

## Short Communication

## NK/T cell ratios associate with interleukin-2 receptor alpha chain expression and shedding in multiple sclerosis

Max Mimpfen<sup>a</sup>, Linda Rolf<sup>a</sup>, Anne-Hilde Muris<sup>a</sup>, Oliver Gerlach<sup>b</sup>, Geert Poelmans<sup>c</sup>, Raymond Hupperts<sup>a,b</sup>, Joost Smolders<sup>d,e</sup>, Jan Damoiseaux<sup>f,\*</sup>

<sup>a</sup> School for Mental Health and Neuroscience, Maastricht University, Maastricht, the Netherlands

<sup>b</sup> Department of Neurology, Zuyderland Medical Center, Sittard, the Netherlands

<sup>c</sup> Department of Human Genetics, Radboud University Medical Center, Nijmegen, the Netherlands

<sup>d</sup> MS center Erasmus, Departments of Neurology and Immunology, Erasmus University Medical Center, Rotterdam, the Netherlands

<sup>e</sup> Department of Neuroimmunology, Netherlands Institute for Neuroscience, Amsterdam, the Netherlands

<sup>f</sup> Central Diagnostic Laboratory, Maastricht University Medical Center, Maastricht, the Netherlands

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## ABSTRACT

NK/T-cell ratios predict disease activity in relapsing remitting multiple sclerosis (RRMS). We investigated in 50 RRMS patients whether interleukin-2 receptor alpha-chain (IL-2R $\alpha$ ) expression and shedding associates with NK/T-cell balance, as suggested by daclizumab-trials in RRMS. A subsample ( $N = 31$ ) was genotyped for *IL2RA*-associated MS risk SNPs. CD56<sup>bright</sup> NK-cell/IL-17A<sup>+</sup>CD4<sup>+</sup> T-cell ratios correlated negatively with plasma and PBMC-culture supernatant sIL-2R $\alpha$ -levels [ $R = -0.209$ ;  $p = 0.038$  and  $R = -0.254$ ;  $p = 0.012$ , resp.], and with CD4<sup>+</sup> T-cell CD25 MFI [ $R = -0.341$ ;  $p = 0.001$ ]. Carriers of the rs3118470 risk-allele showed higher sIL-2R $\alpha$ -levels ( $P = 0.031$ ) and a lower CD56<sup>bright</sup> NK-cell/IL-17A<sup>+</sup>CD4<sup>+</sup> T-cell ratio ( $P = 0.038$ ). Therefore, IL-2R $\alpha$  may be involved in the interplay between NK-cells and T-cells.

## 1. Introduction

Multiple sclerosis (MS) is an immune-mediated inflammatory disease of the central nervous system. (Dobson and Giovannoni, 2019) Although not fully elucidated, many factors and pathways have been suggested to be relevant for MS disease activity. One of these pathways is the interleukin-2 (IL-2) / interleukin-2 receptor (IL-2R) pathway. (Carrieri et al., 1992; Sorensen, 2005) IL-2 is a cytokine with both pro- and anti-inflammatory effects on the immune system, well known as a survival cytokine for activated T cells, but also as important in the biology of regulatory T (Treg) cells and natural killer (NK) cells. (Gaffen and Liu, 2004) The composition of the IL-2R is variable. Incorporation of the  $\alpha$ -chain (IL-2R $\alpha$ , CD25) into a tri-molecular complex renders a receptor with high affinity for IL-2. (Damoiseaux, 2020) Activated immune cells may shed IL-2R $\alpha$  and produce soluble IL-2R $\alpha$  (sIL-2R $\alpha$ ), of which higher circulating levels are associated with MS disease activity. (Sharief and Thompson, 1993) Whether sIL-2R $\alpha$  by itself has a more pro- or anti-inflammatory role, remains controversial. (Damoiseaux, 2020)

The IL-2/IL-2R pathway in MS gained renewed interest after the positive clinical results from an anti-CD25 therapy (daclizumab), aimed

at reducing disease activity in relapsing remitting (RR)MS patients. (Giovannoni et al., 2014; Gold et al., 2013) Daclizumab was shown to increase the NK cell population, specifically the immune-regulatory CD56<sup>bright</sup> NK cell population, which is thought to kill activated T cells. (Elkins et al., 2015) This finding revealed the relevance of the interplay between NK and T cells in RRMS, with the IL-2R as possible mediator. Recently, we have shown in interferon beta-treated RRMS patients that disease activity is associated with lower NK/T-cell ratios, (Mimpfen et al., 2020) which not only points towards an interesting potential prognostic biomarker for disease activity, but also further implicates an influential role of NK/T-cell interplay in RRMS. The role of IL-2R $\alpha$  in these associations has not been explored previously.

