Correlation of different cellular assays to analyze T cell-related cytokine profiles in vitamin D-3-supplemented patients with multiple sclerosis

Citation for published version (APA):

Document status and date:
Published: 01/01/2019

DOI:
10.1016/j.molimm.2018.12.001

Document Version:
Publisher's PDF, also known as Version of record

Document license:
Taverne

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Download date: 15 Sep. 2023
Correlation of different cellular assays to analyze T cell-related cytokine profiles in vitamin D₃-supplemented patients with multiple sclerosis

Linda Rolf, Joost Smolders, Jody van den Ouweland, Raymond Hupperts, Jan Damoiseaux

Abstract

Different laboratory approaches have been exploited to analyze an effect of vitamin D₃ supplements on T cell cytokine profiles in multiple sclerosis, with poorly reproducible results. We assessed the correlation between intra-cellular flow cytometry analysis of CD4 T cell-enriched CD3⁺CD8⁻ lymphocytes after PMA/ionomycin stimulation directly ex-vivo or after 72 h pre-stimulation with anti-CD3, and cytokine levels excreted in culture supernatants. Pre-stimulation with anti-CD3 resulted in higher proportions of cells positive for IFN-γ, IL-17A, IL-4, IL-10 and GM-CSF (all P < 0.001), but not TNF-α. Positive correlation between approaches was highly variable, but most eminent for IFN-γ and IL-4 (R = 0.608-0.612 and R = 0.677-0.777, resp., all P < 0.001). No effect of 16-weeks vitamin D₃ supplements on any outcome was found except for a decreased TNF-α concentration in culture supernatants. Choice of immune-assay is, apparently, a relevant confounder for the reproducibility of individual studies.

1. Introduction

Cytokines are major effector molecules of CD4⁺ T cells, which can drive or suppress inflammatory conditions dependent on concentration and context. Concentrations of cytokines present in plasma or culture supernatants can be quantified by enzyme-linked immune-sorbent assays (ELISA) or addressable laser bead immune assays (ALBIA), while cellular proportions producing the respective cytokines can be quantified by intra-cellular flow cytometry (iFACS) or Elispot assays (Carter and Swain, 1997). These approaches reveal different characteristics of cytokines and can be used in complementary ways (Beebe and Orr, 2017). However, most often researchers chose the assay which fits their laboratory workflow best and this may introduce misinterpretation when comparing results across different methods.

In multiple sclerosis (MS) research, T cell-related cytokines have been assessed as biomarkers of inflammatory disease activity (Becher et al., 2017). Vitamin D₃ (cholecalciferol) is the precursor of a potent immunomodulatory molecule (1,25(OH)₂D₃) in vitro and in animal models of MS, which gained interest for this component in MS research the past decades (Smolders et al., 2016a). A poor circulating vitamin D status has been associated with a higher risk for developing MS and with an increased risk of higher disease activity in relapsing remitting MS (RRMS) (Ascherio et al., 2014; Munger et al., 2006). Since effects of vitamin D on CD4⁺ T cell responses have been suggested as likely underlying mechanism (Smolders et al., 2008), several groups studied whether vitamin D₃ supplements associate with T cell related cytokine profiles in patients with MS. However, results reported are heterogeneous and poorly reproducible. This heterogeneity can be explained by small power of individual studies, but also different patients characteristics and different MS disease modifying treatments in patients. As laboratory approaches, investigators measured circulating cytokine levels ((Åivo et al., 2015; Mahon et al., 2003; Røsjø et al., 2015; Toghianifar et al., 2015), T cell cytokine production with iFACS after short stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Muris et al., 2016; Smolders et al., 2010), T cell cytokine production with iFACS after pre-stimulation with anti-CD3/anti-CD28/ specific stimuli followed by PMA/ ionomycin (Mrdal et al., 2017; O’Conne et al., 2017; Sotirchos et al., 2016), or cytokine production in supernatants by peripheral blood mononuclear cells (PBMC) stimulated with anti-CD3 with/without anti-CD28 or other stimuli (Mrdal et al., 2017; O’Conne et al., 2017; Sotirchos et al., 2016).
2017; Muris et al., 2016; O’Connell et al., 2017). Therefore, as different laboratory approaches have been used in these studies, they also may contribute to the heterogeneity in outcomes.

