# Rituximab increases peripheral natural killer cells in patients with rheumatoid arthritis

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## Abstract

### Objective

*The clinical response of rituximab (RTX) is related to the degree of B cell depletion, although other circulating lymphocytes may be affected. We investigated the changes in lymphocyte sub-populations in rheumatoid arthritis (RA) patients treated with RTX and their relationship with the therapeutic response, with attention to natural killer (NK) cells.*

### Methods

*In fifty-one RA patients peripheral blood B and T lymphocytes and NK cells subtypes were counted by flow cytometry before and 3, 6 and 12 months after RTX administration. Patients were evaluated for disease activity with DAS28-CRP and EULAR response criteria at each visit.*

### Results

*RTX significantly increased from baseline values $CD56^{+}3^{-}$ cells (28 %, 19 % and 25 %; $p<0.001$, $p=0.009$ and $p=0.004$ respectively for month 3, 6 and 12) and $CD56^{dim}CD16^{+}$ cells (41%, 24% and 36%; $p<0.001$, $p=0.001$ and $p<0.001$ respectively for month 3, 6 and 12). $CD56^{bri}16^{-}$ cells were unaffected by RTX treatment. The increase in both $CD56^{+}3^{-}$ and $CD56^{dim}CD16^{+}$ cells was significantly greater in patients who were re-treated with another course of RTX at month 6 ($p=0.046$ and $p=0.010$ respectively). An inverse correlation between disease activity score and increase in NK cells was demonstrated. No significant changes were observed in $CD3^{+}$, $CD4^{+}$ and $CD8^{+}$ cells during the whole observation period.*

### Conclusion

*In RA patients, RTX treatment is associated with significant and persistent increase in $CD56^{+}3^{-}$ and $CD56^{dim}CD16^{+}$ NK cells. A correlation with disease activity is probable, although the association with clinical response remains to be proved.*

### Key words

rituximab, natural killer, rheumatoid arthritis

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## Introduction

Rituximab (RTX), a monoclonal chimeric anti-CD20 antibody, recognises a determinant expressed on intramedullary pre-B- to B-memory stage lymphocytes. Depleting the majority of circulating B-cells may induce various clinical response which can last for many months (1, 2).

Flow cytometry analysis of peripheral blood leukocytes after RTX therapy has shown effects on other cell types in addition to B lymphocytes. It was reported to lower the T helper type 17 (Th17) cells in rheumatoid arthritis (RA) and Sjögren syndrome (SS) (3), to change the migratory pattern of peripheral blood $CD4^+$ cells from RA (4), and, more recently, to partially deplete T cell lineages (5).

Recent studies have implicated human natural killer (NK) cells in the pathogenesis of RA (6). NK cells comprise a significant fraction of lymphocytes (8 to 25%) in the synovial fluid of RA patients and can be detected in the joint early during the disease course (7). In contrast to the accumulation of activated $CD56^+$ NK cells in the synovium, patients with RA have decreased circulating NK cells in their peripheral blood. In addition to the numeric deficit, peripheral blood NK cells in RA patients have decreased cytotoxicity on a per-cell basis (8). $CD56^{dim}$ constitutes 90% of the total NK cell population in peripheral blood and these express a low-affinity receptor for the constant region of immunoglobulin G, FcγRIIIa (CD16) (9).

In this study, we investigated lymphocyte count changes after RTX therapy with particular attention to NK cells and examined the association between changes in lymphocyte counts and clinical response to RTX in RA patients.

## Material and methods

The study includes fifty-one consecutive patients with RA who received RTX treatment over the last 4 years in our Unit. Diagnosis was confirmed according to the ACR 1987 criteria. Patients received a 1,000-mg infusion of RTX preceded by a 100 mg methylprednisolone intravenous pulse, at baseline, week 2 and then every six months. Disease activity was assessed using the Disease Activity Score in 28 joints (DAS28) before treatment and at each follow-up visit. Data on demographic and clinical variables were collected at each time point and patients were classified for low (DAS28 ≤3.2), moderate (3.2 <DAS28 ≤5.1), or high disease activity (DAS28 >5.1), or reaching remission (DAS28 <2.6), and according to the European League Against Rheumatism (EULAR) criteria (10), as responders (good or moderate), or non-responders.