We studied the association between sIL-2R $\alpha$  and NK/T cell ratios in MS. To further consolidate our finding, we extended this analysis by exploring several other IL-2R related markers, as well as the genetic status of our participants with regard to two known *IL2RA*-associated MS risk alleles.

\* Corresponding author at: Central Diagnostic Laboratory, Maastricht University Medical Center, PO box 5800, Maastricht 6202 AZ, the Netherlands.

E-mail address: [jan.damoiseaux@mumc.nl](mailto:jan.damoiseaux@mumc.nl) (J. Damoiseaux).

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## 2. Methods and materials

### 2.1. Patients

This study is a post-hoc extended analysis of the SOLARIUM study, which was a sub-study of the SOLAR study. The SOLAR study evaluated disease activity in interferon beta-treated RRMS patients using high dose vitamin D<sub>3</sub> supplements compared to placebo. The SOLARIUM study investigated the effect of high dose vitamin D<sub>3</sub> supplementations on the immune system composition. In- and exclusion criteria for the SOLAR and SOLARIUM studies are described elsewhere. (Hupperts et al., 2019; Muris et al., 2016) For inclusion in this analysis, all 50 participants of whom data regarding NK cells, T cells and IL-2R $\alpha$  were available were included. Retrospectively, 31 of these 50 participants provided consent for genetic analysis. Written informed consent was acquired and the SOLARIUM study was approved by the Ethical Committee METC-Z (11-T-03; Heerlen, the Netherlands).

### 2.2. PBMC isolation

The acquirement and analysis of peripheral blood mononuclear cells (PBMCs) is described elsewhere. (Muris et al., 2016) In summary, peripheral blood samples were collected from patients at baseline and at week 48 of treatment. Blood was collected in a 10 mL sodium heparin blood sampling tube (BD Biosciences, Breda, The Netherlands) and transported to Maastricht University Medical Center, the Netherlands, at room temperature. Within 24 h PBMCs were isolated as described previously.

### 2.3. IL-2 receptor alpha-chain gene expression, protein expression and shedding

Measurement of sIL-2R $\alpha$  is described elsewhere. (Rolf et al., 2018) In short, sIL-2R $\alpha$  levels in plasma were measured by a chemiluminescent assay, using a commercially available kit for quantitative measurement of sIL-2R $\alpha$  on the Immulite 2000 (Siemens; 95% reference range 158–623 U/mL;  $N = 50$ ). Additionally, sIL-2R $\alpha$  was measured in culture supernatant of PBMCs (100,000/well) which were cultured and stimulated by phytohaemagglutinin (PHA; 25  $\mu$ g/mL) for 72 h ( $N = 50$ ). In addition, the process of obtaining gene expression data by PCR ( $N = 39$ ) and staining of PBMC for flow cytometry is described elsewhere. (Muris et al., 2016; Rolf et al., 2018) Flow cytometry was used to obtain MFI values of IL-2R $\alpha$  (CD25) on CD4<sup>+</sup> T cells ( $N = 50$ ). The gating strategy is shown in Fig. 1A. Genotyping the polymorphisms rs2104286 and rs3118470 was done using extracted DNA from buffy coats. This DNA was analysed on the Infinium PsychArray-24v1.3\_A1 BeadChip (Infinium array technology) and the polymorphisms of interest were defined ( $N = 31$ ).

