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Prognostic value of natural killer cell/T cell ratios for disease activity in multiple sclerosis

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Abstract

Background and purpose: Natural killer (NK) cells may play a role in multiple sclerosis (MS). Ratios of NK cells to $CD4^+$ T cells have been proposed as a biomarker for the therapeutic effect of stem cell transplantation in MS. The objectives here were to explore the relevance of this ratio in MS patients by analysing NK and T cell subsets, as well as their prognostic value for disease activity.

Methods: Baseline peripheral blood mononuclear cells of 50 relapsing–remitting MS patients, participating in our vitamin D supplementation study (SOLARIUM), were analysed with flow cytometry. Disease activity was measured as new magnetic resonance imaging lesions, relapses and mean plasma neurofilament light chain levels after 48 weeks of follow-up.

Results: The proportion of NK cells correlated negatively with $CD4^+$ T cells ($R = -0.335$, $p = 0.001$) and interleukin 17A (IL-$17A^+$) $CD4^+$ T cells ($R = -0.203$, $p = 0.043$). Participants with magnetic resonance imaging activity or relapses displayed lower NK/IL-$17A^+$ $CD4^+$ T cell ratios ($p = 0.025$ and $p = 0.006$, respectively). The NK/IL-$17A^+$ $CD4^+$ T cell ratio correlated negatively with neurofilament light chain levels ($R = -0.320$, $p = 0.050$). Vitamin D supplementation did not affect these ratios.

Conclusions: Our data suggest a protective role of an expanded NK cell compartment compared to the $CD4^+$ T cell subset fractions in relapsing–remitting MS patients. $NK/CD4^+$ T cell ratios may be a prognostic biomarker for disease activity in MS.

KEYWORDS
MRI, multiple sclerosis, neurofilament light chain, NK cells, T cells

INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated, disabling disease which is most common in young adults [1]. The pathogenesis and mechanism of action of MS have been partially elucidated, although many facets still remain unclear. Classically, $CD4^+$ T cells

and interferon gamma $(IFN$-$\gamma)^+$ $CD4^+$ T (T helper type 1, Th1) cells in particular have been named as the main perpetrator in causing MS lesions in the central nervous system [2]. However, other immunological subsets, like interleukin 17 $(IL$-$17^+)$ $CD4^+$ T cells [3,4] $CD8^+$ T cells [5,6] and B cells [5,7] have also been linked to MS.

More recently, natural killer (NK) cells are under investigation in MS [8]. NK cells show a strong enrichment for expression of the MS

susceptibility genes as reported in a recent genome-wide association study [9]. In the experimental autoimmune encephalomyelitis model of neuro-inflammation, both direct neurotoxic effects of infiltrating NK cells [10] as well as a regulatory role of NK cells in controlling autoreactive T cell activation [11,12] have been postulated. During the treatment of people with MS with the anti-CD25 antibody daclizumab, a drug with notable effects in reducing relapses and magnetic resonance imaging (MRI) activity, an expansion of the CD56^{bright} subset of NK cells was observed [13]. This subset is generally considered to fulfil an immuno-regulatory role, with one of its functions being the killing of (autoreactive) activated T cells [13,14]. As such, an inverse correlation between NK cells and pathogenic T cells is hypothesized and supported by the findings of daclizumab trials. Interestingly, Darlington et al. [15] investigated the re-emergence of Th17 in relapsing–remitting (RR) MS patients after autologous hematopoietic stem cell transplantation. Although the absolute number of NK cells did not predict IL-17A^{+} Th cell levels, the ratio between NK cells and CD4^{+} T cells did correlate with lower IL-17A^{+} Th cell levels after 3 weeks, 3 months, 12 months and 21 months. As IL-17A^{+} T cells are considered to contribute to the pathogenic T cell response in MS [3,4] the group hypothesized that a high NK/CD4^{+} T cell ratio is a biomarker of a protective constitution of the lymphocyte compartment in MS [15]. However, no subsets of NK cells or CD4^{+} T cells were analysed, so little can be said about the role of, for example, CD56^{bright} NK cells and IL-17A^{+} CD4^{+} T cells in this hypothesis.

The aim is to expand upon this hypothesis by exploring the correlation between NK cells (and their subsets) and CD4^{+} T cells (and their subsets), and by exploring the association of their ratios with subsequent inflammatory disease activity in a cohort of IFN-β-treated RRMS patients. Disease activity was defined according to commonly used markers, namely new or enlarging MRI lesions and clinical relapse. Additionally, serum levels of neurofilament light chain (NfL), a new biomarker for axonal damage, were measured [16].

MATERIALS AND METHODS

Patients

The complete set of inclusion and exclusion criteria for the SOLAR study and its substudy SOLARIUM are described elsewhere [17,18].

In short, the SOLAR study (NCT01285401) recruited patients aged between 18 and 55 years diagnosed with RRMS (according to the McDonald criteria 2005) confirmed by typical MS findings on MRI. The first clinical event must have been within 5 years prior to screening and signs of active disease must have been present in the last 18 months, but no relapse in 30 days before inclusion. Patients were excluded if they consumed more than 1000 IU (25 μg) of vitamin D_3 supplements. All patients received IFN-β1a 44 μg subcutaneously three times weekly. They used IFN-β1a for at least 90 days but no longer than 18 months. After randomization, the patients received either IFN-β1a and a placebo or IFN-β1a

and vitamin D_3 supplements (cholecalciferol, Vigantol® Oil, Merck KGaA) of 7000 IU daily for 4 weeks, followed by 14,000 IU daily up to week 48.

