Immunology Letters 216 (2019) 63–69

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

CD3$^-$CD56$^+$ NK cells display an inflammatory profile in RR-MS patients

Ilhan Tahrali[a], Umut Can Kucuksezer[a], Nilgun Akdeniz[a], Ayse Altintas[b,c], Ugur Uygunoglu[c], Esin Aktas-Cetin[a], Gunnur Deniz[a,*]

[a] Istanbul University, Aziz Sancar Institute of Experimental Medicine, Department of Immunology, Istanbul, Turkey
[b] Koc University, Faculty of Medicine, Department of Neurology, Istanbul, Turkey
[c] Istanbul University Cerrahpasa, Cerrahpasa Faculty of Medicine, Department of Neurology, Istanbul, Turkey

ARTICLE INFO

Keywords:
IL-22
Multiple sclerosis
Natural killer cells
NKG2A
NKG2D
NKp44

ABSTRACT

Multiple Sclerosis (MS) is an immune-mediated and neurodegenerative disease of central nervous system. Relapsing-remitting (RR)-MS occurring with acute attacks and remissions, is the most common clinical type of MS. There are different strategies applied in first-line treatment of RR-MS patients such as interferon-beta (IFN-β) and glatiramer acetate. In this study, activating and inhibitory receptor expressions and interleukin (IL)-22 levels of NK cells were investigated in RR-MS patients with or without IFN-β therapy. Activating receptor expression and IL-22 levels of NK cells were increased in RR-MS patients under IFN-β therapy. Elevated NK cells with activating profile and increased IL-22 under IFN-β therapy suggest that IFN-β treatment might direct NK cells toward a pro-inflammatory status.

1. Introduction

Multiple Sclerosis (MS) is an immune-facilitated, chronic, neurodegenerative and demyelinating inflammatory central nervous system (CNS) disorder. The subgroups of MS are defined by the clinical course [1]. Relapsing-remitting (RR)-MS constitutes about 85–90% of MS patients and it occurs with acute attacks (relapses) and remissions [2]. The majority of MS patients initially present a single attack known as clinically isolated syndrome (CIS) [3]. Although the pathogenesis of MS is not well defined, autoreactive CD4$^+$ T cells are thought to be responsible for attacking myelin antigens that cause tissue damage and inflammatory responses resulting in demyelination and neuronal damage [4,5]. Also, several studies revealed the contribution of the cells of innate immunity including Natural Killer (NK) cells in MS pathogenesis [6]. Because of the regulatory and cytotoxic functions of NK cells, their roles in MS pathogenesis has gained importance in recent years.

NK cells comprising 5–15% of leukocytes in healthy individuals are characterized phenotypically by the expression of CD56 but not CD3 molecule on their surface membrane. CD56 expression of NK cells is increased upon activation [7–9] suggesting CD56 as an activation marker [10] whereas immunosuppressive agents reduce their frequencies [11].

NK cells are well known by their cytolytic capabilities in early defense against tumor cells as well as pathogens, while having immunoregulatory properties through cytokine secretion revealing their importance in innate immunity [12]. Cellular functions of NK cells are governed by the balance between signals of activating and inhibitory receptors present on NK cells [13,14]. Activating receptors include NKG2D (Natural killer group 2, member D), 2B4 and the natural cytotoxicity receptors (NCRs) such as NKp30, NKp44 and NKp46. These receptors bind ligands induced by infection, tumors or cellular stress [15] and deliver activating signals to NK cells for cytotoxicity or cytokine secretion. NK cell activation is mainly regulated by the inhibitory receptors specific for MHC class I molecules [16]. Killer-cell immunoglobulin-like receptors (KIRs) and CD94-NKG2A heterodimer are the main groups of inhibitory MHC class I–specific receptors [17].

NK cells might be classified by their cytokine secretion profiles such as NK1, NK2 or NK22 cells. NK22 cells produce interleukin-22 (IL-22) upon stimulation with IL-12, IL-18 and IL-23 [18,19] whereas IL-27 suppresses the secretion of IL-22 from CD4$^+$ T cells [20]. IL-22 is a pro-inflammatory cytokine with a protective role in the regeneration of epithelial tissues and host defence in mucosal barriers such as intestine, oral mucosa or lung [21]. There are also many studies demonstrating the possible contribution of IL-22 in neurological diseases [22]. However, contradictory findings were obtained from the studies investigating the involvement of IL-22 in autoimmune diseases [23–25]. IL-22RA2 was defined as a risk gene [26] and an inflammatory role was

* Corresponding author at: Istanbul University, Aziz Sancar Institute of Experimental Medicine, Department of Immunology, Vakif Gureba St., 34093, Istanbul, Turkey.
E-mail address: gdeniz@istanbul.edu.tr (G. Deniz).

https://doi.org/10.1016/j.imlet.2019.10.006
Received 26 June 2019; Received in revised form 22 September 2019; Accepted 3 October 2019
Available online 04 October 2019

attributed to IL-22 in MS [27,28]. Recently, we have demonstrated a tendency to increase in IL-22 levels of NK cell subsets in RR-MS patients in response to cytokine stimulations [29]. However, the possible contribution of IL-22 in MS pathogenesis still needs to be elucidated.

In this study, to investigate the activation status of $CD3^{-}CD56^{+}$ NK cells in treated and untreated RR-MS in comparison with CIS patients, expression of NKG2A, NKG2D and NKp44 were analysed. In addition, plasma IL-22 levels as well as IL-22 content of $CD3^{-}CD56^{+}$ NK cells in response to IL-2, IL-10, IL-12 and IL-27 were measured for exploring the possible contribution of IL-22 in RR-MS pathogenesis.

2. Materials and methods

2.1. Subjects

Istanbul University Cerrahpasa, Cerrahpasa Faculty of Medicine, Department of Neurology followed up the patients and directed them for enrollment to this study. This study was approved by Local Ethical Committee of Istanbul University Cerrahpasa, Cerrahpasa Faculty of Medicine, in compliance with Helsinki declaration and written informed consents were taken from all patients subsequent to their enrollment. The study group consisted of untreated (n = 9) and interferon-beta (IFN-β) treated (at least 6 months) RR−MS patients (n = 11) in remission phase fulfilling 'McDonald' criteria for diagnosis of MS [30]. CIS patients (n = 10) were enrolled to this study at least 3 months after the last steroid intervention (Table 1). Neither of the patients received steroid treatment before or during blood sampling. Patients were negative for hepatitis B virus and did not have any comorbities.

