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RORγT is overexpressed in iNKT and γδ T cells during relapse in relapsing-remitting multiple sclerosis

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ABSTRACT

The aim of the current study is to evaluate IL-17 production and RORγT, and IL-23R expression by iNKT, Th17 and γδ T cells in the peripheral blood of relapsing-remitting multiple sclerosis patients. Samples of peripheral blood from 21 relapse patients and 12 remission patients, and 15 healthy volunteers were stained with monoclonal antibodies for flow cytometry analysis. No significant differences in iNKT, γδ T and Th17 percentages were noted. The significant overexpression of RORγT was observed in all three subpopulations – therefore, iNKT, γδ T and Th cells may be an important source of IL-17 shortly prior to the relapse.

1. Introduction

The invariant NKT (iNKT) type I cells are a small subsets of innate T cells expressing specific TCR – Vα24-Jα18. Once stimulated, they are capable of both potent cytotoxic activity and rapid cytokine production with IL-17A among them (Podbielska et al., 2018). There are limited data on the contribiution of iNKT cells to the pathogenesis of multiple sclerosis (Van Kaer et al., 2015). Nevertheless, a decrease in iNKT percentage has been observed in multiple sclerosis (MS) patients (Araki et al., 2003; Illés et al., 2000). The therapy with IFN-β1 seems to restore the percentage of iNKT in peripheral blood of MS patients (Gigli et al., 2007). Those studies focused rather on the regulatory potential of iNKT cells than on the possible pathogenic role. The γδ T cells comprise up to 5% of the total T cells in peripheral blood and they have also been remarkably more studied in multiple sclerosis. In an animal model of MS, even up to 80% of central nervous system infiltrating γδ T cells are IL-17+ (Sutton et al., 2009). The results of in vivo experiments on animal model of multiple sclerosis suggest both the importance of γδ T cells for disease pathogenesis and the ambivalent function therein (Clark and Lingenheld, 1998; Odyniec et al., 2004; Ponomarev et al., 2004; Ponomarev and Dittel, 2005).

Although the significance of IL-17 for the pathogenesis of multiple sclerosis is not yet fully understood and the opinions vary from necessary to dispensable (Waisman et al., 2015), the results of clinical trial of an anty-IL-17A monoclonal antibody, secukinumab, in multiple sclerosis indicate the importance of IL-17 in the pathogenesis of relapsing-remitting multiple sclerosis (Ochi, 2014).

The aim of the current study is to evaluate IL-17 production and RORγT, and IL-23R expression by iNKT, Th17 and γδ T cells in the peripheral blood of relapsing-remitting multiple sclerosis patients. The hypothesis is that the IL-17+ percentage of those three populations is increased in MS patients compared to healthy controls and that it is also higher during relapse than remission.

2. Materials and methods

2.1. Study group

The current study included patients diagnosed according to revised MacDonald criteria, who were hospitalized in the Clinic of Neurology, Medical University of Lublin (Lublin, Poland) and fulfilled the inclusion criteria: 1) no other autoimmunological disease diagnosed, 2) no glucorticoid treatment during 4 weeks prior to blood collection, 3) no history of any neoplasm or any neurosurgical treatment. The control group was comprised of healthy volunteers who: 1) were not hospitalized for 6 weeks prior to blood collection, 2) were not diagnosed with any autoimmune, neurological or oncologic disease, 3) had not undergone neurosurgical treatment in the past, 4) had no I degree relative with diagnosis of MS. All subjects signed written informed consent. A 10 ml sample of peripheral blood from each participant was collected

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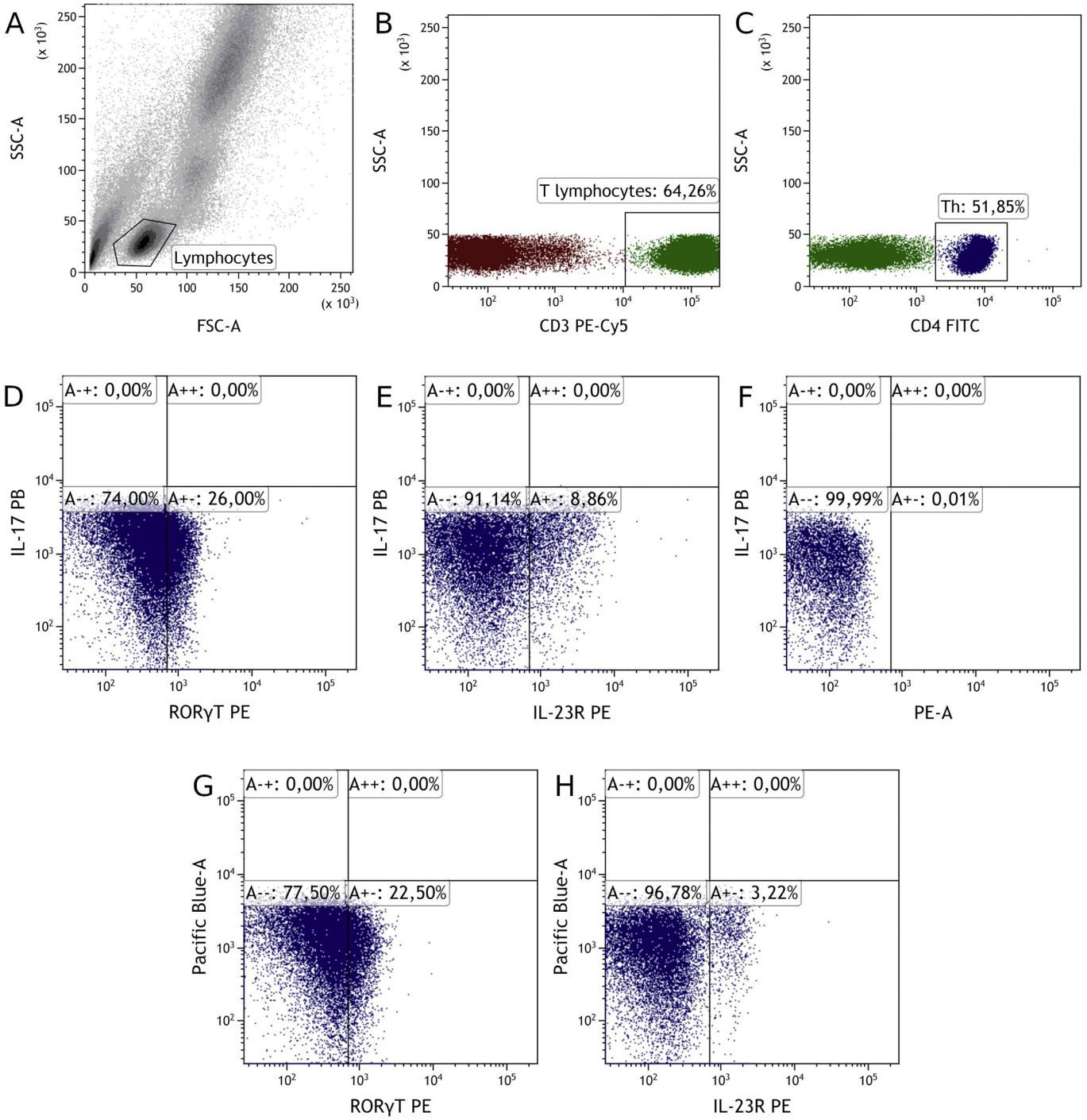

