ORIGINAL ARTICLE

Delayed B cell repopulation after rituximab treatment in multiple sclerosis patients with expanded adaptive natural killer cells

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Abstract

Background and purpose: The aim was to evaluate whether adaptive NKG2C+ natural killer (NK) cells, characterized by enhanced antibody-dependent cell cytotoxicity (ADCC), may influence time to B cell repopulation after rituximab treatment in multiple sclerosis (MS) patients.

Methods: This was a prospective observational study of MS patients treated with rituximab monitoring peripheral B cells for repeated doses. B cell repopulation was defined as CD19+ cells above 2% of total lymphocytes, classifying cases according to the median time of B cell repopulation as early or late (≤9 months, >9 months, respectively). Basal NK cell immunophenotype and in vitro ADCC responses induced by rituximab were assessed by flow cytometry.

Results: B cell repopulation in 38 patients (24 relapsing–remitting MS [RRMS]; 14 progressive MS) was classified as early (≤9 months, $n = 19$) or late (>9 months, $n = 19$). RRMS patients with late B cell repopulation had higher proportions of NKG2C+ NK cells compared to those with early repopulation (24.7% ± 16.2% vs. 11.3% ± 10.4%, $p < 0.05$), and a direct correlation between time to B cell repopulation and percentage of NKG2C+ NK cells (R 0.45, $p < 0.05$) was observed. RRMS cases with late repopulation compared with early repopulation had a higher secretion of tumor necrosis factor α and interferon γ by NK cells after rituximab-dependent NK cell activation. The NK cell immunophenotype appeared unrelated to B cell repopulation in progressive MS patients.

Conclusions: Adaptive NKG2C+ NK cells in RRMS may be associated with delayed B cell repopulation after rituximab, a finding probably related to enhanced depletion of B cells exerted by NK-cell-mediated ADCC, pointing to the use of personalized regimens with anti-CD20 monoclonal antibody therapy in some patients.

KEYWORDS
B cell repopulation, multiple sclerosis, NK cells, rituximab

Antía Moreira and Elvira Munteis contributed equally to the manuscript.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disorder traditionally considered to be mediated by T cells. However, the striking efficacy of anti-CD20 monoclonal antibodies (MAbs) in MS patients has revolutionized the biological paradigm and therapeutic approach of this disease, relocating B cells as a crucial lymphocyte involved in MS immunology. First evidence supporting the benefits of anti-CD20 MAbs in MS was provided by observational and pilot studies using rituximab, a genetically engineered chimeric MAb, showing a reduction in the number of magnetic resonance imaging gadolinium-enhanced lesions in relapsing–remitting MS (RRMS) [1,2] and decreased disability progression in a subgroup of younger progressive MS (PMS) patients characterized by active magnetic resonance imaging [3]. However, a rituximab license in MS is not supported by phase III clinical trials, in contrast to other anti-CD20 MAbs (e.g., ocrelizumab, ofatumumab). Nevertheless, rituximab is frequently used off-label in MS based on several observational and retrospective studies performed in large cohorts of patients [4–7].

Beyond the clinical efficacy of anti-CD20 MAbs in MS, their long-term safety profile remains to be defined. Phase III clinical trials evaluating anti-CD20 MAbs were performed using fixed-dose administration [8,9]. In contrast, the rituximab dosing strategy is not uniform. Some studies support a schedule maintenance based on B cell repopulation [10,11]. Under this personalized approach, treatment efficacy is intended to be maintained whilst reducing the risk of adverse events. In this context, the study of B cell repopulation kinetics after anti-CD20 MAbs might allow an individualized dose regimen of these biological therapies in MS patients.

Rituximab induces B cell death by several mechanisms including antibody-dependent cellular cytotoxicity (ADCC) [12]. Amongst the main effector cells mediating ADCC are NK cells, which are lymphocytes with immunoregulatory properties involved in MS immunopathology [13,14]. The ADCC function of NK cells is markedly influenced by their adaptive immunophenotype, characteristically defined in humans by the expression of the activating receptor NKG2C [15]. Adaptive NKG2C+ NK cells have enhanced capability to mediate ADCC and release cytolytic granules containing perforin and granzymes and to produce pro-inflammatory cytokines upon target recognition through the Fc receptor CD16 (FcγRIII) [15]. The expansion of NKG2C+ NK cells is variable, influenced by the *NKG2C* gene copy number [16] and promoted in some individuals by chronic cytomegalovirus (CMV) infection [17], a beta-herpesvirus recently related with low MS risk [18–20]. Based on the mechanism of action of rituximab, it was hypothesized that adaptive NK cells, characterized by enhanced ADCC function, might influence B cell repopulation kinetics after anti-CD20 MAb therapy. On this premise, the aim was to evaluate whether baseline adaptive NK cells are related to time to B cell repopulation in MS patients treated with a protocolized regimen of rituximab.