2.2. Flow cytometry

Ex vivo frequencies of NK cells and NK cell receptors were determined from fresh heparinized blood samples of donors. $CD3^{-}CD56^{+}$ NK cells were quantified by fluorochrome-labeled monoclonal antibodies (mAbs); anti-CD3-FITC, -CD56-PE-Cy7 and isotype controls (all from Biolegend, San Diego, CA). In separate tubes, PE-labeled anti-NKG2A (R&D System, Minneapolis CA), -NKG2D (Bio-Rad AbD Serotec) and -NKp44 (Biolegend, San Diego CA) mAbs were used to detect NK cell receptors. Following staining, a FACS Lysing Solution (BD Biosciences, San Jose, CA) was utilized for erythrocyte lysing, and cells were washed with phosphate buffered saline (PBS). All incubations were executed in the dark at room temperature. BD FACSCalibur II flow cytometer running Cell Quest Software (BD Bioscience, San Jose, CA) was utilized for cell acquisition and data analyses.

2.3. Cell preparation and culture

Peripheral blood mononuclear cells (PBMCs) were purified from heparinized blood samples which were obtained by Ficoll-Hypaque (Sigma Chem. Co., St. Louis, MO) density gradient centrifugation. Obtained PBMCs were suspended in RPMI 1640 culture media (Gibco, Life Technologies, UK) enriched with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml) and gentamicin (50 mg/ml) (all obtained from Sigma Chem. Co., St. Louis, MO). PBMCs at a concentration of (1×10^{6} cells/ml/well) were cultured for 120 h with the absence or existence of human recombinant (hr)IL-2 (20 ng/ml), hrIL-10 (25 ng/ml), hrIL-12p70 (15 ng/ml) and hrIL-27 (50 ng/ml). Brefeldin A (BFA; eBioscience, San Diego, CA), a Golgistop, was added to the culture wells during the final 4 h of cell culture for intracellular cytokine measurement.

2.4. Intracellular staining

After the cell culture, PBMCs were collected and washed with PBS. Anti-CD3-FITC (Biolegend, San Diego, CA) and -CD56-PE-Cy7 (Biolegend, San Diego, CA) mAbs were used to label $CD3^{-}CD56^{+}$ NK cells prior to the intracellular cytokine staining. Stained cells washing with PBS were fixed and permeabilized in turn with Intracellular Fixation & Permeabilization Buffer Set (eBioscience, San Diego, CA). During permeabilization, anti-IL-22-PE (Biolegend, San Diego, CA) was added to detect intracellular IL-22 levels of $CD3^{-}CD56^{+}$ NK cells. After washing, samples were measured on a FACSCalibur II flow cytometry running Cell Quest software (BD Biosciences, San Jose, CA).

2.5. ELISA

Plasma samples were separated from heparinized peripheral blood samples and stored at -80^{0}C till the measurement of IL-22 levels by enzyme-linked immunosorbent assay (ELISA) (Gen-Probe Inc., San Diego, USA). Samples were assessed according to the manufacturer's protocols. Supplied standards were used to generate the standard curves. The sensitivity of IL-22 was higher than 5 pg/ml.

2.6. Statistical analyses

Statistical analyses were performed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Distribution normality of the groups was analyzed by Kolmogorov Smirnov test and all groups were found to be normally distributed. One-way ANOVA was used for comparison of multiple groups and Tukey's test was used for post-hoc evaluations. Pearson test was used for correlation analyses. ($p < 0.05$) was accepted as the statistical significance level. All p values expressed were the results of Tukey's post hoc test following ANOVA. Graphs were created using Graphpad Prism Software. Data were presented as mean ± standard error mean (SEM).

3. Results

3.1. Ex vivo frequencies of NK cells and activating/inhibitory receptors

Peripheral blood samples obtained from patients were freshly stained for CD3 and CD56 molecules for detecting $CD3^{-}CD56^{+}$ NK

Table 1
Clinical and demographic features of enrolled patients. Table reveals demographic and clinical features of the patient groups. CIS: Clinically Isolated Syndrome, RR-MS: Relapsing-remitting Multiple Sclerosis.

Number	CIS 10	Untreated RR-MS 9	Treated RR-MS 11	Total RR-MS 20
Age (Mean, Range)	28.30 ± 4.90 (21–38)	39.33 ± 13.12 (17–57)	39.27 ± 10.61 (19–51)	39.3 ± 11.48 (17–57)
Sex Ratio (Female To Male)	7:3	4:5	8:3	9:8
Age Of Disease Onset (Mean, Range)	27.70 ± 5.17 (21–38)	35.56 ± 9.65 (17–47)	30.64 ± 8.10 (18–45)	32.85 ± 8.95 (17–47)
Total Number Of Attacks In First 2 Years (Mean, Range)	1.30 (1–3)	2.00 (1–5)	1.91 (1–5)	1.95 (1–5)
Disease Duration At The Time Of Sampling (Year)	0.60 (0–4)	3.78 (0–15)	8.64 (1–16)	6.45 (0–16)
EDSS At The Time Of Sampling	1.25 (0–3)	2.22 (0–6.5)	1.59 (0–4)	1.88 (0–6.5)
Progression Index	0.24 ± 0.33(0–0.83)	0.58 ± 0.38 (0–1.16)	0.23 ± 0.29 (0–1)	0.39 ± 0.37 (0–1.16)

