



Impact of Natural Killer (NK) Cells on Immune Reconstitution, and Their Potential as a Biomarker of Disease Activity, in Alemtuzumab-Treated Patients with Relapsing Remitting Multiple Sclerosis: An Observational Study

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Abstract

Background Defining immune mechanisms leading to multiple sclerosis (MS) is difficult, due to the great inter-individual difference in immune system responses. The anti-CD52 antibody alemtuzumab transiently abolishes differences in immune parameters among individuals, allowing analysis of subsequent immune cell repopulation patterns, and their possible role in MS.

Objective To evaluate the correlation between innate and adaptive immune cell subsets and disease activity in MS in the context of treatment with alemtuzumab.

Methods A two-center observational cohort of patients treated with alemtuzumab underwent immune profiling of T, B, and natural killer (NK) cells, biomarker, clinical and radiological follow-up.

Results After treatment, the percentage of NK and B cells increased; NK, T- and B-cell populations underwent a profound rearrangement. Within the effector T-cell compartment, treatment led to a transient decrease, followed by an increase, of T-helper 1 cells, and to a transient decrease of T-helper 17 cells. Within the T-regulatory compartment, naïve T-regulatory cells increased. Within the B-cell compartment, memory B cells and mature B cells decreased, whereas transitional B cells increased. Within the NK cell compartment, CD56^{bright} NK cells increased. Subjects without disease activity had a greater decrease in serum NFL and greater NK cell/CD3+ T cell ratio. NK cell numbers at baseline and after treatment influenced reconstitution of T and B cells, being inversely correlated with the reconstitution of proinflammatory CD3+ T cells and mature B cells, and directly correlated to the increase in transitional B cells.

Conclusions The results of this study provide novel evidence that NK cells influence reconstitution of adaptive immune cells upon alemtuzumab and that patients with a successful response to alemtuzumab have an early immune reconstitution dominated by NK cells.

1 Introduction

A great inter-individual difference in immune system responses exists in humans, making it difficult to define common mechanisms leading to human autoimmune diseases as compared to animal models [1]. Some treatments, however, due to a powerful effect on the immune system, transiently abolish differences in immune parameters among individuals

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Key Points

Efficacy of alemtuzumab for multiple sclerosis (MS) is believed to be mediated by its effect on adaptive T and B cells.

Results of this observational study evaluating immune profiles of people with MS treated with alemtuzumab show that responders have increased natural killer (NK) cells, and that NK cells, at baseline and in later follow-up, influence subsequent reconstitution of T and B cells.

NK cells regulate the adaptive immune response in MS after treatment with alemtuzumab.

and thus make it easier to open a window on common pathways leading to human autoimmunity. The anti-CD52 monoclonal antibody alemtuzumab, approved for the treatment of relapsing multiple sclerosis (MS), has such an effect. Disentangling the role of B and T lymphocytes in MS has always been difficult [2]. Alemtuzumab transiently depletes almost all circulating T and B lymphocytes—an effect followed by cell-specific repopulation patterns, which can highlight the role of immune subsets in autoimmunity. For instance, the early repopulation of B cells, which occurs in alemtuzumab-treated patients, has been hypothesized to cause both disease reactivation and the occurrence of another autoimmune disease (“secondary autoimmunity”) in a percentage of treated patients [3]. However, this is debated given studies that show that absolute numbers of T and B cells after alemtuzumab treatment do not correlate with MS activity or autoimmunity [4]. One possible explanation for the observed differences is that most studies on the effect of alemtuzumab do not take into account a third player of immune responses in humans, innate natural killer (NK) cells, the role of which is continually emerging in the control of adaptive immune responses [5–7]. Earlier studies have suggested that NK cells are, indeed, affected by alemtuzumab and that treatment leads to an increase in their regulatory subset, CD56^{bright} NK cells [8]. Moreover, NK cells are involved in mediating the effect of alemtuzumab through antibody-dependent cytotoxicity (ADCC) [9].

As such, the objectives of this study were: (1) to study the innate and adaptive immune reconstitution kinetics, and their possible mutual relationships, of alemtuzumab in MS, and (2) to study whether such kinetics are correlated to recurrence of MS disease activity or development of another autoimmune disease.

2 Methods

2.1 Longitudinal Follow-Up

Thirty-three subjects followed at two Italian clinical centers (31 at the MS center, Clinica Neurologica, Ospedale Policlinico San Martino, Genova, and two at the MS center, Ospedale di Crema) who started treatment with alemtuzumab were enrolled. Two patients became pregnant shortly after enrolment and withdrew from the study. Patients underwent clinical evaluation, with clinical examination and EDSS (Expanded Disability Status Scale) score, at baseline and every 3 months; contrast-enhanced 1.5 Tesla (T) magnetic resonance imaging (MRI) every 6 months; immune profiling with standard flow cytometry at baseline, end of treatment, and 1 month, 3 months, every 3 months until 12 months after treatment, 1 month after the second infusion,

and 15 months and then every 3 months until 24 months after treatment start. A subset of 21 patients underwent immune profiling for effector and regulatory T-, B-, and NK-cell subsets and serum sampling for neurofilament light chain (NfL) test throughout the first year of treatment. To track the possible occurrence of other autoimmune diseases, clinical and laboratory monitoring was performed during the entire duration of follow-up time, including dosage of thyroid function hormones, anti-thyroid antibodies, and absolute number of platelets every 3 months, as per standard clinical practice. Figure 1a summarizes the study flow and analyses.

2.2 Immune Profiling

Standard flow cytometry data were obtained from hospital records. For profiling of immune-cell sub-populations, in a subgroup of patients followed at the MS center, Neurology Clinic, Ospedale Policlinico San Martino, Genova, peripheral blood mononuclear cells (PBMCs) were isolated through gradient density centrifugation and frozen in fetal bovine serum with 10% dimethyl sulfoxide in liquid nitrogen until examination. For flow cytometry, after adequate rest time upon thawing, PBMCs were stained in Becton Dickinson Lyotubes Vials™ (BD Biosciences) as detailed in Supplementary Table 1 and Supplementary Fig. 1 (Electronic Supplemental Material, ESM) and as previously described [10]. In detail, classic Th1 cells were defined as CD3+CD4+CCR6-CD161-CXCR3+; non-classic Th1 cells were defined as CD3+CD4+CCR6-CD161+CXCR3+; Th17 cells were defined as CD3+CD4+CCR6+CD161+CCR4+; Th1/17 cells were defined as CD3+CD4+CCR6+CXCR3^{hi}CCR4^{low}CD161+; CD4+ and CD8+ regulatory T cells were defined as CD3+CD4+CD25^{high}CD127- cells (naïve: CD45RA+; effector: CD45RA-) and CD3+CD8+CD28-CD127-, respectively. B-cell subsets were defined as: CD3-CD19+CD38^{int}, CD24^{int} (mature B cells); CD3-CD19+CD38^{int}CD24^{hi} (memory B cells); CD3-CD19+CD38^{neg}CD24^{hi} (memory atypic B cells); CD3-CD19+CD38^{hi}CD24^{hi} (regulatory B cells). NK cells were defined as CD3-CD16+CD56^{dim} (CD56^{dim} NK cells); CD3-CD16+/-CD56^{bright} (CD56^{bright} NK cells). Viability staining ensured staining of viable cells. Flow cytometry was performed with a BD FACS Canto II flow cytometer. Analysis of flow cytometric data was performed with FACS Diva (Becton Dickinson, Franklin Lakes, NJ, USA), FCS Express 6™ (DeNovo Software, Pasadena, CA, USA), and FlowJo™ (Flowjo LLC, Ashland, OR, USA).