METHODS

Study population

Multiple sclerosis patients receiving at least one infusion of rituximab were prospectively evaluated from January 2017 to December 2021 at the Neurology Department, Hospital del Mar, Barcelona, Spain. Patients fulfilled the McDonald criteria 2017, classifying MS form as RRMS or PMS [21]. Rituximab was indicated as induction therapy for active MS in the case of suboptimal response to previous disease-modifying therapy (DMT) or aggressive MS (≥two relapses in less than 1 year); rituximab was used in PMS patients in the case of increased disability progression in the previous year [11]. Exclusion criteria from the study were corticosteroid treatment in the previous 30 days or severe concomitant diseases. The study was approved by the local ethics committee (Hospital del Mar Research Institute, IMIM, 2017/7460/I), providing all patients written informed consent.

Rituximab initiation consisted of a first intravenous infusion of 1000 mg followed, in the case of no severe adverse events, by a second dose of 1000 mg after 2 weeks. Routine premedication included intravenous paracetamol 1000 mg, diphenhydramine 5 mg and methylprednisolone 100 mg. Peripheral blood mononuclear cells (PBMCs) were collected by venous puncture previous to the first rituximab dose and every 3 months after rituximab onset; in the case of no detection of B cell repopulation after 6 months, PBMCs were obtained every month. B cell repopulation was defined as any number of B cells above 2% of the total lymphocyte count, using the median time to repopulation in our cohort (288 days) to classify patients in early or late B cell repopulation (≤9 months or >9 months, respectively). Patients received a new dose of 1000 mg rituximab when B cell repopulation was detected.

Flow cytometry analysis of NK cell immunophenotype

Peripheral blood mononuclear cells were isolated from blood samples collected in ethylenediaminetetraacetic acid tubes using Ficoll–Hypaque density gradient centrifugation and cryopreserved in fetal calf serum with 10% dimethyl sulfoxide until analysis, following methods previously reported [22]. Sample staining for flow cytometry analysis was performed using the following fluorochrome-conjugated antibodies: anti-CD3-PerCP (BD Pharmigen), anti-CD56-BV510 (BD Pharmigen), antiCD16-eFluor450 (eBioscience), NKG2C-PE (R&D Systems) and anti-NKG2A-PEvio770 (Miltenyi-Biotec). NK cells were defined as CD3(−) CD56(+) lymphocytes. Samples were acquired with an LSRFortessa (BD Biosciences), analyzing data using FlowJo software (Tree Star).

Functional assessment of antibody-dependent NK cell activation

After overnight incubation of PBMCs at 37°C with recombinant interleukin 2 (IL-2) (200 U/ml), the response of NK cells to the HLA class I-defective 721.221 cell line with or without rituximab (50 ng/ml) was assessed in representative samples (early B cell repopulation, $n = 5$; late B cell repopulation, $n = 5$) following a 4 h incubation (effector to target ratio 1/1), as previously described [23]. Cell degranulation was assessed with anti-CD107a-FITC (BD Bioscience) during incubation together with monensin (GolgiStop®; BD) and brefeldin (GolgiPlug®; BD); subsequently, cultures were stained with anti-CD3-PerCP (BD Pharmigen) and anti-CD56-BV510 (BD Biosciences), fixed, permeabilized and stained intracellularly to assess the production of pro-inflammatory cytokines with anti-tumor necrosis factor α (anti-TNF-α) CFP-Blue (labeled by Immunostep) and anti-interferon γ (anti-IFN-γ) PE (BD Bioscience), as previously reported [23]. Data acquisition was performed with an LSRFortessa cytometer (BD Biosciences).

Analysis of the *NKG2C* genotype

The *NKG2C* gene copy number was evaluated in DNA samples as previously reported [22]. Briefly, after DNA isolation using the Puregene BloodCore Kit B (Quiagen), *NKG2C* copy number was assessed by polymerase chain reaction using two set of primers which amplified a 411-bp fragment in the absence of *NKG2C* and a second set of primers that produced a 201-bp fragment in the presence of *NKG2C*.

Statistical analysis

The Kolmogorov–Smirnov test assessed normal distribution, providing mean and standard deviation for parametric variables and median and interquartile range for non-parametric variables. Student's *t* test and the Mann–Whitney *U* test were used to analyze parametric and non-parametric values, respectively, calculating Pearson or Spearman correlation indices for pairwise continuous variables. Multivariate logistic regression analysis determined clinical predictors for B cell repopulation time. Results were considered significant at the two-sided level of 0.05. Data analysis was performed using GraphPad Prism 6.0 software and SPSS v.23 software.