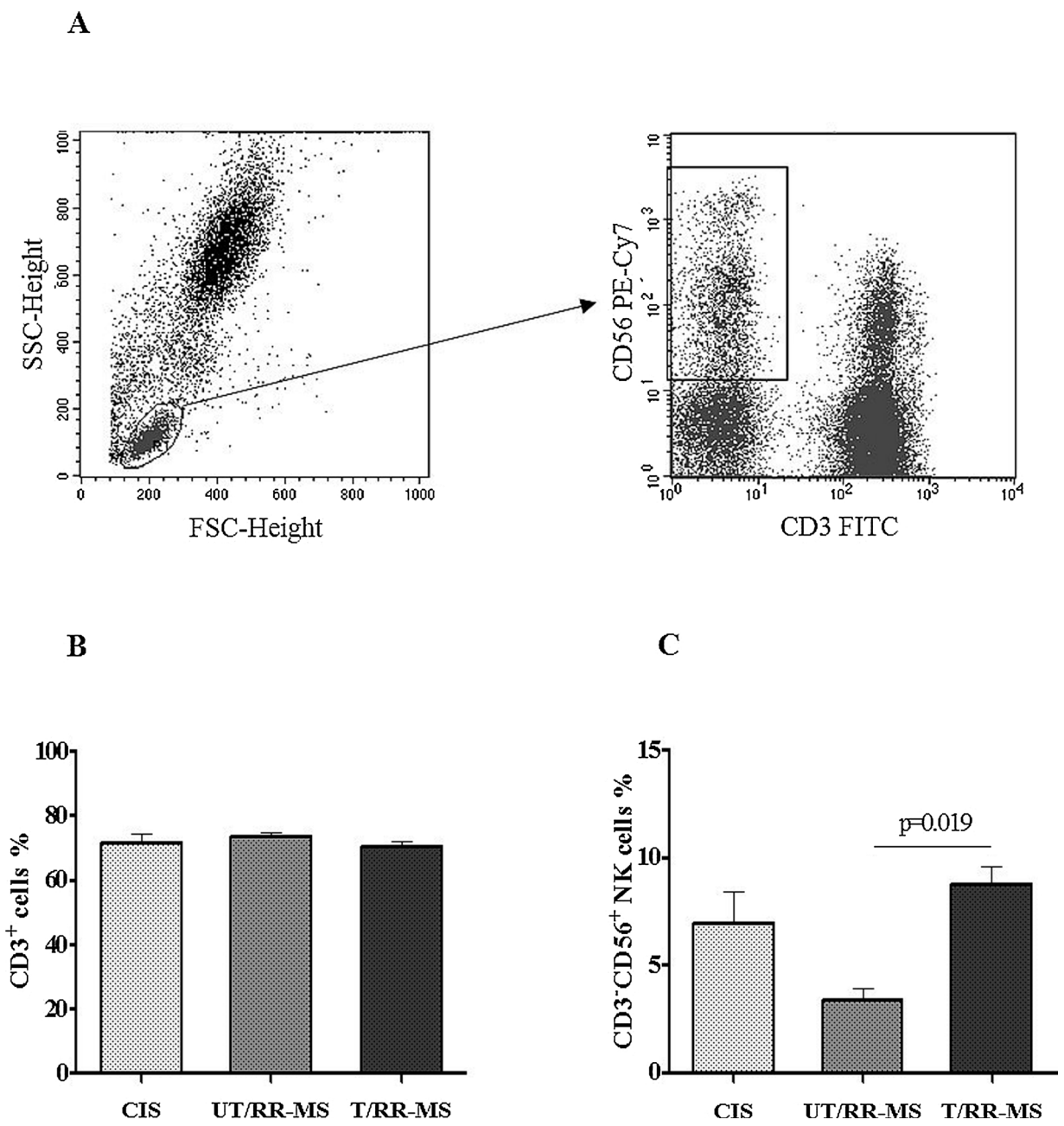

Fig. 1. *Ex vivo*frequencies of CD3$^-$CD56$^+$NK cells. A) The gating strategy of CD3$^-$CD56$^+$ NK cells in peripheral blood samples of donors. B) The frequencies of CD3$^+$ T and C) CD3$^-$CD56$^+$ NK (right) cells in patients. CIS: Clinically isolated syndrome, UT/RR-MS: Untreated RR-MS and T/RR-MS: Treated RR-MS patients. Bar graphs express (Mean ± SEM) of the relevant groups. $p < 0.05$ was accepted as statistical significance level, p values were obtained from TUKEY's post hoc test following ANOVA.

cells (Fig. 1A) prior to measurement of their activating and inhibitory receptor expressions. *Ex vivo* CD3$^-$CD56$^+$ NK cell frequencies of treated RR-MS patients were significantly increased in comparison with untreated RR-MS patients ($p = 0.019$) although the percentage of CD3$^+$ T cells were similar (Fig.1B).

In addition, the percentage of NKG2D$^+$CD3$^-$CD56$^+$ NK cells of untreated RR-MS and CIS patients were significantly lower than treated RR-MS patients ($p = 0.0001$, $p = 0.0001$, respectively) (Fig. 2A). Also, there was a negative correlation between NKG2D expression and EDSS values of total RR-MS patients (Fig. 2B, left). However, NKp44 expressions of CD3$^-$CD56$^+$ NK cells in untreated RR-MS and also treated RR-MS patients were significantly decreased in comparison with CIS patients ($p = 0.001$, $p = 0.003$, respectively). NKG2A levels of CD3$^-$CD56$^+$ NK cells also showed similar results to NKp44 expressions. NKG2A$^+$CD3$^-$CD56$^+$ NK cell expression of untreated and treated RR-MS patients were significantly lower than CIS patients ($p = 0.003$, $p = 0.043$, respectively). Similar to NKG2D, NKG2A expressions of total RR-MS patients were also shown to be negatively correlated with their EDSS values (Fig. 2B, right).

3.2. Intracellular IL-22 content of CD3$^-$CD56$^+$ NK cells

Intracellular IL-22 content of CD3$^-$CD56$^+$ NK cells were determined by flow cytometry after 120 h cell culture. Spontaneous IL-22 contents of CD3$^-$CD56$^+$ NK cells in untreated RR-MS patients were significantly lower than treated RR-MS and CIS patients ($p = 0.0001$, $p = 0.0001$, respectively) (Fig. 3). IL-12 stimulation induced a similar result; IL-22$^+$CD3$^-$CD56$^+$ NK cells of untreated RR-MS patients were