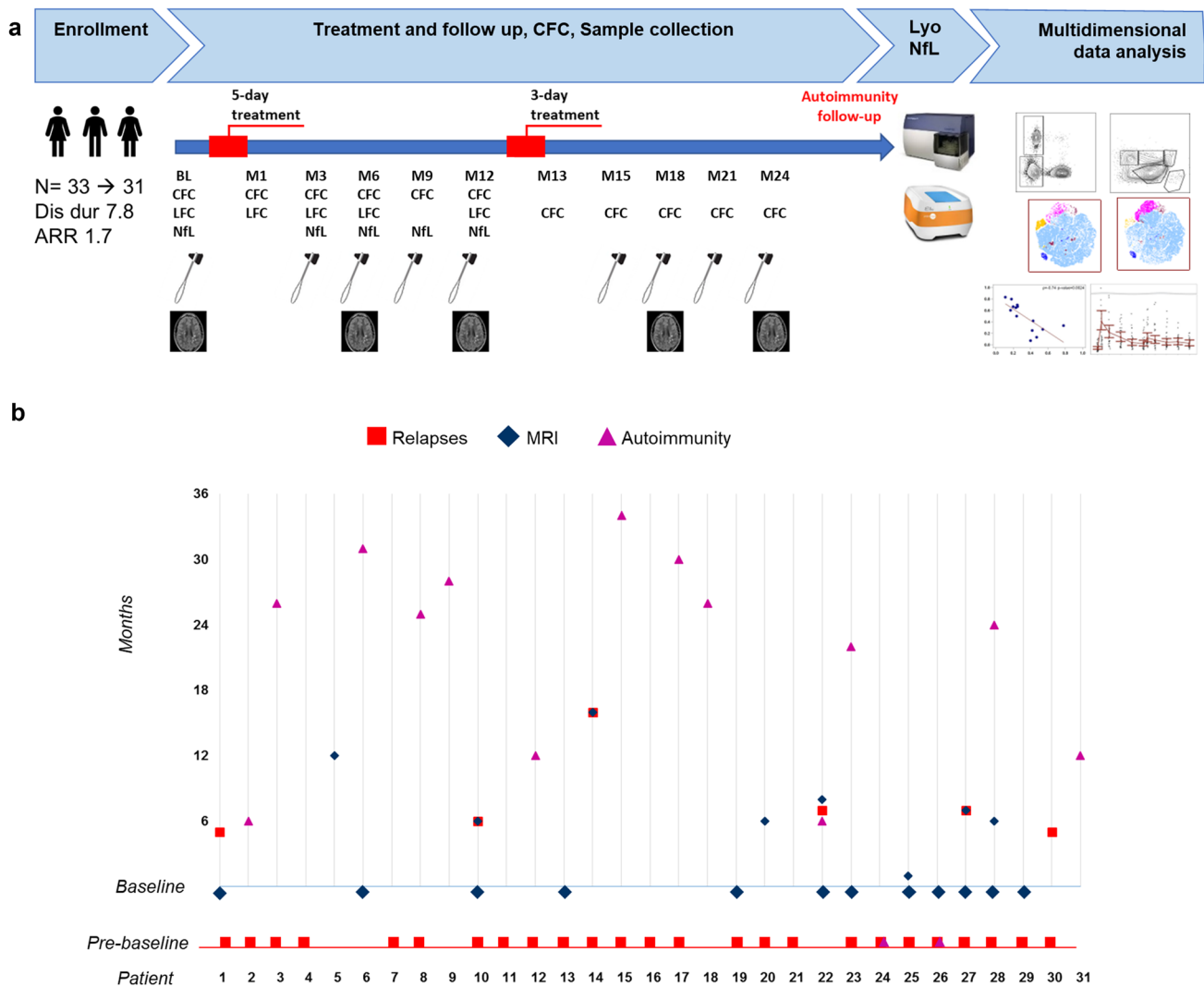


Fig. 1 Study flow and follow-up. **a** Study flow and analyses. Panels in the upper right side represent the type of conducted analyses, and not specific findings. Reflex hammers represent timepoints at which clinical disease was assessed. MRI symbols indicate timepoints at which the imaging assay was performed. The main immune cell populations were tested at baseline, months 1, 3, 6, 9, 12, 13, 15, 18, 21, and 24 with conventional flow cytometry (CFC). The immune cell subsets were tested with BD Lyotubes Vials® (LFC) at baseline,

and at months 3, 6, 9, and 12. Neurofilament light chain (NfL) was tested at baseline, and at months 3, 6, 9, and 12. **b** Timeline of clinical, MRI, and autoimmunity events before baseline (pre-baseline), at baseline and during follow-up (months) for every patient included in the study ($n = 31$). ARR annualized relapse rate, BL baseline, CFC main immune subsets flow cytometry, Dis dur disease duration, LFC lyotube flow cytometry, MRI magnetic resonance imaging, NfL serum neurofilament light chain

2.3 NfL Measurement

Serum samples from patients followed at the MS center, Clinica Neurologica, Ospedale San Martino, Genova, were stored at -80°C until analysis. On the day of analysis, samples were thawed and the concentration of NfL was measured through the ELLA™ platform (Protein Simple, USA), according to the manufacturer's instructions [11]. The NfL assay we employed has a lower limit of quantification = 2.7 pg/ml, an upper limit of quantification of 10,290 pg/ml, and a limit of detection of 1.09 pg/ml, according to the manufacturer.

2.4 Statistical Analysis

Longitudinal assessment of each cell population was performed by means of a linear mixed model with random intercept. Longitudinal differences on cell reconstitution according to disease or MRI activity and development of another autoimmune disease were tested including into the model the interaction between disease or MRI activity and the time. To account for missing data over follow-up, a multiple imputation by chained equations approach was used. Ten complete imputed datasets were generated and used for the subsequent

analyses. In each analysis only patients with available data for the baseline were considered. For this reason, the number of analyzed patients was different in each subset of analyses. Additionally, we performed a sensitivity analysis, using complete data, to check the robustness of the results.

Regarding NfL, due to the presence of just one observation between baseline and the first month, the two time points were gathered. Spearman's correlation coefficient was used to correlate the different cell populations at baseline and during follow-up. A p value < 0.05 was considered statistically significant. SAS (version 9.4) (SAS, Cary, NC, USA) was used for the computation.

3 Results

3.1 Clinical Results and Evaluation of NfL

Demographic, baseline, and follow-up clinical data and duration of follow-up are shown in Fig. 1b and Table 1. In particular, treatment was associated with residual MRI activity in 7/26 (27%) patients in the first year and 1/26 (3.8%) patients in the second year. In the first year of treatment, five patients had a relapse, while only one patient had a relapse in the second year. Median EDSS at baseline was 3.0 [interquartile range (IQR) 1.0–4.5], median EDSS at year 1 and 2 was 2.0 (IQR 1.5–4.0) and 2.0 (IQR 1.0–3.5), respectively. Of the patients, 13/31 (42%) developed another autoimmune disease during follow-up.

