlying pathogenic mechanisms of this still poorly-understood disease and to make treatments more effective while minimizing the associated serious side effects. Genetic (International Multiple Sclerosis Genetics et al., 2011), epidemiological (Mowry et al., 2010; Simpson et al., 2010), clinical (Burton et al., 2010; Runia et al., 2012; Soilu-Hanninen et al., 2005; Soilu-Hanninen et al., 2008) and immunological (Correale et al., 2009; Smolders et al., 2008) data suggest a beneficial role of vitamin D in MS risk, and disease activity. Since most MS patients have low vitamin D levels, vitamin D supplementation may be an effective and safe (add-on) treatment option in MS. The SOLAR study assessed the efficacy of high dose vitamin D_3 as add-on therapy

Abbreviations: BFA, brefeldin A; BMI, body mass index; Breg, regulatory B cell; CNS, central nervous system; EDSS, expanded disability status scale; GMCSF, granulocyte macrophage colony-stimulating factor; HC, healthy control; IFNβ-1a, interferon beta-1a; IL, interleukin; iTreg, inducible Treg; LAP, latency activated protein of TGFβ; MS, multiple sclerosis; nTreg, natural Treg; PBMC, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; RCT, randomized controlled trial; RR, relapsing remitting; SOLAR, supplementation of VigantoL®Oil *versus* placebo as add-on in patients with relapsing remitting multiple sclerosis receiving Rebif® treatment; SOLARIUM, SOLAR Immune Modulating effects; Th cell, T Helper; TNF, tumor necrosis factor; Treg, regulatory T cell; WBC, white blood cell count.

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to interferon beta-1a (IFNβ-1a) on clinical outcome, *i.e.* disease activity free status, in 232 patients with early relapsing remitting MS (RRMS) (NCT01285401). (Smolders et al., 2011) In this paper we present the results on the immune modulating effects investigated in the Dutch substudy SOLARIUM (SOLAR **I**mm**U**ne **M**odulating effects).

MS is a devastating inflammatory disease of the central nervous system (CNS). In the most prevalent phenotypic entity, RRMS, the disease course is characterized by subacute neurological deterioration (relapses), followed by periods of recovery (remissions). In particular, this subtype is considered to be of autoimmune origin, but neither the target nor the exact aetiology has been unravelled to date. (Bar-Or, 2008; Hemmer et al., 2002; Smolders and Damoiseaux, 2011) Although classically regarded as a T helper 1 (Th1; mainly IFNγ producing Th cells) mediated disease (Gutcher and Becher, 2007), more recently, Th17 cells, producing interleukin 17 (IL17) (Gutcher and Becher, 2007; Park et al., 2005), and GMCSF producing Th cells (Codarri et al., 2011; Noster et al., 2014) are held responsible for the encephalitogenic reaction. Outside the CNS, in the circulating immune system, MS is characterized by a disturbed lymphocyte homeostasis, with a diminished function of regulatory T cells (Treg) (Venken et al., 2010; Viglietta et al., 2004), a decrease in the number of regulatory B cells (Breg) (Knippenberg et al., 2011; Viglietta et al., 2004) and a disrupted equilibrium between pro- and anti-inflammatory immune cells resulting in disturbed cytokine networks (Amedei et al., 2012; Opdenakker and Van Damme, 1994).

The function of vitamin D as an immune regulator may explain its proposed disease modulating effects (Muris et al., 2013), since immune cells metabolize and respond to vitamin D. (Baeke et al., 2010; Peelen et al., 2011b; Smolders and Damoiseaux, 2011) *In vivo* association studies suggest that vitamin D can promote immune homeostasis in autoimmune diseases by promoting the number and/or suppressive function of the Treg compartment (Muris et al., 2013; Smolders et al., 2009), inhibiting T cell proliferation (Muris et al., 2013) and pro-inflammatory cytokine production (including IFNγ), promoting anti-inflammatory cytokines (including IL4 and IL10) (Smolders et al., 2009), and shifting the balance between T cell subsets (less $IL17^+$ Th cells) (Peelen et al., 2011a; Smolders et al., 2009), while lower serum vitamin D levels were not correlated with Breg proportion (Knippenberg et al., 2011). GMCSF production by Th cells was differentially inhibited by vitamin D in an *in vitro* study in MS patients and healthy controls (HC). (Peelen et al., 2015) In HC, *in vivo* high dose vitamin D_3 supplementation studies (mostly uncontrolled) have shown an increased number of Tregs without any effect on Treg function or on other immune cells (Muris et al., 2013; Prietl et al., 2014), and a reduced $IL17^+$ Th cell frequency with an increased production of the regulatory cytokine IL10 by non-T cells (Allen et al., 2012). In patients with MS, vitamin D_3 supplementation resulted in a relative increase of $IFN\gamma^+$ to $IL4^+$ Th cells and an increase in inducible Treg (iTreg) as proportion of total Th cells, whereas the number and function of natural Treg (nTreg) was not affected. (Smolders et al., 2010) In general, data on vitamin D and the immune system are not consistent and studies were often underpowered, but they do suggest that vitamin D promotes anti-inflammatory/regulatory immune parameters and reduces pro-inflammatory immune parameters.

