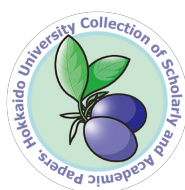




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Original Article

Anti-cyclic citrullinated peptide antibody titers decrease in rheumatoid arthritis patients treated with tocilizumab: A pilot study

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Abstract

Objectives. To analyze the effects of tocilizumab on peripheral B-cell subpopulation and its ability to produce anti-cyclic citrullinated peptide (CCP) antibody in patients with rheumatoid arthritis (RA).

Methods. Thirteen consecutive RA patients initiated with tocilizumab were enrolled in our prospective study. Anti-CCP antibody titers and clinical parameters were evaluated during treatment. Peripheral blood B-cell subsets were analyzed using flow cytometry according to the Human Immunology Project.

Results. Disease activity was significantly improved and anti-CCP antibody titers significantly decreased at week 24 compared to baseline. The percentages of post-switch memory B cells in CD19⁺ cells transiently increased at week 12, but there was no significant difference in any of the investigated B-cell subpopulations at week 24 compared to baseline. The ratios of post-switch memory to naïve B cells (post-switch/naïve) correlated negatively with anti-CCP antibody titers regardless of the time-points.

Conclusions. Our study indicated that tocilizumab has a potential to reduce anti-CCP antibody production presumably by affecting post-switch/naïve ratio, and that anti-CCP antibody titers reflect B-cell distribution/subpopulation. As anti-CCP antibodies are produced in lymph nodes or ectopic lymphoid structures in synovial tissues, not in circulation, transient increment of post-switch memory B cells after tocilizumab treatment may reflect the altered balance of B-cell distribution between circulation and arthritic joints, resulting in suppressed production of anti-CCP antibody in situ.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation, autoantibody production, and cartilage and bone destruction [1]. Although the etiology of RA is considered multifactorial, proinflammatory cytokines including tumor necrosis factor- α , interleukin (IL)-1 β , and IL-6, are known to play a crucial role in the pathogenesis of RA [2]. The changes in the synovium in patients with RA are characterized by neovascularization, infiltration of inflammatory cells and synovial hyperplasia, resulting in pannus formation [3]. IL-6 is a pleiotropic proinflammatory cytokine with a variety of biologic effects such as angiogenesis via production of vascular endothelial growth factor, osteoclast maturation and activation via receptor activator of nuclear factor kappa-B ligand from synovial tissues, autoantibody production via maturation of B cells, and production of C-reactive protein from the liver, which all contribute to the destructive arthritis [4].

IL-6 was originally identified as a B-cell differentiation factor that induces differentiation of B cells into mature plasma cells that secrete immunoglobulins [5]. The ability of IL-6 to promote humoral immunity has been linked to its effects on follicular helper T cells, a specialized subset of CD4⁺ T cells that express the chemokine receptor CXCR5 and localize to B cell follicles, where they promote B cell proliferation and immunoglobulin class switching [6]. The excessive activity of IL-6 causes polyclonal B cell activation, hyperglobulinemia, and autoantibody production [7]. In the pristane induced model of lupus, the direct role of IL-6 in controlling autoantibody production was demonstrated [8]. In RA, however, the effect of IL-6 on B-cell differentiation and autoantibody production has not been fully understood. Roll P et al indicated that tocilizumab in RA patients led to reductions in total IgG and IgA levels as well as reductions in pre- and post-switch memory B cells, but the results of anti-cyclic citrullinated peptide (CCP) antibody levels were not mentioned [9]. Carbone G et al showed that the serum levels of IgG4 anti-CCP antibodies were decreased but no reductions of IgG1 anti-CCP antibodies were observed after treatment with tocilizumab [10]. They demonstrated IL-21 is a powerful inducer of IgG4-specific anti-CCP antibodies in RA patients, but the effects of tocilizumab on B-cell subpopulation including autoantibody-producing components in RA were not discussed in the study.

B-cell subpopulation that we investigate on this study can briefly be described as follows. Naïve B cells are B cells that have not been exposed to antigens. These cells express the B-cell receptor such as IgM and IgD

molecules and then catch and recognize antigens in the first immune response [11]. Memory B cells are characterized as expressing CD27 surface antigen. They carry immunoglobulin somatic hypermutation and generate immunoglobulins rapidly and vigorously in the second immune response. This population is heterogenous, comprising mutated pre-switch memory B cells (IgD+CD27+) and post-switch memory B cells (IgD-CD27+). Pre-switch memory B cells are derived from naïve B cells responding to T-cell-dependent antigen as well as memory B cells participating in T-cell independent response [12]. Post-switch memory B cells are post-germinal center highly mutated memory B cells. Plasmablasts are generated in germinal centers along with memory B cells and can also originate from the differentiation of reactivated memory B cells or from early T-B cell interactions outside the germinal center [13]. They produce antibodies containing variable degrees of somatic hypermutation and of different immunoglobulin classes. Some of them will differentiate into long-lived plasma cells and migrate to the bone marrow to provide sustained serum antibody titers[14].

Tocilizumab is a recombinant humanized monoclonal antibody that binds with high affinity to both the soluble and membrane-bound forms of the IL-6 receptor. It has been recognized as one of the highly effective therapeutic agents in RA. To investigate the role of IL-6 in B-cell differentiation and autoantibody production in RA, we conducted a prospective clinical study to investigate the effect of tocilizumab on B-cell subpopulation and the production of anti-CCP antibody in our RA patients initiated with tocilizumab.