The serum concentration of NfL decreased significantly from baseline in the overall cohort and remained low throughout the follow-up (Fig. 2a). Baseline NfL values were significantly lower in patients with subsequent MRI activity than in patients who did not have MRI activity ($p = 0.042$), despite higher clinical activity in the pre-treatment period. Significantly different trends during follow-up on NfL concentrations between active and not active MRI patients were observed (p value for interaction = 0.003; Fig. 2b). In detail, the concentration of serum NfL increased in the first 6 months after treatment and then stabilized in those with active MRI, whereas in those without active MRI it decreased in the first 3 months and then stabilized.

The longitudinal concentration of NfL did not show any significant association with the occurrence of secondary autoimmunity (p value for interaction = 0.66) (ESM Fig. 2).

3.2 Analysis of Reconstitution of Main Lymphocyte Subsets After Treatment With Alemtuzumab

At baseline, data on standard immune profiling were available for the whole cohort (Table 2).

Flow cytometry revealed a decrease in the absolute counts of total T lymphocytes, CD4+ T cells, CD8+ T cells, and total B lymphocytes, as well as in the absolute counts of total NK cells, after the first and second infusion of alemtuzumab, followed by repopulation (Fig. 3). The percentage of immune cells among lymphocytes changed significantly after alemtuzumab infusion (Fig. 4): In the overall population, total T lymphocytes, CD4+ and CD8+ T lymphocytes decreased after the treatment and remained low over the time of observation. In contrast, after the initial decrease, the percentage of B lymphocytes markedly increased at month 3 after the first infusion, remaining at significantly higher levels compared to baseline during the first and second years of treatment. The percentage of NK cells markedly increased at month 1 and remained significantly higher than baseline throughout the first and second years of treatment; B- and NK-cell percentages had a similar repopulation pattern in the second year of treatment (Fig. 4). The timeline for immune profiling is shown in Table 2.

No significant correlations (ranging between -0.17 and -0.27) were observed between baseline NfL concentration and baseline lymphocyte subsets concentration. Similarly, no significant correlations were observed longitudinally (correlations ranging between -0.02 and -0.13) (ESM Table 2).

3.3 Analysis of Reconstitution of Effector and Regulatory T-, B-, and NK-Cell Subsets After Treatment With Alemtuzumab

Next, we evaluated immune reconstitution of T-cell, B-cell, and NK-cell subsets in the first year after treatment. Baseline data were available in 15 patients.

Among CD4+ T-effector cell subsets, we found an increase in T-helper 1 (Th1) cells (ESM Fig. 1a) at 3 months post treatment, followed by a gradual return to the baseline proportion, and a transient decrease in Th17 cells. Th1/Th17 were unchanged after treatment (Fig. 5a, b, ESM Table 3). Among T-regulatory cells, we found a decreased proportion of naïve T-regulatory cells (CD3+CD4+CD127-CD25^{high}-CD45RA+) (ESM Fig. 1b) upon treatment, followed by partial repopulation and an increase of CD8+CD28-CD127-regulatory T cells. Total CD4+T regs and effector CD45RA- CD4+ T-regulatory cells were not significantly

Table 1 Demographic characteristics, MRI activity and autoimmunity of the 31 enrolled subjects at baseline

Sex, <i>n</i> (%)	
Males	7 (23%)
Females	24 (77%)
Age, mean (SD) (years)	33.7 (8.9)
Duration of follow-up, median (range) (months)	42 (25–66)
EDSS 1 year before, median (IQR)	2.5 (1–3.5)
Number of relapses in the previous year, mean (SD)	1.7 (1.8)
EDSS baseline, median (IQR)	3.0 (1–4.5)
EDSS 1 year after first course, median (IQR)	2.0 (1.5–4.0)
EDSS 2 years after first course, median (IQR)	2.0 (1.0–3.5)
Disease duration, mean (SD)	7.8 (6.1)
Disease course, <i>n</i> (%)	
Relapsing-remitting multiple sclerosis (RRMS)	27/31 (87%)
Secondary-progressive multiple sclerosis (SPMS)	4/31 (13%)
MRI activity at baseline, <i>n</i> (%)	12/31 (39%)
Autoimmunity at baseline, <i>n</i> (%)	2/31 (6%)
Alemtuzumab courses, <i>n</i> (%)	
1	2 (6%)
2	29 (94%)
MRI activity [§] in the first year, <i>n</i> (%)	7/26 (27%) [§]
MRI activity in the first year, month (since baseline), median (IQR)	6 (6–8)
MRI activity in the second year, <i>n</i> (%)	1/26
MRI activity in the second year, month (since baseline)	16
Development of secondary autoimmunity, <i>n</i> (%)	13/31 (42%)
Thyroid autoimmunity	11/31 (35%)
Idiopathic thrombocytopenic purpura	2/31 (6%)
Skin psoriasis	1/31 (3%)

EDSS Expanded Disability Status Scale, IQR interquartile range, MRI magnetic resonance imaging, *N* patient count, SD standard deviation

[§]Five missing

affected by the treatment (Fig. 5c, d, ESM Table 3). Treatment resulted in profound changes in the B-cell compartment, with a decrease in CD24^{high}CD38^{int/neg} B memory cells and in mature, naïve CD24^{int}CD38^{int} B cells, and an increase in transitional, CD24^{hi}CD38^{hi} B cells (ESM Fig. 1c). These cells have been previously characterized for their regulatory properties and have been termed “regulatory” B cells [12]. A decrease in B-memory cells was persistent throughout the first year, whereas mature B cells repopulated starting from month 3 after treatment. At months 1 and 3, transitional B cells constituted about the 30% of the circulating B-cell pool (Fig. 5e, f, ESM Table 3).

Finally, among the NK-cell subsets, CD56^{bright} regulatory NK cells were increased at month 6 after treatment (Fig. 5g, h, ESM Table 3). Proportions of CD56^{dim}CD16+ and CD56^{dim}CD16- NK cell subsets did not change significantly (ESM Table 3).

3.4 Mixed-effect Model Analysis of Lymphocyte Reconstitution According to Response to Alemtuzumab Treatment in the First Year

Given the profound changes in immune cells upon immune reconstitution, we analyzed whether immune cell subsets at baseline or during reconstitution were associated with persistent MRI disease activity, defined as development of new T2 lesions and/or presence of contrast-enhancing lesions in the first year. Seven of 26 subjects (27%) had detectable MRI disease activity in the first year, whereas 19/26 (73%) did not have MRI activity.