In this observational study of a randomized placebo-controlled trial (RCT) in patients with RRMS, we assess the effects of vitamin D_3 supplementation as add-on therapy to IFNβ treatment on the circulating lymphocyte compartment. In line with our hypothesis, *i.e.* vitamin D being an important player in the immune regulation and Th cells being the most relevant cells of the immunological cascade in the immune cell compartment of patients with MS (International Multiple Sclerosis Genetics et al., 2011), we first report the vitamin D_3 effects on regulatory lymphocytes, followed by the effects on cytokine production by Th cells and peripheral blood mononuclear cells (PBMC).

2. Methods

2.1. Patients

In- and exclusion criteria and the study design of the SOLAR study are described elsewhere. (Smolders et al., 2011) In short, study participants, aged between 18 and 55 years, had a diagnosis of RRMS (according to McDonald criteria 2005) confirmed by typical MS findings on MRI. They had a first clinical event within 5 years prior to screening and active disease in the prior 18 months, but no relapse 30 days before inclusion. Patients were excluded if they took more than 1000 IU (25 μg) of vitamin D supplements. All patients received IFNβ-1a (Rebif®, Merck Serono S.A., Darmstadt, Germany), 44 μg three times weekly s.c. for a minimum of 90 days and not longer than 18 months. After being randomized, patients received either IFNβ-1a and placebo or IFNβ-1a and vitamin D_3 (cholecalciferol, Vigantol® Oil, Merck KGaA, Darmstadt, Germany) 7000 IU daily for 4 weeks, followed by 14,000 IU daily up to week 48.

Patients for the SOLARIUM substudy were recruited in four of the five participating centers in the Netherlands (Zuyderland Medical Center (Sittard), Maasstad Hospital (Rotterdam), Groene Hart Hospital (Gouda) and St. Antonius Hospital (Nieuwegein)), without additional inclusion or exclusion criteria. They were eligible when they agreed with participation in this sub-study. The study protocol was approved by the Medical Ethical Committee Zuyderland Zuyd (11-T-03, Heerlen, the Netherlands). Researchers performing the immunological analyses were blinded for treatment and patient details.

2.2. PBMC isolation

Peripheral blood samples from study subjects were collected at baseline (wk0) and after 48 weeks (wk48) of treatment (placebo or vitamin D_3). Blood was collected in a 10 mL sodium heparin blood sampling tube (BD Bioscience, Breda, the Netherlands) and kept at room temperature during transportation to Maastricht University Medical Center, the Netherlands. PBMC were isolated within 24 h using a Ficoll-density gradient (Histopaque; Sigma Aldrich, Zwijndrecht, the Netherlands) and centrifugation, performed as described before. (Smolders et al., 2009).

2.3. Staining for detection of T and B cell subtypes

Directly after isolation, PBMC were stained with a cocktail of monoclonal antibodies to identify nTreg according to the following definitions $CD25^+CD127^-$ $CD4^+$ T cells ($CD25^+CD127^-$ nTreg), $CD25^+FoxP3^+$ $CD4^+$ T cells ($CD25^+FoxP3^+$ nTreg) and $CD25^+CD127^-FoxP3^+$ $CD4^+$ T cells ($CD25^+CD127^-FoxP3^+$ nTreg), as described before (Peelen et al., 2011a; Smolders et al., 2010) (Fig. 1A). Within the $CD25^+CD127^-$ nTreg, subsets were defined based on expression of CD45RA (naïve/memory nTreg) and CD39 ($CD39^+$ nTreg, being associated with the suppression of $IL17^+$ Th cells (Fletcher et al., 2009)).

Cytokine expression of IL4, IFNγ, IL17, IL22, GMCSF and TNFα by $CD3^+CD8^-$ T lymphocytes, which are further referred to as Th cells (Smolders et al., 2010), was assessed after a 5 h *in vitro* activation with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, Sigma Aldrich) and ionomycin (1 μg/mL, Sigma Aldrich) in the presence of monensin (1.25 μg/mL, BD Biosciences). Expression of IL10, which was used to define $IL10^+$ Th cells/iTreg, was assessed after similar activation, but without the addition of monensin. (Muris et al., 2012) Cells were intracellularly stained after fixation and permeabilization (Cytofix/Cytoperm, BD Biosciences). Antibodies used, are described before (Peelen et al., 2015; Smolders et al., 2010), except for IL22 (anti-IL22-efluor-660 (eBioscience, Vienna, Austria)) and TNFα (anti-TNFa-PE-Cy7 (eBioscience)). Gating strategies are shown in Fig. 1B.

To identify Breg, PBMC were stimulated with CpG oligodeoxynucleotide 2006 (0.1 μM, Invivogen, Toulouse, France) for