The objective of our study is to determine to what extent different laboratory approaches to quantify cytokines contribute to heterogeneity in outcomes. We explored the correlation between iFACS performed directly ex-vivo or after 72 h pre-stimulation with anti-CD3, and cytokine levels excreted in supernatants of PBMC culture with anti-CD3 for several frequently studied T cell-related cytokines. Furthermore, we explored an effect of vitamin D3 supplements on cytokine-producing T cells and cytokine levels in culture supernatants.

2. Materials and methods

2.1. Study design and procedure

This study reports a single-center sub-study of a multi-center, randomized, double-blind, placebo-controlled study, which was conducted between October 2014 and November 2016 (ClinicalTrials.gov NCT02096133, EudraCT 2014-000728-97) (Rolf et al., 2018b). This study was approved by the local ethical research committee ‘METC-Z’, and written informed consent was obtained from all study participants. In short, participants were randomly allocated in a 1:1 ratio to the vitamin D3 or placebo group. Participants in the vitamin D3 group received 4000 IU/day vitamin D3 drops (Vigantol Oil, Merck, Darmstadt, Germany), patients in the placebo group received matched placebo. The dose of 4000 IU/day was chosen because this is considered the tolerable upper intake level of vitamin D according to the Dietary Reference Intakes developed by the Food and Nutrition Board and the Institute of Medicine. Moreover, we have shown that this dose induced 25(OH)D levels exceeding 100 nmol/L in most patients (Rolf et al., 2018b), which has been advocated by a recent ECTRIMS workshop report to be beneficial for MS related outcomes (Amato et al., 2017). Randomization was performed by the pharmacy. Study personnel, participants and care providers were blinded to the study interventions. Study visits for serum sampling were performed at baseline and at approximately 16 weeks.

2.2. Participants

All participants were female patients with relapsing-remitting MS (RRMS), according to the McDonald criteria (Polman et al., 2011). Other inclusion criteria were age > 18, and treatment with injectable or oral disease modifying drugs (DMD; interferon-beta, glatiramer acetate, dimethyl fumarate, teriflunomide or fingolimod) or no DMD treatment, and no DMD-changes 3 months prior to baseline visit. Also, participants had to be premenopausal with a perceptible menstrual cycle. Oral contraception was allowed. Exclusion criteria were all contraindications for vitamin D3 supplementation, relapse within 6 weeks prior to study initiation, use of systemic glucocorticoids within 8 weeks prior to study initiation, use of vitamin D supplements > 1000 IU/day, current (treatment for) major depression, pregnancy and glucocorticoid treatment for relapses during the trial.

2.3. Cell isolation and culture

Peripheral blood samples were collected in sodium heparin at baseline and at approximately 16 weeks (BD Biosciences, Breda, the Netherlands). PBMC were directly isolated using a standard Ficoll-density gradient (Histopaque; Sigma Aldrich, Zwijndrecht, the Netherlands) and centrifugation. PBMC were frozen in liquid nitrogen for batch-wise analysis at the end of the study. Paired samples were analyzed in the same batch. After thawing cells were kept in culture medium (RPMI-1640 medium supplemented with 10% fetal calf serum, 2% penicillin-streptomycin, 1% sodium pyruvate, and 1% non-essential amino acids). The same culture medium was used for ex-vivo as well as 72 h culture conditions. For the direct ex-vivo analysis, we activated cells 5 h in vitro with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, Sigma Aldrich) and ionomycin (1 μg/mL, Sigma Aldrich). Monensin (1.25 μg/mL, BD Biosciences) was added at the start of the culture. For the prolonged stimulation, cells were cultured for 3 days in a U-bottom 96-wells plate and stimulated with soluble anti-CD3 (2.0 pg/mL, WT32 IgG2a monoclonal antibody, kindly provided by dr. W Tax, Nijmegen, The Netherlands (Tax et al., 1983)). This culture was followed by a re-stimulation under the same conditions as ex-vivo activation. All cell cultures were performed in a 37°C incubator with a humidified atmosphere containing 95% air and 5% CO2. Cells were intracellularly stained after fixation and permeabilization (Cytofix/ Cytobind, BD Biosciences) with anti-IFN-γ-FITC, anti-CD3-horizon 450, anti-CD8-APC-H7 (all BD Biosciences, Breda, the Netherlands), anti-GM-CSF-PE, anti-IL-10-APC and anti-IL-17A-PerCP-Cy5.5 (all Biolegend, Uithoorn, the Netherlands) and anti-TNFα-PECy7 (ebiScience, Vienna, Austria). Flow cytometry was performed on a FACS Canto II flow cytometer (BD Biosciences) and data were analyzed with FACS Diva software version 6.1.2. (BD Biosciences). Cytokine expression was analyzed in CD3+CD8− T cells, because stimulation with PMA and ionomycin interferes with CD4 expression. Although CD3+CD8− T cells will also contain TCRγδ T cells, the cells are enriched for CD4+ T cells (Smolders et al., 2010). In the supernatants of the 3 day prolonged stimulation cultures, concentrations of IL-4, IL-10, IL-17A, IFN-γ, TNF-α and GM-CSF were measured in duplicates with an in-house developed and validated multiplex immunoassay (ISO9001:2008 certified, Laboratory of Translational Immunology, University Medical Center Utrecht, the Netherlands) based on Luminex technology (xMAP, Luminex, Austin, TX) (de Jager et al., 2005; Schipper et al., 2008). Data were analyzed using Bio-Plex Manager software, version 6.1.1 (Biorad laboratories, Hercules, CA). Plasma samples were stored at −80 °C in which 25(OH) D levels were measured at the end of the study using LC-MS/MS as described previously (van den Ouweland et al., 2014).