Baseline lymphocytes numbers and percentages did not differ in those who later developed disease activity compared to stable patients (ESM Table 4). Similarly, no significant differences were observed for the NK/T cell ratio of responders versus non-responders ($p = 0.506$). With regard

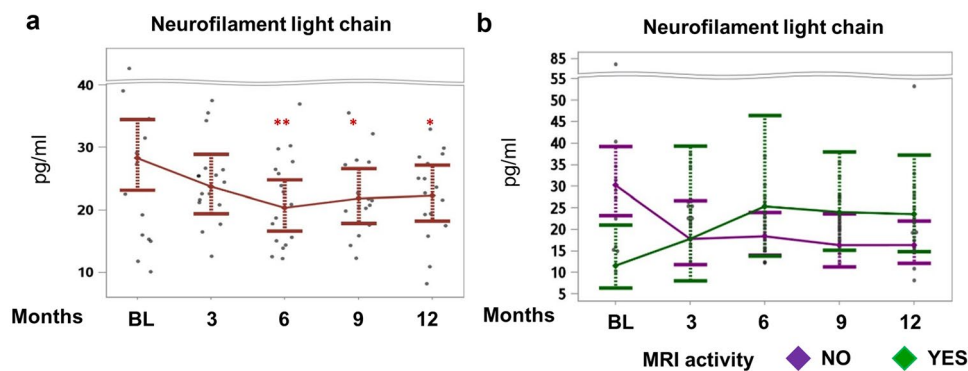


Fig. 2 Serum neurofilament light chain (NfL) during treatment. **a** Concentration of NfL in serum before and after immune reconstitution treatment with alemtuzumab. BL includes month 0 and month 1. $n = 17$, mixed-effect model $*p < 0.05$ compared to baseline; $**p < 0.01$ compared to baseline. **b** Concentration of NfL in serum accord-

ing to response to treatment defined by MRI activity over follow-up ($n=15$; two patients without MRI data). Mixed-effect model $*p < 0.05$ compared to baseline; $**p < 0.01$ compared to baseline, BL baseline, MRI magnetic resonance imaging

Table 2 Timepoints for flow cytometry

	Baseline	Month 1	Month 3	Month 6	Month 9	Month 12	Month 13	Month 15	Month 18	Month 21	Month 24
Total	31	27	25	27	26	24	15	23	20	20	15
Standard immune profiling	15	13	13	14	-	8	-	-	-	-	-
Immune subsets profiling	6	5	6	7	-	-	-	-	-	-	-
Standard immune profiling	4	4	3	4	-	-	-	-	-	-	-
Immune subsets profiling	11	16	16	17	-	-	-	-	-	-	-
Standard immune profiling	6	9	10	10	-	-	-	-	-	-	-
Immune subsets profiling	7	12	11	12	11	10	7	9	9	10	10
Standard immune profiling [§]	4	7	8	8	-	4	-	-	-	-	-
Immune subsets profiling	14	15	14	15	15	14	8	14	11	10	5
No secondary autoimmunity [§]	6	7	6	7	-	5	-	-	-	-	-
Immune subsets profiling											

Numbers indicate the number of patients whose samples were available at each timepoint

*MRI activity in the first year

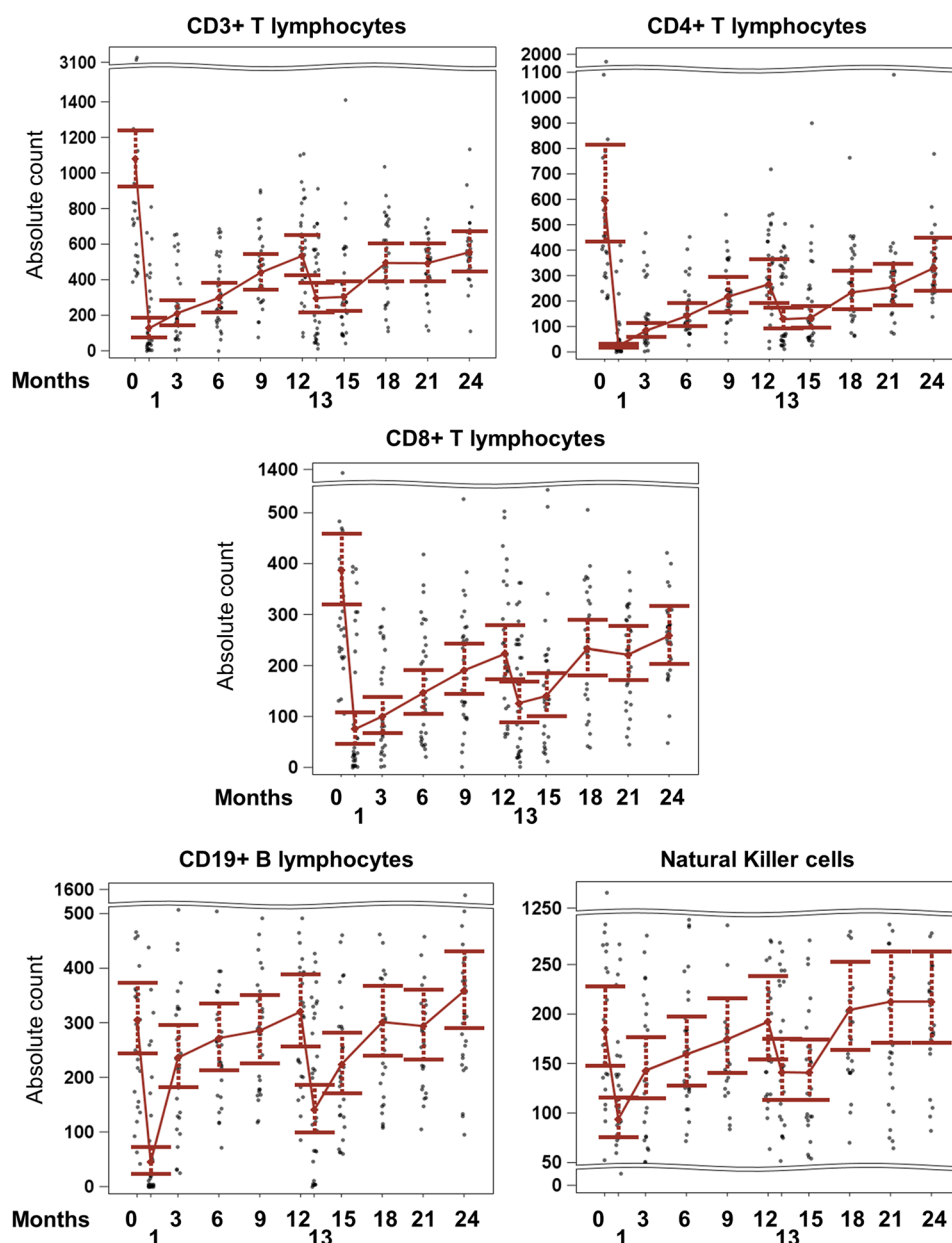
§ At any time point after first course

to immune cell reconstitution, changes in the absolute counts of lymphocytes were not associated with disease recrudescence. However, we found that patients without disease activity had a higher percentage of NK cells at month 1 and a low percentage of total CD3+ T cells, and vice versa, followed by a return to a similar proportion at the following time-points in both groups (Fig. 6a). This result did not change when the dataset was analyzed using non-imputed data. We analyzed whether the CD56^{bright} or CD56^{dim} ratio to T cells was different in responders versus non-responders, and we did not find any significant difference at 1 month after treatment ($p = 0.8650$ and $p = 0.7353$, respectively). Such a result may be explained by the lower number of patients where the percentage of CD56^{bright} and CD56^{dim} NK cell subsets were available ($n = 15$). In the overall population, treatment was associated with profound changes in the NK-cell/T-cell ratios at all timepoints (Fig. 6b). T-, B-, or NK-cell subset reconstitution did not differ in those who developed disease activity and those who did not (ESM Table 4).