The SOLARIUM substudy recruited patients from four of the five participating centres in the Netherlands without adding additional inclusion or exclusion criteria; the patients were eligible when they agreed to participate in the substudy. Peripheral blood samples were collected at baseline and after 48 weeks and analysed using flow cytometry. Written informed consent was acquired and the SOLARIUM study was approved by the Ethical Committee METC-Z (11-T-03).

This is a further analysis of the data obtained in the SOLARIUM study, with the only requirement being a full measure of NK cell related markers at baseline and after 48 weeks.

For our measures of disease activity, the MRI and relapse findings as described in the SOLAR study were used [18] as well as the NfL levels as measured from available material from the SOLAR study [19]. Since only 3/53 patients had more than one new MRI lesion within 48 weeks and 4/53 patients had more than one relapse within 48 weeks, data regarding MRI activity and clinical relapse were collected as a dichotomous yes/no outcome. Since this study included RRMS patients with a short disease duration and follow-up of 48 weeks, no clinically relevant disability progression was expected and therefore no correlations with Expanded Disability Status Scale progression were analysed. Plasma NfL levels were measured at baseline and 48 weeks using a single molecule array [19] in a subgroup of patients where samples were available. Since the baseline and week 48 levels were highly correlated (Spearman rho 0.615, $p < 0.001$), the mean NfL level was calculated, where available, to report the most representative level for the 48 week follow-up.

Peripheral blood mononuclear cell isolation

The acquirement and analysis of peripheral blood mononuclear cells (PBMCs) is described elsewhere [17]. In summary, peripheral blood samples were collected from patients at baseline and week 48 of treatment. Blood was collected in a 10 ml sodium heparin blood sampling tube (BD Biosciences) and transported to Maastricht University Medical Centre, The Netherlands, at room temperature. Within 24 h PBMCs were isolated as described in previous publications [17,20].

Flow cytometry for NK cells, T cells and subtypes

Immediately after isolation, PBMCs were stained with a cocktail of monoclonal antibodies. The T cell staining is described elsewhere [17]. In short, PBMCs were stained with a cocktail of monoclonal antibodies to define, amongst others, CD3^{+} T cells, CD3^{+} CD4^{+} T cells and T_{reg} cells. Cytokine expression of IFN-γ and IL-17A by CD3^{+} CD4^{+} T cells was assessed after a 5 h in vitro activation with

phorbol 12-myristate 13-acetate (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 IL-10 was assessed after similar activation but without the addition of monensin [21].

The NK cell staining consisted of NKp46-Horizon (Biolegend); CD56-PE (BD Biosciences); CD3-PerCP (BD Biosciences); NKG2D-APC (Biolegend); and CD16-PE-Cy7 (BD Biosciences). For fluorescence-activated cell sorting (FACS) analysis (FACS Canto II flow cytometer, BD Biosciences), NK cells were analysed for 100,000 events in the lymphocyte gate.

FACS DIVA software (BD Biosciences) was used to analyse the flow cytometry data. To identify both NK cell subsets, first CD3⁻NKp46⁺ cells (NK cells) were gated by accepting some contamination with CD3⁻NKp46⁻ cells, because there is some overlap between both CD3⁻ subsets. In order to adjust for this contamination, the total NK cell population was defined as the sum of CD56^{bright} CD16⁻ and CD56^{dim} CD16⁻ NK cells. Gating strategies are shown in Figure 1a.

Additional laboratory findings

During the SOLARIUM study, total white blood cell counts were gathered to calculate absolute numbers of lymphocyte subsets (Covance) [17]. These white blood cell counts were now used to calculate absolute cell numbers for subtypes. NfL levels were measured in plasma in duplicate with a single molecule array (Simoa) [19].