Materials and methods

Study design

Fourteen consecutive RA patients initiated with tocilizumab between December 2013 and September 2015 were enrolled in our prospective study. Indication for the initiation of tocilizumab was determined by attending rheumatologists and patients based on the clinical and social conditions of the patients. All patients met 2010 ACR/EULAR classification criteria for RA and were treated with 162mg of tocilizumab subcutaneously every other week. Peripheral blood samples were collected, and B-cell subsets were analyzed by flow cytometry during tocilizumab treatment at baseline, week 12 and week 24, according to the protocols suggested by Human Immunology Project [15]. Clinical parameters, including disease activity and the serum levels of autoantibodies, were also evaluated in these time points. The protocol of this study was approved by both ethics committees of

Hokkaido University Hospital and Chugai Pharmaceutical Co., Ltd.. Written informed consent was obtained from each patient. The present study complied with the Declaration of Helsinki.

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density-gradient centrifugation using Ficoll-Paque Plus (GE healthcare Life Sciences, Piscataway, NJ). PBMC were stained with CD19-PerCP-CyTM5.5, CD27-phycoerythrin (PE), CD38-Allophycocyanin (APC) and IgD-fluorescein isothiocyanate (FITC). B-cell subsets were defined as follows; naïve B cells [CD19+CD27-], pre-switch memory B cells [CD19+CD27+CD38-IgD+], post-switch memory B cells [CD19+CD27+CD38-IgD-] and plasmablasts [CD19+CD27+CD38+IgD-]. All antibodies were purchased from BD Biosciences (San Jose, CA, USA). Flow cytometry analysis was performed using BD FACS Aria II (BD Biosciences).

Statistical analysis

Continuous variables are expressed as mean $\pm$ SD (standard deviation) for those normally distributed or otherwise as medians and interquartile ranges (IQR). p-values were calculated for comparison between baseline and follow-up periods (week 12 or week 24) using paired *t*-test or Wilcoxon signed-rank test. Correlation coefficient was assessed by Pearson's correlation or Spearman rank method. p-values less than 0.05 were considered significant. All statistical analyses were performed using IBM SPSS Statistics (version 22.0.0, Inc., Armonk, NY, USA).

Results

Clinical parameters

Fourteen patients were enrolled, with one dropped out because of missing data. Remaining 13 patients were analyzed at the baseline, at week 12 and at week 24 (Table). Eight (62%) patients were female, the mean age was 55.8 ± 13.2 and the median disease duration was 44 (IQR: 24 - 60) months. Tocilizumab was started as a

first biological agent in 9 (69%) patients. Disease activity score 28-erythrocyte sedimentation rate (DAS28-ESR) and Health Assessment Questionnaire (HAQ) significantly improved at week 24 compared to baseline ($p<0.001$, $p=0.036$, respectively). Anti-CCP antibody titers significantly decreased at week 24 compared to baseline ($p=0.033$, Figure 1). Moreover, those with higher anti-CCP titers tended to result in decrement of this autoantibody in larger magnitude, compared with those with relatively low-positive anti-CCP.

Flow cytometry analysis

In flow cytometry analysis, the percentages of post-switch memory B cells in the population of CD19 positive cells were significantly increased at week 12 than those at baseline ($p=0.011$, Figure 2), but returned to the baseline at week 24 although there was a tendency to increment in this subpopulation in many of the patients. The other B-cell subsets including naïve, pre-switch memory, and plasmablasts showed no significant changes from baseline to week 12 or those to week 24. Intriguingly, the changes in naïve B cells and those in post-switch memory B cells from baseline to week 24 were inversely correlated ($r=-0.703$, $p=0.007$, Figure 3A): Namely, the increment of peripheral post-switch memory B cells was associated with the decrement of naïve B cells during treatment with tocilizumab.

The relationship between anti-CCP antibody titers and B-cell subsets

To clarify the effect of tocilizumab on the ability of B cells to produce anti-CCP antibody, we next examined the relationship between anti-CCP antibody titers and B-cell subpopulation. Interestingly, the ratios of peripheral post-switch memory to naïve B cells (post-switch/naïve) correlated negatively with anti-CCP antibody titers regardless of the examined time-points ($r=-0.621$, $p=0.024$ at baseline, $r=-0.623$, $p=0.023$ at week 12, and $r=-0.643$, $p=0.018$ at week 24, Figure 3B). However, there were no relationships between post-switch/naïve ratio and rheumatoid factor (RF) or disease activity of RA (data not shown). We also examined the relationship between IgG level and B-cell subsets, and between the ratio of anti-CCP antibody titers to IgG (anti-CCP/IgG) and B-cell subsets, as well as between anti-CCP antibody titers and B-cell subsets, at baseline and at week 24. We found the percentage of post-switch memory B cells in CD19 positive cells (post-switch/CD19+) negatively correlated with anti-CCP antibody titers at baseline and at week 24. IgG positively

correlated with the percentage of naïve B cells in CD19 positive cells (naïve/CD19+) at week 24, but we observed no other significant correlation between B-cell subsets and IgG nor anti-CCP/IgG at either time points (Supplementary Figure 1, 2).