3.5 NK Cell Numbers at Baseline and During Follow-Up Influence the Reconstitution of T and B Cells

Because of the differences in NK cell percentages among active and non-active subjects and given that MS is a disease mediated by a dysfunction of adaptive cell populations, we investigated whether there was a correlation between NK-cell percentage at baseline and the reconstitution of T and B cells (ESM Table 5). We found that baseline NK cell percentage was inversely correlated with CD3+ T cells at 1 month after treatment (Fig. 6c). Interestingly, at 3 months after treatment, the percentage of NK cells negatively correlated with total B cells (ESM Table 5); however, within the B-cell subset, we found a negative correlation between the percentage of NK cells and the percentage of mature B cells and a positive correlation between the percentage of NK cells and the percentage of transitional, “regulatory” B cells at the same timepoint (Fig. 6d, e, ESM Table 5). This result may suggest that NK cells modulate B-cell subsets differently. Additionally, for all the results reported above we performed the same analyses before multiple imputation and results were consistent with those reported.

Fig. 3 Effect of immune reconstitution treatment on absolute number of main immune cell populations. Results are shown as mean \pm 95% confidence interval ($n = 31$). Longitudinal observation, absolute numbers of indicated main immune cell subsets, before and after immune reconstitution treatment with alemtuzumab. Mixed-effect model: CD3+ T lymphocytes $p < 0.0001$ for months 1, 3, 6, 9, 12, 13, 15, 18, 21, and 24 compared to baseline. CD4+ T lymphocytes: $p < 0.0001$ for months 1, 3, 6, 9, 12, 13, 15, 18, and 21 compared to baseline; $p = 0.0045$ for month 24 compared to baseline. CD8+ T lymphocytes: $p < 0.0001$ for months 1, 3, 6, 9, 12, 13, 15, 18, and 21 compared to baseline; $p = 0.0012$ for month 24 compared to baseline. CD19+B lymphocytes: $p < 0.0001$ for month 1 compared to baseline; $p \leq 0.0001$ for month 13 compared to baseline; $p = 0.0288$ for month 15 compared to baseline. Natural killer cells: $p < 0.0001$ for month 1 compared to baseline; $p = 0.0468$ for month 3 compared to baseline; $p = 0.0377$ for month 13 compared to baseline; $p = 0.0349$ for month 15 compared to baseline.



3.6 Reconstitution of Immune Cell Subsets and the Development of Secondary Autoimmunity

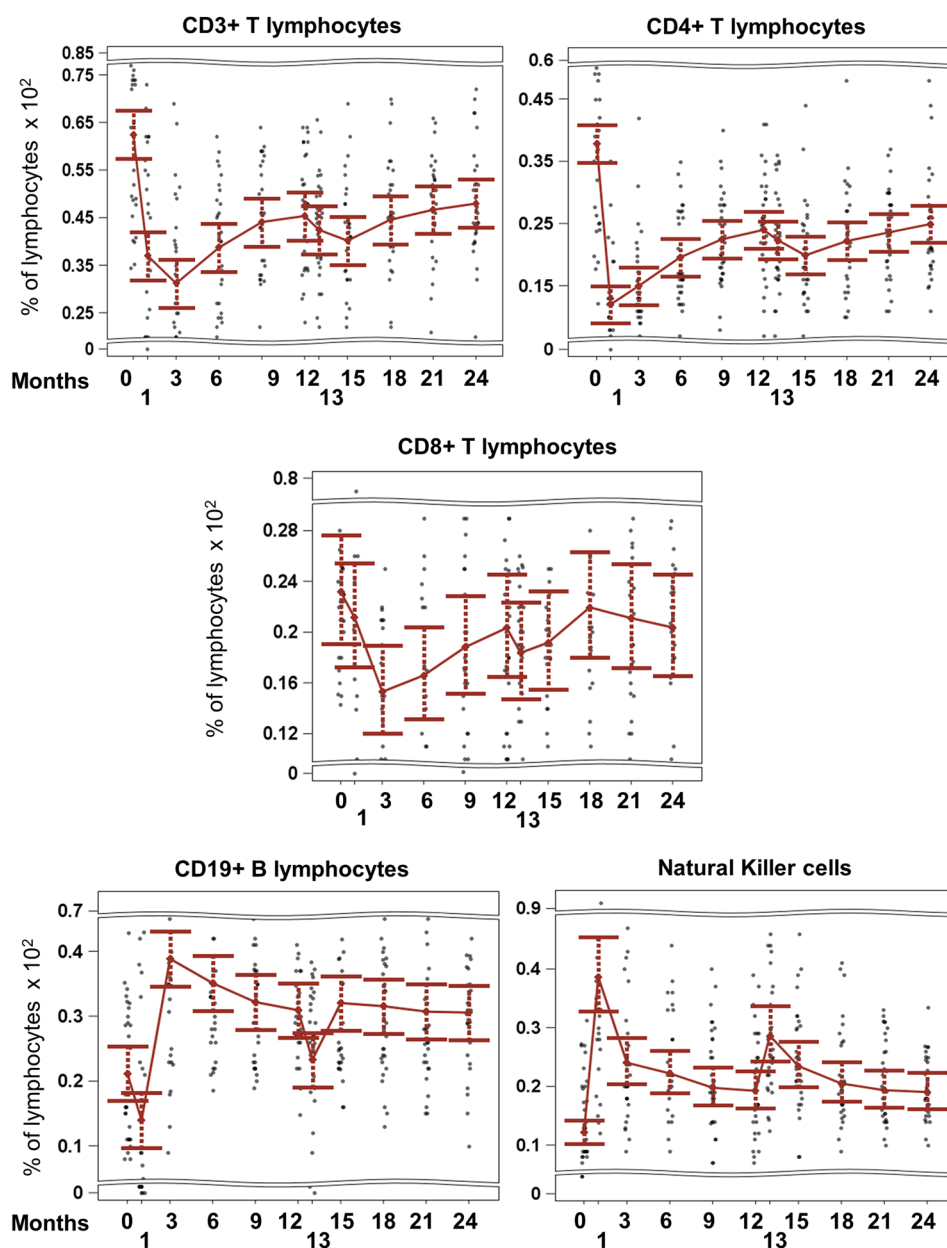
Since 13/31 (42%) subjects developed another autoimmune disease during follow-up, we evaluated whether baseline immune populations and/or immune reconstitution could be associated with autoimmune disease. No differences in the main immune cell subsets at baseline nor in the reconstitution patterns of immune cells were observed between

patients who later developed secondary autoimmunity and those who did not (ESM Table 4).