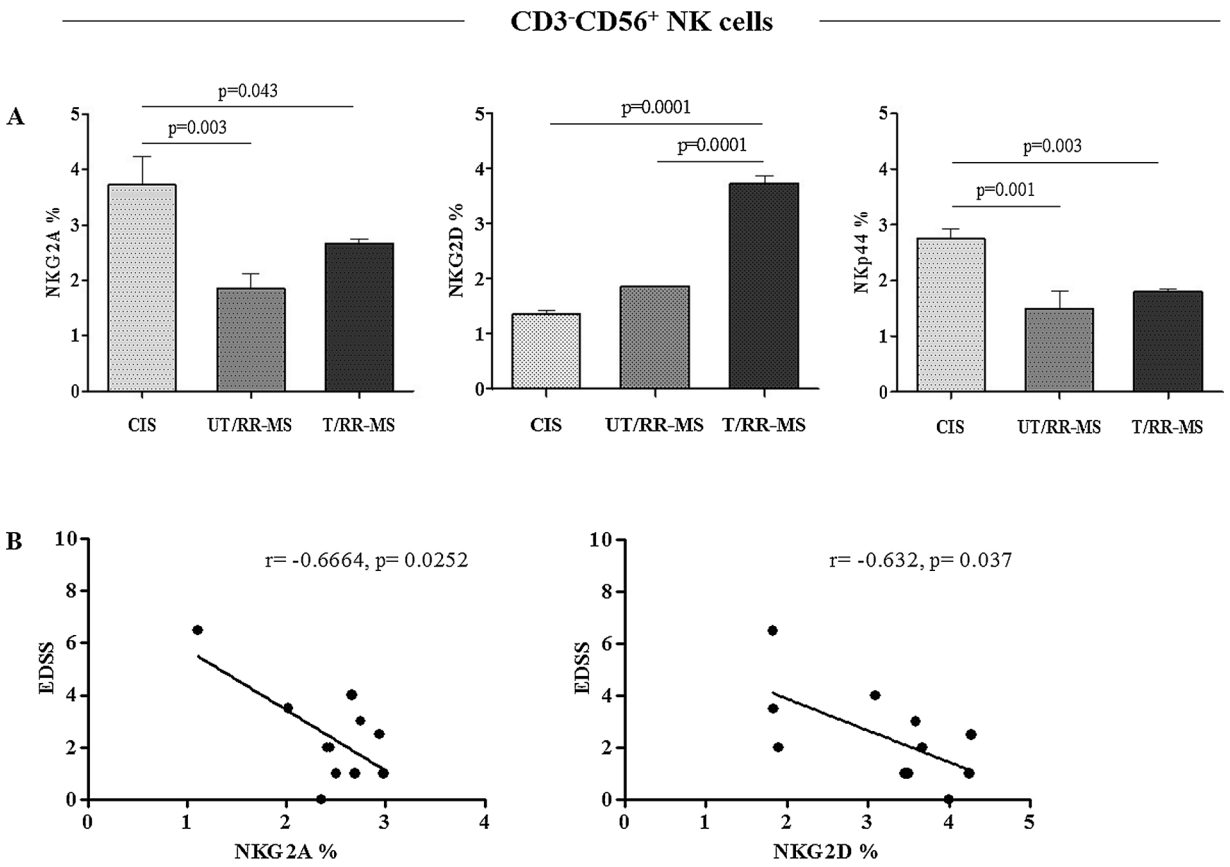

Fig. 2. ***Ex vivo*****frequencies of NK receptors on CD3$^-$CD56$^+$NK cells.** A) NKG2A, NKG2D and NKp44 expressions of CD3$^-$CD56$^+$ NK cells. B) The correlations between NKG2A/EDSS (left) and NKG2D/EDSS (right) values of total RR-MS patients. CIS: Clinically isolated syndrome, UT/RR-MS: Untreated RR-MS and T/RR-MS: Treated RR-MS patients. Bar graphs express (Mean ± SEM) of the relevant groups. $p < 0.05$ was accepted as statistical significance level, p values were obtained from TUKEY's post hoc test following ANOVA. Pearson Test was used for evaluation of correlation.

significantly decreased in comparison with treated RR-MS and CIS patients ($p = 0.0001$, $p = 0.010$, respectively). However, same sub-population was significantly increased in treated RR-MS patients than CIS patients at the same condition ($p = 0.0001$). Following IL-2 stimulation, IL-22 contents of CD3$^-$CD56$^+$ NK cells in CIS patients were significantly diminished in comparison with untreated and treated RR-MS patients ($p = 0.001$, $p = 0.003$, respectively). Conversely, IL-10 stimulated CD3$^-$CD56$^+$ NK cells of CIS patients were significantly increased when compared with that of untreated and treated RR-MS patients ($p = 0.0001$, $p = 0.001$, respectively). There was no significant difference between IL-22 levels of CD3$^-$CD56$^+$ NK cells of patient groups following IL-27 stimulation.

3.3. IL-22 measurement by ELISA

In addition to the intracellular staining of CD3$^-$CD56$^+$ NK cells, IL-22 levels were also measured in plasma samples by ELISA to explore systemic IL-22 levels in patients. IL-22 levels in plasma samples of untreated RR-MS patients have tendency to be increased in comparison with CIS patients without statistical significance (Fig. 4). No significant correlation was found among IL-22 levels versus EDSS or PI values of RR-MS patients.

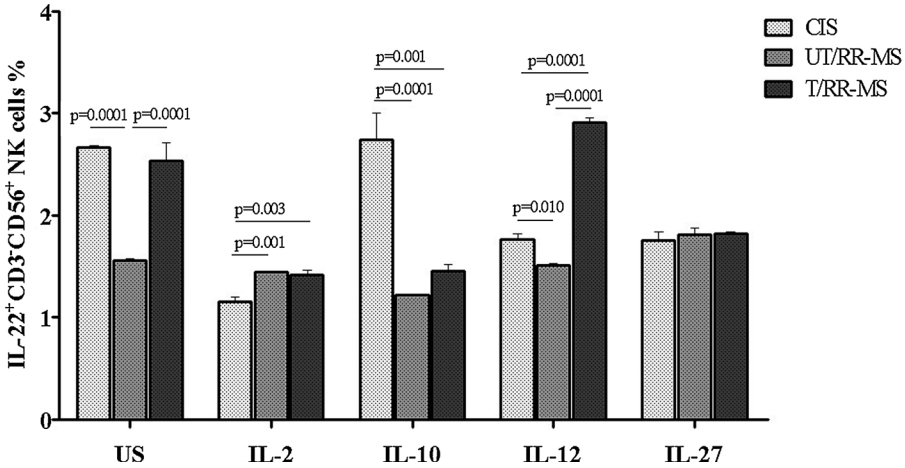

Fig. 3. Intracellular IL-22 levels of CD3$^-$CD56$^+$NK cells after 120 h cell culture with/without cytokine stimulations. US: Unstimulated, CIS: Clinically isolated syndrome, UT/RR-MS: Untreated RR-MS and T/RR-MS: Treated RR-MS patients. Bar graphs express (Mean ± SEM) of the relevant groups. $p < 0.05$ was accepted as statistical significance level, p values were obtained from TUKEY's post hoc test following ANOVA.

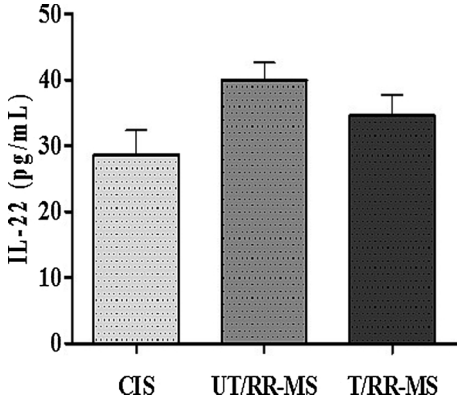

Fig. 4. Plasma IL-22 measurements of patient groups by ELISA. US: Unstimulated, CIS: Clinically isolated syndrome, UT/RR-MS: Untreated RR-MS and T/RR-MS: Treated RR-MS patients. Bar graphs express (Mean ± SEM) of the relevant groups. $p < 0.05$ was accepted as statistical significance level, p values were obtained from TUKEY's post hoc test following ANOVA.