RESULTS

Demographic and clinical characteristics of MS patients

Forty-seven MS patients treated with at least one dose of rituximab in the study period were evaluated. Nine patients were excluded due to loss of follow-up ($n = 4$) or because they were receiving a fixed dose of rituximab every 6 months regardless of B cell repopulation ($n = 5$). The remaining 38 MS patients constituted the study cohort (mean age 44.7 years ± 11.7; females, $n = 15$; RRMS, $n = 24$, PMS, $n = 14$). Thirty-two MS patients received rituximab for active MS (failure to previous DMT, $n = 18$; aggressive MS onset, $n = 14$) and six patients for disability progression in the previous year.

Table 1 shows clinical and demographic characteristics of MS patients classified according to B cell repopulation after rituximab as early ($n = 19$) and late ($n = 19$) (mean time to B cell repopulation 233 days ± 53 vs. 383 days ± 89, respectively, $p < 0.001$). No significant differences between patients with early and late B cell repopulation were observed for age, sex, body mass index, MS form, disease activity or disability. At the time of rituximab indication, patients with late B cell repopulation had a shorter MS duration and were more frequently DMT-naïve compared with those cases with early B cell repopulation (Table 1). However, after logistic regression analysis, no significant associations were found for time to B cell repopulation (MS duration, B −0.017, exp(B) 0.983, 95% confidence interval 0.903–1.070; DMT-naïve, B −1.578, exp(B) 0.206, 95% confidence interval 0.035–1.215).

Adaptive NKG2C+ NK cells are associated with late B cell repopulation after rituximab in RRMS patients

The NK cell compartment was studied in MS patients previous to rituximab onset according to time to B cell repopulation. Total NK cell numbers, CD56^{dim} and CD56^{bright} NK cell subsets did not differ in MS patients according to B cell repopulation after rituximab (Table S1). Evaluating the expression of NK cell receptors in the whole MS cohort, proportions of NKG2C+ NK cells were significantly higher in MS patients with late B cell repopulation compared to those cases with early repopulation (19.9% ± 14.9% vs. 11.8% ± 9.3%, $p < 0.05$; no additional differences were observed for the expression of the inhibitory receptor NKG2A, the Fc receptor CD16 or the expression of CD57 as a marker of mature NK cells (Table S1).

Since NKG2C expression by NK cells is known to be lower in PMS compared to RRMS [22], NK receptors were subsequently evaluated according to MS form. A significantly higher NKG2C expression by NK cells was found in RRMS patients with late B cell repopulation compared to early repopulation (24.7% ± 16.2% vs. 11.3% ± 10.4%, $p < 0.05$), with no additional differences perceived for other NK cell receptors (Figure 1). In addition, a direct correlation between time to B cell repopulation and proportions of NKG2C+ NK cells was observed in RRMS patients ($R_{Spearman}$ 0.45, $p < 0.05$; Figure 2a). In contrast, the expression of NK receptors in PMS patients was not associated with B cell repopulation kinetics (Figures 1 and 2b). CMV serology, characteristically associated with higher NKG2C expression [17], did not differ in MS patients according to time to B cell repopulation and MS form (CMV seropositivity in RRMS patients with early B cell repopulation 79% vs. late B cell repopulation 70%, $p = 0.325$; CMV seropositivity in PMS patients with early B cell repopulation 60% vs. late B cell repopulation 89%, $p = 0.247$). No additional clinical variables were associated with NK cell immunophenotype (data not shown).

TABLE 1 Clinical and demographic characteristics of multiple sclerosis (MS) patients classified according to B cell repopulation after rituximab

	Early B cell repopulation (n = 19)	Late B cell repopulation (n = 19)	p value
Age (years)	42.8 ± 13.0	46.5 ± 10.4	0.333
Sex (female)	8 (42.1%)	7 (36.8%)	0.740
BMI	22.0 (20.0–25.5)	23.8 (21.6–27.8)	0.172
CMV(+) serology	14 (73.7%)	15 (78.9%)	0.703
MS duration (years)	17.0 (4–22)	5.0 (0.6–13)	<0.05
ARR	0.61 (0.27–0.82)	0.5 (0–1.0)	0.806
2-year RR	0.5 (0–1)	0.5 (0–1.0)	0.297
Time to last relapse (months)	2.0 (1.0–5.25)	5.0 (1.0–7.0)	0.659
Gd-enhanced MRI lesions	1 (0–8)	0 (0–1)	0.245
MS form: RRMS (n = 24)	14	10	
PMS (n = 14)	5	9	0.110
EDSS	3.95 ± 2.39	3.5 ± 2.49	0.531
MSSS	3.93 (2.79–5.63)	3.86 (2.44–8.49)	0.935
DMT-naïve	5 (26.3%)	13 (68.4%)	<0.01