Statistical analysis

SPSS software (IBM SPSS, version 25.0) was used to assess the correlation between various NK cell subsets and T cell subsets, as well as the prognostic value of NK/T ratios and their subtypes for clinical outcomes and NfL levels. Normality of data was assessed by visual inspection of histograms with normal curves, skewness and kurtosis. Potential significant outliers were found using scatterplots with regression lines where a Cook's distance >1 was defined as a significant outlier. When a significant outlier was found, an additional analysis was performed without the outlier. Assessment of correlations between NK cell and CD4⁺ T cell subsets was done using Pearson r analyses or Spearman rho analyses, depending on normality of the data distribution. To assess the association between ratios and MRI outcome/relapse outcome, independent t tests or Mann–Whitney U tests were performed based on the distribution of data. Since in the SOLAR trial the presence of combined unique active (CUA) lesions was influenced by treatment arm [18] a logistic regression analysis was conducted to correct the association between NK/T cell ratios and MRI end-point for treatment arm allocation. If data were not normally distributed, ratios were logarithmically transformed in
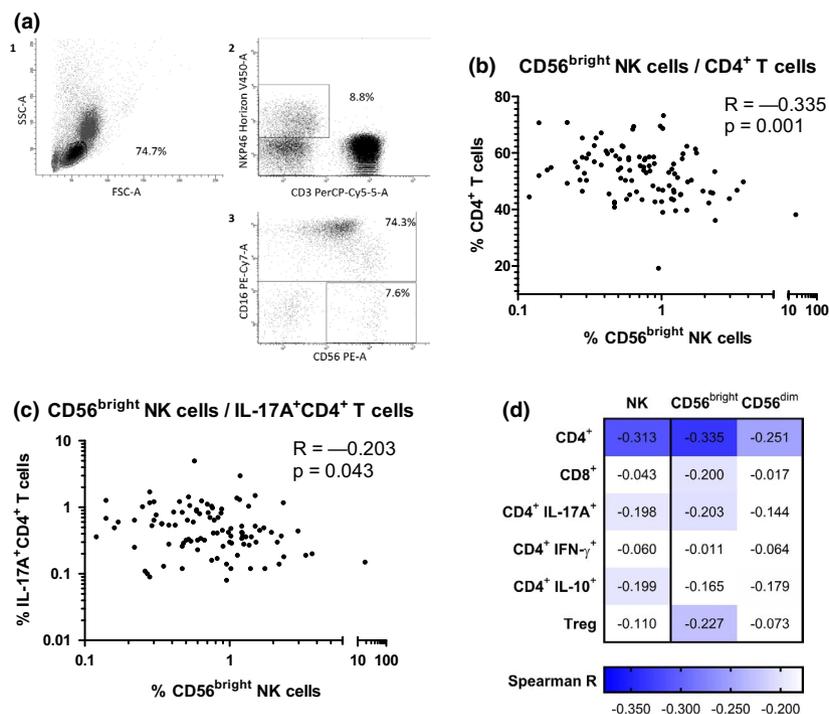

FIGURE 1 (a) Gating strategy used to analyse NK cells and subsets. Step 1 shows the gating of lymphocytes from the PBMC population. Step 2 shows the gating of NK cells, defined as CD3⁻ NKp46⁺. Step 3 shows the differentiation between the CD56^{dim} CD16⁺ NK cells (above) and CD56^{bright} CD16⁻ NK cells (below). (b) Correlation between the percentage of CD56^{bright} NK cells and the percentage of CD4⁺ T cells. R is Spearman's rho. N = 100. (c) Correlation between the percentage of CD56^{bright} NK cells and the percentage of IL17-A⁺ CD4⁺ T cells. R is Spearman's rho. N = 100. (d) Heatmap of correlations between NK cells (subsets) and various T cell subsets. Correlations with a tinted background are statistically significant. The associations between NK cells and T cell subsets seem to be mainly caused by the CD56^{bright} NK cells, despite them making up a small portion of the total NK cells. N = 100 [Colour figure can be viewed at wileyonlinelibrary.com]

order to create normally distributed data. The presence of relapses [18] and plasma NfL levels [19] were no different between treatment arms, so no correction for these end-points was applied. The correlation between ratios and NfL levels was assessed via the use of Pearson r or Spearman rho analyses, again based on data distribution. A p value of ≤0.05 was considered statistically significant.

RESULTS

Patient characteristics

The SOLARIUM study included 53 patients, but due to incomplete staining at baseline or week 48 regarding NK cell related markers, three patients were excluded from the current study, leaving 50 patients for analysis. Baseline patient characteristics of both treatment arms are described in Table 1.

Additionally, three patients did not undergo an MRI examination at week 48, leaving 47 to be analysed for MRI activity. Clinical disease activity markers were equally distributed between patients with and without MRI activity at week 48 (Table 1). NfL levels were measured for 35 patients at baseline and 38 patients at week 48.

Natural killer cells correlate with CD4$^+$ and IL-17A$^+$ CD4$^+$ T cells

To explore the relationship between circulating NK and T cells, correlations were calculated between total NK cells, CD56^{bright} NK cells and CD56^{dim} NK cells, on the one hand, and CD4$^+$ T cells, CD8$^+$ T cells, IL-17A$^+$ CD4$^+$ T cells, IFN-γ$^+$ CD4$^+$ T cells, IL-10$^+$ CD4$^+$ T cells and T$_{reg}$s on the other. Most notably, CD56^{bright} NK cells correlated negatively with CD4$^+$ T cells ($R = -0.335$, $p = 0.001$) and CD4$^+$ IL-17A$^+$ T cells ($R = -0.203$; $p = 0.043$) (shown in Figure 1b,c). Correlations between the CD56^{bright} NK cell fraction and T cell subsets were more prominent compared to the CD56^{dim} NK cell fraction (Figure 1d). These findings suggest that there is a biological association between NK and T cell proportions, supporting the assessment of ratios as proposed by Darlington et al. [15] Furthermore, these data suggest that the CD56^{bright} NK cell subset is the most influential subset in these associations.

