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Killing of CLL and NHL cells by rituximab and ofatumumab under limited availability of complement.

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Abstract

Rituximab and ofatumumab are anti-CD20 antibodies applicable to treatment of non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL). Effectiveness of both immunotherapeutics may depend on exhaustible complement system. To model the efficacy of complement usage by ofatumumab and rituximab under limited complement availability, we compared complement-dependent cytotoxicity (CDC) exerted by these antibodies at low (5% and 10%) and physiological (50%) serum concentration in twelve CD20-positive cell lines and six freshly isolated CLL cells. Simultaneously, we assessed expression of CD20 and membrane-bound complement inhibitors. Ratios of CD20 to CD59 and/or CD55 distinguished highly sensitive cells lysed equally efficient by both antibodies from the moderately sensitive cells, which were killed more efficiently by ofatumumab.

Keywords: complement, CD20, non-Hodgkin's lymphoma, CLL, ofatumumab, rituximab

Introduction

The complement system is a part of the innate immunity aiming to recognize and subsequently eliminate pathogenic microorganisms without harming constituents of own host. Existence of several activation pathways and pattern-recognition molecules as well as multiple effector mechanisms including opsonisation, anaphylaxis and direct cell lysis ensure wide spectrum of direct and indirect cytocidal activities of complement [1]. Although tumor cells also display molecular patterns recognized by complement-activating molecules [2, 3], numerous endogenous factors and mechanisms protect them from spontaneous complement attack [4]. Such protection may be overrun by tumor-targeted monoclonal antibodies (mAbs), which enhance the efficacy of complement-dependent cytotoxicity (CDC, attributable to lysis of target cells due to membrane attack complex formation) and lead to establishment of successful therapies. Therapeutic mAbs may exert at least two more modes of target cell killing: antibody-dependent cellular cytotoxicity (ADCC) or direct effects such as apoptotic cell death upon antibody binding [5]. Moreover, phagocytes engulf cells opsonized by antibodies and/or complement more readily. Number of mAbs approved for treatment of B cell malignancies recognize CD20 molecule, which is present on the surface of most tumor B cells [6]. Studies aiming at pointing out the effector mechanism pivotal for therapeutic effect of CD20 mAbs ended up with seemingly contradictory results. These discrepancies (reviewed in a recent review [5]) suggest that the role of CDC in the therapeutic effect of certain mAbs must be further examined in extended experiments.

Rituximab (RTX) is the first, clinically approved anti-CD20 mAb and it is widely used in treatment of non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL). However, limited single agent efficacy in patients with CLL [7] (usually expressing less CD20 than NHL, [8]) raised the need for improvement of current immunotherapeutics. One such novel agent is ofatumumab (OFA), which targets the small extracellular loop of CD20 molecule and thus binds more proximally to cell membrane than rituximab [9]. Recently, several studies describing *in vitro* efficacy of RTX and OFA were published. The absolute number of CD20 molecules necessary to exert CDC in NHL cells was significantly lower for OFA (reviewed in [9]). Similarly, lower concentration of OFA efficiently eliminated CLL cells comparing to RTX and the killing in full blood was dependent on CDC but not ADCC [10].

As shown for both OFA and RTX, increase of mAb concentration gradually increased deposition of complement component C3b but did not increase CDC over the saturation level achieved already at 25 times lower mAb concentration needed for maximal C3b deposition [11]. Conversely, high doses may lead to exhaustion of early complement components, which renders the next round of drug infusion less effective. Aforementioned study highlights the need for optimization of dosing and schedule CD20 mAbs but also the need for mAbs capable of efficient usage of available complement for induction of CDC. The latter may be particularly important when complement availability is affected already at initial therapeutic step, e.g. by deficiency of complement components described in number of CLL patients [12] or in case of possible gene dependent alterations in complement, as recently suggested in patients with follicular lymphoma [13]. Therefore, we decided to study parameters never compared before, i.e. how proficient RTX and OFA are when CDC is limited by complement availability. To do so, we analyzed the differences in CDC exerted by RTX and OFA at low serum concentrations (5% and 10%) and 50% (physiological) serum concentration. In order to create simplified mathematical model describing the changes in complement usage from low to high serum concentrations, we calculated the trendlines of CDC obtained for each mAb by linear regression. To eliminate direct effects of mAbs as confounding factors, time interval for this experiment was limited to 30 minutes, unlike 4 hours in other study [10]. We applied measurement of CDC by chromium release assay, since it eliminates false positive results possible to obtain by other methods. For

example, tests based on detection of dye-permeable cells will also detect cells already necrotic or late-apoptotic. Except of CD20, malignant B cells are usually equipped with a set of membrane-bound complement inhibitors; CD46, CD55 and CD59 and controversy remains whether killing of B cells induced by anti-CD20 mAbs depends exclusively on CD20 expression level or combination of CD20 and complement inhibitors (reviewed in [5]). Thus, CDC readout was accompanied by analysis of all four proteins performed at the same time as CDC assay to exclude possible day-to-day variations of expression and to assure accurate correlations between cell killing and actual phenotype.