4 Discussion

The results of this study reveal the important role of NK cells as a biomarker of disease remission during immune reconstitution mediated by alemtuzumab in MS. First, we provide novel evidence that NK cells influence the reconstitution

Fig. 4 Effect of immune reconstitution treatment on percentage of main immune cell populations. Results are shown as mean \pm 95% confidence interval ($n = 31$). Longitudinal observation, percentage of indicated main immune cell subsets among lymphocytes, before and after immune reconstitution treatment with alemtuzumab. Mixed-effect model: CD3+ T lymphocytes $p < 0.0001$ for months 1, 3, 6, 9, 12, 13, 15, 18, 21, and 24 compared to baseline. CD4+ T lymphocytes $p < 0.0001$ for months 1, 3, 6, 9, 12, 13, 15, 18, 21, and 24 compared to baseline. CD8+ T lymphocytes $p = 0.0003$ for month 3 compared to baseline; $p = 0.0031$ for month 6 compared to baseline; $p = 0.0569$ for month 9 compared to baseline; $p = 0.0348$ for month 13 compared to baseline. CD19+ B lymphocytes $p < 0.0001$ for months 3, 6, 9, 12, 15, 18, 21, and 24 compared to baseline; $p = 0.021$ for month 1 compared to baseline. NK cells: $p < 0.0001$ for all time points compared to baseline



of T and possibly B cells, after alemtuzumab; second, we demonstrate that patients with a successful response to alemtuzumab have a very early immune reconstitution dominated by NK cells over T cells, and vice versa.

There is agreement on the involvement of the adaptive immune system in the pathogenesis of MS, but the role of innate lymphocytes, particularly NK cells, is less defined. Earlier studies in small numbers of patients suggested that MS disease activity may be associated with a reduced number/function of in NK cells [13–15], although evidence pointing to a beneficial role of NK cells upon different treatments emerged more recently. NK cells, particularly

regulatory CD56^{bright} NK cells, increase upon treatment with dimethyl fumarate, interferon-beta, and are higher in responders to fingolimod, and become more numerous than T cells after autologous hematopoietic stem cell transplantation (AHSCT) [16].

Many patients treated with alemtuzumab develop secondary autoimmunity, although the reason is still unclear [17]. In this cohort, alemtuzumab treatment led to disease control associated with a well-defined pattern of immune reconstitution, with most patients without MRI disease activity in the first year; the effect of treatment was also confirmed at the level of serum NfL, a well-known biomarker, the decrease of

which correlated with the decrease in MRI activity. Subjects with MRI activity at 6 months had a longitudinal increase in serum NfL in the previous 6 months, confirming previous findings by Agkun et al. [18].

We confirmed previous data showing that alemtuzumab causes a persistent decrease in absolute counts of T lymphocytes, involving both the CD4+ and CD8+ subsets, and a transient decrease of B lymphocytes that repopulate earlier than T cells, reaching numbers higher than baseline at 1 year after treatment [3, 4, 19, 20]. Early reports, particularly from the animal model, suggested that alemtuzumab spares the innate compartment [9]. On the contrary, Gross et al. and Gilmore et al. found that treatment with alemtuzumab increases CD56^{bright} NK cells starting from 5 to 6 months after the first course [8, 21].

We found that alemtuzumab causes a transient decrease in NK cells, followed by a repopulation starting from month 3, reaching absolute counts even higher than baseline during the second year of observation.

We observed that such changes in lymphocyte numbers are associated with profound changes in the percentages of lymphocytes. In particular, percentages of T cells fell and remained lower than baseline, whereas percentages of NK cells and of B cells among lymphocytes steadily increased starting from month 1 and month 3, respectively.

T-, B-, and NK-cell populations each comprise subsets with effector or regulatory functions. In MS, a dysfunction of regulatory subsets of all populations exists, supporting the view that a defect in peripheral immune regulatory mechanisms eventually contributes to autoimmunity [22–25]. In order to assess whether an effective treatment may potentiate immune regulatory functions, we evaluated the reconstitution in T-, B-, and NK-cell subsets after treatment, and found different rearrangements among main populations. In particular, within the T compartment, immune reconstitution was associated to an increase in supposedly proinflammatory Th1 lymphocytes and a decrease in Th17 lymphocytes. Among regulatory T cells, naïve CD4+ T cells decreased, but CD8+ CD28- regulatory cells increased. On the contrary, within the B compartment, treatment led to a persistent decrease in proinflammatory B memory cells, to a transient decrease in mature, naïve B cells, and to an increase in transitional B cells, which have a regulatory function [12]. Within the NK-cell compartment, regulatory CD56^{bright} NK cells increased 6 months after treatment. Such differential reconstitution curves are not fully explained by differences in the expression of CD52 on immune cells, since other studies have shown that absolute counts of all T CD4+ and B-cell subsets reach near to zero values after treatment, suggesting that the immediate response to treatment is similar for all lymphocyte subsets [4]. It has been previously reported that

inter-individual variability in immune depletion and immune reconstitution after alemtuzumab exists [26, 27]. Since NK cells mediate the effect of alemtuzumab through ADCC [9], we hypothesized that a difference in baseline NK cells could lead to different effect of the treatment. Accordingly, we report here for the first time that a higher number of NK cells at baseline correlates with a low number of CD3+ T cells 1 month after treatment. We did not find an association between baseline NK cell numbers and other T-cell subsets, nor with B-cell subsets.

In line with previous studies, we did not find differential reconstitution of absolute counts of immune cells in patients with or without MRI disease activity in the first year of treatment [4]. We hypothesized that if immune reconstitution repairs the peripheral defective regulatory function that characterizes MS, changes in the relative proportion of immune cells, rather than in absolute counts, may differ among full responders and suboptimal responders. Indeed, many regulatory mechanisms within the immune system involve cell-to-cell contact and may be enhanced by increasing the numbers of regulatory cells, as was previously shown with the anti-CD25 antibody daclizumab [28]. Accordingly, we found that patients without MRI disease activity had higher proportions of NK cells, and lower proportions of T cells, at 1 month after treatment. Compared to what has been previously reported by others [8, 21], the results of this study show for the first time that the repopulation of NK cells starting as early as at 1 month after treatment can predict future MS disease activity.

We and others have previously shown that NK cells in general, and the CD56^{bright} population in particular, have a regulatory function that is defective in untreated MS and that such a defect is reversed by treatment [29, 30]. Interestingly, an increase in the NK/T-cell ratio has been observed after AHSCT, another extremely effective treatment leading to a substantial immune reset [31].

Interestingly, here we demonstrate for the first time that the NK cell proportion not only at baseline, but also at later follow-ups, correlated with T- and B-cell reconstitution, at the same time point and at later observations, supporting the hypothesis that NK cells may contribute to the regulation of adaptive regulatory responses. Indeed, Darlington and coauthors have shown that NK cells regulate adaptive immune responses after AHSCT, through direct cytotoxicity of proinflammatory T cells [31]. We have previously shown that activated, but not resting, CD4+ T cells are susceptible to killing by CD56^{bright} NK cells [29]. Other mechanisms involved in the regulation of adaptive responses by NK cells may include secretion of cytokines, as demonstrated by several studies [32]. We cannot exclude the possibility that other cells belonging to the innate immune system, such