Fig. 1. The gating strategy for T helper cells. The Th17 cells were gated as $CD3^+$ $CD4^+$ $IL\text{-}17^+$. Panels F—H present the FMO controls. The Th17 cells were gated as $CD3^+$ (B), $CD4^+$ (C) and $IL\text{-}17^+$ (D). The percentage of $IL\text{-}17^+$ Th cells was calculated as the sum of two upper quadrants (D), while for the $IL\text{-}23R^+$ and $ROR\gamma T^+$ cells both right quadrants were summed up. The double positive population (either $IL\text{-}17^+/ROR\gamma T^+$ or $IL\text{-}17^+/IL\text{-}23R^+$) was gated as the upper right quadrant (D, E). Panel F shows the FMO used to set the gate for IL-23R and RORγT, panel G and H depicts FMO controls for IL-17 either with RORγT or IL-23R.

into EDTA-tube. The study protocol was approved by the Bioethical Committee at the Medical University of Lublin (KE-0254/293/2016).

2.2. Flow cytometry

The following monoclonal antibodies were used: anti-CD3 PE-Cy5 (BioLegend, San Diego, CA, USA), anti-CD4 FITC (BioLegend, San Diego, CA, USA), anti-IL-17A PE (BioLegend, San Diego, CA, USA), anti-IL-17A Pacific Blue (BioLegend, San Diego, CA, USA), anti-TCRγδ FITC (BioLegend, San Diego, CA, USA), anti-RORγT PE (BioLegend, San Diego, CA, USA), anti-IL-23R PE (BioLegend, San Diego, CA, USA), anti-iNKT FITC (BioLegend, San Diego, CA, USA). An adequate amount of monoclonal antibodies against surface antigens were added to 100 µl of whole blood and left in darkness for 20 min of incubation. Two ml of FACS Lysing Solution (Becton Dickinson, Franklin Lakes, NJ, USA) were added thereafter to lyse the erythrocytes, permeabilise and fix the cells (Bekkema et al., 2008). The samples were then centrifuged for 5 min at 700 xg. The supernatant was discarded and the precipitate was suspended in 2 ml of PBS and centrifuged for 5 min at 700 xg. The last step was repeated twice. The antibodies against intercellular antigens were