### 2.4. Statistical analysis

SPSS software (IBM SPSS, version 25.0. Chicago, IL) was used to assess the correlation between baseline and week 48 IL-2R $\alpha$  markers, as well as the correlation between IL-2R $\alpha$  markers and NK/T ratios. Normality of data was assessed by visual inspection of histograms with normal curves, skewness and kurtosis. Both correlations between baseline and week 48 IL-2R $\alpha$  markers, and IL-2R markers and NK/T ratios were performed using a Pearson  $r$  correlation or Spearman  $\rho$  correlation test, depending on distribution of data. Differences in continuous variables between carriers of genetic risk-alleles were analysed with a Mann Whitney  $U$  test. A  $p$ -value of  $<0.05$  was considered statistically significant.

## 3. Results

### 3.1. Strong correlation between baseline and week 48 plasma sIL-2R $\alpha$ levels

We analysed data on serum sIL-2R $\alpha$  levels, supernatant sIL-2R $\alpha$  levels, *IL2RA* gene expression and CD4<sup>+</sup> T cell IL-2R $\alpha$  protein expression at baseline and at 48 weeks follow-up (Fig. 1). While sIL-2R $\alpha$  levels in serum at baseline and week 48 correlated strongly ( $R = 0.836$ ;  $p \leq 0.001$ ), other IL-2R $\alpha$  related markers showed relatively low or statistically non-significant correlations (Fig. 1B). Since these variables all show a biological variability, which also applies to the NK/T-cell ratios, we pooled our data of both time points.

### 3.2. Lower NK/T cell ratios associate with higher IL-2R $\alpha$ protein expression and shedding

As NK/IL-17A<sup>+</sup>CD4<sup>+</sup> T cell ratios were most strongly associated with disease activity in our previous work, (Mimpfen et al., 2020) and IL-17<sup>+</sup> T cells have been proposed as a pathogenic T cell subset in MS, (Li et al., 2017) this ratio was focus for our IL-2R $\alpha$  related analyses. We included ratios of the total, CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cell subsets, to explore effects of these different subsets.

Most notably, all ratios involving IL-17A<sup>+</sup>CD4<sup>+</sup> T cells showed a negative correlation with serum sIL-2R $\alpha$  levels (Fig. 2A), as well as sIL-2R $\alpha$  shedding by PHA-stimulated PBMCs (Fig. 2B). Since sIL-2R $\alpha$  has been hypothesized to be mostly shedded by activated CD4<sup>+</sup> T cells, (Brusko et al., 2009) we also explored the correlation of NK/T-cell ratios with CD4<sup>+</sup> T cell IL-2R $\alpha$  (CD25) MFI. Although IL-2R $\alpha$  MFI on Treg CD4<sup>+</sup> cells did not correlate with NK/T-cell ratios (Fig. 2C), IL-2R $\alpha$  MFI on non-Treg CD4<sup>+</sup> T cells correlated negatively with all NK/T-cell ratios investigated (Fig. 2D). *IL2RA* gene expression levels in total PBMCs were available but did not correlate with NK/T-cell ratios (Fig. 2E). We conclude that in MS, IL-2R $\alpha$  protein expression and shedding by T cells correlate consistently with a, relative to NK cells, increased proportion of circulating (pathogenic) CD4<sup>+</sup> T cells.