4. Discussion

As we have recently confirmed [29], IFN-β treatment was demonstrated to increase $CD3^-CD56^{bright}$ NK cell subset frequencies of RR-MS patients in various studies [31]. In this study, total $CD3^-CD56^+$ NK cells of RR-MS patients were also shown to be increased following IFN-β treatment though $CD3^+$ T cell frequencies did not vary between patient groups. This finding might indicate that IFN-β treatment directly affect NK cell ratio, regardless of whether $CD3^+$ T cell frequencies decrease. To explore the functional capacity of these cells, the activating and inhibitory receptor molecule expressions of $CD3^-CD56^+$ NK cells were investigated prior to cytokine measurement.

NKG2D is a molecular stress sensor and a potent activating receptor [32] mainly expressed on NK cells, $CD8^+$ and $\gamma\delta^+$ T cells [33] though there are studies showing the high levels of rare $NKG2D^+CD4^+$ cells in various diseases [34,35]. NKG2D poorly binds several self-ligands on healthy cells and it is up-regulated in response to increased expression of stress molecules, such as MICA/B (MHC class-I-related chain A/B) and ULBP (UL-16 binding proteins) 1–6 in humans [36]. In the studies of MS, elevated levels of MICB was shown in sera of patients [37] and an association was found between MICB*004 allele and increased MS susceptibility [38]. Increased levels of $NKG2D^+CD4^+$ T cells were shown in cerebrospinal fluid and lesions of MS patients [39]. In humans, NK cells expressing NKG2D were demonstrated to efficiently kill activated $CD4^+$ T cells which upregulate their NKG2D ligands [40,41]. Also, NK cells were shown to lyse oligodendrocytes, microglia and astrocytes by a NKG2D-dependent mechanism [42]. All these data suggest an association between NKG2D and MS development though there are nominal studies about NKG2D expressions of NK cells in MS pathogenesis. In our study, NKG2D expression of $CD3^-CD56^+$ NK cells in treated RR-MS patients was found to be increased compared to untreated RR-MS and CIS patients. Although the increase in NKG2D ligand expression means a danger signal to trigger NK cell activation, decreased NKG2D levels could also point a problem in some situations. NKG2D expression is down-modulated with chronic exposure to NKG2D ligands which results in decreased cytotoxicity toward targeted cells [43–46]. Thus, diminished levels of NKG2D in untreated RR-MS and CIS patients might reflect a similar condition which could be correlated with decreased NK cytotoxicity in these patient groups demonstrated in our previous study [29]. In RR-MS patients under IFN-β treatment, elevated NKG2D levels were displayed which was negatively correlated with EDSS values of total RR-MS patients. This correlation suggests a beneficial role of NKG2D increased by IFN-β treatment in RR-MS patients, however it should be confirmed in order to prove this association is not coincidental. As type I IFNs are known to trigger an anti-viral state, increased activity of NK cells might be a consequence of IFN-β treatment. NK cells could exert cytotoxicity toward both T cells and also myeloid cells [47–49], thus elevated NKG2D might point a regulatory attempt in MS pathophysiology. While viral infections are suggested as possible predisposing factors of MS [50,51], increased activation of NK cells as defenders against viruses by their cytotoxicity could be investigated together with viral load of RR-MS patients receiving therapy.

In contrast to NKG2D which were similar in CIS and untreated RR-MS patients, NKp44 and NKG2A expressions were varied between CIS and both RR-MS patient groups. NKp44 is an activating receptor differed from the others by being only expressed in activated NK cells able to initiate a cytotoxic response immediately [52]. However, NKp44 inhibits NK cell functions depending on the bound-ligand [53]. This could explain the difference between NKG2D and NKp44 expressions on $CD3^-CD56^+$ NK cells of patient groups in our study. Furthermore, the inhibitory receptor NKG2A expression profiles of patients were similar to NKp44 expression which was lower in $CD3^-CD56^+$ NK cells of treated and untreated RR-MS patients than CIS patients. All these findings suggests an inhibitory role for NKp44 in $CD3^-CD56^+$ NK cells of CIS and RR-MS patients.

Unlike KIRs recognizing HLA-C molecules, NKG2A is an inhibitory receptor binding non-classical HLA-E molecules. HLA-E which prevents $NKG2A^+$ NK cells to lyse target cells [54] was shown to be increased in active but not chronic lesions of MS patients [55]. There are studies demonstrating that blocking the interaction between NKG2A and its ligand on the target cells results in decreased inflammation in EAE (the animal model of MS) via killing T cells and microglial cells [56,57]. Following the blockage of the increased HLA-E expression on $CD4^+$ T cells, $CD56^{bright}$ NK cells were shown to suppress the proliferation of autologous $CD4^+$ T cells effectively, which are thought to be responsible for the inflammation in MS. Thus, it was suggested that $CD56^{bright}$ NK cell cytotoxicity on target cells might be inhibited by the binding of HLA-E molecules on T cells to inhibitory NKG2A on NK cells [58]. In this study, $NKG2A^+CD3^-CD56^+$ NK cells of untreated and treated RR-MS patients were decreased in comparison with CIS patients concluding that the activation status of $CD3^-CD56^+$ NK cells of RR-MS patients might be higher than CIS patients depending on the ligand expressions. Nevertheless, the negative correlation between NKG2A expression and EDSS values of total RR-MS patients might indicate that NKG2A is also involved in regulation of autoreactive T cells. At this point, it is necessary to clarify how the increase of inhibitory NKG2A could provide a regulatory function while NK cell activation seems to be beneficial for the healing of MS.

In addition to activating and inhibitory receptor expression profiles, intracellular IL-22 levels of $CD3^-CD56^+$ NK cells were investigated. There are several studies demonstrating the contribution of IL-22 in autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and psoriasis as well as MS [59]. The role of IL-22 can vary as contributing inflammation, chemotaxis, host defense or tissue protection, wound healing and cell survival depending on the target and cytokine milieu [60–62]. It has been suggested that IL-22 enables lymphocyte entrance into the CNS by compromising the blood brain barrier integrity in MS [63]. However, there are studies which propose a protective role for IL-22 in MS pathogenesis [64,65]. Previously, we have demonstrated IL-22 secretion of $CD3^-CD16^-CD56^{bright}$ and $CD3^-CD16^+CD56^{dim}$ NK cell subsets in CIS and RR-MS patients - especially patients under IFN-β treatment were elevated upon IL-2 and IL-4 stimulation, respectively, compared to healthy subjects [29]. This study showed increased IL-22 content of $CD3^-CD56^+$ NK cells in treated RR-MS patients at unstimulated and also IL-12 stimulated culture conditions. Although this might suggest a beneficial role for IL-22, absence of correlation between IL-22 levels and EDSS or PI values of treated RR-MS patients argues against this assertion. Thus elevated IL-22 production of $CD3–CD56^+$ NK cells is presumably an undesired side effect of IFN-β treatment. However, this effect seems to be restricted to NK cells since the plasma IL-22 levels of patients are similar. IL-10 as an

inhibitory cytokine, had a suppressor effect on IL-22 secretion in both treated and untreated RR-MS patients but not in CIS patients.