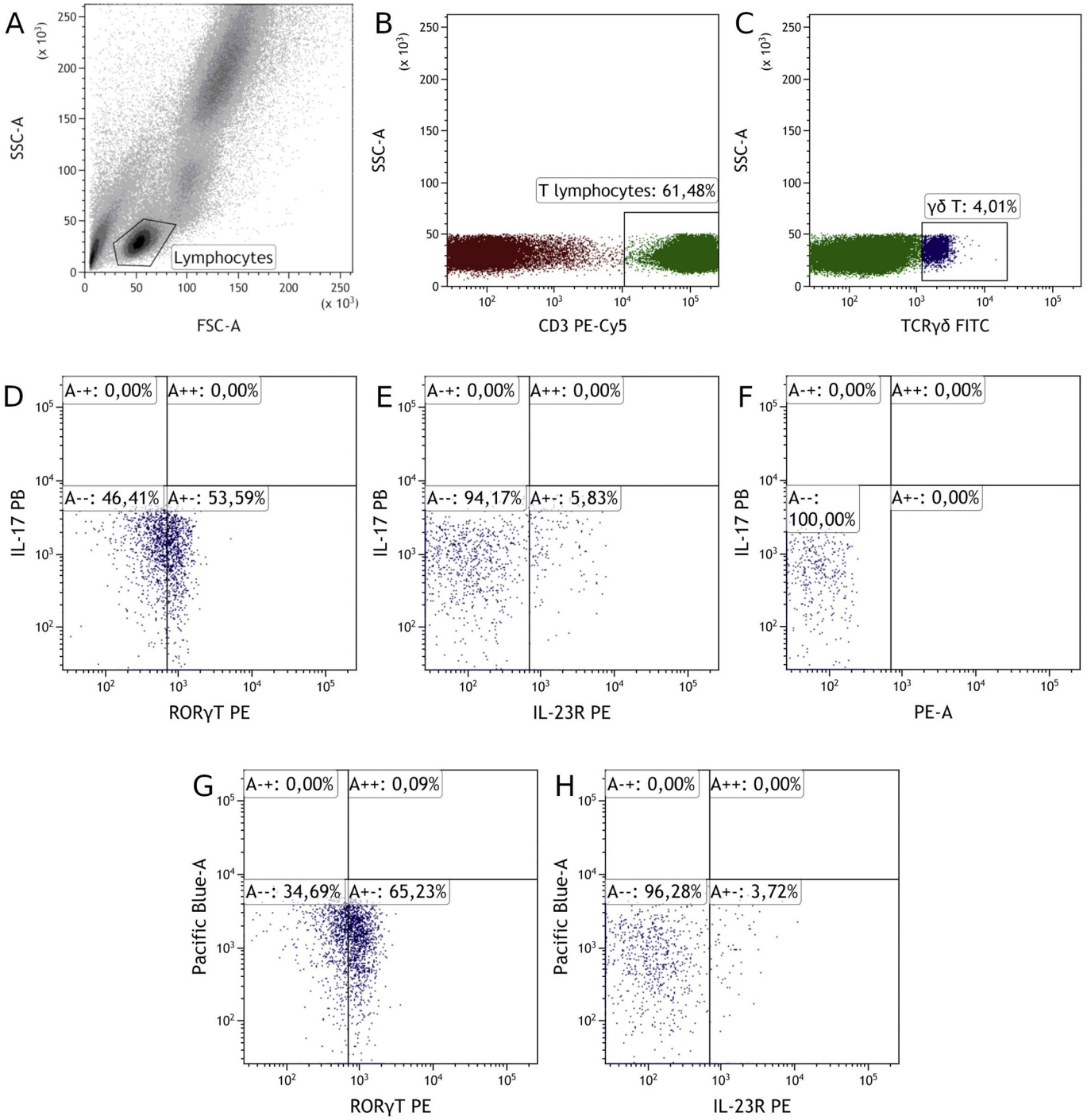

Fig. 2. The gating strategy for γδ T cells. Panels F–H present the FMO controls. The percentage of IL-17$^+$ γδ T cells was calculated as the sum of two upper quadrants (D), while for the IL-23R$^+$ and RORγT$^+$ cells both right quadrants were summed up. The double positive population (either IL-17$^+$/RORγT$^+$ or IL-17$^+$/IL-23R$^+$) was gated as the upper right quadrant (D, E). Panel F shows the FMO used to set the gate for IL-23R and RORγT, panel G and H depicts FMO controls for IL-17 either with RORγT or IL-23R.

added thereafter and the samples were again incubated for 20 min. Finally, two ml of PBS were added to each sample prior to final 5 min centrifugation at 700 xg. The flow cytometry was performed with either BD FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA) or BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescence minus one (FMO) controls were used to set proper gates. Kaluza 2.1.1 (Beckman Coulter, Miami, FL, USA) was used for the data analysis. The gating was performed as depicted in Figs. 1–3. The results of flow cytometry are presented as the percentage of positive cells.

2.3. Cell sorting

The peripheral blood mononuclear cells (PBMC) were isolated from the whole blood by density gradient centrifugation in Gradisol L (Aqua-Med, Łódź, Poland). PBMC were then incubated for 20 min with anti-TCRγδ FITC (BioLegend, San Diego, CA, USA), anti-CD4 PE-Cy5 (BioLegend, San Diego, CA, USA) and anti-iNKT PE (BioLegend, San Diego, CA, USA) antibodies. The excess of antibodies were then washed out by adding PBS and centrifuging for 5 min at 700 x g. The iNKT, γδT and Th cells were then sorted with BD FACS Aria II (Becton Dickinson,