Note: B cell repopulation was defined as detection of CD19+ cells >2% of total lymphocytes after rituximab treatment, classifying MS patients as early B cell repopulation (≤9 months, n = 16) or late B cell repopulation (>9 months, n = 19). Values are expressed as mean ± SD or median (first–third quartile). Previous treatment in patients with early B cell repletion: interferon beta (n = 2), teriflunomide (n = 2), dimethyl fumarate (n = 1), azathioprine (n = 3), fingolimod (n = 4), natalizumab (n = 1), glatiramer acetate (n = 1). Previous treatment in patients with late B cell repopulation: interferon beta (n = 3), fingolimod (n = 2), natalizumab (n = 1).

Abbreviations: ARR, annualized relapse rate; BMI, body mass index; CMV, cytomegalovirus; DMT, disease-modifying therapy; EDSS, Expanded Disability Status Scale; Gd, gadolinium; MRI, magnetic resonance imaging; MSSS, Multiple Sclerosis Severity Scale; PMS, progressive MS; RRMS, relapsing–remitting MS; 2-year RR, relapse in the previous 2 years.

NKG2C genotype and time to B cell repopulation after rituximab

Due to the influence of the *NKG2C* gene copy number in the expression of NKG2C by NK cells [16], an evaluation was made of whether the *NKG2C* genotype in our MS patients influences time to B cell repopulation after rituximab. Twenty-five MS patients were wild-type homozygous carriers ($NKG2C^{+/+}$), 11 patients had a hemizygous deletion ($NKG2C^{+/del}$) and two patients a homozygous deletion ($NKG2C^{del/del}$). In MS patients with early B cell repopulation, the frequency of homozygous deletion was 10.5% (n = 2), whereas 21% of cases (n = 4) had a hemizygous deletion (Figure 3); amongst patients with late B cell repopulation, a hemizygous deletion was observed in 36.8% (n = 7) without observing cases with homozygous deletion (Figure 3). No significant differences were observed for *NKG2C* genotype according to time to B cell repopulation.

Natural killer cell mediated ADCC in RRMS patients with late B cell repopulation after rituximab

Whether time to B cell repopulation after rituximab was associated with ADCC function mediated by NK cells was subsequently evaluated. Representative samples of RRMS patients were selected according to B cell repopulation (early repopulation, n = 5, %NKG2C+ NK cells 10.4 ± 5.8; late repopulation, n = 5, %NKG2C+ NK cells 30.5 ± 21). Neither MS patients with a homozygous NKG2C deletion nor PMS cases were included in the assessment. MS patients with late B cell repopulation, compared with patients with early B cell repopulation, showed a higher secretion of TNF-α and IFN-γ by NK cells after stimulation with the 721.221 lymphoblastoid cell line in the presence of rituximab (Figure 4). No differences were observed in NK cell degranulation between patients with early and late B cell repopulation (Figure 4). In line with previous studies [15,23], our results may suggest a higher ADCC function by NKG2C+ NK cells.

Clinical outcome of MS patients treated with rituximab

Once B cells reached 2% of peripheral lymphocytes after rituximab, patients received a new dose of 1000 mg. After a mean follow-up of 19.3 ± 8.4 months (range 6–35 months), the mean number of rituximab infusions was 2.7 ± 0.8. At the last follow-up, none of the patients presented clinical or radiological activity. Disability decreased in RRMS patients compared to the basal evaluation (Expanded Disability Status Scale 2.1 ± 2.2 vs. 2.51 ± 2.1, respectively, p < 0.05) but increased in PMS patients (Expanded Disability Status Scale 6.1 ± 1.4 vs. 5.8 ± 1.3, p < 0.05). Rituximab