NK/CD4$^+$ T cell subset ratios are lower in patients with new MRI lesions

To explore the relevance of NK/T cell subset ratios in the course of MS, the association of these ratios with the presence of new or enhancing MRI lesions (CUA) were assessed after 48 weeks' follow-up as the most sensitive marker for inflammatory disease activity [22]. When focusing on total CD4$^+$ T cells, the total NK/CD4$^+$ T cell ratio and the CD56^{dim}/CD4$^+$ T cell ratio were significantly lower in the group with MRI activity ($p = 0.050$, corrected for treatment arm $p = 0.071$, and $p = 0.050$, corrected for treatment arm $p = 0.061$; Figure 2a). Similarly, when analysing IL-17A$^+$ CD4$^+$ T cells, patients with CUA lesions had a lower total NK/IL-17A$^+$ CD4$^+$ T cell ratio ($p = 0.025$, corrected for treatment arm $p = 0.029$) and CD56^{dim} NK/

TABLE 1 Baseline characteristics of study participants, as well as a comparison of baseline characteristics between patients with and without MRI activity

	RRMS patients ($N = 50$)	No MRI activity	MRI activity	p value
Sex (N, %)				
Female	33 (66)	25 (69)	5 (45)	0.171
Male	17 (34)	11 (31)	6 (55)	
Age (years, median, interquartile range)	37.2 (31.7–44.3)	40.0 (32.8–45.3)	36.2 (29.6–43.9)	0.291
Body mass index (N, %)				
≥25 kg/m^2	28 (56)	19 (53)	7 (64)	0.731
<25 kg/m^2	22 (44)	17 (47)	4 (36)	
Disease duration (months, median, interquartile range)	7.4 (4.5–12.3)	7.3 (4.6–12.8)	8.4 (4.3–12.3)	0.851
Attacks during past 2 years at baseline (N, %)				
>1	16 (32)	11 (31)	4 (36)	0.725
≤1	34 (68)	25 (69)	7 (64)	
Duration since last attack at baseline (months, median, interquartile range)	7.6 (5.0–10.5)	7.4 (4.4–10.4)	8.0 (7.2–11.3)	0.386
Treatment (N, %)				
Placebo	21 (42)	11 (31)	8 (73)	0.018
Vitamin D3	29 (58)	25 (69)	3 (27)	

Note: Analysis of continuous data is done with a Mann–Whitney U test. Analysis of dichotomous data is done with Fischer's exact test.

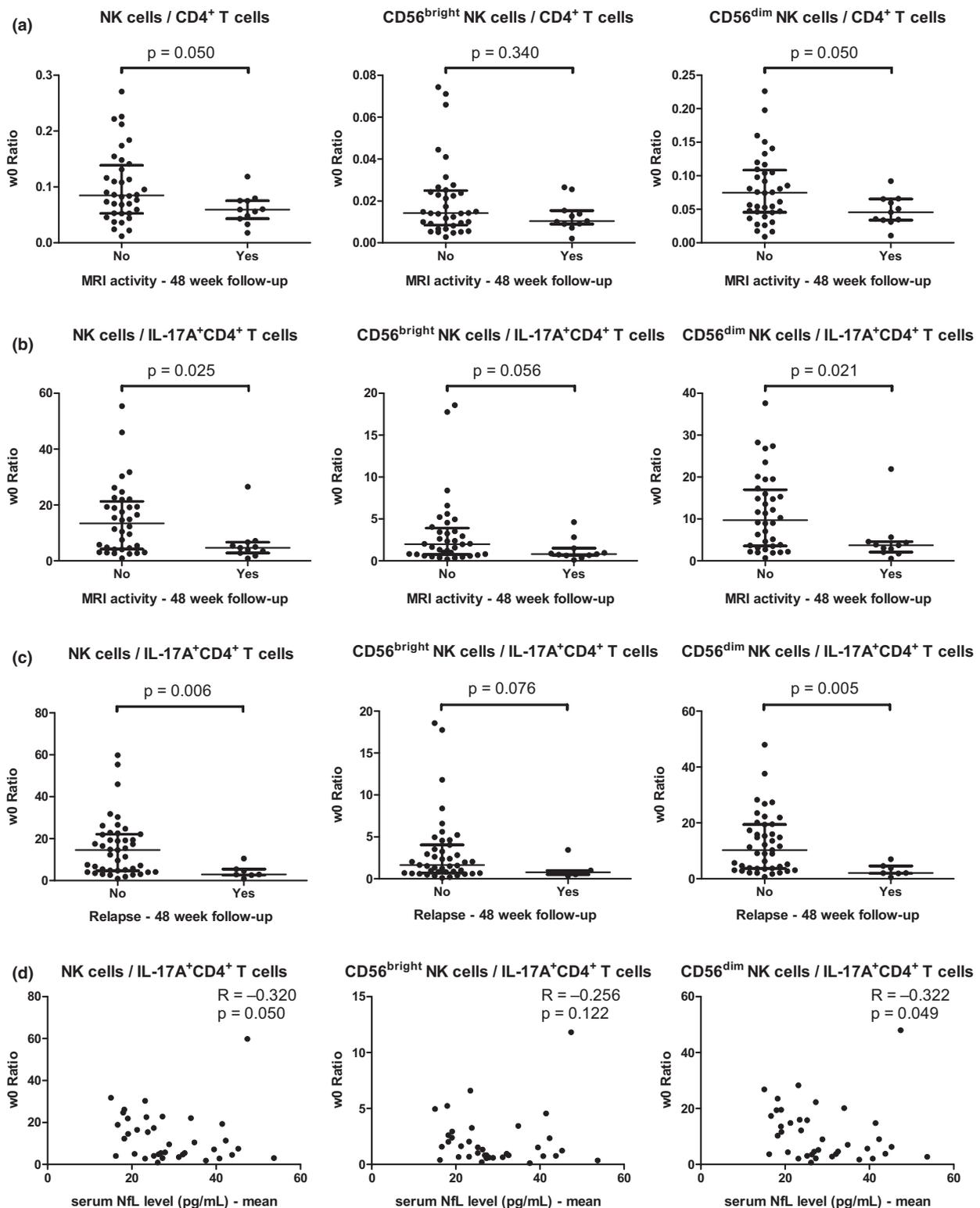Abbreviations: MRI, magnetic resonance imaging; RRMS, relapsing–remitting multiple sclerosis.