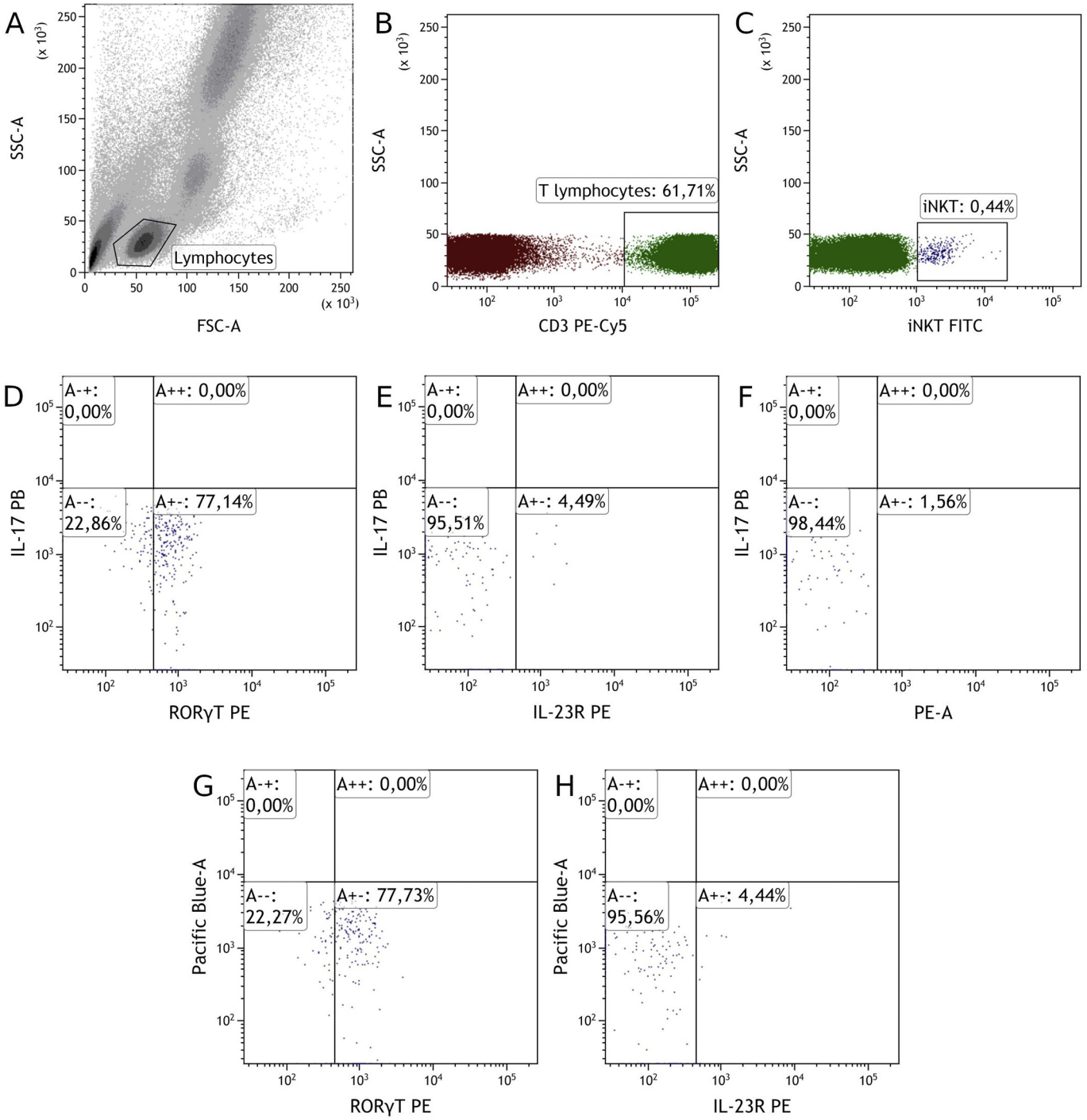

Fig. 3. The gating strategy for γδ T cells. Panels F–H present the FMO controls. The percentage of IL-17$^+$ iNKT cells was calculated as the sum of two upper quadrants (D), while for the IL-23R$^+$ and RORγT$^+$ cells both right quadrants were summed up. The double positive population (either IL-17$^+$/RORγT$^+$ or IL-17$^+$/IL-23R$^+$) was gated as the upper right quadrant (D, E). Panel F shows the FMO used to set the gate for IL-23R and RORγT, panel G and H depicts FMO controls for IL-17 either with RORγT or IL-23R.

Table 1
Sociodemographic data of MS patients. The data is presented as median (IQR).

Group	Age	EDSS	ARR	Years after diagnosis
MS	42.5 (IQR 23.25)	3.5 (IQR 2.875)	1 (IQR 2)	8.5 (IQR 9.246)
Control	48 (IQR 36)			

Franklin Lakes, NJ, USA). The sorting strategy is depicted in Supplement Fig. 1.

2.4. RT-qPCR

Sorted cells were immediately used for RNA isolation. The High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) was used, the standard manufacturer protocol was followed. The isolated RNA was then stored at -80 °C. Next, the reverse transcription was performed with the Transcriptor FirstStrand cDNA Synthesis Kit (Roche