FIGURE 1 NK cell subsets in MS patients according to clinical form and time to B cell repopulation after rituximab. Proportions of the main NK cell subsets CD56^{dim} and CD56^{bright} are depicted in (a) and (b), respectively. NKG2C expression by NK cells was higher in RRMS patients characterized by late B cell repopulation after rituximab (c). No differences were observed for NKG2A (d), CD16 (e) and CD57 (f) regarding clinical form and time to B cell repopulation. Bars represent mean with standard deviation. EP, early B cell repopulation (white dots); LP, late B cell repopulation (black dots); PMS, progressive MS; RRMS, relapsing–remitting MS. *p value <0.05
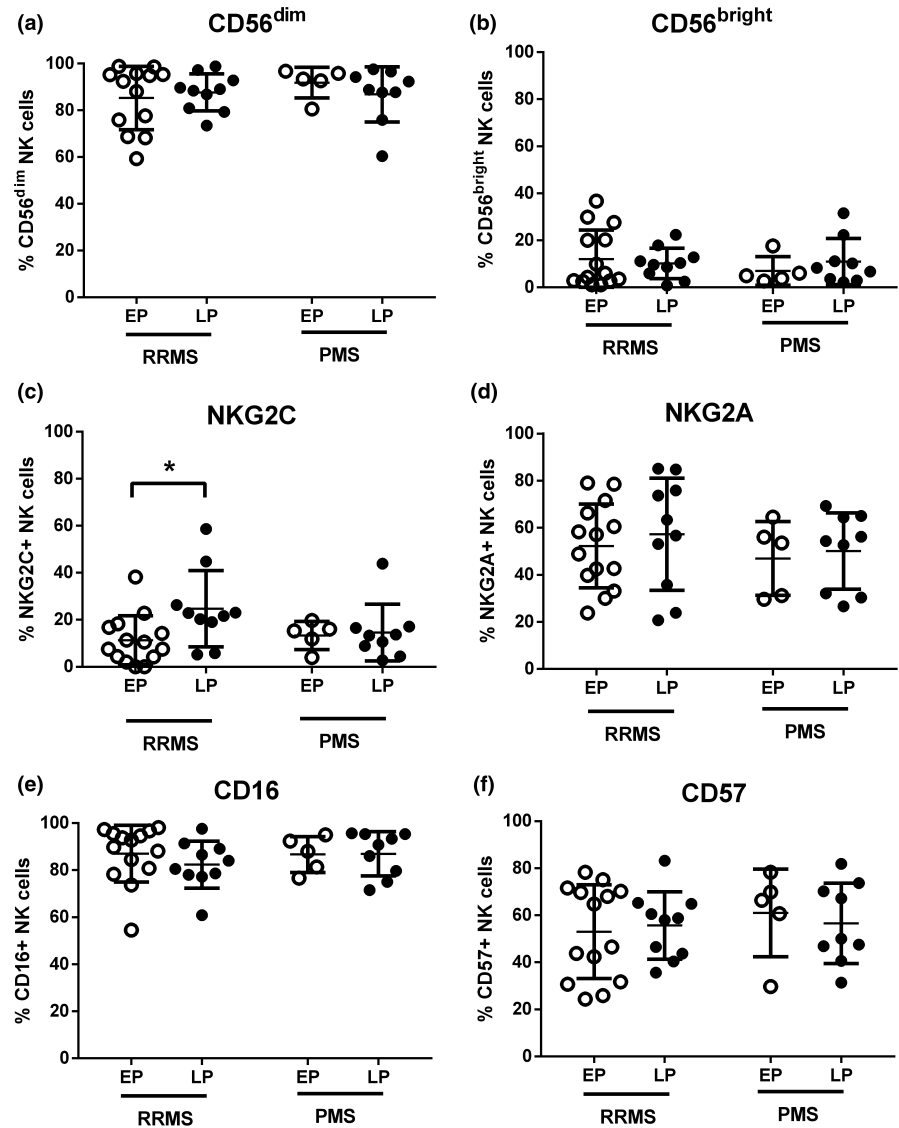
administration was well tolerated except for a patient who presented severe arthralgias after the first dose, without fever or rash, requiring rituximab discontinuation. No infections were observed in the follow-up period except for a case of COVID-19 pneumonia needing hospitalization due to low oxygen saturation; this patient recovered completely, and rituximab was continued after 3 months with no further consequences.

DISCUSSION

This study found an influence of baseline adaptive NK cells on B cell repopulation time in RRMS patients treated with rituximab. Those patients with higher proportions of NKG2C+ NK cells had a delayed time to B cell repopulation, a finding probably related to increased rituximab-dependent depletion of B cells by a higher ADCC function exerted by these lymphocytes. Our results point to the potential development of immunological biomarkers based on the mechanism of action of anti-CD20 MAbs, aiming to personalize B cell monitoring

and re-dosing to improve long-term efficiency and safety of these therapies in MS patients.