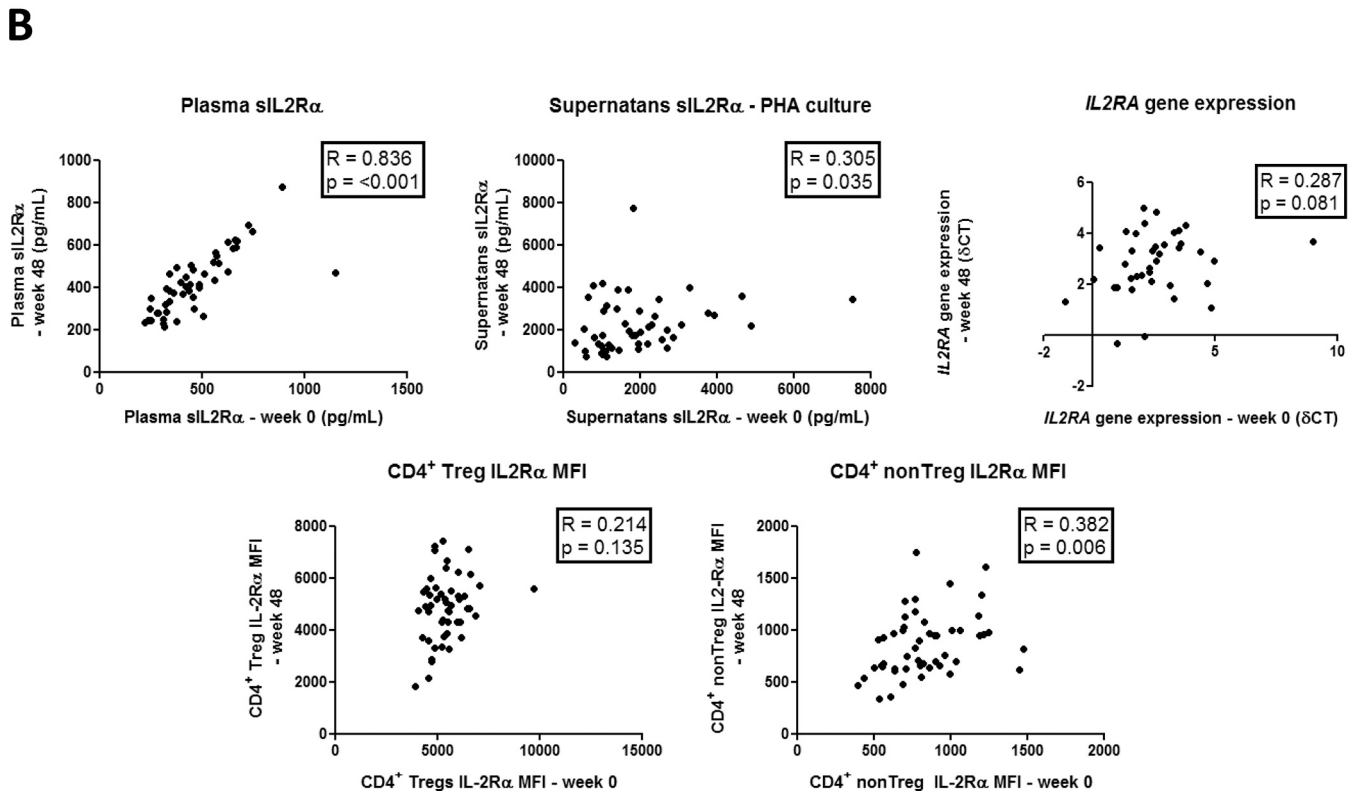
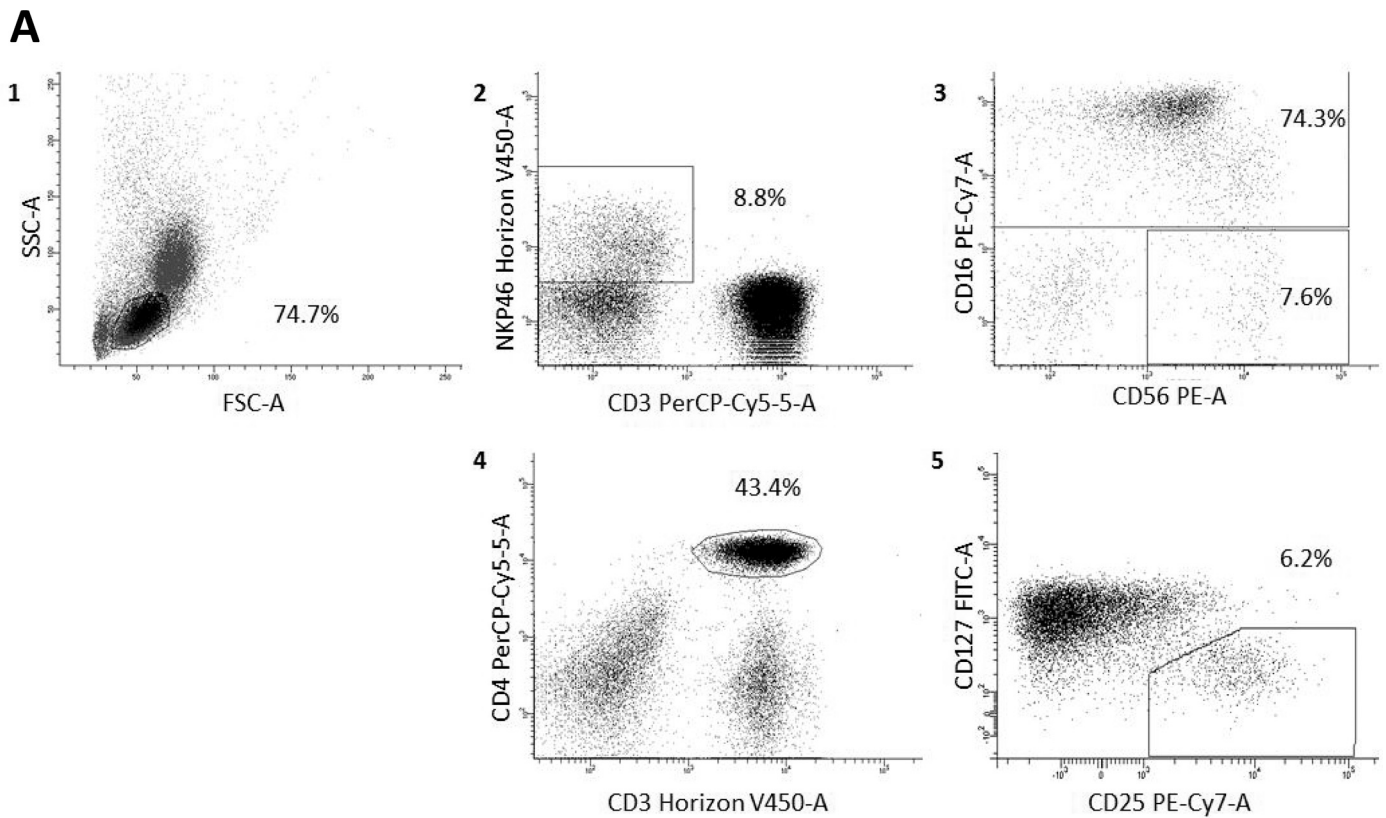
### 3.3. Presence of risk allele of rs3118470 is linked with higher sIL-2R $\alpha$ levels