This study is part of a large project on the monitoring of biological agents in RA, approved by our Ethics Committee and an informed consent was obtained from all patients. All patients gave their written informed consent to participate in the study.

### *Lymphocyte phenotyping by flow cytometry*

White blood cells (WBC) and differential cell counts were performed by an automated haematology analyzer (Advia 2120i Siemens, Malvern, PA). Fifty microliters of blood was distributed into each tube by the automated BD FACS Sample Prep Assistant II (Becton Dickinson, Mountain View, CA), a mixture of monoclonal antibodies conjugated with different fluorochromes (FITC, PE, PerCP, PE-Cy7, APC, APCCy7; BD Biosciences, San Diego CA) was added, red blood cells were lysed, and finally the cells were fixed (BD FACS Lysing Solution). Lymphocytes were analysed by flow cytometry (BD FACSCanto, Becton Dickinson) using BD FACS Diva software. Lymphocytes were isolated using CD45 *versus* SSC as a gating strategy. Different subsets of T cells were counted using the following monoclonal antibodies: APC-conjugated anti-CD3, FITC-conjugated anti-CD4, PE-Cy7-conjugated anti-CD8. The CD T cells were counted in the samples of $CD3^+$ T lymphocytes stained with anti-TCR c/d-PE. Natural killer (NK) cells were counted using APC-CY7-conjugated anti-CD16 and PE-conjugated antiCD56. B cells were counted using APC-CY7-conjugated anti-CD19. The laboratory used UK NEQAS (www.ukneqas.org.uk) for leu-

**Table I.** Baseline characteristics of patient population.*

| | | |
|---|---|---|
| Females, n (%) | 45 | (88) |
| RF, n (%) | 36 | (71) |
| ACPA, n (%) | 40 | (79) |
| Previous DMARDs | 4 | (2) |
| Previous biological drugs | 2 | (1) |
| Biologic drug naïve, n (%) | 11 | (22) |
| Age at baseline, y | 59 | (20) |
| Disease duration, y | 12 | (14) |
| Steroid usage, n (%) | 45 | (88) |
| Steroid dosage, mg | 5 | (2,5) |
| DMARD, n (%) | 31 | (61) |
| NSAID, n (%) | 40 | (78) |
| ESR, mm/h | 48 | (44) |
| CRP, mg/l | 10 | (22) |
| TJC(28) | 10 | (8) |
| SJC(28) | 3 | (4) |
| DAS28(CRP) | 6.03 | (1.60) |
| WBC, cells/μl | 7580 | (3790) |
| Lymphocytes, cells/μl | 1997 | (865) |
| $CD19^+$, cells/μl | 118 | (94) |
| $CD3^+$, cells/μl | 1710 | (683) |
| $CD4^+$, cells/μl | 1121 | (558) |
| $CD8^+$, cells/μl | 546 | (313) |
| $CD56^+3^-$, cells/μl | 94 | (74) |
| $CD56^{dim}16^+$, cells/μl | 87 | (67) |
| $CD56^{bri}16^-$, cells/μl | 6 | (8) |

*Except where indicated otherwise, data are expressed as medians (interquartile range).

kocyte immunophenotyping to ensure external quality.

*Statistical analysis*
Patient characteristics are described as numbers and percentages for categorical variables and median and interquartile range (IQR) for continuous variables. Correlation between continuous variables was evaluated by Spearman's correlation coefficient. The change in proportions of cell types at 3, 6 and 12 months was assessed by Wilcoxon signed-rank test. The comparisons of percentages and absolute lymphocyte changes between categories at each time point were assessed by Mann-Whitney test. Statistical analyses were performed using SPSS v. 20 with two-tailed tests. *p*-values <0.05 were considered statistically significant.