Rituximab is frequently indicated in MS as an off-label therapy under a considerable heterogeneity of protocols regarding induction doses and maintenance schedules. Rituximab re-dosing in inflammatory disorders is often performed based on monitoring B cell repopulation in order to avoid an intense depletion of the B cell compartment. However, there is no uniform criterion to define B cell repopulation after anti-CD20 MAbs. Some studies establish a threshold based on memory B cells (>0.05% of PBMCs) [24], whereas others use a variable B cell percentage (>1% or >2% of PBMCs) to indicate new doses of rituximab [10,11,25]. Nevertheless, safety concerns regarding MS reactivation are raised when anti-CD20 MAb maintenance is adapted according to B cell monitoring. In our study, an individualized protocol adjusting new doses of rituximab once B cell repopulation reached 2% of PBMCs was well tolerated and safe in the period evaluated (19.3 ± 8.4 months), without detecting new MS clinical or radiological activity.
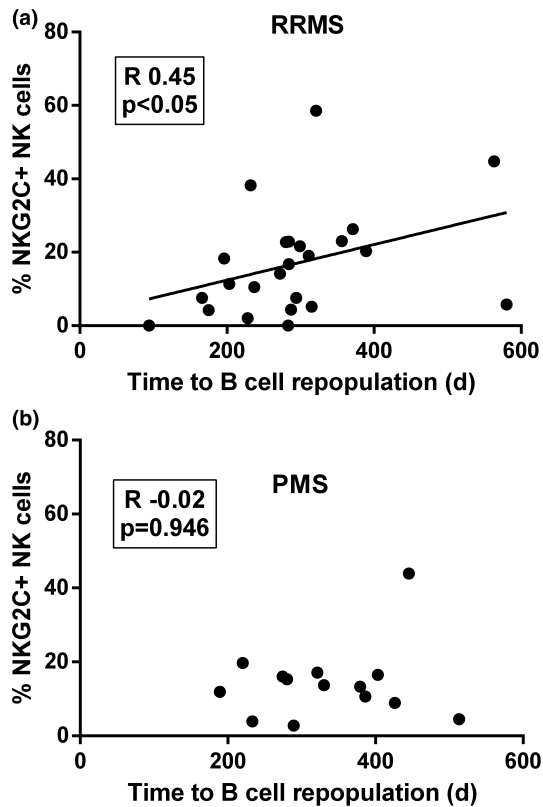
FIGURE 2 Correlation between NKG2C expression by NK cells and time to B cell repopulation after rituximab in MS patients: (a) RRMS, relapsing–remitting MS ($n = 24$); (b) PMS, progressive MS ($n = 14$). R, Spearman's correlation coefficient; d, days
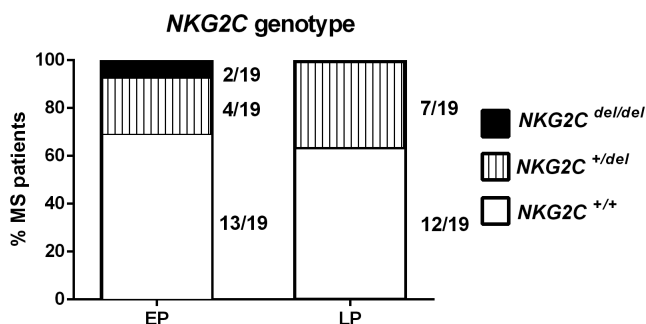

FIGURE 3 *NKG2C* genotype in MS patients according to B cell repopulation time after rituximab. EP, early B cell repopulation; LP, late B cell repopulation; *NKG2C*del/del, homozygous deletion; *NKG2C*+/del, hemizygous deletion; *NKG2C*+/+, wild-type homozygous carriers

Considering B cell repopulation kinetics after anti-CD20 MAbs, an increase in proportions of B cells producing pro-inflammatory cytokines (e.g., granulocyte-macrophage colony-stimulating factor and IL-6) was recently detected after 6 months of ocreli-zumab, suggesting the need for retreatment before complete B cell repopulation [26]. Nevertheless, B cell reconstitution at early stages is mainly formed by immature lymphocytes [27]and charac-terized by a remarkable reduction of memory B cells, which may

be prolonged for as long as 5 years, as noted by some authors [28,29]. In this regard, clinical and radiological evidence does not support MS rebound activity as a general characteristic of the im-munological reconstitution period after anti-CD20 MAb therapy [30–32], in contrast to other immunomodulatory therapies (i.e., natalizumab, fingolimod). It should be noted that adjusting doses of anti-CD20 MAbs according to B cell monitoring may not be applicable in the setting of other inflammatory diseases such as neuromyelitis optica spectrum disorder, in which the B cell com-partment may have a different state of activation and migratory capability compared to MS [10], requiring a continuous admin-istration or adjusting new doses of anti-CD20 MAbs to a lower threshold of B cell repopulation.