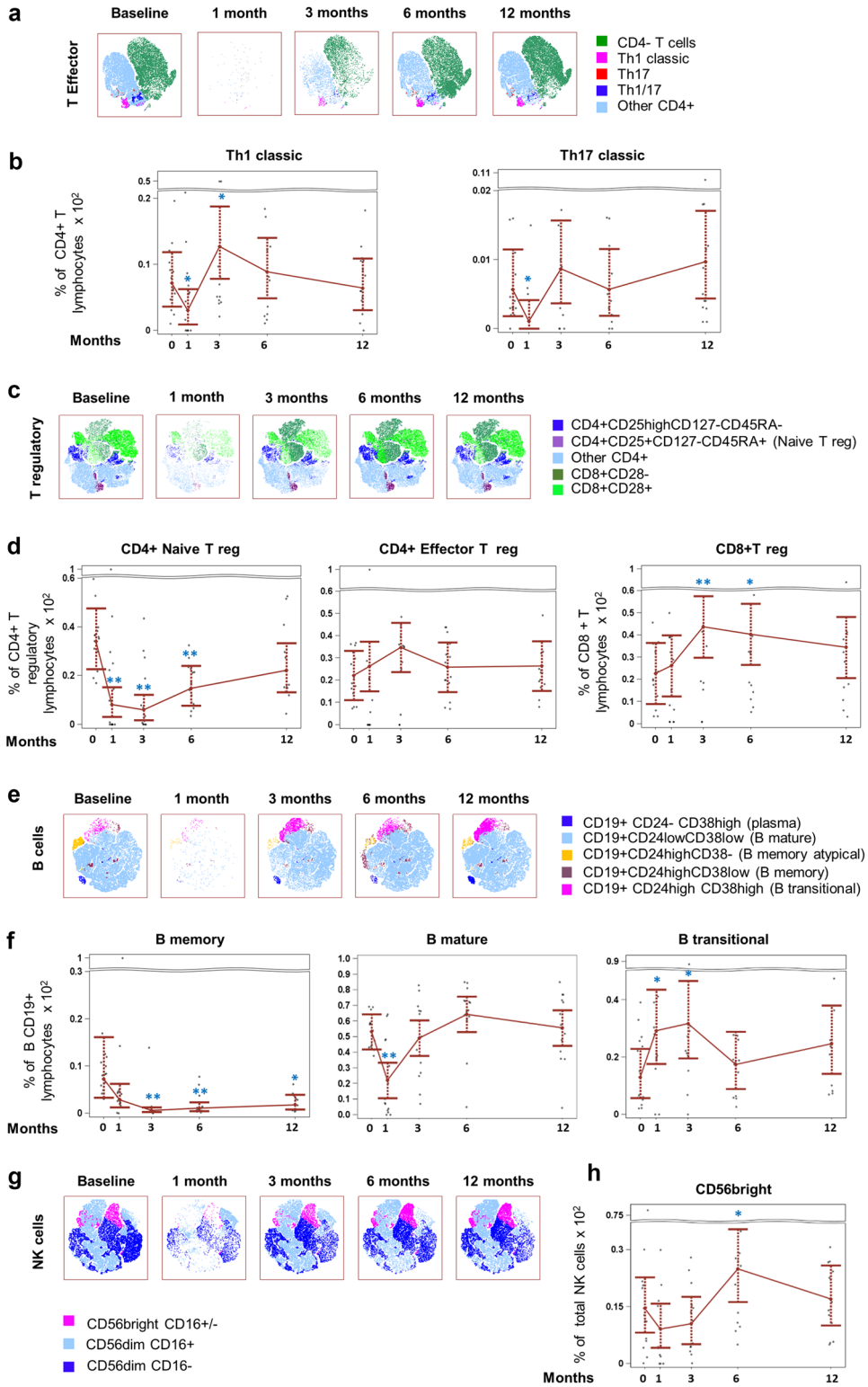


Fig. 5 Effect of immune reconstitution treatment on T-, B-, and natural killer (NK)-cell subsets. T-distributed stochastic neighbor embedding (tSNE) analysis was conducted on data obtained from a subgroup of six patients whose samples were available for all time points throughout the year after the first treatment. **a** tSNE plots of effector T-cell subsets clustering among CD3+ T cells before and during the first year after immune reconstitution treatment with alemtuzumab ($n = 6$). T-cell subsets clusters are represented in different colors as indicated in the legend. **b** Longitudinal follow-up of percentage of Th1 cells (left) and Th17 cells (right) among T lymphocytes before and during the first year after immune reconstitution treatment ($n = 14$). Results are shown as mean \pm 95% confidence interval. Mixed-effect model $*p \leq 0.05$. Results are shown as mean \pm 95% confidence interval. **c** tSNE plots of regulatory CD3+ T-cell subsets before and during the first year after immune reconstitution treatment with alemtuzumab ($n = 6$). **d** Longitudinal follow-up of percentage of CD4+ naïve T regulatory cells (left), CD4+ effector T regulatory cells (middle) and CD8+ CD28- regulatory cells (right) among T lymphocytes before and during the first year after immune reconstitution treatment ($n = 15$). Mixed-effect model $**p < 0.01$ compared to baseline. Results are shown as mean \pm 95% confidence interval. **e** tSNE plots of CD19+ B cells before and during the first year after immune reconstitution treatment with alemtuzumab ($n = 5$). One outlier was removed from the figure. B-cell subsets clusters are represented in different colors as indicated in the legend. **f** Longitudinal follow-up of percentage of B memory cells (left), naïve mature B cells (center), and transitional B cells (right) among B cells before and during the first year after immune reconstitution treatment ($n = 14$). Mixed-effect model $*p < 0.05$, $**p < 0.01$ compared to baseline. Results are shown as mean \pm 95% confidence interval. **g** tSNE plots of CD56+ NK cells before and during the first year after immune reconstitution treatment with alemtuzumab ($n = 6$). NK-cell clusters are represented in different colors as indicated in the legend. **h** Longitudinal follow-up of the percentage of CD56^{bright} NK cells among total NK cells before and during the first year after immune reconstitution treatment ($n = 15$). Mixed-effect model $*p < 0.05$ compared to baseline. Results are shown as mean \pm 95% confidence interval

as dendritic cells, may contribute to indirectly mediating the effect of NK cells on adaptive T- and B-cell subsets.

Interestingly, high NK cell/IL-17 CD4+ T cell ratio was recently reported to be associated with stable disease upon treatment with interferon-beta, supporting the hypothesis that the NK/T-cell ratio may be beneficial in MS in treatment settings that extend beyond immune reconstitution [33].

Importantly, the rise in B-cell numbers and proportion was not linked to disease activity in this cohort, in line with what has been observed previously [4], but not with what was suggested by case reports [34, 35]. Nor was the B-cell rise associated with secondary autoimmunity, as hypothesized earlier [3]. Indeed, the percentage of transitional B cells, which are known to have a regulatory function, increased with treatment, suggesting that B cells enhanced by treatment with alemtuzumab may not be pro-inflammatory.

Finally, we confirm previously reported data regarding no association between early reconstitution of immune cell subsets and later development of secondary autoimmunity [4]. Longer follow-up of immune cell reconstitution may be required to detect such an effect.