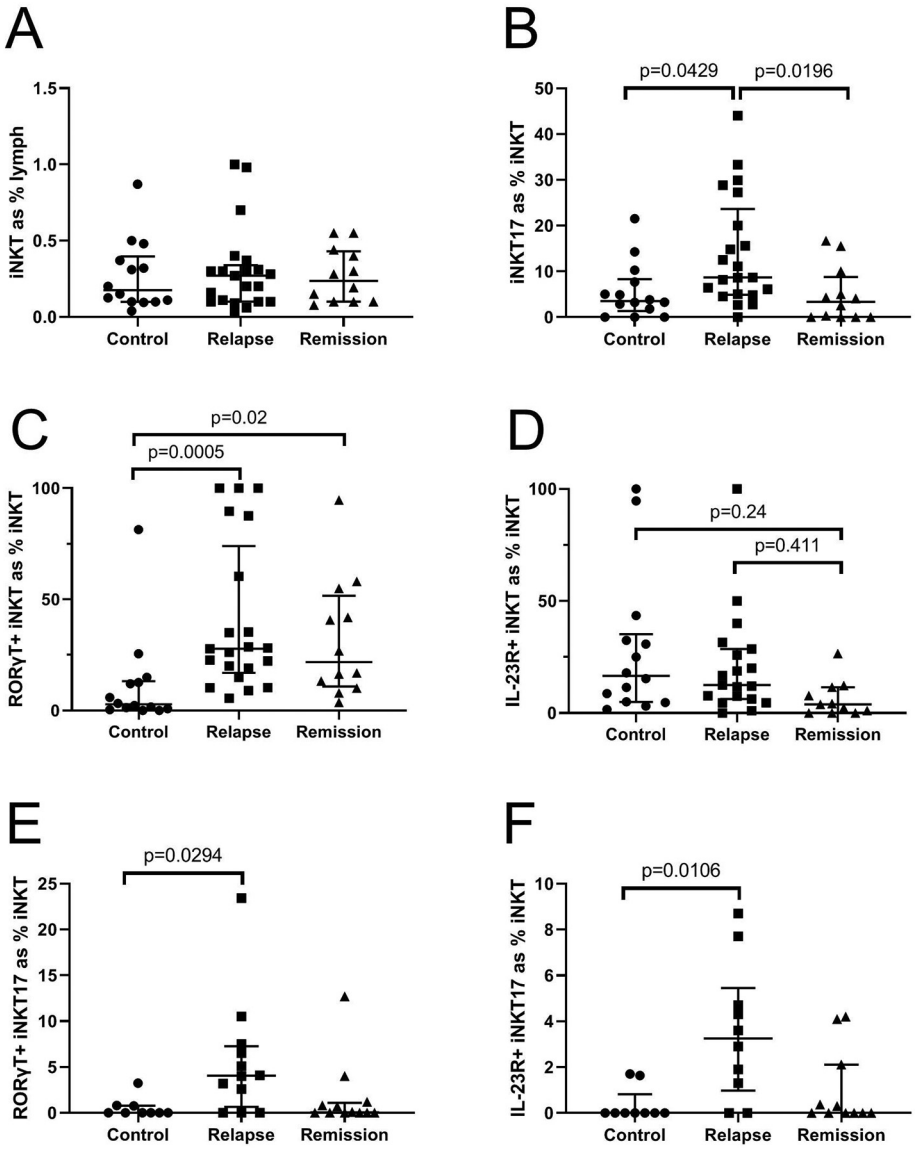

Fig. 4. iNKT cells by groups. The data is presented as median and IQR.

Applied Science, Mannheim, Germany). The cDNA was then stored at -20 °C for further use. Finally, the qPCR was performed. The TaqMan FastAdvanced MasterMix (Applied Biosystems, Austin, TX, USA) TaqMan Gene Expression RORC (Applied Biosystems, Austin, TX, USA, Hs01076112_m1), TaqMan Gene Expression IL-23R (Applied Biosystems, Austin, TX, USA, Hs00332759_m1) and TaqMan Gene Expression IL-17A (Applied Biosystems, Austin, TX, USA, Hs00174383_m1) were used. Human ACTB (Beta Actin) Endogenous Control (Applied Biosystems, Austin, TX, USA, 4326315E) was used for gene expression normalisation, The 20 µl protocol with TaqMan FastAdvanced MasterMix was followed. Briefly – the polymerase was activated by 2 min hold in 95 °C, followed by 45 cycles of 3 s denaturation in 95 °C and 30 s annealing in 60 °C. The Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Austin, TX, USA) was used. The relative expression was calculated as $2^{-\Delta CT}$.

2.5. Statistical analysis

The statistical analysis was performed with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The distribution of data was determined with the Shapiro Wilk test. The Kruskal-Wallis test with Dunn correction was used for the calculation of statistical significance of flow cytometry data and U Mann-Whitney test for RT-qPCR results. The level of significance was set as $p < .05$. The data are presented as the median and IQR. The Spearman's rho was employed for the correlation analysis, rho values between 0.30 and 0.59 were considered as fair correlation, between 0.60 and 0.79 as moderate correlation and above 0.80 as very strong correlation as proposed by Chan (Akoglu, 2018; Chan, 2003).

3. Results

3.1. Study group

A total of 33 MS patients and 15 healthy individuals had been included in the current study. The former group comprised 21 patients during relapse and 12 during remission. All participants were women. The socio-demographic data are presented in Table 1. More than one third of patients was receiving no persistent treatment, similar percentage of patients was taking natalizumab, the remaining one third was taking one of the following: daclizumab, glatiramer acetate, dimethyl fumarate, cladribine or interferon beta-1b.