FIGURE 2 (a) Differences in NK cell/CD4$^+$ T cell ratio, CD56^{bright} NK cell/CD4$^+$ T cell ratio and CD56^{dim} NK cell/CD4$^+$ T cell ratio between patients with and without MRI activity after 48 weeks of follow-up. *p* value is calculated using a Mann–Whitney *U* test. Bars represent median with interquartile range. *N* = 47. (b) Differences in NK cell/IL-17A$^+$ CD4$^+$ T cell ratio, CD56^{bright} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio and CD56^{dim} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio between patients with and without MRI activity after 48 weeks of follow-up. *p* value is calculated using a Mann–Whitney *U* test. Bars represent median with interquartile range. *N* = 47. (c) Differences in NK cell/IL-17A$^+$ CD4$^+$ T cell ratio, CD56^{bright} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio and CD56^{dim} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio between patients suffering one or more relapses or no relapse during the 48 week follow-up period. *p* value is calculated using a Mann–Whitney *U* test. Bars represent median with interquartile range. *N* = 50. (d) Correlations between NK cell/IL-17A$^+$ CD4$^+$ T cell ratio, CD56^{bright} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio and CD56^{dim} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio at baseline and mean NfL values measured in pg/ml. *R* is Spearman's rho. *N* = 38
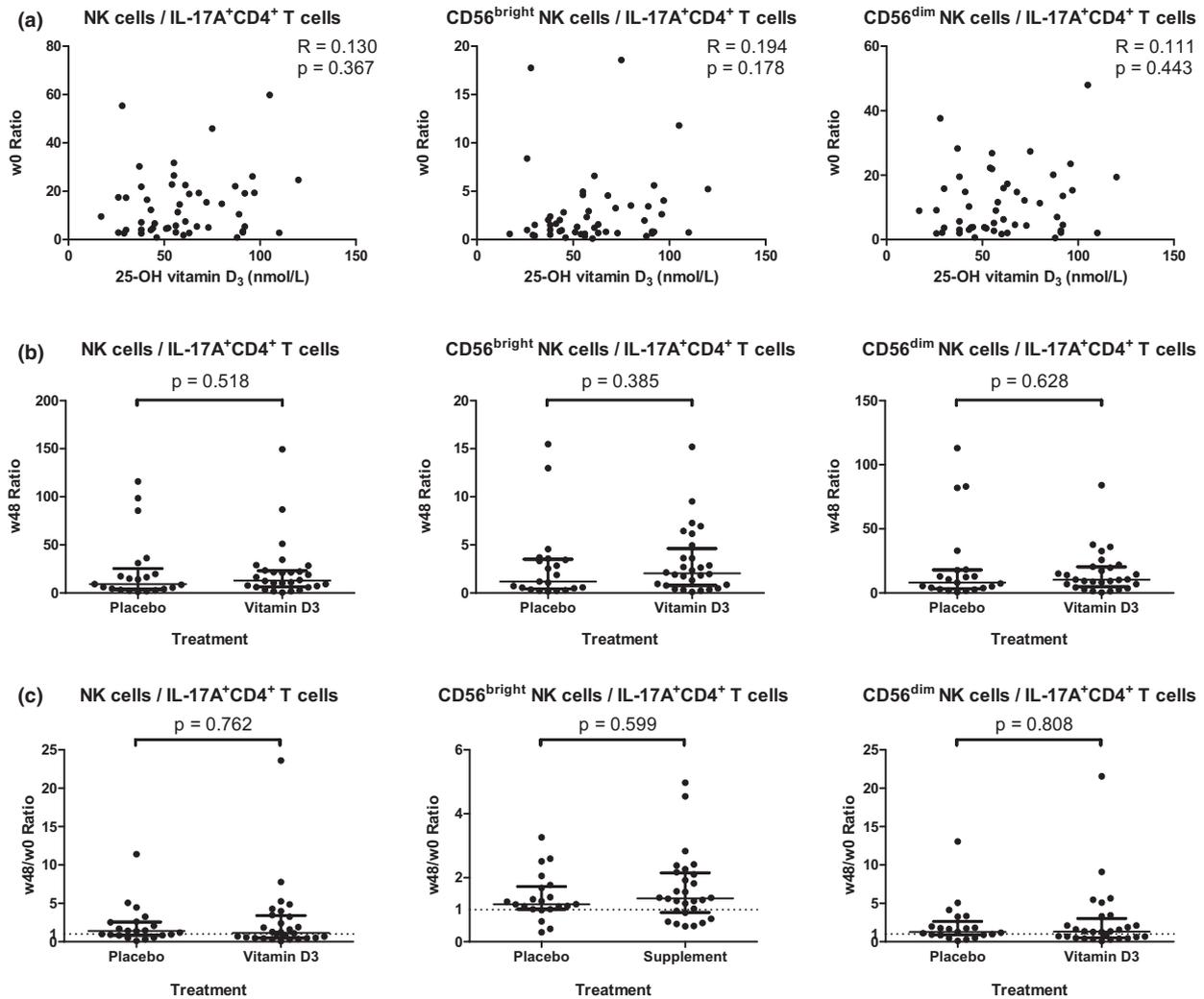
FIGURE 3 (a) Correlation between 25(OH)D levels at baseline and NK cell/IL-17A$^+$ CD4$^+$ T cell ratio, CD56^{bright} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio and CD56^{dim} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio. *R* is Spearman's rho. *N* = 50. (b) Comparison of NK cell/IL-17A$^+$ CD4$^+$ T cell ratio, CD56^{bright} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio and CD56^{dim} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio at week 48 between patients receiving a placebo or vitamin D3 supplements. *p* value is calculated using a Mann–Whitney *U* test. Bars represent median with interquartile range. *N* = 50. (c) Comparisons of the evolutions of NK cell/IL-17A$^+$ CD4$^+$ T cell ratio, CD56^{bright} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio and CD56^{dim} NK cell/IL-17A$^+$ CD4$^+$ T cell ratio. Evolution was calculated by dividing the ratio at week 48 by the ratio at baseline. An evolution >1 means the ratio has increased after 48 weeks, whereas a ratio <1 means the ratio has decreased. *p* value is calculated using a Mann–Whitney *U* test. Bars represent median with interquartile range. *N* = 50