Discussion

Many clinical trials have shown that tocilizumab reduces disease activity and inhibits joint destruction in patients with RA [16-18]. In this prospective study we demonstrated tocilizumab significantly reduced the titers of anti-CCP antibody as well as disease activity, suggesting the role of IL-6 in production of anti-CCP antibody in patients with RA. The effects of IL-6 inhibition on B cell subpopulations in circulation have been demonstrated in several studies. Roll P et al demonstrated that tocilizumab treatment affected the frequency of pre-switch and post-switch memory B cells in patients with RA and they found a correlation between changes in the DAS28 scores and the relative numbers of pre-switch memory B cells [9]. Moura RA et al demonstrated that the frequency of double negative (IgD-CD27-) B cells in circulation as well as IgM expression by double negative B cells were significantly decreased after treatment with tocilizumab [19]. However, no previous data have shown the relationship between the changes in B cell subsets and those in antibody titers in patients with RA who had received tocilizumab treatment.

We revealed tocilizumab has a potential to reduce anti-CCP antibody and to transiently increase the percentage of post-switch memory B cells in CD19+ cells (post-switch/CD19+). Although there was no significant difference in post-switch/CD19+ between baseline and week 24, continuous increment of post-switch/CD19+ was observed in some patients. The mechanism of transient impact of IL-6 inhibition on the increment of post-switch memory B cells in many of the patients is unknown, but the effect of IL-6 on autoantibody production via B-cell maturation may be associated with the altered balance of B-cell distribution in circulation and in synovial tissues. Stohl et al. showed the initial increment and gradual returning to baseline of memory B cells in the blood after treatment with belimumab in patients with systemic lupus erythematosus [20]. They demonstrated it may be secondary to their release from germinal centers where these B cells reside or may be due to inhibition of their return to these lymphoid tissues. We support their speculation because both tocilizumab and belimumab have a potential to inhibit autoantibody production even though the mode of action is different between these two biologics. Furthermore, the change in post-switch memory B cells was negatively

correlated with the change in naïve B cells during treatment with tocilizumab, suggesting that tocilizumab has a potential to reduce anti-CCP antibody production by affecting the balance of B cell subpopulation in circulation and in lymphoid tissues. As shown in Table 1, we demonstrate a marked decreased titer of anti-CCP antibody at Week 24, but not at Week 12. It seems there is a time discrepancy between the increment of post-switch/CD19+ and the decrement of anti-CCP antibody titers, but we speculate there would be a delay in anti-CCP antibody titer compared to the change of B cell subpopulation because of the half-life of IgG molecules. Wunderlich et al. [21] prospectively investigated the effect of biologic disease-modifying anti-rheumatic drugs (bDMARDs) on the levels of anti-CCP2 antibody. In this study, 20 patients treated with tocilizumab did not show significant reduction in anti-CCP2 antibody titers, although mean titers still substantially decreased. The reason for the discrepancy between this study and ours is unknown, but larger sized study would be awaited.

We also found post-switch/naïve ratio is a pivotal marker that reflects anti-CCP antibody production. Post-switch/naïve ratio negatively correlated with anti-CCP antibody titers regardless of the time points during treatment with tocilizumab. It seems paradoxical because memory B cells highly contribute to autoantibody production compared to naïve B cells, but we speculate B cell subsets travel to-and-fro between circulation and ectopic lymphoid structures in the synovial tissues or lymph nodes where anti-CCP antibodies are produced. These data indicated that anti-CCP antibody titers reflect B-cell distribution and that tocilizumab has a potential to modulate the production of anti-CCP antibody by altering peripheral post-switch/naïve ratio.

In the synovial tissues of active RA, ectopic lymphoid structures (ELS) are frequently observed [22]. Citrullinated proteins are reported to be abundant in RA synovium [23]. Thus, in such unique lymphoid tissues, antibodies against several citrullinated proteins including histone2A, histone2B and vimentin, are actively produced and secreted [24]. van Oosterhout et al. [25] showed that the number of leukocytes (especially lymphocytes) in peripheral blood was decreased in anti-CCP-positive patients, and anti-CCP-positive patients had a higher number of infiltrating lymphocytes in synovial tissue than anti-CCP-negative patients. They also demonstrated a preferential homing of leukocytes into the inflamed joints of anti-CCP-positive patients. In addition, citrullinated proteins are also abundant in oral mucosa, lung and gut in RA patients and anti-citrullinated protein antibody is considered to be produced in these local organs [26, 27]. Taken together, we speculate that the increment of post-switch memory B cells at relatively early phase after initiation of tocilizumab may reflect the altered balance of B-cell distribution between circulation and lymphoid structures in arthritic joints and other inflamed tissues, which results in suppressed production of anti-CCP antibody.

Certainly, our study comprises several limitations. First, this is a single-centred pilot study with relatively small number of patients. Second, only B-cell subpopulations were investigated. Finally, observation period was not long enough. It would be desirable to expand the patient number as well as observation period in the follow-up study. Ideally, evaluation of synovial tissue would give us some clue to the effect of tocilizumab on ELS in RA patients.

In conclusion, we demonstrate that IL-6 is one of the key cytokines which promote anti-CCP antibody production in patients with RA, and that tocilizumab has a potential to reduce anti-CCP antibody production by affecting the distribution of B cell subsets in circulation.

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Conflict of interest

T.A. received grants and personal fees from Astellas, grants and personal fees from Takeda, grants and personal fees from Mitsubishi Tanabe, grants and personal fees from Chugai, grants and personal fees from Pfizer, grants and personal fees from Daiichi Sankyo, grants from Otsuka, personal fees from Eisai, personal fees from AbbVie, outside the submitted work. S.Y. received grants and personal fees from Chugai Pharmaceutical, grants and personal fees from Bristol Myers Squibb, personal fees from Tanabe-Mitsubishi Pharmaceutical, grants from Novartis Pharmaceutical, personal fees from Pfizer, outside the submitted work. The other authors have declared no conflict of interest associated with this manuscript. Y.M. and M.S. are employees of Chugai pharmaceutical.