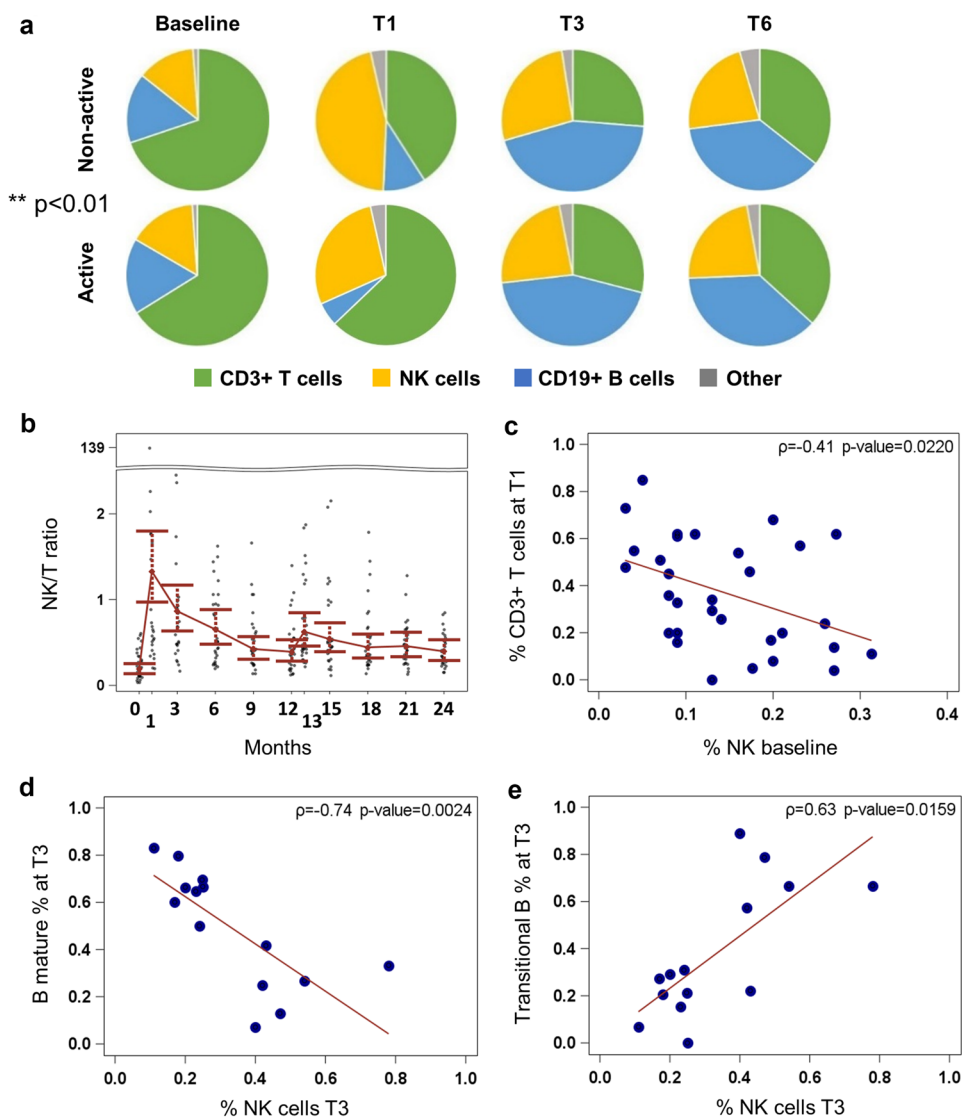
4.1 Limitations

This study has some limitations: first of all, the study cohort included a limited number of subjects and some analyses were not appropriately powered; second, samples for extended immune cell phenotype were available for a subset of patients while NfI was available only in the first year after alemtuzumab initiation. Moreover, immune cell subsets were defined by means of surface markers, and their effector or regulatory function was not demonstrated through functional studies. Finally, evaluation of innate immunity was limited to NK cells, and did not include other cells potentially involved in the immune response, such as for instance monocytes.

5 Conclusions

In conclusion, the results of our study highlight the potential use of NK cells as a biomarker of disease remission for alemtuzumab, and potentially for other depleting treatments followed by immune reconstitution, in MS, and suggest

Fig. 6 Reconstitution of NK cells and its relationship with disease activity and reconstitution of T and B cells. **a** Percentage of T cells, B cells, NK cells, and other cells among lymphocytes before and in the first 6 months after immune reconstitution treatment, stratified according to MRI disease activity. Mixed-effect model test: $p = 0.01$ for CD3+ T cells and $p = 0.01$ for NK cells. Results are shown as mean \pm 95% confidence interval ($n = 31$). **b** NK/T-cell ratios before and during 24-month follow-up after immune reconstitution treatment ($n = 31$). Mixed-effect model test, $**p < 0.01$. **c** Spearman correlation between NK cell percentages at baseline and percentage of CD4+ T cells (left) at 1 month after the first treatment ($\rho = -0.41$, $p = 0.02$; $n = 31$). **d** Spearman correlation between percentage of NK cells and percentage of mature B cells at 3 months after immune reconstitution treatment ($\rho = -0.74$; $p = 0.00$, $n = 15$). **e** Spearman correlation between percentage of NK cells and percentage of transitional B cells at 3 months after immune reconstitution treatment ($\rho = 0.63$; $p = 0.010$, $n = 15$). *MRI* magnetic resonance imaging, *NK* natural killer



that NK cells play an important role in regulating adaptive immune responses in MS.

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Declarations

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Conflict of Interest S. Palmeri reports no disclosures relevant to the article; M. Ponzano reports no disclosures relevant to the article; F. Ivaldi reports no disclosures relevant to the article; A. Signori reports no disclosures relevant to the article; C. Lapucci received research grants from Sanofi Genzyme and Roche; V. Casella reports no disclosures relevant to the article; M. T. Ferrò reports no disclosures relevant to the article; T. Vigo reports no disclosures relevant to the article; M. Inglese received research grants from NIH, NMSS, DOD, MS Society, FISM, INAIL, Italian Ministry of Health, European Union; received honoraria or consultation fees from Biogen, Roche, Merck, Genzyme, Novartis; G. L. Mancardi reports no disclosures relevant to the article; A. Uccelli received grants and contracts from FISM, Novartis, Biogen, Merck, Fondazione Cariplo, Italian Ministry of Health; received honoraria or consultation fees from Biogen, Roche, Teva, Merck, Genzyme, Novartis; A. Laroni received grants from Fondazione Italiana Sclerosi Multipla, Italian Ministry of Health, Italian Ministry of University; received honoraria or consultation fees from Biogen, Roche, Merck, Genzyme, Novartis.

Ethics Approval The study was approved by the Ethics Committee of IRCCS Ospedale Policlinico San Martino, Genova, protocol 190/12.

Consent to Participate Patients gave written consent to participate to the study.

Consent for Publication Not applicable

Availability of Data and Material (Data Transparency) Data are available upon reasonable request to the authors.

Code Availability (Software Application or Custom Code) Not applicable


Author Contributions SP: Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. MP: Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data; additional contributions: graphic presentation. FI: Major role in the acquisition of data; revision of the manuscript for content. AS: Drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data. CL: Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. VC: Major role in the acquisition of data; revision of the manuscript for intellectual content. MTF: Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. TV: Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data. MI: Drafting/revision of the manuscript for content, including medical writing for content. GLM: Drafting/revision of the manuscript for content, including medical writing for content. AU: Drafting/revision of the manuscript for content, including medical writing for content. AL: Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. All authors read and approved the final submitted paper and agree to be accountable for this work.

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