In summary, this study demonstrates the increased NK cell frequencies which have activating profile and elevated IL-22 levels under IFN-β therapy which suggest that IFN-β treatment might direct NK cells toward a pro-inflammatory status. Whether these pro-inflammatory NK cell subsets might have an anti-MS action needs to be further elucidated. NKG2A and NKG2D expression levels might serve as possible prognostic biomarkers in the distinction between CIS and RR-MS, however it deserves to be intensively studied and illuminated. The activation status and cytotoxic functions of NK cells in RR-MS are also worth to be further investigated in more detail based on the existing data. The difference of the frequencies and activation status of NK cells or IL-22 levels between CIS and untreated RR-MS patients might suggest that these diseases have distinct developmental pathways. Pathogenic mechanisms of MS might possibly vary in different stages of MS such as CIS, RR-MS and progressive stages. Alterations in cell surface receptor expression and cytokine production patterns of NK cells during the disease course might be one of the reasons underlying treatment resistance to immuno-modulating medications in advanced stages of MS.

Declaration of Competing Interest

All authors declare that they have no financial or non-financial competing interests.

Acknowledgments

We thank Erdem Tuzun, MD for critical reading of the manuscript. This study was funded by Scientific Research Projects Coordination Unit (BAP) of Istanbul University, Turkey (Project number: 18423) and The Scientific and Technological Research Council of Turkey (TUBITAK, Project number: 112S005).