To further consolidate the association of IL-2R $\alpha$  related endpoints with NK/T-cell ratios, we genotyped a subset of participants for *IL2RA*-associated MS risk SNPs rs2104286 and rs3118470. (International Multiple Sclerosis Genetics C, 2019) Notably, the rs2104286 risk-allele has been associated with higher serum sIL-2R $\alpha$  levels in MS. (Buhelt et al., 2017) Of the  $N = 31$  participants of whom genetic data were available,  $N = 30$  were carriers of the rs2104286 risk allele. Comparing carriers and non-carriers of the rs3118470 MS risk allele ( $N = 13$  and  $N = 18$ , respectively), carriers had a significantly higher level of sIL-2R $\alpha$  at baseline (Fig. 3A), as well as a lower CD56<sup>bright</sup> NK/IL-17A<sup>+</sup>CD4<sup>+</sup> T cell ratio (Fig. 3C), with differences between other NK/T-cell ratios not being statistically significant (Fig. 3B and D).

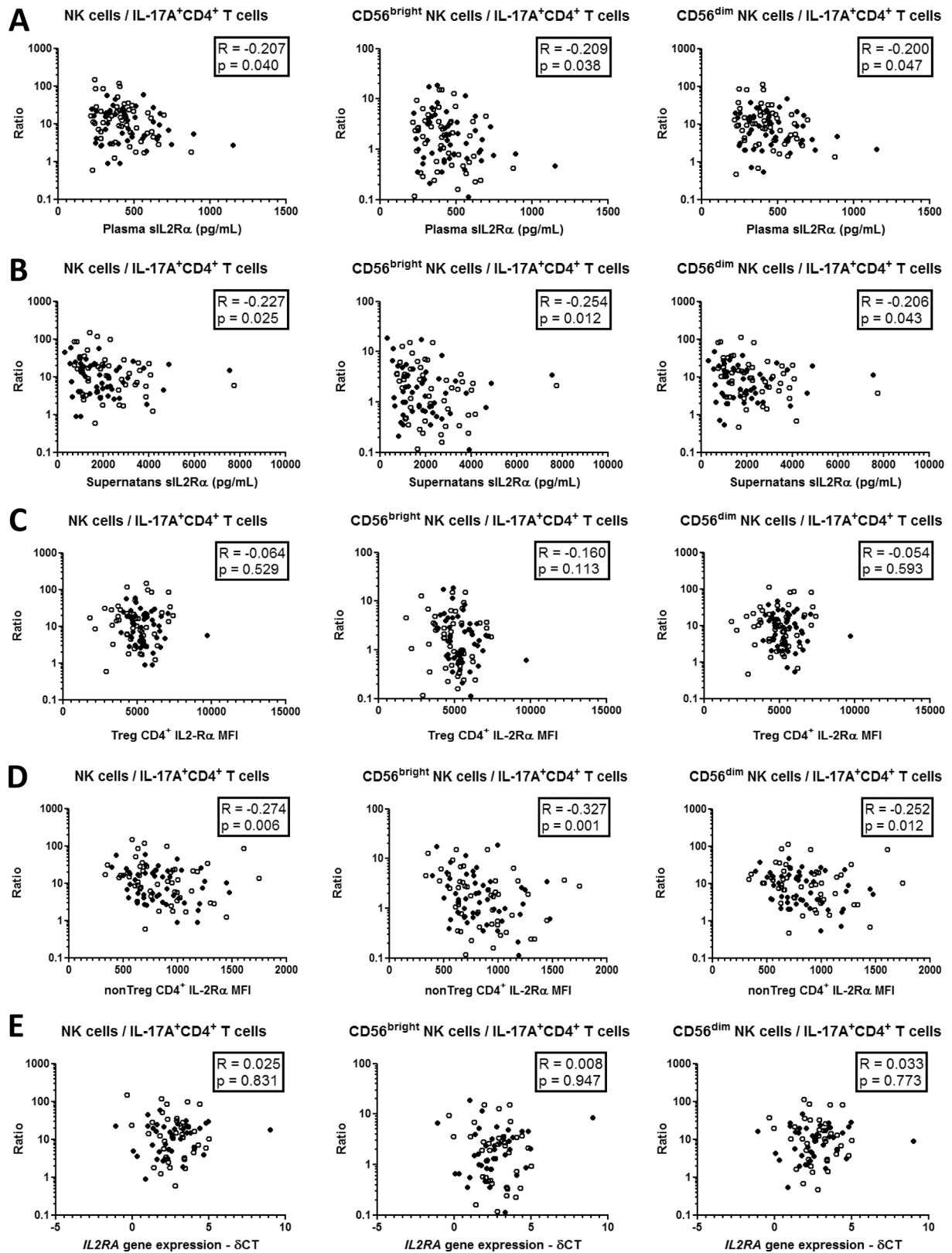
## 4. Discussion

We investigated the role of IL-2R $\alpha$  in the interplay between NK and T cells in a cohort study using a homogenous group of interferon beta-treated RRMS patients. We report associations between NK/IL-17A<sup>+</sup>CD4<sup>+</sup> T cell ratios and sIL-2R $\alpha$  protein shedding in vivo and in vitro, and IL-2R $\alpha$  protein expression by non-regulatory CD4<sup>+</sup> T cells. Furthermore, higher baseline serum sIL-2R $\alpha$  levels and lower CD56<sup>bright</sup>/IL-17A<sup>+</sup>CD4<sup>+</sup> T cell ratios associate with the rs3118470 risk allele.