2.4. Statistical analyses

Descriptive statistics of continuous data are provided as medians with interquartile ranges (IQR, 25th – 75th percentile), categorical data are presented as n (%). Comparisons of continuous variables between two unpaired groups were performed using Mann-Whitney U tests and of multiple unpaired groups with the Friedman test. Within group comparisons were performed using Wilcoxon signed-rank tests for paired data. Correlations were explored by calculating Spearman correlation coefficients. To explore an effect of vitamin D3 supplements on cytokine outcomes, ratios of baseline (T = 0) and end-of-study (T = 1) proportions or supernatant concentrations were compared between treatment arms (Mann-Whitney U test). Figures were constructed with GraphPad Prism version 7 (GraphPad Software Inc., La Jolla CA), statistical analyses were conducted with SPSS version 23.0 (IBM Corp., Armonk, NY). Since 6 different cytokines were assessed, a P-value ≤ 0.008 (P ≤ α/n = 0.05/6) was considered significant.

3. Results

3.1. Polyclonal stimulation with anti-CD3 affects proportions of CD4+ T cell-enriched CD3+ CD8− lymphocytes positive for individual cytokines to a different extent when compared to directly ex-vivo

Of N = 27 RRMS patients, two blood samples were acquired with an interval of approximately 16 weeks. First, we analyzed the effect of pre-stimulation with anti-CD3 on the proportions of CD3+CD8− T cells positive for IFN-γ, IL-4, IL-10, IL-17A, GM-CSF and TNF-α as measured with iFACS (Fig. 1A). For IFN-γ, IL-4, IL-10, IL-17A and GM-CSF, pre-stimulation with anti-CD3 resulted in increased proportions of CD3+CD8− T cells expressing the respective cytokines (Fig. 1B–E & G). Proportions CD3+CD8− T cells positive for TNF-α were not affected by
stimulation with anti-CD3 (Fig. 1F). For all cytokines tested, the proportional differences between analysis-protocol with and without pre-stimulation with anti-CD3, differed from each other (Fig. 1H, P < 0.001). There was an average 6.9-fold increase in cells positive for IL-17A, with only an average 2.1 to 3.4-fold increase of IL-4 and IFN-γ, respectively. The proportions of cells positive for IFN-γ, IL-4, IL-17A, GM-CSF and TNF-α as measured directly ex-vivo or after polyclonal stimulation showed a moderate to strong positive correlation (Fig. 2A,B,D–F), which was most pronounced for IL-4 and TNF-α. There was no significant correlation between proportions positive for IL-10 (Fig. 2C).

3.2. Proportions of cells positive for individual cytokines show a variable correlation with concentrations in supernatants after polyclonal stimulation with anti-CD3

In the supernatants of the cultures pre-stimulated with anti-CD3, all cytokines could be measured with Luminex technology. There was a strong positive correlation between the proportions of IFN-γ and IL-4 positive CD3⁺CD8⁻ T cells and supernatant cytokine concentrations (Fig. 3A–B), and a moderate positive correlation for GM-CSF (Fig. 3F). The proportions of CD3⁺CD8⁻ T cells positive for IL-10, IL-17A and TNF-α did not correlate with the supernatant levels of these cytokines (Fig. 3C–E).