Table. Patients' characteristics

Demographics at baseline (n = 13)					
Female, n (%)	8 (62)				
Age, mean $\pm$ SD, years	55.8 $\pm$ 13.2				
Disease duration, median (IQR), months	44 (24 - 60)				
First biological agent, n (%)	9 (69)				
Methotrexate use, n (%)	10 (77)				
Methotrexate dose, mean $\pm$ SD, mg/week	8.5 $\pm$ 5.3				
Corticosteroids use, n (%)	10 (77)				
Prednisolone dose, mean $\pm$ SD, mg/day	3.9 $\pm$ 3.2				
Clinical parameters	Baseline (n = 13)	Week 12 (n = 13)	p-value	Week 24 (n = 13)	p-value
DAS28-ESR, mean $\pm$ SD	5.00 $\pm$ 1.39	2.42 $\pm$ 1.14	< 0.001	2.20 $\pm$ 1.00	< 0.001
DAS28-CRP, median (IQR)	3.79 (3.57 - 5.17)	2.05 (1.45 - 2.58)	0.001	2.15 (1.17 - 2.44)	0.001
SDAI, mean $\pm$ SD	24.16 $\pm$ 14.98	8.70 $\pm$ 6.97	< 0.001	7.60 $\pm$ 6.76	< 0.001
CDAI, mean $\pm$ SD	21.28 $\pm$ 13.52	8.48 $\pm$ 6.44	0.002	7.57 $\pm$ 6.76	0.001
HAQ, mean $\pm$ SD	0.63 $\pm$ 0.53	0.46 $\pm$ 0.39	0.089	0.40 $\pm$ 0.35	0.036
Anti-CCP, median (IQR), U/mL	123.7 (55.9 - 234.8)	114.8 (49.9 - 224.3)	0.075	99.6 (45.1 - 255.0)	0.033
RF, median (IQR), IU/mL	74.3 (27.2 - 112.5)	42.8 (17.4 - 100.5)	0.028	61.5 (16.4 - 82.9)	0.133

p-values were calculated for comparison between baseline and week 12, or week 24 using either paired t-test or Wilcoxon signed-rank test. SD, standard deviation; IQR, interquartile range; DAS28-ESR, disease activity score based on 28-joint-erythrocyte sedimentation rate; DAS28-CRP, disease activity score based on 28-joint-C-reactive protein; SDAI, simple disease activity index; CDAI, clinical disease activity index; HAQ, health assessment questionnaire; CCP, cyclic citrullinated peptide; RF, rheumatoid factor.

Figure legends

Figure 1. Change in anti-CCP antibody titers during treatment. Data are shown as symbols and lines as well as box plots, representing minimum, first quartile, median, third quartile and maximum. p-values were calculated for comparison between baseline and week 24, using Wilcoxon signed-rank test.

Figure 2. Change in the percentage of B-cell subsets to CD19-positive cells during treatment. p-values were calculated by Wilcoxon signed-rank test. Naïve/CD19+, Plasmablasts/CD19+, Pre-switch/CD19+ and Post-switch/CD19+ represent the percentage of naïve B cells, plasmablasts, pre-switch and post-switch memory B cells to CD19-positive cells, respectively.