## Results

*Baseline characteristics of the patients*
Fifty-one patients were evaluated and analysed by flow-cytometry before administration of RTX at baseline, and then after 3, 6 and 12 months. Baseline characteristics are summarised in Table I.
An inverse correlation was demonstrated between $CD19^+$ cell counts and age at baseline (r=-0.374; *p*=0.007). Other correlations between lymphocytes subsets and baseline variables (disease duration, prednisone use and previous treatments) were analysed but none resulted significant (data not shown).

*Response to therapy and adverse events*
Response to therapy at each time point was evaluated according to EULAR response criteria and EULAR remission criteria for DAS28 values.
At month 6, twenty-four patients (47%) were classified as responders (good or moderate). A total of 34 patients (67%) were treated with another course of RTX. At the end of the study (month 12), 10 patients (20%) were in remission; there were 36 (71%) responders.
During the twelve months of follow-up, fourteen patients (27%) experienced infections, twelve of which were minor events; one patient had pneumonia and one had sepsis. Five patients had serious adverse events (meningoencephalytis, MALT lymphoma, acute cardiac failure and gastric ulcer) and discontinued therapy for this reason. Minor laboratory abnormalities were seen during the study, mostly increase in liver enzymes; only one patient had a decrease in lymphocyte counts <1000/μl at month 3, which increased above inferior limit in a repetition of the exam two weeks later.

*RTX induced an early and persistent increase in NK cell counts*
$CD56^+3^-$ NK cells showed a significant increase from baseline values at month 3, 6 and 12 (28% [50], 19% [71] and 25% [87]; *p*<0.001, *p*=0.009 and *p*=0.004 respectively; Fig. 1B). Similarly, the $CD56^{dim}16^+$ NK cells were significantly increased at each time point (41% [57], 24% [112] and 36% [85]; *p*<0.001, *p*=0.001 and *p*<0.001 respectively; Fig. 1C). Conversely, we could not demonstrate any variation in absolute or percentage values for $CD56^{bri}16^-$ cells (Fig. 1D).

*A second administration of RTX led to a further increase in NK cell counts*
An increasing trend was noticed for the $CD56^+3^-$ and $CD56^{dim}16^+$ NK cell percentage changes between month 6 and 12. Therefore, we compared the chang-

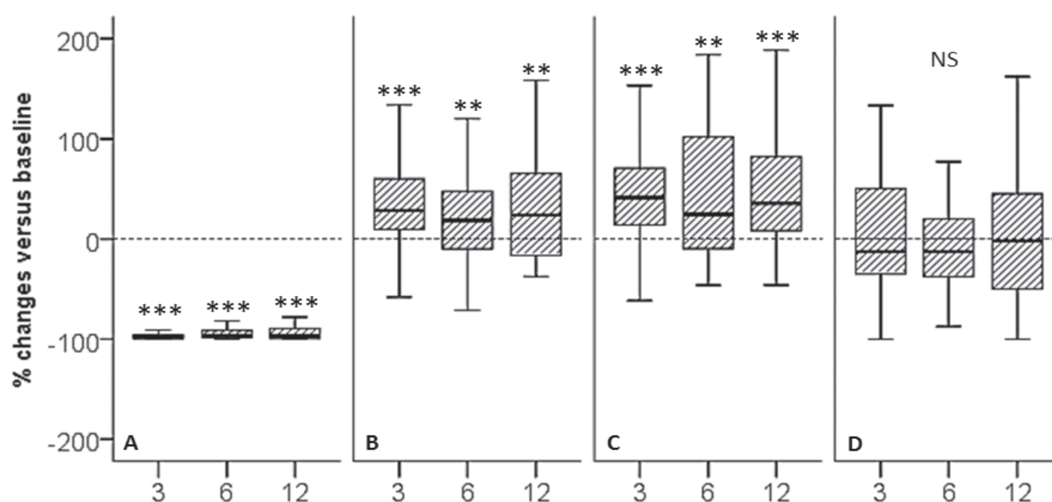


**Fig. 1.** Percentage changes *vs.* baseline value at each time point (month 3, month 6 and month 12) in $CD19^+$ (**A**), $CD56^+3^-$ (**B**), $CD56^{dim}16^+$ (**C**) and $CD56^{bri}16^-$ cells (**D**). Data represented by box plot. Each plot represents the first and third quartile; midline represen the median. Whiskers represent the mininum and maximum. *p<0.05, **p<0.01, ***p<0.001 *vs.* baseline.