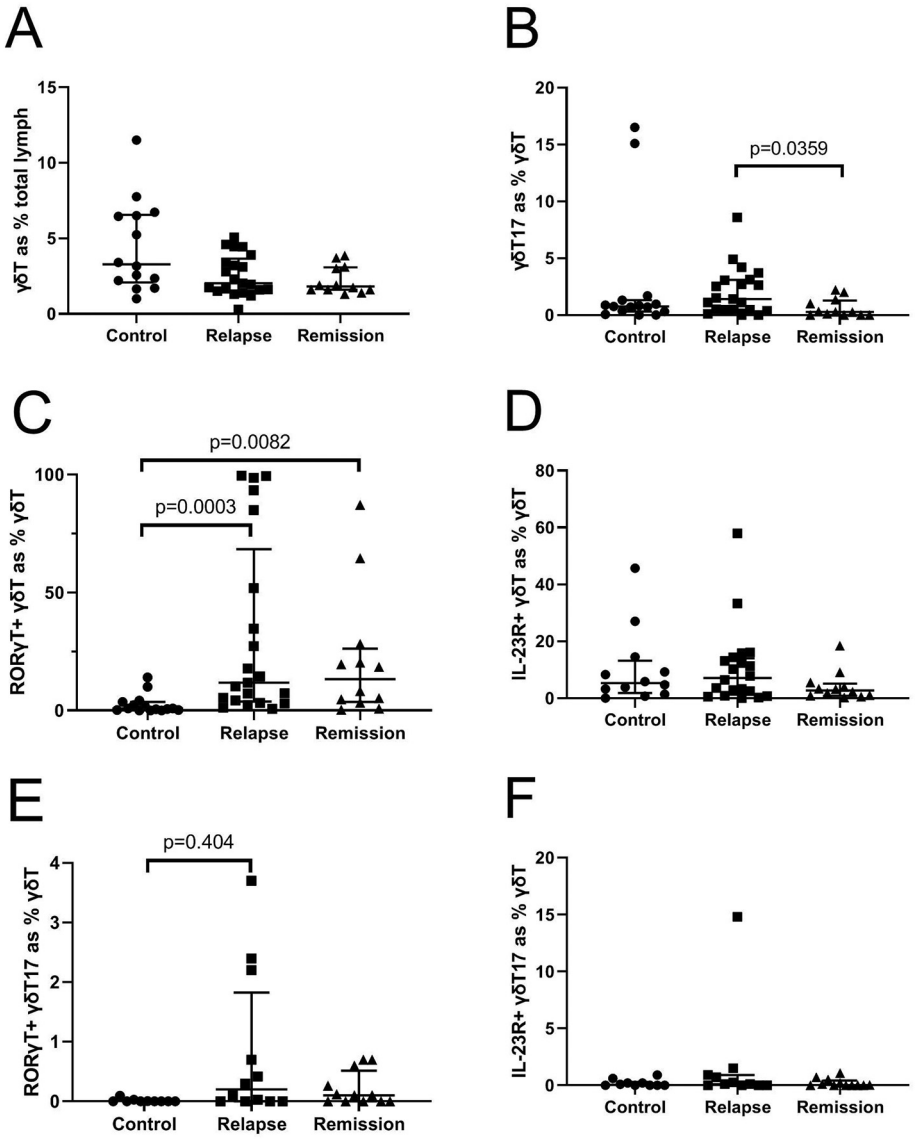

Fig. 5. γδ T cells by groups. The data is presented as median and IQR.

3.2. The RORγT is overexpressed in iNKT, γδ T and Th cells in MS patients

A statistically higher expression of RORγT in iNKT cells was observed during both relapse and remission comparing to the control group (Fig. 4C). MS patients had statistically lower expression of IL-23R in iNKT cells when compared to the control group (Fig. 4D). The percentage of IL-17+ cells was higher during relapse than both in control and remission (Fig. 4B). Similarly, a higher percentage of IL-23R+ or RORγT+ iNKT17 cells was observed during relapse than in control (Fig. 4E, F).

The percentage of γδ T cells among the total lymphocytes was insignificantly lower both in remission and relapse when compared to the control group (Fig. 5A). The percentage of IL-17+ γδ T cells was significantly higher during relapse than in remission (Fig. 5B) while the IL-17+ RORγT+ γδ T cells were significantly increased during relapse comparing to control group (Fig. 5E). Similarly, the percentage of γδ T cells expressing RORγT was significantly higher in both groups of patients when compared to the healthy individuals (Fig. 5C). No statistical significance was observed in other measurements (details in Fig. 5).

No significant difference was observed in the case of Th cells as a percentage of total lymphocytes, RORγT or IL-23R positive Th cells, Th17 as a percentage of Th cells and expression of IL-23R in Th17 cells. The percentage of RORγT$^+$ Th17 was significantly higher in remission and relapse when compared to the control group (Fig. 6E). Moreover, it was also higher in Th cells during relapse compared to control group (Fig. 6C).

3.3. RORC in γδ T cells is significantly overexpressed in MS patients

To confirm the results of the flow cytometry, we analysed the relative expression of RORC in all three subsets of cells. No significant difference was noted in iNKT (Fig. 7B) and Th (Fig. 7C) cells, but a significant ovexpression in γδ T cells was observed (Fig. 7A). The relative expression of IL-23R in iNKT cells was significantly higher in MS patients (Fig. 7E). IL-17A was significantly overexpressed in MS patient Th cells (Fig. 7 I).

3.4. iNKT17 percentage moderately correlates to ARR and EDSS

To assess whether the clinical indicators of disease activity correlate with the immunological parameters the Spearman's correlation coefficient (rho) was calculated. A moderate positive correlation was noted for iNKT17 percentage and EDSS, and ARR values. Similar correlation was also observed for the percentage of iNKT17 RORγT+ cells and