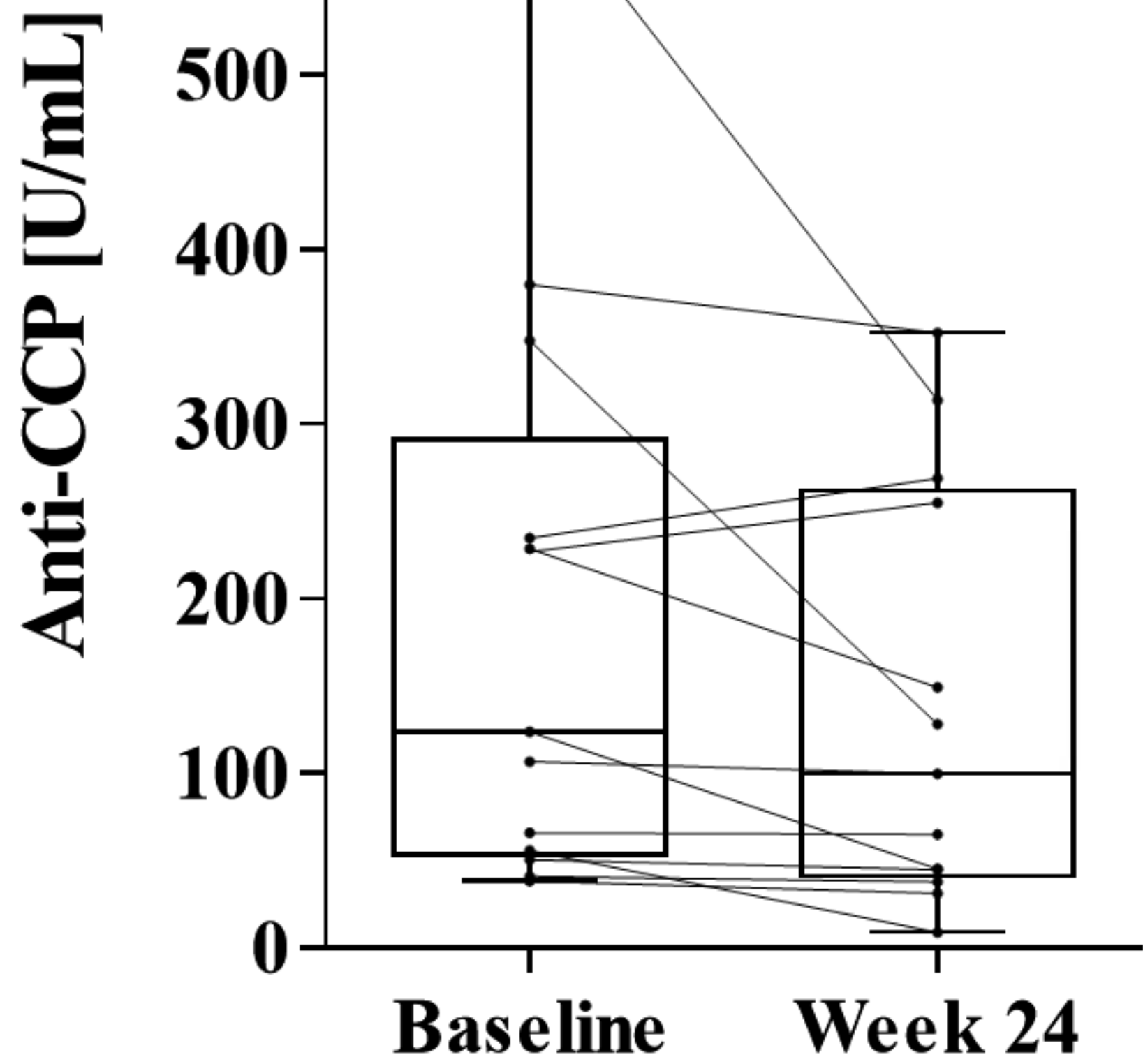
Figure 3. (A) Correlation between the changes in naïve/CD19+ and post-switch/CD19+ from baseline to week 24. Δ Naïve/CD19+ and Δ Post-switch/CD19+ represent the changes in the percentages of naïve B cells and post-switch memory B cells in the population of CD19-positive cells, respectively. (B) Correlation between anti-CCP antibody titers and post-switch/naïve ratios at baseline, week 12 and week 24. Post-switch/Naïve represents the ratios of post-switch memory to naïve B cells. Correlation coefficient was assessed by Pearson's correlation or Spearman rank method.