es at month 12 between patients who were re-treated with another course of RTX at month 6, and found that this increase was mostly sustained by patients who underwent a second course of RTX (no retreatment *vs.* retreatment, percentage of variations between month 6 and 12: - 18% [35] *vs.* 24% [68], $p$=0.046 and - 7% [31] *vs.* 23% [70], $p$=0.010 respectively for $CD56^{+}3^{-}$ and $CD56^{dim}16^{+}$ NK cells).

*Inverse correlation between the increase in NK subsets and response to RTX*

Firstly, we aimed to elucidate correlations between absolute values of NK cells and DAS28-CRP in the entire study population (the 51 patients).

At baseline, we did not find any correlation between NK subsets and disease activity (DAS28-CRP). Similarly, values of NK cells at baseline were not significantly correlated with changes in DAS28-CRP evaluated at different time points. Therefore, in our study, baseline NK cells were not predictive of response to RTX.

We then looked at the percentage variations of NK cell counts and DAS28-CRP values from baseline. As soon as three months after therapy, a poor and negative correlation between $CD56^{+}3^{-}$ NK cells changes and DAS28-CRP was shown (r=-0.191, $p$=0.121). However, the same correlation resulted significant at six months from baseline (r=-0.329, $p$=0.018). Therefore, after six months of therapy the patients who had greater increases in NK cells showed also higher disease activity.

This result led us to compare lymphocyte changes in responders and non-responders to RTX therapy at month 6. As expected, the non-responders had greater increases both in $CD56^{+}3^{-}$ and $CD56^{dim}16^{+}$ NK populations, although results did not reach statistical significance. The same data was confirmed at month 3 and 12.

Thereafter, we proceeded to analyse the responders separately. In the 24 responders at month 6 we found that the higher DAS28-CRP values were correlated with the higher counts of $CD56^{+}3^{-}$ NK cells (r=0.470, $p$=0.020). This result was confirmed for the $CD56^{dim}16^{+}$ subset (and r=0.446, $p$=0.029, respectively).

Finally, we analysed the lymphocyte changes in the 36 patients who were classified as responders after 12 months. Again, we demonstrated that the decrease in DAS28-CRP values were lower in those who showed the greater increases in $CD56^{+}3^{-}$ NK cells (r=0.403, $p$=0.015; Fig. 2).

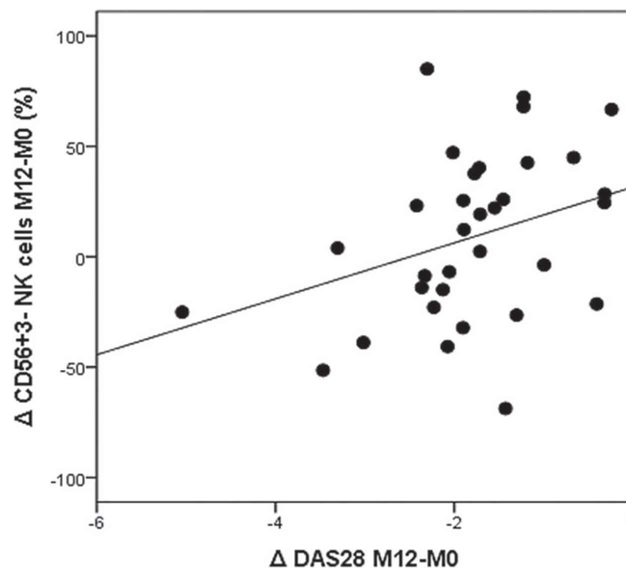


**Fig. 2.** Correlation of the percentage changes of $CD56^{+}3^{-}$ NK cells with absolute DAS28 changes at month 12 *vs.* baseline, in 36 responders.