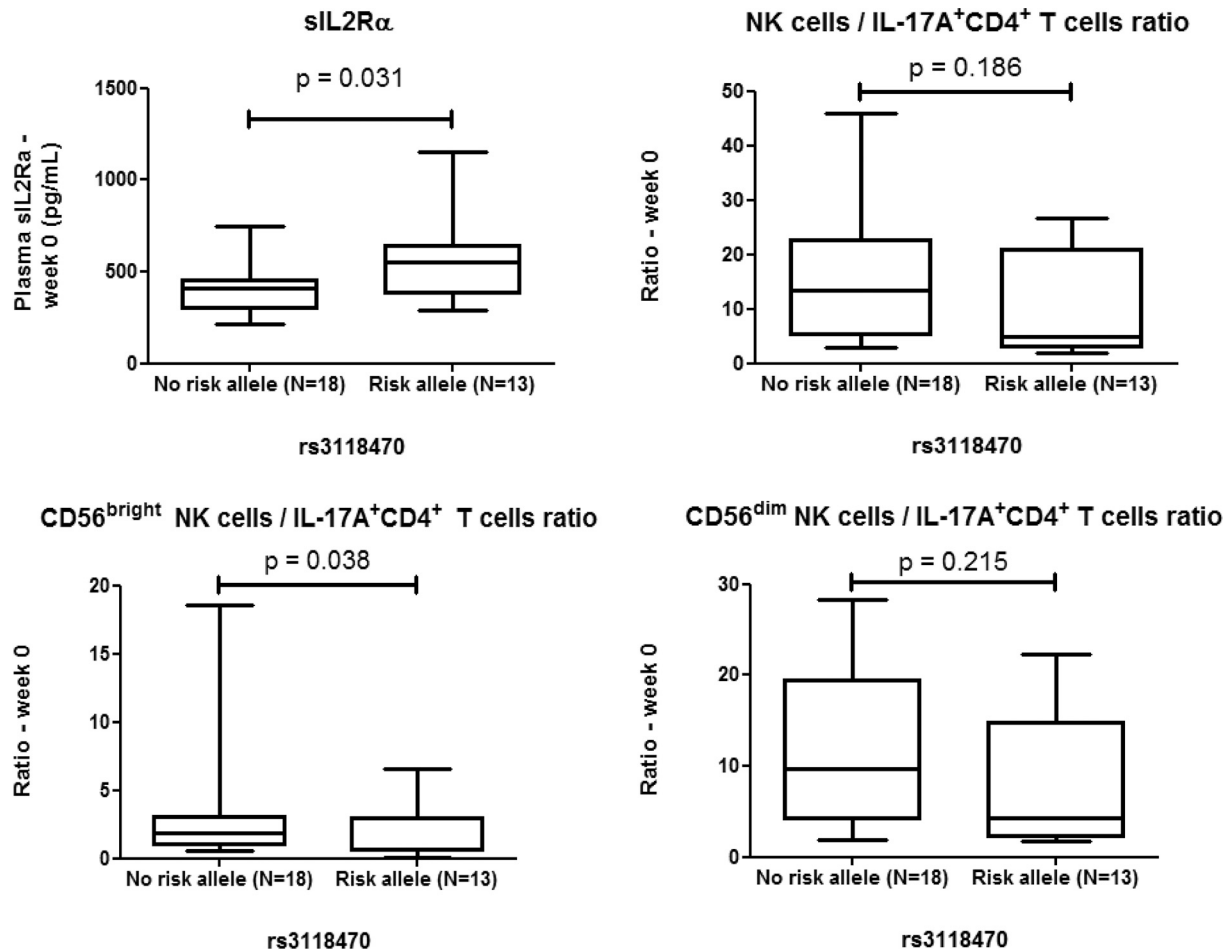
Our results may be interpreted in different ways. First, a higher sIL-2R $\alpha$  level may simply be the result of a lower NK/ T cell ratio. As a lower ratio would imply a reduced regulation of activated T cells by NK cells,



**Fig. 1.** A: Gating strategy used to analyse NK cells, T cells and their respective subsets. Step 1 shows the gating of lymphocytes from the PBMC population. Step 2 shows the gating of NK cells, defined as CD3<sup>-</sup>NKp46<sup>+</sup>, from the lymphocyte population. Step 3 shows the differentiation between the CD56<sup>dim</sup>CD16<sup>+</sup> NK cells (above) and CD56<sup>bright</sup>CD16<sup>-</sup> NK cells (below). Step 4 shows the gating of CD4<sup>+</sup>T cells, defined as CD3<sup>+</sup>CD4<sup>+</sup>, from the lymphocyte population. Step 5 shows the differentiation between regulatory T cells (Treg), defined as CD25<sup>+</sup>CD127<sup>-</sup> and other CD4<sup>+</sup> T cells (nonTreg). FSC: forward scatter; SSC: side scatter. B: Correlations between baseline and week 48 values of soluble IL-2 receptor alpha-chain (sIL2Rα) in serum, sIL2Rα in PBMC PHA culture, *IL2RA* gene expression, IL-2Rα MFI on Tregs and IL-2Rα MFI on nonTregs. R and p-value shown are based on Spearman rho analyses. MFI: mean fluorescence index; Treg: regulatory T cell; PHA: phytohaemagglutinin.