Our results point to the mechanism of action of rituximab to differentiate some MS patients according to B cell repopulation time. Rituximab induces the death of target cells by several cyto-toxic mechanisms, including complement-dependent cytotoxicity, antibody-dependent cell-mediated phagocytosis and Fc-receptor-mediated depletion through cellular effector mechanisms such as ADCC mediated by NK cells. In the last few years, several studies have described an enhanced capability of NK cells to mediate ADCC in relation to the acquisition of adaptive epigenetic modifications in-duced by cytomegalovirus infection, the expansion of NKG2C+ NK cells being the hallmark of this adaptive immunophenotype [17]. In our study, a higher secretion of TNF-α and IFN-γ by NK cells after antibody-dependent NK cell activation was related to the expansion of NKG2C+ NK cells in RRMS cases, in line with previous studies in the field [23]. No differences in CD107 as a marker of NK cell degranulation in the ADCC functional evaluation was perceived re-garding time of B cell repopulation, in contrast to pro-inflammatory cytokines (IFN-γ, TNF-α), a finding probably related to the low sam-ple size evaluated in our study ($n = 10$) in contrast to previous works performed in large cohorts of MS patients [23]. However, it is im-portant to note that adaptive NK cell responses and ADCC are not exclusively mediated by NKG2C+ NK cells, as shown by individu-als with the homozygous *NKG2C* deletion [33]. In this regard, two *NKG2C*del/del MS patients were detected who had a complete deple-tion of B cells after rituximab, underlying the existence of alternative mechanisms in addition to ADCC to explain the biological effect of anti-CD20 MAbs. Interestingly, early B cell repopulation was appre-ciated in both patients with *NKG2C* homozygous deletion, although any relation between the *NKG2C* copy number and time to B cell re-population was limited by the low sample size evaluated in our study.

Adapting rituximab re-dosing to B cell repopulation opens an in-teresting approach to MS therapy under the scope of personalized treatment regimens [29]. In this regard, the COVID-19 pandemic and its higher risk of severe infection observed in some MS patients treated with anti-CD20 MAbs may lead to consideration of an ad-justed dose according to individualized monitoring of B cell repopu-lation [32]. Mean time of B cell repopulation after rituximab usually varies between 6 and 9 months, but with a high interindividual and intraindividual variability [34,35]. However, studies evaluating B cell repopulation after anti-CD20 MAbs are limited, mainly explained by
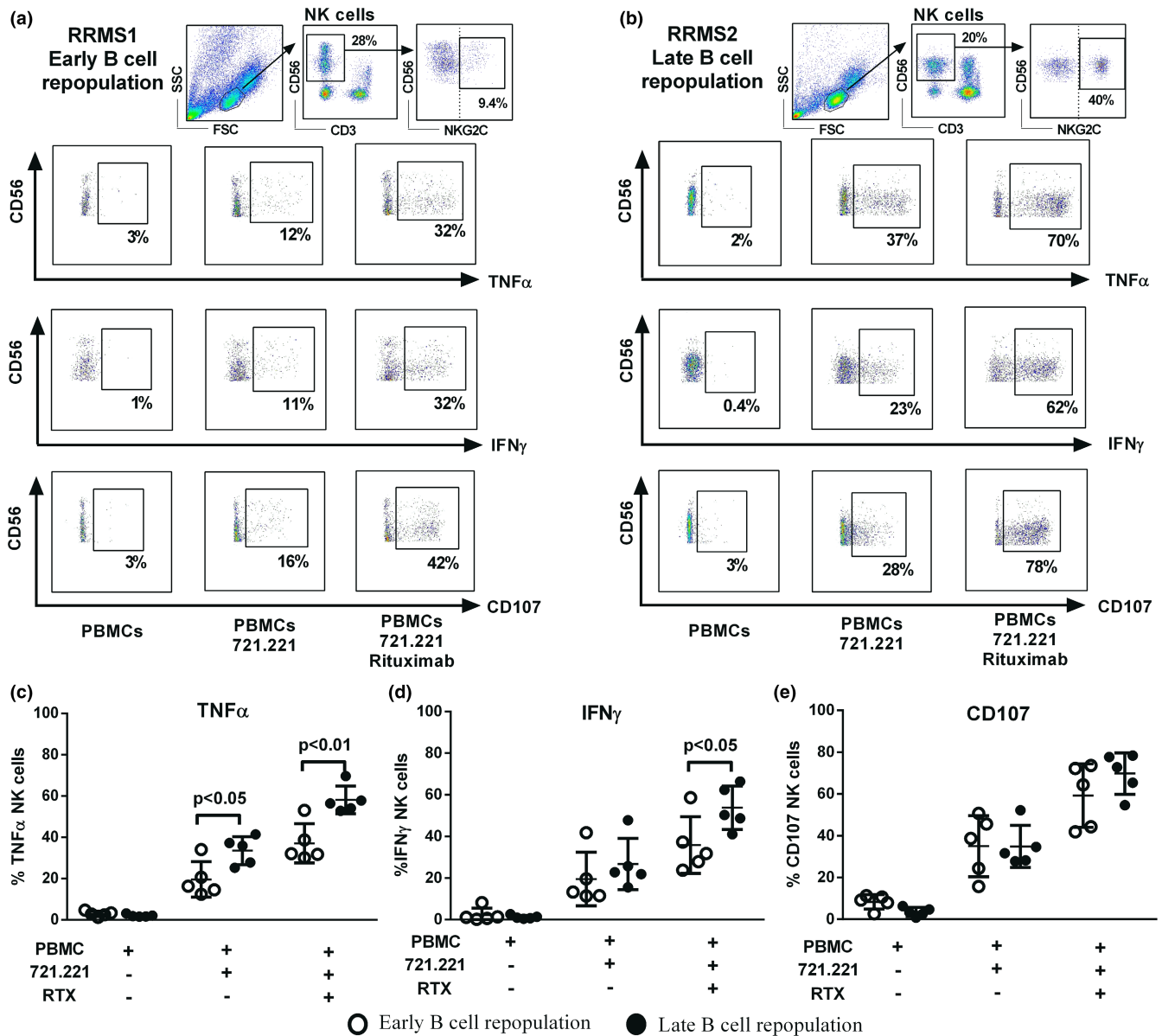
FIGURE 4 NK-cell-mediated ADCC function in RRMS patients and time to B cell repopulation after rituximab. NK cells were incubated with the 721.221 lymphoblastoid cell line with or without rituximab. ADCC responses were evaluated determining the proportion of NK cells producing pro-inflammatory cytokines (TNF-α, IFN-γ) and expressing the CD107a surface marker of NK cell degranulation. Representative examples of cases with early B cell repopulation and late B cell repopulation are depicted in (a) and (b), respectively, showing the gating strategy on NK cells and NKG2C expression in the upper right side of each figure. The mean production of intracellular TNF-α (c), IFN-γ (d) and CD107a surface expression (e) in different experimental conditions including PBMCs, 721.221 cells and rituximab is shown below (early B cell repopulation, $n = 5$; late B cell repopulation, $n = 5$). FSC, forward scatter; RTX, rituximab; SSC, side scatter; 721.221, lymphoblastoid cell line 721.221 [Colour figure can be viewed at wileyonlinelibrary.com]