In non-responders, the opposite inverse correlations between DAS28-CRP and NK subsets were found, although without reaching statistical significance (data not shown).

*Effects of RTX on B and T cells*

As expected, $CD19^{+}$ cell counts fell at month 3 (percentage of variations with respect to baseline values, medians [interquartile range]: -98% [4]; $p$<0.001), and the decrease persisted substantially unchanged at month 6, and month 12 (Fig. 1A) irrespectively to re-treatment at month 6 (data not shown). T lymphocyte counts (both $CD4^{+}$ and $CD8^{+}$ subsets) remained unchanged during the entire follow-up (data not shown).

**Discussion**

Many reports on B lymphocyte depletion during treatment with RTX in RA have been published, but the changes in other lymphocyte subsets by flow-cytometry are limited to $CD4^{+}$ T and $CD8^{+}$ T cells from the peripheral blood.

Mélet *et al.* observed a transient decrease of $CD4^{+}$ T cells (naive and memory cells) and $CD8^{+}$ T cells from the peripheral blood of patients treated with RTX, which was predictive of response to therapy (5). In a comment to this article, it was suggested that the depletion of $CD4^{+}$ T cells observed after RTX treatment might be due to direct effects of RTX on the small population of T cells co-expressing CD20, which is thought to represent a population that drives auto-reactive T cells (11). We did not observe any change in $CD4^{+}$ T and $CD8^{+}$ T cells from the peripheral blood, even by analysing separately the best responders (results not shown), and we did not find a severe decrease in lymphocyte numbers. It should be mentioned that the results from Mélet *et al.* were not confirmed by a number of small studies on T cell subtype counts in peripheral blood (12-14) or in synovial tissue (14) or bone marrow (12, 14) of patients treated with RTX.

To our knowledge, this is the first study to show that RTX induces significant and persistent increase in peripheral circulating NK cells.

Previously, it was reported that patients with RA have decreased circulating NK cells in their peripheral blood, and also they have a decreased cytotoxicity (8). Our data suggest that RTX in RA patients could restore those deficits probably by increasing CD16 expression on NK cells or $CD56^{dim}16^{+}$ cells themselves.

These high cytotoxic cells constitutes 90% of the total NK cell population in peripheral blood and constitutively express CD16 (FcRγIII), which is a low-