References

[1] W.J. Brownlee, T.A. Hardy, F. Fazekas, D.H. Miller, Diagnosis of multiple sclerosis: progress and challenges, Lancet 389 (10076) (2017) 1336–1346.
[2] F.D. Lublin, S.C. Reingold, J.A. Cohen, G.R. Cutter, P.S. Sorensen, A.J. Thompson, J.S. Wolinsky, L.J. Balcer, B. Banwell, F. Barkhof, B. Bebo Jr, P.A. Calabresi, M. Clanet, G. Comi, R.J. Fox, M.S. Freedman, A.D. Goodman, M. Inglese, L. Kappos, B.C. Kieseier, J.A. Lincoln, C. Lubetzki, A.E. Miller, X. Montalban, P.W. O'Connor, J. Petkau, C. Pozzilli, R.A. Rudick, M.P. Sormani, O. Stuve, E. Waubant, C.H. Polman, Defining the clinical course of multiple sclerosis: the 2013 revisions, Neurology 83 (3) (2014) 278–286.
[3] D.H. Miller, D.T. Chard, O. Ciccarelli, Clinically isolated syndromes, Lancet Neurol. 11 (2) (2012) 157–169.
[4] R. Weissert, The immune pathogenesis of multiple sclerosis, J. Neuroimmune Pharmacol. 8 (4) (2013) 857–866.
[5] A.P. Jones, A.G. Kermode, R.M. Lucas, W.M. Carroll, D. Nolan, P.H. Hart, Circulating immune cells in multiple sclerosis, Clin. Exp. Immunol. 187 (2) (2017) 193–203.
[6] H.L. Weiner, A shift from adaptive to innate immunity: a potential mechanism of disease progression in multiple sclerosis, J. Neurol. 255 (Suppl 1) (2008) 3–11.
[7] H.J. Bontkes, D. Kramer, J.J. Ruizendaal, C.J. Meijer, E. Hooijberg, Tumor associated antigen and interleukin-12 mRNA transfected dendritic cells enhance effector function of natural killer cells and antigen specific T-cells, Clin. Immunol. 127 (3) (2008) 375–384.
[8] Y. Liu, H.W. Wu, M.A. Sheard, R. Sposto, S.S. Somanchi, L.J. Cooper, D.A. Lee, R.C. Seeger, Growth and activation of natural killer cells ex vivo from children with neuroblastoma for adoptive cell therapy, Clin. Cancer Res. 19 (8) (2013) 2132–2143.
[9] S. Anguille, H.H. Van Acker, J. Van den Bergh, Y. Willemen, H. Goossens, V.F. Van Tendeloo, E.L. Smits, Z.N. Berneman, E. Lion, Interleukin-15 dendritic cells harness NK cell cytotoxic effector function in a contact- and IL-15-Dependent manner, PLoS One 10 (5) (2015) e0123340.
[10] H.H. Van Acker, A. Capsomidis, E.L. Smits, V.F. Van Tendeloo, CD56 in the Immune System: More Than a Marker for Cytotoxicity? Front. Immunol. 8 (2017) 892.
[11] K.S. Reiners, D. Topolar, A. Henke, V.R. Simhadri, J. Kessler, M. Sauer, M. Bessler, H.P. Hansen, S. Tawadros, M. Herling, M. Kronke, M. Hallek, E. Pogge von Strandmann, Soluble ligands for NK cell receptors promote evasion of chronic lymphocytic leukemia cells from NK cell anti-tumor activity, Blood 121 (18) (2013) 3658–3665.
[12] M.J. Robertson, J. Ritz, Biology and clinical relevance of human natural killer cells, Blood 76 (12) (1990) 2421–2438.
[13] L.L. Lanier, Up on the tightrope: natural killer cell activation and inhibition, Nat. Immunol. 9 (5) (2008) 495–502.
[14] E.O. Long, H.S. Kim, D. Liu, M.E. Peterson, S. Rajagopalan, Controlling natural killer cell responses: integration of signals for activation and inhibition, Annu. Rev. Immunol. 31 (2013) 227–258.
[15] C. Bottino, R. Castriconi, L. Moretta, A. Moretta, Cellular ligands of activating NK receptors, Trends Immunol. 26 (4) (2005) 221–226.
[16] E.O. Long, Negative signaling by inhibitory receptors: the NK cell paradigm, Immunol. Rev. 224 (2008) 70–84.
[17] S. Kumar, Natural killer cell cytotoxicity and its regulation by inhibitory receptors, Immunology 154 (3) (2018) 383–393.
[18] M. Cella, A. Fuchs, W. Vermi, F. Facchetti, K. Otero, J.K. Lennerz, J.M. Doherty, J.C. Mills, M. Colonna, A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity, Nature 457 (7230) (2009) 722–725.
[19] H. Guo, D.J. Topham, Interleukin-22 (IL-22) production by pulmonary Natural Killer cells and the potential role of IL-22 during primary influenza virus infection, J. Virol. 84 (15) (2010) 7750–7759.
[20] H. Wang, Z. Li, B. Yang, S. Yu, C. Wu, IL-27 suppresses the production of IL-22 in human CD4(+) T cells by inducing the expression of SOCS1, Immunol. Lett. 152 (2) (2013) 96–103.
[21] J.A. Dudakov, A.M. Hanash, M.R. van den Brink, Interleukin-22: immunobiology and pathology, Annu. Rev. Immunol. (2015).
[22] N. Xin, M.P. Namaka, C. Dou, Y. Zhang, Exploring the role of interleukin-22 in neurological and autoimmune disorders, Int. Immunopharmacol. 28 (2) (2015) 1076–1083.
[23] H.F. Pan, X.F. Zhao, H. Yuan, W.H. Zhang, X.P. Li, G.H. Wang, G.C. Wu, X.W. Tang, W.X. Li, L.H. Li, J.B. Feng, C.S. Hu, D.Q. Ye, Decreased serum IL-22 levels in patients with systemic lupus erythematosus, Clin. Chim. Acta 401 (1–2) (2009) 179–180.
[24] F. Cheng, Z. Guo, H. Xu, D. Yan, Q. Li, Decreased plasma IL22 levels, but not increased IL17 and IL23 levels, correlate with disease activity in patients with systemic lupus erythematosus, Ann. Rheum. Dis. 68 (4) (2009) 604–606.
[25] L.F. da Rocha Jr, A.L. Duarte, A.T. Dantas, H.A. Mariz, R. Pitta Ida, S.L. Galdino, M.G. Pitta, Increased serum interleukin 22 in patients with rheumatoid arthritis and correlation with disease activity, J. Rheumatol. 39 (7) (2012) 1320–1325.
[26] A.D. Beyeen, M.Z. Adzemovic, J. Ockinger, P. Stridh, K. Becanovic, H. Laaksonen, H. Lassmann, R.A. Harris, J. Hillert, L. Alfredsson, E.G. Celius, H.F. Harbo, I. Kockum, M. Jagodic, T. Olsson, IL-22RA2 associates with multiple sclerosis and macrophage effector mechanisms in experimental neuroinflammation, J. Immunol. 185 (11) (2010) 6883–6890.
[27] B. Almolda, M. Costa, M. Montoya, B. Gonzalez, B. Castellano, Increase in Th17 and T-reg lymphocytes and decrease of IL22 correlate with the recovery phase of acute EAE in rat, PLoS One 6 (11) (2011) e27473.
[28] S. Rolla, V. Bardina, S. De Mercanti, P. Quaglino, R. De Palma, D. Gned, D. Brusa, L. Durelli, F. Novelli, M. Clerico, Th22 cells are expanded in multiple sclerosis and are resistant to IFN-beta, J. Leukoc. Biol. 96 (6) (2014) 1155–1164.
[29] I. Tahrali, U.C. Kucuksezer, A. Altintas, U. Uygunoglu, N. Akdeniz, E. Aktas-Cetin, G. Deniz, Dysfunction of CD3(-)CD16(+)CD56(dim) and CD3(-)CD16(-)CD56(bright) NK cell subsets in RR-MS patients, Clin. Immunol. 193 (2018) 88–97.
[30] C.H. Polman, S.C. Reingold, B. Banwell, M. Clanet, J.A. Cohen, M. Filippi, K. Fujihara, E. Havrdova, M. Hutchinson, L. Kappos, F.D. Lublin, X. Montalban, P. O'Connor, M. Sandberg-Wollheim, A.J. Thompson, E. Waubant, B. Weinshenker, J.S. Wolinsky, Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria, Ann. Neurol. 69 (2) (2011) 292–302.
[31] J.E. Martinez-Rodriguez, M. Lopez-Botet, E. Munteis, J. Rio, J. Roquer, X. Montalban, M. Comabella, Natural killer cell phenotype and clinical response to interferon-beta therapy in multiple sclerosis, Clin. Immunol. 141 (3) (2011) 348–356.
[32] M. Babic, C. Romagnani, The role of natural killer group 2, member d in chronic inflammation and autoimmunity, Front. Immunol. 9 (2018) 1219.
[33] L.L. Lanier, NKG2D receptor and its ligands in host defense, Cancer Immunol. Res. 3 (6) (2015) 575–582.
[34] V. Groh, A. Bruhl, H. El-Gabalawy, J.L. Nelson, T. Spies, Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis, Proc. Natl. Acad. Sci. U. S. A. 100 (16) (2003) 9452–9457.
[35] M. Allez, V. Tieng, A. Nakazawa, X. Treton, V. Pacault, N. Dulphy, S. Caillat-Zucman, P. Paul, J.M. Gornet, C. Douay, S. Ravet, R. Tamouza, D. Charron, M. Lemann, L. Mayer, A. Toubert, CD4+NKG2D+ T cells in Crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions, Gastroenterology 132 (7) (2007) 2346–2358.
[36] D.H. Raulet, S. Gasser, B.G. Gowen, W. Deng, H. Jung, Regulation of ligands for the NKG2D activating receptor, Annu. Rev. Immunol. 31 (2013) 413–441.
[37] J.L. Fernandez-Morera, S. Rodriguez-Rodero, C. Lahoz, A. Tunon, A. Astudillo, O. Garcia-Suarez, J. Martinez-Borra, A. Lopez-Vazquez, L. Rodrigo, S. Gonzalez, C. Lopez-Larrea, Soluble MHC class I chain-related protein B serum levels correlate with disease activity in relapsing-remitting multiple sclerosis, Hum. Immunol. 69 (4–5) (2008) 235–240.
[38] J.L. Fernandez-Morera, S. Rodriguez-Rodero, A. Tunon, J. Martinez-Borra, J.R. Vidal-Castineira, A. Lopez-Vazquez, L. Rodrigo, P. Rodrigo, S. Gonzalez, C.H. Lahoz, C. Lopez-Larrea, Genetic influence of the nonclassical major histocompatibility complex class I molecule MICB in multiple sclerosis susceptibility, Tissue Antigens 72 (1) (2008) 54–59.
[39] T. Ruck, S. Bittner, C.C. Gross, J. Breuer, S. Albrecht, S. Korr, K. Gobel, S. Pankratz, C.M. Henschel, N. Schwab, O. Staszewski, M. Prinz, T. Kuhlmann, S.G. Meuth,