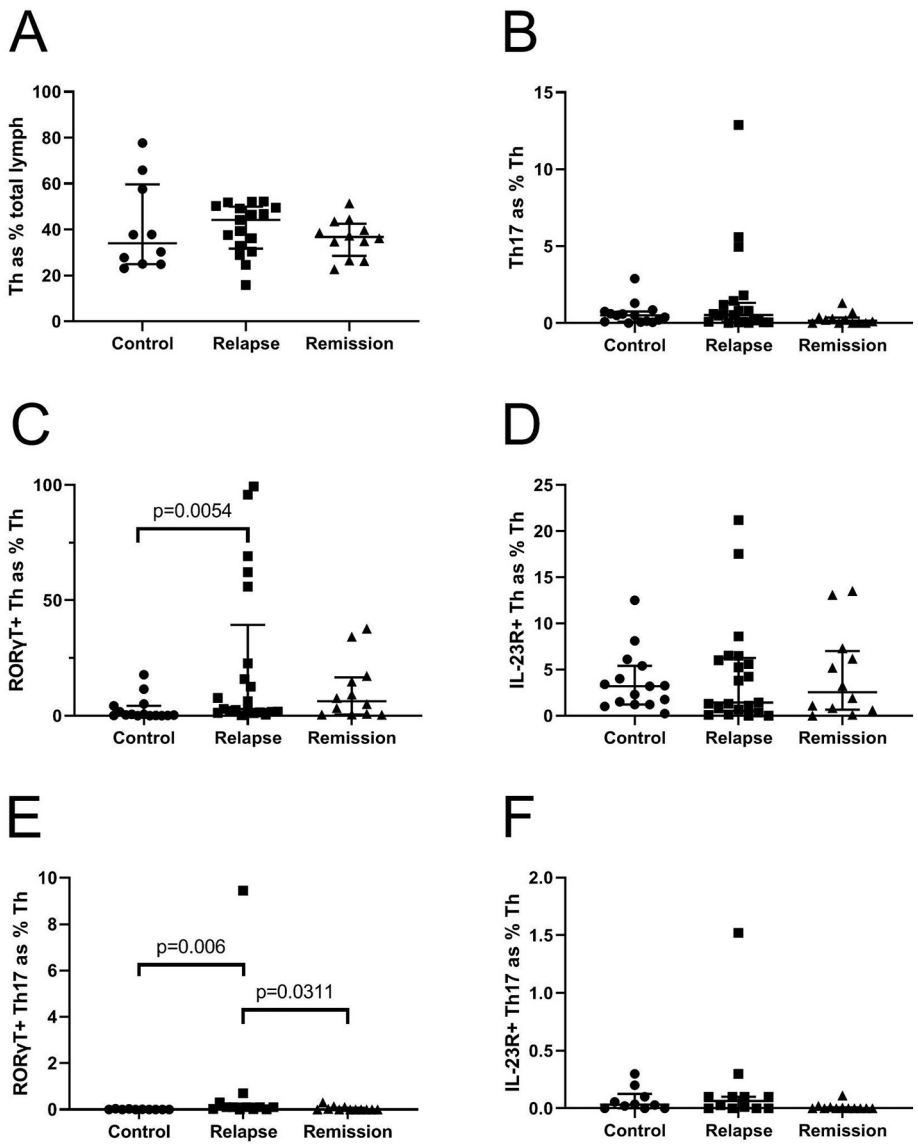

Fig. 6. Th cells by groups. The data is presented as median and IQR.

ARR. A moderate negative correlation was observed for Th17 IL-23R + cell percentage and years since diagnosis. A moderate negative correlation was also noted for the percentage of IL-23R + Th and Th17 cells on the one hand and patient age on the other. The full matrix of correlation coefficients is presented in Supplementary Table 1.

4. Discussion

No significant difference was observed in the sole percentage of the iNKT cells between groups, which diverges from the results of Illés Z. et al. and van der Vliet HJ et al., who observed significant reduction of iNKT cells (Illés et al., 2000; Van Der Vliet et al., 2001). In the former study, a different methodology was used, and in the latter study the authors did not clearly state the form of MS. Both issues may be the reasons for the different results. Araki M. et al. have observed a statistically insignificant reduction of iNKT cell percentage in relapse and significant in remission (Araki et al., 2003), which is in contrary to the results of the current study, as a noticeable (but insignificant) increase in both remission and relapse were observed. It is possible that the changes in iNKT cells are not related to their percentages, but rather to their functional state, as suggested by the clonal expansion of iNKT cells during relapse reported by Démoulins T. et al. (Démoulins et al., 2003). While there are limited studies on the role of iNKT in multiple sclerosis, numerous papers have been published regarding those cells in an animal model of the disease. Those, however, are highly inconclusive and in many cases present opposite results (Van Kaer et al., 2015). The results of the present study indicate that iNKT cells may be involved in the pathogenesis of MS relapses. The significant increase in the percentage of iNKT IL-17 + (iNKT17) cells (Fig. 1B) shows that the iNKT17 cells may be partially responsible for relapses. This is further supported by the significantly higher percentage of RORγT expression in iNKT cells, as the latter is a IL-17-associated transcription factor.

An insignificant decrease in γδ T cell percentage was observed in the current study, which is in line with the studies by Nick et al. and Paź et al. (Nick et al., 1995; Paź et al., 1999). Nevertheless, an increase was noted by Stinissen et al. and Schirmer et al., and significant decrease was observed by Ramos et al.(Ramos et al., 2016; Schirmer et al., 2013; Stinissen et al., 1995)(Nick et al., 1995; Paź et al., 1999; Schirmer et al., 2013; Stinissen et al., 1995). Despite different results obtained by Schirmer et al. in terms of the percentage of total γδ T cells, they, similarly to the current study, observed no significant difference in γδ T IL-17 + cells in peripheral blood between control subjects and MS patients. Moreover, a significantly increased RORγT expression in γδ T cells was noted in the current study. This corresponds to the data on the