affinity receptor for the constant region of immunoglobulin G (9). The recognition of antibody-coated cells is associated with a potent signal to NK cells, which eliminate targets through direct killing and cytokine production (15).
RTX and other anti-CD20 monoclonal antibodies are known to promote down-modulation of CD16, which is the IgG receptor they use to mediate their effector functions (16). Importantly, CD16 cell-surface expression is down-modulated following NK cell activation and, in particular, exposure to stimulatory cytokines (IL-2 or IL-15) and metallo-proteinases (17).
However, the effect of anti-CD20 mAb-coated target cells on NK-cell expression of CD16 has been studied *in vitro,* whilst clinical and flow-cytometric data are currently lacking. Therefore, our results might not be in contrast with those previous findings. An increase of NK cells could have followed B-cell depletion by a homeostatic mechanism, but their increase might dump the efficacy of RTX on inflammatory processes.
Interestingly, CD20 is not internalised from the plasma membrane following monoclonal antibody treatment, allowing sustained immunologic action from complement and innate immunity (18). This peculiarity might help to explain the increase in peripheral NK cells after anti-CD20 therapy, as primary effectors of RTX-mediated citotoxicity.
NK cells cytotoxicity is promoted by type I interferons (IFNs), that are expressed and have a major role in several autoimmune conditions, in particular in systemic lupus erythematosus (SLE) (19). In RA, previous studies suggest that a type I IFN signature is possibly associated with a lesser response to rituximab treatment (20, 21). Our results provide new insights into this hypothesis. In fact, though we could not analyse the expression levels of IFN response genes or their serum levels, the increase in NK cells after rituximab we demonstrate could underlie a major activation of the type I IFN molecules.
Interestingly, clinical trials of RTX in SLE patients failed to show efficacy, although the explanation for those negative findings is still a subject of controversy for many reasons (22). However, in SLE, the function of NK cells is down-regulated with a shift from the $CD56^{dim}$ population to the $CD56^{bright}$ subset, and a reduced cytotoxicity (23, 24). In our study, we demonstrate a RTX-induced increase in the $CD56^{dim}$ subset. Though whether this effect will modify the clinical response to RTX in SLE patients is currently unpredictable, we suggest that future research of RTX in SLE should consider also the NK cell counts and their changes during treatment.
Moreover, there is evidence that rituximab resistance could be correlated to increased levels of B-cell activating factor (BAFF, or B lymphocyte stimulator, BLyS) (25). Since BAFF is dependently produced by activated NK cells (26), we suggest that BAFF levels could be increased in nonresponder patients to RTX therapy as a consequence of increased $CD56^{dim}$ cells. Belimumab, a BAFF-neutralising monoclonal antibody currently approved for SLE and under investigation also for RA, could restore sensitivity to rituximab-induced NK citotoxicity according to a recent study (26). Therefore, it has been proposed that B-cell depletion should be swiftly followed by BAFF inhibition has been (25). Our results are substantially in agreement with this theory.
The clinical response to RTX had been correlated with serum markers of B cell activation and with the proportion of some $CD19^{+}$ cell subsets (27). However, our results underline the possibility of a relationship between NK cells and disease activity during anti-CD20 therapy. We found correlations between DAS28 and absolute or percentage values of both $CD56^{+}CD3^{-}$ or $CD56^{dim}CD16^{+}$ cells at different time points during the study. Indeed, in our study population, the increase in NK cells was associated with the persistence of high disease activity.
We found greater increase of NK cell counts in nonresponders than in responders. The above mentioned RTX-dependent down-modulation of CD16 might explain why the greater increases were seen in nonresponders. However, the reason why a lack of response might result in an increase of NK cells is unclear and needs more robust data. In responders, NK cell numbers were significantly correlated with disease activity scores and baseline values were correlated with a greater decrease in DAS28 after RTX therapy (results not shown). We suggest that absolute numbers of NK cells could predict outcome in RA patients treated with RTX.
The increase after the second course of RTX is another original finding of our work. Rituximab showed an early impact on NK cells with a substantial increase already after three months from the first administration. The patients who were not re-treated with a second administration of RTX showed a decline in NK cell numbers at the final evaluation, whilst the others resulted in a stability or further increase compared to month 6 values. This result confirms that the increase in NK cells is dependent on the effect of RTX and self-limiting over time.
Our study has several limitations. Firstly, our patients had a very long disease course, resulting in disability and a clinical difficulty in reaching noteworthy decreases in disease activity. In fact, only one in five patients were in remission after twelve months of therapy. This could make it harder to highlight significant differences when analysing the correlation between lymphocyte changes and disease activity.
The second limitation is the presence of previous or concomitant therapy, which could have a role as a determinant in $CD16^{+}$ NK cell counts. However, the small number of biological drug naïve patients did not permit to perform a subanalysis. We did not find differences for glucocorticoid or methotrexate concomitant use (results not shown).
Finally, it has to be proved if the changes in NK cell numbers directly translate in a variation of cytotoxicity, which is considered the hallmark of their activation. Further studies are needed to correlate the CD16 cell-surface expression of NK cells in patients treated with RTX and their cellular activity.

## Conclusions
In RA patients RTX treatment is associated with important increases in NK populations but the interactions between anti-CD20 antibodies and NK cells, and the clinical significance, remain unclear.

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