IL-17A$^+$ CD4$^+$ T cell ratio (*p* = 0.021, corrected for treatment arm *p* = 0.026) (Figure 2b).

NK/IL-17A$^+$ CD4$^+$ T cell ratios are lower in patients with subsequent relapses

To explore the consistency of this finding, other markers of disease activity were analysed. First, patients who experienced a clinical exacerbation during the 48 week follow-up period were compared with patients who did not experience a relapse (Figure 2c). Focusing on the ratios most clearly associated with radiological disease activity, a trend towards a lower CD56^{bright} NK/IL-17A$^+$ CD4$^+$ T cell ratio was found, whereas significantly

lower total NK/IL-17A$^+$ CD4$^+$ T cell (*p* = 0.006) and CD56^{dim} NK/IL-17A$^+$ CD4$^+$ T cell ratios (*p* = 0.005) were observed in patients with exacerbations of disease.

NK/CD4$^+$ T cell subset ratios are negatively correlated with NfL

Plasma NfL is a measure of clinical and MRI disease activity in MS, and strongly correlates with enhancing MRI lesions [16,23]. Baseline total NK and CD56^{dim} NK/IL-17A$^+$ CD4$^+$ ratios were negatively correlated with mean NfL levels (*R* = −0.320, *p* = 0.050; and *R* = −0.322, *p* = 0.049; Figure 2d). Notably, the same data point in all three correlation plots is a significant outlier (Cook's distance >1). Correlation

analyses without this significant outlier were more clearly supportive of a negative correlation for IL-17A$^+$ CD4$^+$ in a ratio with NK cells (R = −0.422, p = 0.009), CD56^{bright} cells (R = −0.352, p = 0.033) and CD56^{dim} cells (R = −0.424, p = 0.009).

NK/CD4$^+$ subset ratios are not correlated with vitamin D

Low serum 25-hydroxyvitamin D (25(OH)D) levels are a biomarker for a higher risk of subsequent relapses [24]. First, baseline 25(OH)D did not significantly correlate with baseline NK/CD4$^+$ T cell subset ratios (Figure 3a). Then, in order to assess an effect of vitamin D supplementation on NK/CD4$^+$ T cell subset ratios, ratios at week 48 were compared between treatment arms, which did not show any influence of vitamin D3 supplementation (Figure 3b). Additionally, the relative increase or decrease in these ratios during 48 weeks of follow-up was analysed, stratified for treatment arm. There was no significant difference in distribution of any of the NK/CD4$^+$ T cell subset ratios between vitamin D3 and the placebo arm (Figure 3c). Also, the relative and absolute NK cell (subset) proportions were not different between the treatment arms (Figure S1).

DISCUSSION

The prognostic value of NK/CD4$^+$ T cell subset ratios was investigated for disease activity in a cohort study of a homogeneous group of INF-β-treated early RRMS patients.