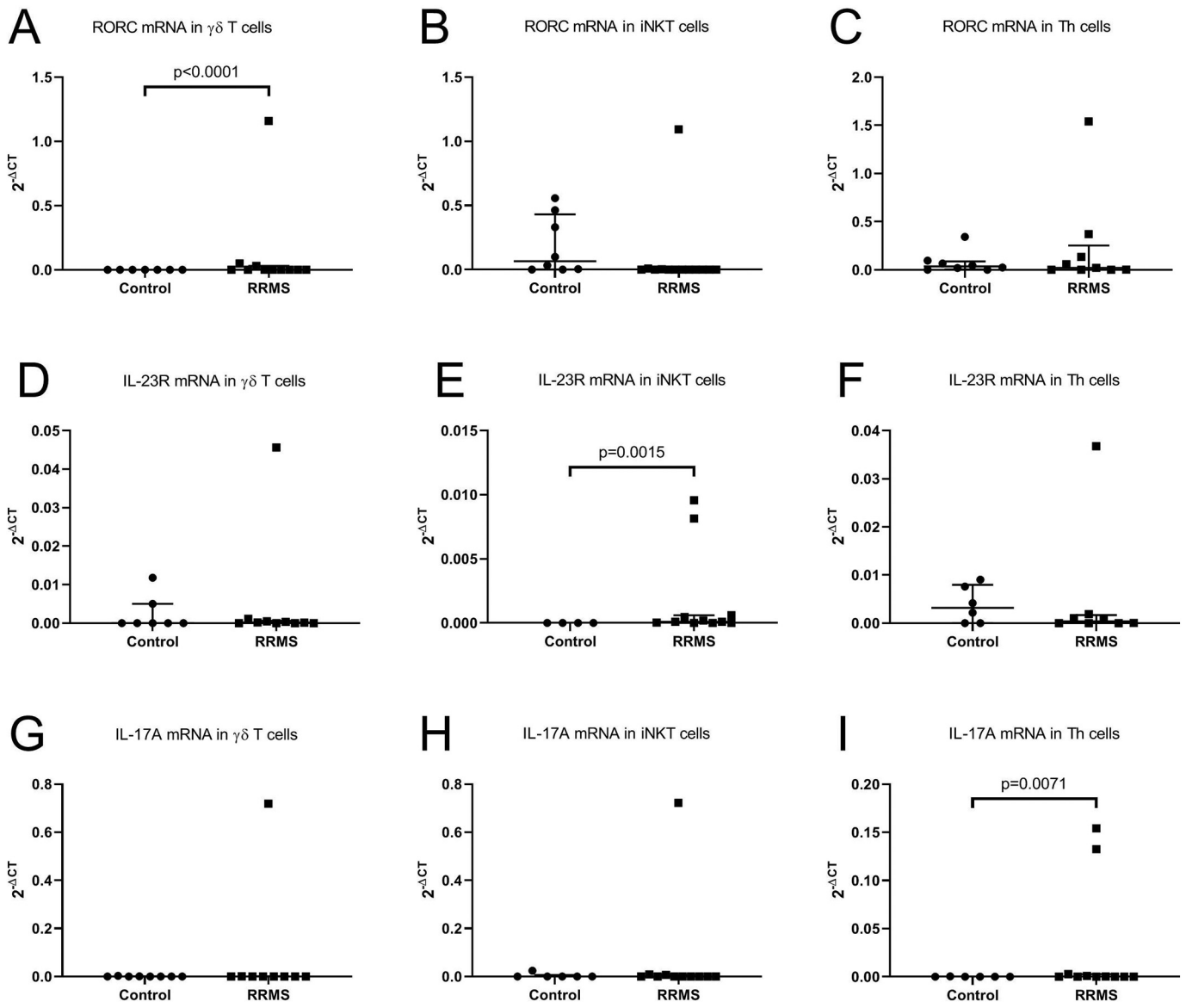

Fig. 7. The RORC, IL-23R and IL-17A relative expression in γδ T, iNKT and Th cells. The data is presented as median and IQR.

role of γδ T cells in the pathogenesis of experimental autoimmune encephalitis (EAE). Lalor et al. observed a significantly higher percentage of IL-17+ γδ T cells at the onset of disease (Lalor et al., 2011), and according to Sutton et al., γδ T cells are an important source of IL-17, needed to promote production thereof by Th17 cells and amplify the response (Sutton et al., 2009). The overexpression of RORγT along with no significant difference in IL-17+ γδ T cell percentage may be a sign of a prior involvement in the initiation of relapse.

The expression of RORγT is significantly up-regulated in multiple sclerosis (Etesam et al., 2016). Moreover, the RORγT overexpression in transgenic mice is linked with more severe course of experimental autoimmune encephalitis (Martinez et al., 2014). RORγT is a major transcription factor of Th17 and other IL-17-producing cells and its expression is at least partially dependent on IL-23 – IL-23R interaction (Chen and Shannon, 2013). The expression of RORγT is also regulated by microRNAs, the disregulation thereof was observed in multiple sclerosis (Majd et al., 2018; Zhu et al., 2014). Moreover, retinol and retinoic acid are probably also involved therein (Mohammadzadeh Honarvar et al., 2013; Mousavi Nasl-Khameneh et al., 2018; Raverdeau et al., 2016).

A statistically significant overexpression of RORγT in all three studied subpopulations suggests their involvement in the pathogenesis of multiple sclerosis. It may be possible that all three subsets produce IL-17 during the initiation of relapse.

4.1. Conclusions

As a significant intergroup differences in IL-17+ iNKT cells was observed, it seems that the role of iNKT cells in IL-17 production may be of importance for the pathogenesis of relapse. Even though there was no significant difference in IL-17+ positive cells among Th and γδ T lymphocytes, the RORγT seemed to be overexpressed in all three populations during MS course. Therefore, it seems that γδ T, iNKT and Th cells as IL-17 source are of major importance shortly prior to the relapse.

4.2. The limitations of the study

The studied group was homogenous, but limited in size. There was no measurement of serum IL-17 levels, nor was there any assessment of cerebrospinal fluid circulating cells.

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Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.577046.

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