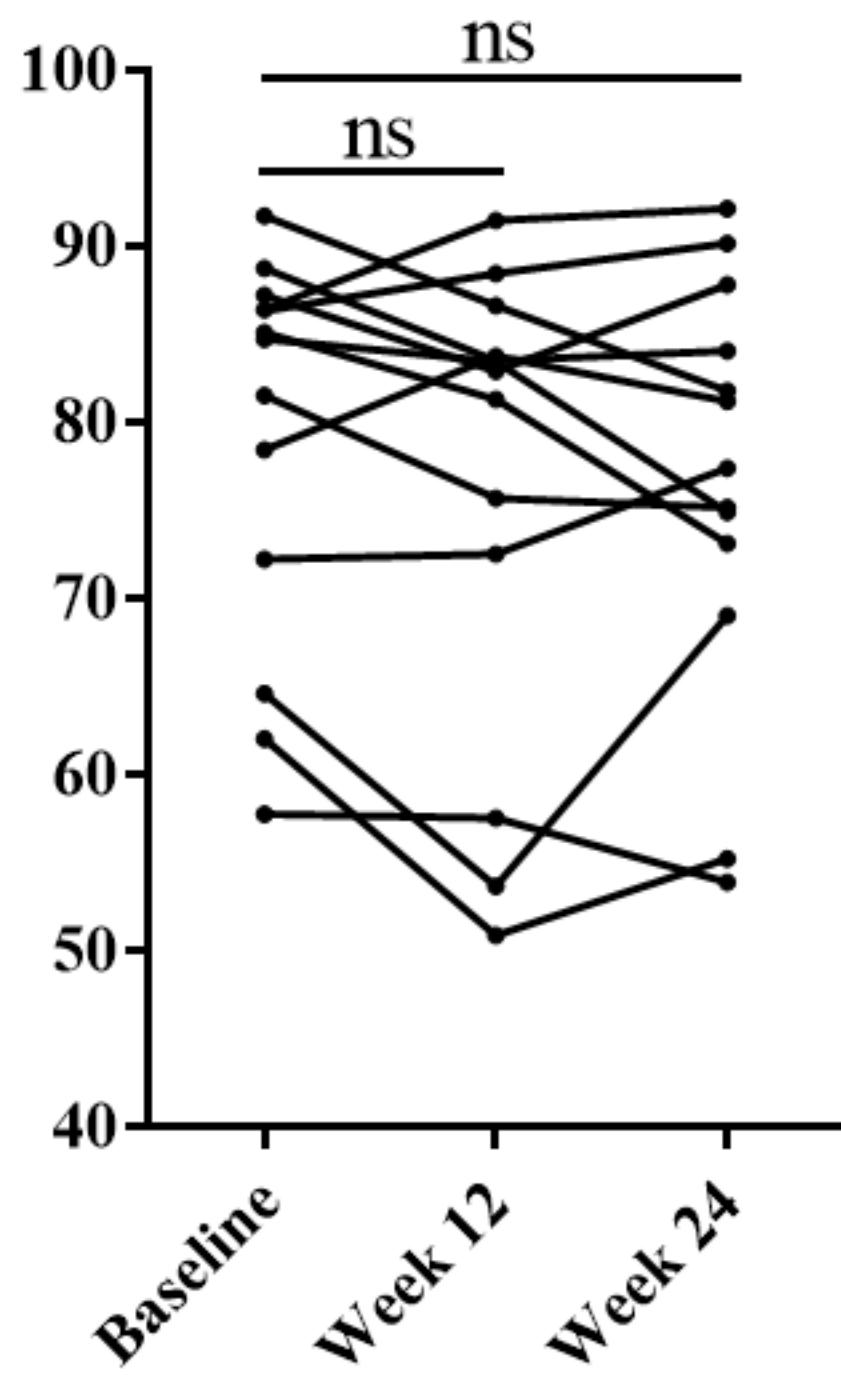
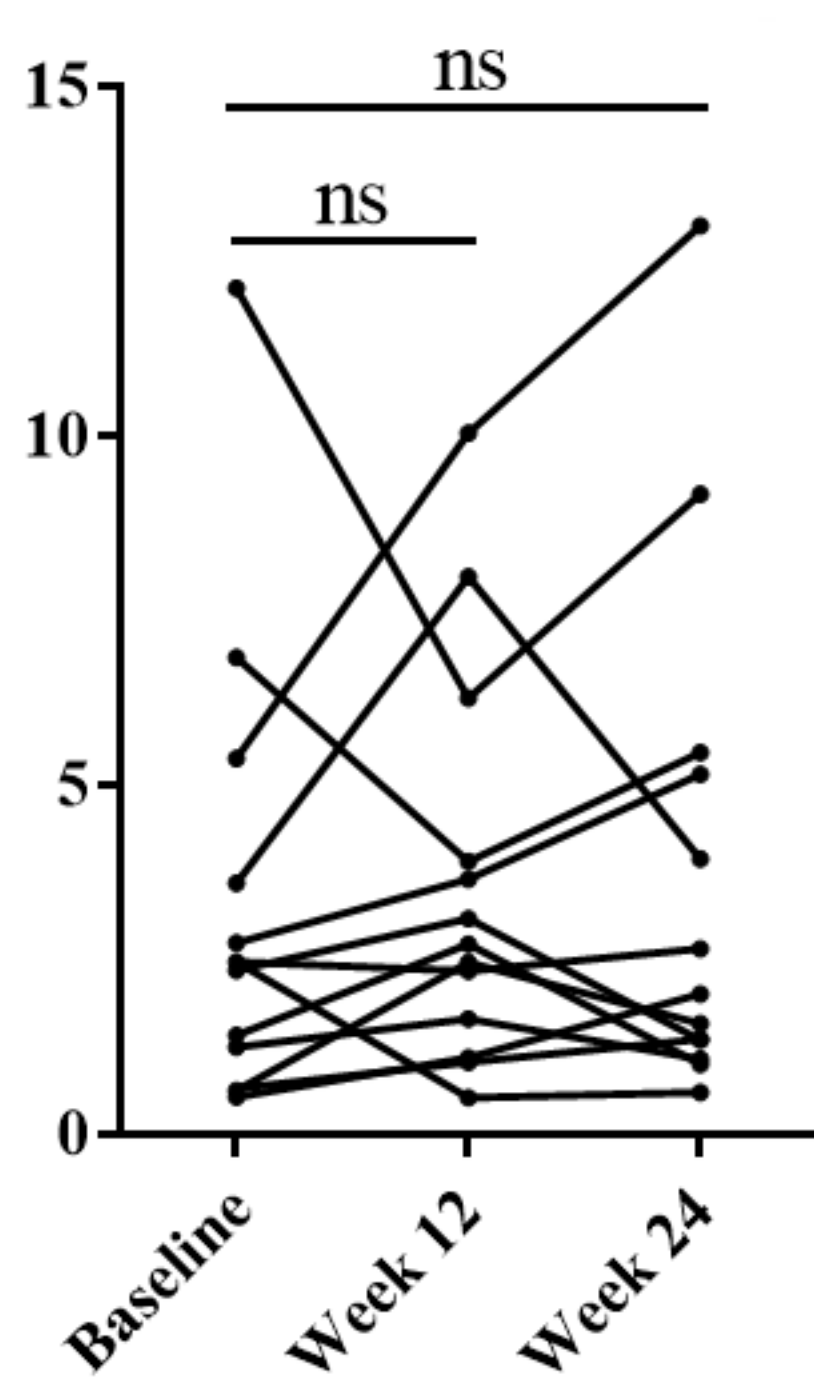
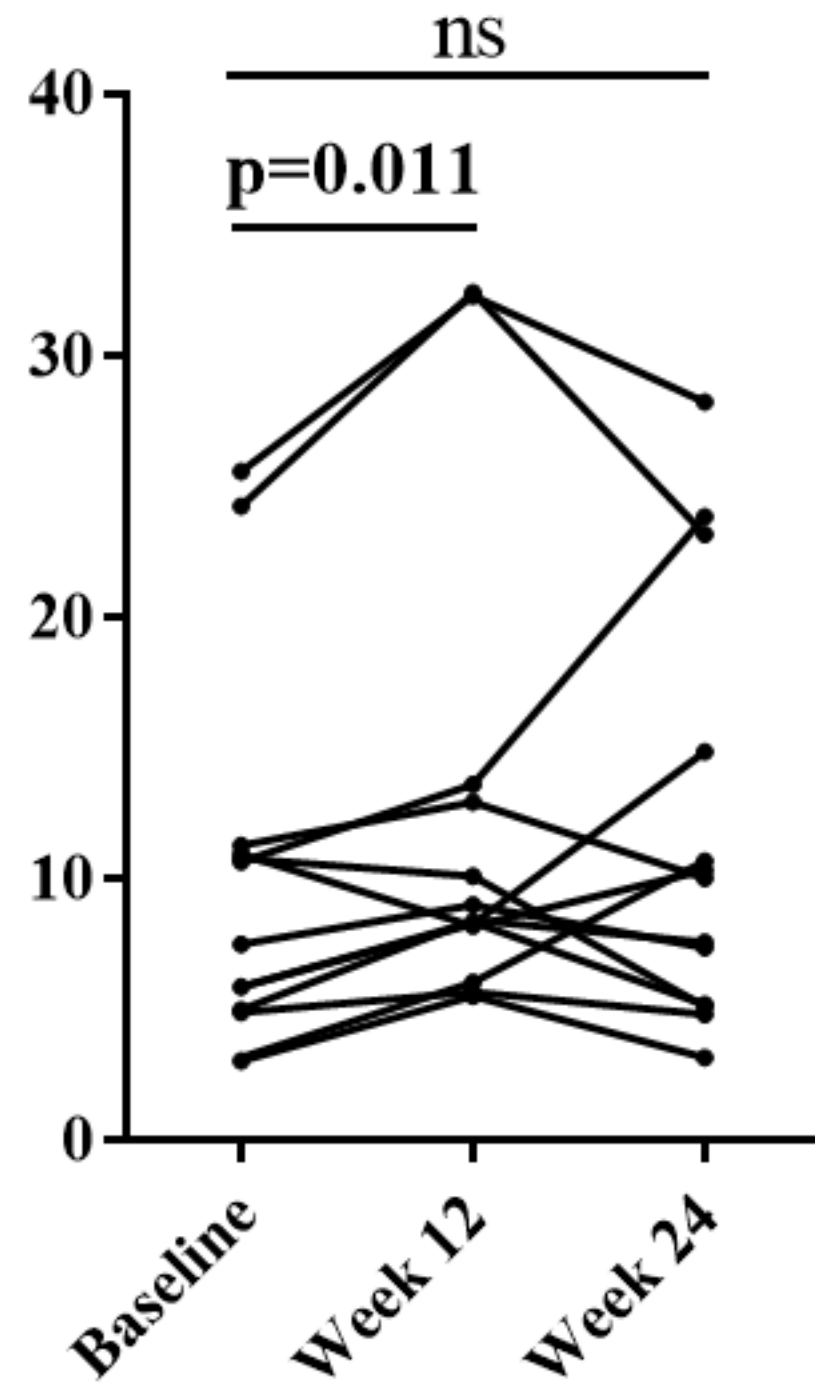