First, an association was found between the relative presence of NK cells and the relative presence of CD4$^+$ T cells and IL-17A$^+$ CD4$^+$ T cells. This association is also found with NK cell subsets, where the CD56^{bright} subset of NK cells shows a stronger association than the CD56^{dim} subset, despite it making up a relatively small portion of the total NK cell population. This stronger association with CD56^{bright} NK cells may support the hypothesis of CD56^{bright} NK cells fulfilling an immuno-regulatory role in MS by suppressing (autologous) activated T cells, as seen in daclizumab trials [13].

Secondly, the relative presence of NK cells and subsets compared to CD4$^+$ T cells and IL-17A$^+$ CD4$^+$ T cells, expressed as a ratio, seems to be relevant for disease activity. Indeed, NK/CD4$^+$ T cell subset ratios are lower in patients with new and/or enlarging MRI lesions after 48 weeks of follow-up. This effect is seen in ratios including CD4$^+$ T cells and, perhaps more specifically, in ratios including IL-17A$^+$ CD4$^+$ T cells. IL-17A$^+$ CD4$^+$ T cells have been argued to constitute a subset involved in the pathogenesis of MS [25–28] although other subsets based on other cytokines and surface markers have been shown to be involved as well. Nevertheless, our findings do reinforce the idea of an IL-17A$^+$ CD4$^+$ T cell contribution to MS disease activity, and also support a regulatory effect of NK cells on IL-17A$^+$ CD4$^+$ T cells, as suggested by Darlington et al. [15] and daclizumab trials [29]. When specifically looking at the NK cell subsets, the prognostic value for

disease activity seems predominantly driven by the CD56^{dim} NK cell ratios. Given that the CD56^{bright} NK cell subset is generally considered to be the regulatory subset and CD56^{bright} NK cells correlate negatively with IL-17A$^+$ CD4$^+$ T cell proportions, our clinical associations seem to be conflicting with this concept [8]. However, not only CD56^{bright} NK cells but also CD56^{dim} NK cells show a capability to kill activated T cells [30]. Over the last few years, CD56^{bright} NK cells have been the main focus for NK cell research in MS, but our findings highlight that the CD56^{dim} population may be an important facet as well.

Our exploratory cohort study has some limitations. First, the SOLAR study was originally designed to investigate the effect of high-dose vitamin D3 treatment [18] whilst the SOLARIUM sub-study was initiated to unravel the effect of high-dose vitamin D3 supplementation on the immune system [17]. This is also the reason for some missing data, in particular for NfL measurements [19]. Furthermore, MS patients included were selected based on having RRMS, short disease duration and treatment with IFN-β. Therefore, extrapolation of the data requires extension of our findings in other MS patient cohorts. Finally, due to the initial research question of the SOLAR study, the number of patients is not based on a power calculation relevant to our question. Hence, our study is referred to as an exploratory study.

Interestingly, it has been shown in daclizumab studies that IL-2 is important in the interplay between NK and T cells [31] and other studies have shown that granulocyte-macrophage colony-stimulating factor (GM-CSF), a pro-inflammatory cytokine, is strongly induced by IL-2 [32]. Unfortunately, it was not possible to evaluate GM-CSF$^+$ CD4$^+$ T cells due to too many missing data points at baseline [17]. Thus, the possible relation between GM-CSF, IL-2 and NK cells could not be investigated in this study.

In conclusion, a relation between NK cells (subsets) and CD4$^+$ T cells (subsets) in RRMS was shown. NK cells seem to exert a protective effect, hypothetically by controlling the T cell population. Not only is the CD56^{bright} NK cell population, known for its immuno-regulatory properties, involved, but also the CD56^{dim} NK cell population seems to play a role in reducing disease activity. More research with independent datasets is needed to confirm the validity and use of NK/CD4$^+$ subset ratios as a prognostic marker in RRMS.

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ETHICAL STATEMENT

Our data have not been submitted or published elsewhere, all authors agree with submission, financial conflicts of interest and funding are disclosed, no animal studies were conducted. Our research is based on human studies, which were approved by the local Ethical Committee. Informed consent from all participating subject was obtained. This study was funded by Nationaal MS Fonds grant OZ2016-001 and an unrestricted grant by Merck.