the need to continually administer rituximab in an onco-hematological scenario. In MS, some authors have described a relationship between B cell repopulation and body surface area [10]. In our study, time to B cell repopulation after rituximab was not independently associated with any clinical or demographic variable. However, a significant association was found between NKG2C expression and time to B cell repopulation in RRMS cases. As previously reported [22,23], baseline MS clinical variables were not related to NKG2C expression by NK cells. Our results suggest that RRMS cases with expanded adaptive NKG2C+ NK cells might be selected for rituximab

administration based on B cell monitoring. In addition, it would be interesting to evaluate in future studies whether MS patients with expanded NKG2C+ NK cells may have a higher number of adverse events when treated with rituximab in fixed-dose administration (i.e., every 6 months), based on the assumption of a higher depletion of the B cell reservoir in these cases. No association between the NK immunophenotype and time to B cell repopulation was found in PMS patients, probably explained by the low expression of NKG2C by NK cells usually found in this MS form compared to RRMS [22], limiting the applicability of our results in progressive MS.

In summary, the assessment of baseline adaptive NK cells in RRMS patients may help in the selection of some RRMS cases in which rituximab may be associated with delayed B cell repopulation. This finding might facilitate the development of personalized regimens of anti-CD20 MAb therapy based on B cell monitoring after rituximab, aimed to reduce long-term adverse events whilst maintaining clinical efficacy. Since ADCC is the main mechanism exerted by other anti-CD20 MAbs (e.g., ocrelizumab and, especially, ublituximab), it would be interesting to evaluate in this setting whether NKG2C+ NK cell determination may allow a more accurate detection of MS patients characterized by a higher efficacy of anti-CD20 MAbs. In addition, the extension of our finding to other inflammatory disorders, which may have different kinetics of the B cell compartment, deserves further investigation.

CONFLICT OF INTEREST

AUTHOR CONTRIBUTIONS

Antia Moreira: Conceptualization (equal); formal analysis (equal). Elvira Munteis Olivas: Conceptualization (equal); formal analysis (equal); investigation (equal). Andrea Vera: Methodology (equal). Adrián Macias Gómez: Formal analysis (equal); methodology (equal). Bernat Bertran Recasens: Data curation (equal); methodology (equal); validation (equal). Miguel Ángel Rubio Pérez: Formal analysis (equal); methodology (equal). Mireia Llop: Methodology (equal); supervision (equal). Jose Enrique Martinez-Rodriguez: Conceptualization (lead); formal analysis (lead); funding acquisition (lead); investigation (equal); validation (equal); writing—original draft (lead); writing—review and editing (lead).

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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