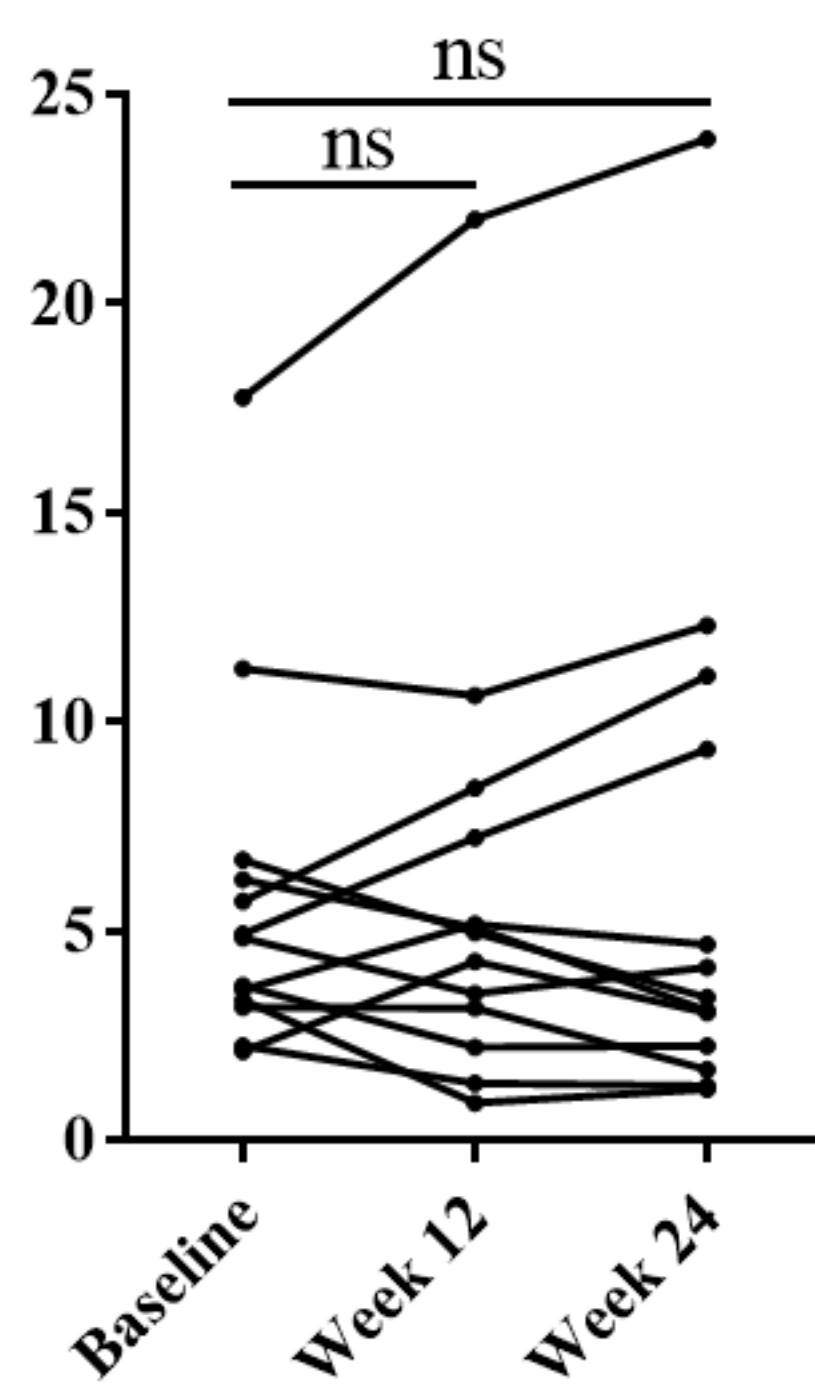
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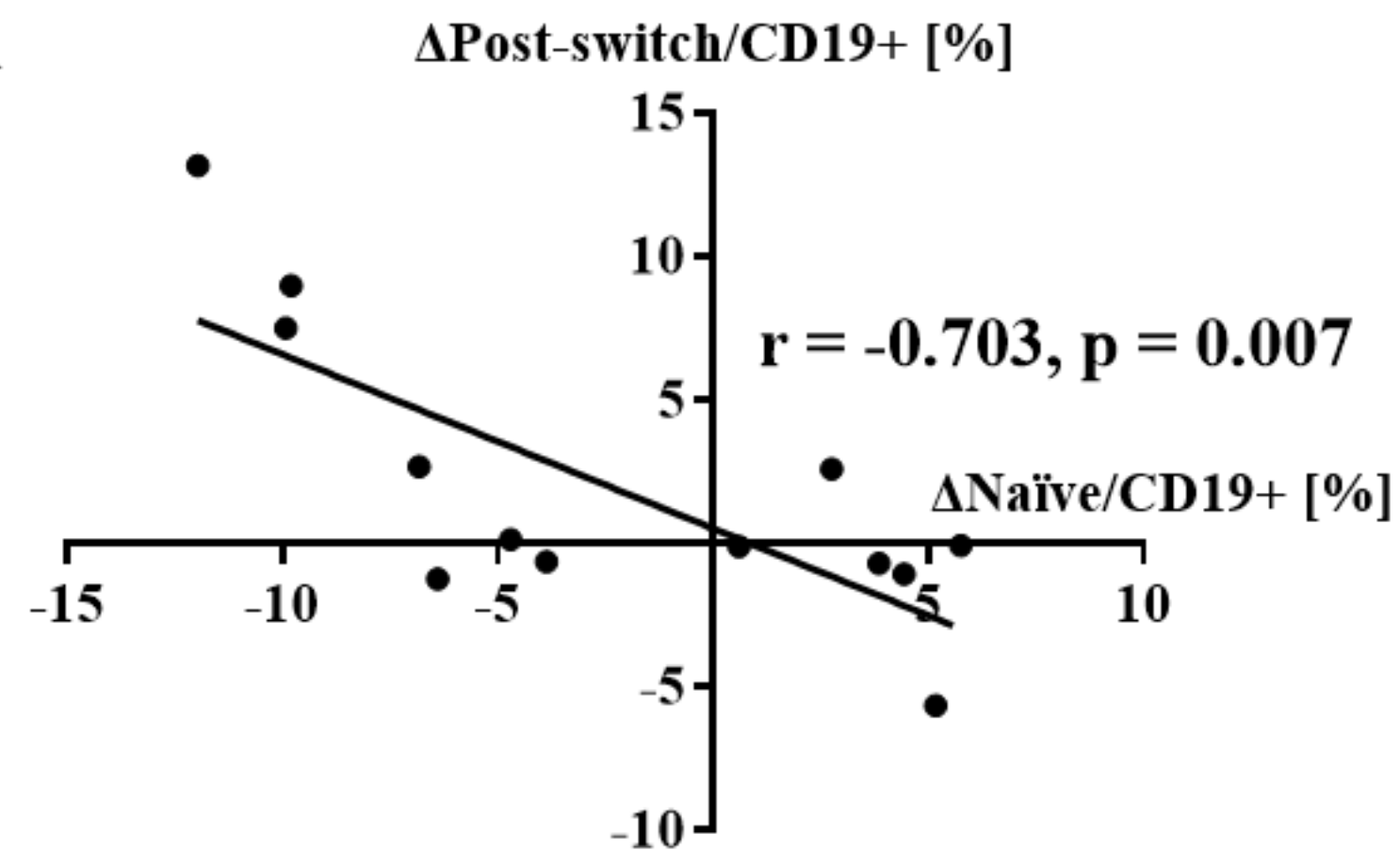
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p = 0.033



Naïve/CD19+ [%]**Pre-switch/CD19+ [%]****Post-switch/CD19+ [%]****Plasmablasts/CD19+ [%]**

A**B**