AUTHOR CONTRIBUTIONS

MM contributed to the writing of the article and statistical analyses performed. JD was involved in writing and gave expert opinions on the immunological basis of the paper. AM and LR were involved in gathering and analysing blood samples and creating the database from which our T cell data is derived. OG was involved in writing and the general outline of the paper. JK was involved in the general outline of the paper and provided expert opinions on the basis of neurofilament light chain. RH contributed in the general outline of the paper and provided financial support. JS contributed to writing, statistics, and the general outlines of the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Dobson R, Giovannoni G. Multiple sclerosis—a review. *Eur J Neurol.* 2019;26:27-40.
2. Gutcher I, Becher B. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J Clin Invest.* 2007;117:1119-1127.
3. Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol.* 2005;6:1133-1141.
4. van Langelaar J, van der Vuurst de Vries RM, Janssen M, et al. T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention. *Brain.* 2018;141:1334-1349.
5. Machado-Santos J, Saji E, Troscher AR, et al. The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. *Brain.* 2018;141:2066-2082.
6. Salou M, Nicol B, Garcia A, Laplaud DA. Involvement of CD8(+) T cells in multiple sclerosis. *Front Immunol.* 2015;6:604.
7. Li R, Patterson KR, Bar-Or A. Reassessing B cell contributions in multiple sclerosis. *Nat Immunol.* 2018;19:696-707.
8. Mimpen M, Smolders J, Hupperts R, Damoiseaux J. Natural killer cells in multiple sclerosis: a review. *Immunol Lett.* 2020;222:1-11.
9. International Multiple Sclerosis Genetics C. Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science.* 2019;365:eaav7188.
10. Liu Q, Sanai N, Jin WN, La Cava A, Van Kaer L, Shi FD. Neural stem cells sustain natural killer cells that dictate recovery from brain inflammation. *Nat Neurosci.* 2016;19:243-252.
11. Leavenworth JW, Schellack C, Kim HJ, Lu L, Spee P, Cantor H. Analysis of the cellular mechanism underlying inhibition of EAE after treatment with anti-NKG2A F(ab')2. *Proc Natl Acad Sci U S A.* 2010;107:2562-2567.
12. Lu L, Ikizawa K, Hu D, Werneck MB, Wucherpfennig KW, Cantor H. Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway. *Immunity.* 2007;26:593-604.
13. Bielekova B, Catalfamo M, Reichert-Scrivner S, et al. Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. *Proc Natl Acad Sci U S A.* 2006;103:5941-5946.
14. Jiang W, Chai NR, Maric D, Bielekova B. Unexpected role for granzyme K in CD56bright NK cell-mediated immunoregulation of multiple sclerosis. *J Immunol.* 2011;187:781-790.
15. Darlington PJ, Stopnicki B, Touil T, et al. Natural killer cells regulate Th17 cells after autologous hematopoietic stem cell transplantation for relapsing remitting multiple sclerosis. *Front Immunol.* 2018;9:834.
16. Kuhle J, Kropshofer H, Haering DA, et al. Blood neurofilament light chain as a biomarker of MS disease activity and treatment response. *Neurology.* 2019;92:e1007-e1015.
17. Muris AH, Smolders J, Rolf L, et al. Immune regulatory effects of high dose vitamin D3 supplementation in a randomized controlled trial in relapsing remitting multiple sclerosis patients receiving IFNbeta; the SOLARIUM study. *J Neuroimmunol.* 2016;300:47-56.
18. Hupperts R, Smolders J, Vieth R, et al. Randomized trial of daily high-dose vitamin D3 in patients with RRMS receiving subcutaneous interferon beta-1a. *Neurology.* 2019;93:e1906-e1916.
19. Smolders J, Mimpen M, Oechtering J, et al. Vitamin D3 supplementation and neurofilament light chain in multiple sclerosis. *Acta Neurol Scand.* 2020;141:77-80.
20. Smolders J, Thewissen M, Peelen E, et al. Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis. *PLoS One.* 2009;4:e6635.
21. Muris AH, Damoiseaux J, Smolders J, Cohen Tervaert JW, Hupperts R, Thewissen M. Intracellular IL-10 detection in T cells by flow cytometry: the use of protein transport inhibitors revisited. *J Immunol Methods.* 2012;381:59-65.
22. Barkhof F, Simon JH, Fazekas F, et al. MRI monitoring of immunomodulation in relapse-onset multiple sclerosis trials. *Nat Rev Neurol.* 2011;8:13-21.
23. Gafson AR, Barthelemy NR, Bomont P, et al. Neurofilaments: neurobiological foundations for biomarker applications. *Brain.* 2020;143:1975-1998.
24. Simpson S Jr, Taylor B, Blizzard L, et al. Higher 25-hydroxyvitamin D is associated with lower relapse risk in multiple sclerosis. *Ann Neurol.* 2010;68:193-203.
25. Durelli L, Conti L, Clerico M, et al. T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta. *Ann Neurol.* 2009;65:499-509.
26. Hu D, Notarbartolo S, Croonenborghs T, et al. Transcriptional signature of human pro-inflammatory TH17 cells identifies reduced IL10 gene expression in multiple sclerosis. *Nat Commun.* 2017;8:1600.
27. Kebir H, Kreymborg K, Ifergan I, et al. Human TH17 lymphocytes promote blood–brain barrier disruption and central nervous system inflammation. *Nat Med.* 2007;13:1173-1175.
28. Tzartos JS, Friese MA, Craner MJ, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is

associated with active disease in multiple sclerosis. *Am J Pathol.* 2008;172:146-155.

29. Bielekova B, Richert N, Herman ML, et al. Intrathecal effects of daclizumab treatment of multiple sclerosis. *Neurology.* 2011;77:1877-1886.

30. Nielsen N, Odum N, Urso B, Lanier LL, Spee P. Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. *PLoS One.* 2012;7:e31959.

31. Bielekova B. Daclizumab therapy for multiple sclerosis. *Cold Spring Harb Perspect Med.* 2019;9:a034470.

32. Hartmann FJ, Khademi M, Aram J, et al. Multiple sclerosis-associated IL2RA polymorphism controls GM-CSF production in human TH cells. *Nat Commun.* 2014;5:5056.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.