**Fig. 2.** Correlations between NK/IL-17A<sup>+</sup>CD4<sup>+</sup> T cells ratio, CD56<sup>bright</sup> NK/IL-17A<sup>+</sup>CD4<sup>+</sup> T cells ratio and CD56<sup>dim</sup> NK/IL-17A<sup>+</sup>CD4<sup>+</sup> T cells ratio, and A: soluble IL-2 receptor alpha-chain (sIL2Rα) in serum. B: soluble IL-2 receptor alpha-chain (sIL2Rα) in stimulated culture. C: *IL2RA* gene expression. D: IL-2 receptor alpha-chain expression on regulatory T cells. E: IL-2 receptor alpha-chain expression on non-regulatory CD4<sup>+</sup> T cells. R and p-value shown are based on spearman rho analyses. Baseline and week 48 values are pooled. Baseline values are represented by black dots, week 48 values are represented by open dots. MFI; mean fluorescence index; sIL2Rα: soluble IL-2 receptor alpha-chain; Treg: regulatory T cell.



**Fig. 3.** Influence of genetic profile on soluble IL-2 receptor alpha-chain (sIL2R $\alpha$ ) levels in serum, NK/IL-17A<sup>+</sup>CD4<sup>+</sup> T cells ratio, CD56<sup>bright</sup> NK/IL-17A<sup>+</sup>CD4<sup>+</sup> T cells ratio and CD56<sup>dim</sup> NK/IL-17A<sup>+</sup>CD4<sup>+</sup> T cells ratio. All shown measurements are baseline values. Box and whiskers plots showing median and 5th-95th percentiles. P-value shown is calculated using Mann-Whitney *U* tests.

this would mean that there are more activated non-regulatory T cells to shed IL-2R $\alpha$  and thus increase sIL-2R $\alpha$  levels. In this scenario, sIL-2R $\alpha$  would be a biomarker of higher T cell activity, causing disease activity, which could then also be expressed as a lower NK/T cell ratio. Alternatively, sIL-2R $\alpha$  could influence NK cells and/or T cells in such a way that the NK/T ratio is altered, thus leading to a reduced regulation of activated T cells and paving the way for disease activity. Unfortunately, interpretation of our results is hindered by the poor understanding of the role of sIL-2R $\alpha$  in immune activation and regulation. (Damoiseaux, 2020) Although our genetic data does not favour one interpretation over the other, it does further strengthen the implication that IL-2R $\alpha$  is involved in NK/T interplay.

This study has some limitations. The initial research question of the SOLARIUM study was focused on vitamin D<sub>3</sub> supplementation, which makes the current analysis exploratory. Additionally, only a limited set of IL-2/IL-2R pathway-related variables was explored, with IL-2 levels lacking. Finally, our cohort consists of patients using interferon beta, that transiently affects IL-2R $\alpha$  expression on CD4<sup>+</sup> T cells, which may have influenced our results. (Ferrarini et al., 1998)

In conclusion, we report a significant association between IL-2R $\alpha$  protein expression and shedding and the interplay between NK cells and T cells. The exact context and influence remain to be elucidated and, as such, more research is needed to investigate the exact mechanism of action in MS.

#### Declaration of Competing Interest

MM has nothing to disclose; LR has nothing to disclose; AH has nothing to disclose; OG received travel support, speaker honorarium and/or served on advisory boards by Biogen, Merck, Sanofi Genzyme and TEVA; GP is director of Drug Target ID, Ltd.; RH received institutional research grants and fees for lectures and advisory boards from Biogen, Merck, and Genzyme-Sanofi; JS received lecture and/or consultancy fees from Biogen, Merck, Sanofi-Genzyme, and Novartis; JD has nothing to disclose.

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