3.3. Exploratory analyses do not reveal effects of vitamin D₃ supplements on cytokine outcomes

We explored whether evolution of cytokine outcomes over 16 weeks differed between patients supplemented with vitamin D₃ (N = 12) or placebo-arm (N = 15) (Table 1). In the placebo group, the median 25(OH)D level at baseline was 78 (71–95) nmol/L, and 81 (61–90) nmol/L at T1. In the vitamin D₃ arm, this was 76 (71–132) nmol/L and 135 (123–144) nmol/L, respectively (difference between groups P = 0.005). In the vitamin D₃ group, 11/12 had 25(OH)D levels exceeding 100 nmol/L at last visit, compared to 0/15 in the placebo-group. For none of the outcomes reported on IFN-γ, IL-4, IL-10, IL-17A, TNF-α, GM-CSF, there was a significant difference in absolute levels/proportions or baseline vs 16-weeks ratios between vitamin D₃ and placebo groups (Tables 2, S1-3). This is except for the levels of TNF-α observed in culture supernatants (Table S3), which were lower after 16 weeks follow-up in the vitamin D₃ (1201.4 (782.6–2564.9) to 983 (540.1–1106.0) pg/mL, P = 0.008) but not the placebo-group (1147.8 (816.4–1625.0) to 1329.6 (850.6–2076.7) pg/mL, P = 0.433).

4. Discussion

In this study, we compared three approaches to analyze cytokine production by CD4 T cell-enriched CD3⁺CD8⁻ lymphocytes in the
context of vitamin D supplementation in patients with MS. We observed that polyclonal stimulation of PBMC with anti-CD3 results in increased proportions of cells expressing several cytokines (IFN-γ, IL-4, IL-10, IL-17A and GM-CSF) as measured with iFACS, but not all (TNF-α). This increase is highly variable between individuals, and on average also different for distinct cytokines. There is a moderate to strong correlation between CD3+CD8− T cell proportions positive for individual cytokines directly ex-vivo or after 72 h pre-stimulation with anti-CD3, except for IL-10. The release of cytokines in supernatants after 72 h stimulation with anti-CD3 showed a moderate to strong positive correlation with the proportion of IFN-γ, IL-4 and GM-CSF-positive cells as measured with iFACS, but not with IL-10, IL-17A and TNF-α. Therefore, especially in the case of small effects and sample sizes, the specific assay to analyze the T cell cytokine profile is likely to be a relevant confounder for the reproducibility of study results.

iFACS is a powerful technique to assess cell-specific cytokine profiles (Freer and Rindi, 2013), which has originally been designed and optimized for the analysis of IFN-γ, IL-2 and IL-4 (Jung et al., 1993) and later also IL-10 (Caraher, 2000). There are several parameters which must be taken care of during design and optimization of an experiment (Lamoreaux et al., 2006). For our experiments, cells are stimulated with PMA/ionomycin to boost cytokine production, which provides a strong T cell receptor independent stimulus. A caveat of intracellular cytokine analysis upon stimulation with PMA and ionomycin is the loss of CD4 expression. Therefore, CD3+CD8− T cells were analyzed although these are known to also include TCRγδ T cells. Stimulation durations up to 6 h have been associated with increased proportions of IFN-γ+ CD4+ T cells (Caraher, 2000). Whether this is most optimal for the kinetics of all cytokines studied in our panel is uncertain. Release of cytokines is blocked by adding golgi-inhibitors brefeldin A or monensin, of which monensin is slightly more toxic to cells (Caraher, 2000). The use of golgi-inhibitors allows accumulation of cytokines in the cells. The timing of monensin addition to the cell culture is also a relevant variable, since longer addition to cultures associates with higher proportions of CD3+CD8− T cells positive for IFN-γ and IL-4 with higher MFI-values (Muris et al., 2012). However, interfering with the golgi-apparatus is not beneficial for the detection of all cytokines. We showed earlier for IL-10 that monensin and brefeldin A or monensin, of which monensin is slightly more toxic to cells (Caraher, 2000). The use of golgi-inhibitors allows accumulation of cytokines in the cells. 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Trachomatis were specifically inhibited by brefeldin A and not monensin (Vicetti Miguel et al., 2012). Since the measurement of most cytokines clearly benefits from monensin, we did not omit this molecule from our iFACS protocol. We are, however, aware that by applying this protocol we measured IL-10 sub-optimally (Muris et al., 2012), and that this may contribute to the reported absence of a correlation between proportions IL10⁺ CD3⁺ CD8⁻ T cells observed directly ex-vivo or after pre-stimulation with anti-CD3. For detection of intracellular cytokines with fluorochrome-labeled antibodies, a fixation and permeabilization step is performed (Freer and Rindi, 2013). Our protocol allowed staining of 6 difference cytokines in 2 tubes. However, of several cytokines, very low proportions of positive cells were found. The more rare the events registered, the poorer the signal-noise ratio can be. This signal-noise ratio can also add to the poor correlation of study results, especially for the proportions of IL-10⁺ and IL-17A⁺ CD3⁺ CD8⁻ T cells. Next, comparison of iFACS data obtained directly ex-vivo or after 72 h pre-stimulation with anti-CD3 may be hampered by several confounding factors, including potential short-term treatment effects in the cells analyzed directly ex-vivo, and/or a change in the pool of T cells upon culturing. Last, the cytokine levels measured in culture supernatants may also deviate from the results obtained by iFACS. First, culture supernatants were obtained after pre-stimulation with anti-CD3, but prior to re-stimulation with PMA and ionomycin. Second, the cytokine levels in culture supernatants are the result of production by all cells present in the culture system. In the case of anti-CD3 stimulated PBMC, these also comprise CD8⁺ T cells, but also, indirectly, B cells, NK-cells and myeloid cells will contribute to the cytokine levels. This also holds for cytokine-levels as measured in the patients circulation (Åivo et al., 2015; Mahon et al., 2003; Røsjø et al., 2015; Toghianifar et al., 2015). We did not perform these analysis in the current study, since concentrations of T cell cytokines in the circulation are low and usually fall within the bottom range of most assay’s detection limits (personal experience).