H. Wiendl, CD4+NKG2D+ T cells exhibit enhanced migratory and encephalitogenic properties in neuroinflammation, PLoS One 8 (11) (2013) e81455.
[40] C. Cerboni, A. Zingoni, M. Cippitelli, M. Piccoli, L. Frati, A. Santoni, Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK- cell lysis, Blood 110 (2) (2007) 606–615.
[41] N. Nielsen, N. Odum, B. Urso, L.L. Lanier, P. Spee, 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 7 (2) (2012) e31959.
[42] P. Saikali, J.P. Antel, J. Newcombe, Z. Chen, M. Freedman, M. Blain, R. Cayrol, A. Prat, J.A. Hall, N. Arbour, NKG2D-mediated cytotoxicity toward oligodendrocytes suggests a mechanism for tissue injury in multiple sclerosis, J. Neurosci. 27 (5) (2007) 1220–1228.
[43] V. Groh, J. Wu, C. Yee, T. Spies, Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation, Nature 419 (6908) (2002) 734–738.
[44] K. Wiemann, H.W. Mittrucker, U. Feger, S.A. Welte, W.M. Yokoyama, T. Spies, H.G. Rammensee, A. Steinle, Systemic NKG2D down-regulation impairs NK and CD8 T cell responses in vivo, J. Immunol. 175 (2) (2005) 720–729.
[45] A. Clayton, J.P. Mitchell, J. Court, S. Linnane, M.D. Mason, Z. Tabi, Human tumor-derived exosomes down-modulate NKG2D expression, J. Immunol. 180 (11) (2008) 7249–7258.
[46] R. Molfetta, L. Quatrini, A. Santoni, R. Paolini, Regulation of NKG2D-Dependent NK cell functions: the yin and the yang of receptor endocytosis, Int. J. Mol. Sci. 18 (8) (2017).
[47] B. Zitti, Y.T. Bryceson, Natural killer cells in inflammation and autoimmunity, Cytokine Growth Factor Rev. 42 (2018) 37–46.
[48] H. Schlums, F. Cichocki, B. Tesi, J. Theorell, V. Beziat, T.D. Holmes, H. Han, S.C. Chiang, B. Foley, K. Mattsson, S. Larsson, M. Schaffer, K.J. Malmberg, H.G. Ljunggren, J.S. Miller, Y.T. Bryceson, Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function, Immunity 42 (3) (2015) 443–456.
[49] G. Ferlazzo, M.L. Tsang, L. Moretta, G. Melioli, R.M. Steinman, C. Munz, Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells, J. Exp. Med. 195 (3) (2002) 343–351.
[50] W. Fierz, Multiple sclerosis: an example of pathogenic viral interaction? Virol. J. 14 (1) (2017) 42.
[51] M. Paroni, V. Maltese, M. De Simone, V. Ranzani, P. Larghi, C. Fenoglio, A.M. Pietroboni, M.A. De Riz, M.C. Crosti, S. Maglie, M. Moro, F. Caprioli, R. Rossi, G. Rossetti, D. Galimberti, M. Pagani, E. Scarpini, S. Abrignani, J. Geginat, Recognition of viral and self-antigens by TH1 and TH1/TH17 central memory cells in patients with multiple sclerosis reveals distinct roles in immune surveillance and relapses, J. Allergy Clin. Immunol. 140 (3) (2017) 797–808.
[52] C. Cantoni, C. Bottino, M. Vitale, A. Pessino, R. Augugliaro, A. Malaspina, S. Parolini, L. Moretta, A. Moretta, R. Biassoni, NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily, J. Exp. Med. 189 (5) (1999) 787–796.
[53] B. Rosental, M. Brusilovsky, U. Hadad, D. Oz, M.Y. Appel, F. Afergan, R. Yossef, L.A. Rosenberg, A. Aharoni, A. Cerwenka, K.S. Campbell, A. Braiman, A. Porgador, Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44, J. Immunol. 187 (11) (2011) 5693–5702.
[54] N. Lee, M. Llano, M. Carretero, A. Ishitani, F. Navarro, M. Lopez-Botet, D.E. Geraghty, HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A, Proc. Natl. Acad. Sci. U. S. A. 95 (9) (1998) 5199–5204.
[55] P.F. Durrenberger, L.V. Webb, M.J. Sim, R.S. Nicholas, D.M. Altmann, R.J. Boyton, Increased HLA-E expression in white matter lesions in multiple sclerosis, Immunology 137 (4) (2012) 317–325.
[56] J.W. Leavenworth, C. Schellack, H.J. Kim, L. Lu, P. Spee, H. Cantor, Analysis of the cellular mechanism underlying inhibition of EAE after treatment with anti-NKG2A F (ab')2, Proc. Natl. Acad. Sci. U. S. A. 107 (6) (2010) 2562–2567.
[57] L. Lu, K. Ikizawa, D. Hu, M.B. Werneck, K.W. Wucherpfennig, H. Cantor, Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway, Immunity 26 (5) (2007) 593–604.
[58] A. Laroni, E. Armentani, N. Kerlero de Rosbo, F. Ivaldi, E. Marcenaro, S. Sivori, R. Gandhi, H.L. Weiner, A. Moretta, G.L. Mancardi, A. Uccelli, Dysregulation of regulatory CD56(bright) NK cells/T cells interactions in multiple sclerosis, J. Autoimmun. 72 (2016) 8–18.
[59] H.F. Pan, X.P. Li, S.G. Zheng, D.Q. Ye, Emerging role of interleukin-22 in autoimmune diseases, Cytokine Growth Factor Rev. 24 (1) (2013) 51–57.
[60] K. Wolk, S. Kunz, E. Witte, M. Friedrich, K. Asadullah, R. Sabat, IL-22 increases the innate immunity of tissues, Immunity 21 (2) (2004) 241–254.
[61] S.J. Aujla, Y.R. Chan, M. Zheng, M. Fei, D.J. Askew, D.A. Pociask, T.A. Reinhart, F. McAllister, J. Edeal, K. Gaus, S. Husain, J.L. Kreindler, P.J. Dubin, J.M. Pilewski, M.M. Myerburg, C.A. Mason, Y. Iwakura, J.K. Kolls, IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia, Nat. Med. 14 (3) (2008) 275–281.
[62] E. Witte, K. Witte, K. Warszawska, R. Sabat, K. Wolk, Interleukin-22: a cytokine produced by T, NK and NKT cell subsets, with importance in the innate immune defense and tissue protection, Cytokine Growth Factor Rev. 21 (5) (2010) 365–379.
[63] H. Kebir, K. Kreymborg, I. Ifergan, A. Dodelet-Devillers, R. Cayrol, M. Bernard, F. Giuliani, N. Arbour, B. Becher, A. Prat, Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation, Nat. Med. 13 (10) (2007) 1173–1175.
[64] H. Muhl, P. Scheiermann, M. Bachmann, L. Hardle, A. Heinrichs, J. Pfeilschifter, IL-22 in tissue-protective therapy, Br. J. Pharmacol. 169 (4) (2013) 761–771.
[65] H. Laaksonen, A.O. Guerreiro-Cacais, M.Z. Adzemovic, R. Parsa, M. Zeitelhofer, M. Jagodic, T. Olsson, The multiple sclerosis risk gene IL22RA2 contributes to a more severe murine autoimmune neuroinflammation, Genes Immun. 15 (7) (2014) 457–465.