To elaborate on the implications of our data, we discuss the example of IL-17. Although there are more cytokines to consider (Smolders et al., 2016b), IL-17 is an important CD4⁺ T cell inflammatory cytokine in the
Intracellular staining of CD3⁺CD8⁻ T cells after 72 h stimulation with anti-CD3, followed by re-stimulation with PMA/ionomycin.

<table>
<thead>
<tr>
<th>Vitamin D₃</th>
<th>Placebo</th>
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<tbody>
<tr>
<td><strong>T0</strong></td>
<td><strong>T1</strong></td>
</tr>
<tr>
<td>IL-17A</td>
<td>1.3 (0.8 – 2.6)</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.4 (0.1 – 1.1)</td>
</tr>
<tr>
<td>IL-4</td>
<td>5.3 (4.1 – 7.8)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>17.1 (10.2 – 24.0)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>38.6 (25.3 – 53.2)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>13.9 (7.5 – 23.0)</td>
</tr>
</tbody>
</table>

Data show median percentage of total CD3⁺CD8⁻ T cells (IQR). *Difference within group comparisons (Wilcoxon Signed Ranks). **Difference between group comparisons (Man Whitney U comparing T1/T0-ratios).
line with this notion, a more MS-relevant immunological outcome to assess in association of vitamin D3 supplements may be circulating anti-Epstein Barr virus nuclear antigen 1 (EBna-1) immunoglobulin G (IgG) antibody levels. Also the Epstein-Barr virus (EBV) has been identified as an environmental risk factor for MS. In particular, higher anti-EBNA-1 IgG levels are associated with an increased risk of MS (Ascherio et al., 2001). A reduction of anti-EBNA-1 IgG has been reproducibly associated with 12–96 weeks vitamin D3 supplements in patients with MS (Disanto et al., 2013; Rolf et al., 2017, [Rolf et al., 2018a]; Rasjo et al., 2017), with one study reporting a rise in anti-EBNA-1 IgG over 6 months following up in N = 13 control and not in N = 27 vitamin D3-supplemented MS patients (Najafipoor et al., 2015).

In our study, we explored the correlations between several approaches to measure CD3+CD8+ T cell cytokine responses in PBMC. Our analysis provides a best-case scenario: flowcytometry analyses were performed in a single-batch using the same reagents for stimulation and antigen-detection, all analyses were performed using the same flowcytometer. These are all factors which may introduce additional variation between studies. We conclude that experimental set-up introduces heterogeneity and may contribute to differences between studies in outcomes with a small effect size. Our data highlight that relatively small immunological studies in MS should be regarded as hypothesis generating. This paper is a call for research groups to perform reproducibility studies.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.molimm.2018.12.001.

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