Methods

Cells: NHL cell lines: Raji, Daudi, Ramos, BL-41, WSU-NHL, SU-DHL-5, SU-DHL-8, ULA and CLL cell lines MEC-2 and EHEB were obtained from German Collection of Microorganisms and Cell Cultures whereas NHL cell lines Namalwa and BJAB were from American Type Culture Collection. Cells were cultured in RPMI with 2 mM L-glutamine (HyClone, Logan, USA) supplemented with 10% or 20% (SU-DHL-5, SU-DHL-8 and ULA) fetal bovine serum (Gibco, Paisley, UK), except for MEC-2 and ULA cells cultured in 1:1 RMPI/DMEM medium. Primary CLL cells (6 in total) were freshly obtained from full, heparinized blood of patients with CLL upon written informed consent and according to permit from regional ethic committee (www.epn.se). Cells were purified on Lymphoprep (R.E.D. Laboratories, Zellik, Belgium) gradient, washed with PBS and cultured for 1 day in 1:1 RPMI:DMEM medium with 10% serum.

CDC was assessed by ^{51}-chromium release assay. Cells (2.5 x10^5 per experimental point) were harvested and incubated with 1 μCi of sodium ^{51}chromate (Perkin Elmer, Groningen, The Netherlands) for 30 minutes at 37 °C. Then cells were washed three times and incubated with normal human serum (prepared as described in [14]) and 50 μg/ml RTX (Roche, Basel, Switzerland) or OFA (GlaxoSmithKline, Brentford, UK) diluted in PBS supplemented with 1 mM Ca^{2+} and Mg^{2+}. Full lysis of given cell type was set up as a readout of cells lysed with 1% Triton X-100 (Merck, Darmstadt, Germany). Chromium-specific counts were analyzed in γ-counter Wizard2 (Perkin Elmer).

Expression of CD20, CD46, CD55 and CD59 was assessed by flow cytometry. Cells (10^5 per experimental point) were harvested and incubated with following primary antibodies: 200 μg/ml RTX, 4 μg/ml of anti CD46 MEM-258, anti CD55 MEM-118, anti CD59 MEM-43 or their isotype controls (all from Immunotools, Friesoythe, Germany) for 1h following by secondary goat-anti mouse $F(ab)_2$ labelled with FITC (Dako, Glostrup, Denmark, 1:75 dilution). Cells were analyzed using CyFlow Space machine (Partec, Munster, Germany) and FlowJo software (TreeStar, Ashland, USA).

Statistical analyses were performed with Prism5 software (GraphPad, La Jolla, USA).

Results

The novelty and merit of our study lies in comparison of mAb efficiency of complement usage and as such was only possible when constant, CDC saturating concentration of antibodies was applied. Hence we chose antibody concentration of 50 μg/ml, which saturated CDC in our experimental conditions since increasing its concentration 10 times did not increase the effect in low and moderately sensitive cells (Fig.1). Additionally, this concentration was also saturating in terms of antibody binding (data not shown). Then, we assessed CDC at three different serum

concentrations (Table 1) and compared the differences in CDC obtained at 5% and 50% serum as well as 10% and 50% serum. In case of CLL cells, increasing serum concentration to physiological value resulted in more efficient CDC exerted by OFA than CDC exerted by RTX (Fig. 2 B,D). In case of NHL cells these differences were statistically significant (by paired T-test) when measured between 5% and 50% of serum (Fig. 2A). Interestingly, there were four cell lines belonging to NHL but no CLL cells, which showed almost maximally attainable sensitivity to both OFA and RTX already at 5% serum (Ramos, Daudi, SU-DHL-5 and ULA, Table 1 and Fig. 2AC, red circles).

In order to extract and statistically analyze the values the efficacy of complement usage upon low and high availability, we calculated the CDC trendlines for each mAb by linear regression. In our simplified model, these trendlines are linear functions $y=ax+b$, where *a* is the slope value and *b* is the Y axis intercept (Fig. 3, top panel). Therefore high *b* (and consequently low *a*) coefficients group the cells, which are highly sensitive to CDC already at low serum concentration whereas high slope value (in the cases with low or moderate b value) reflects the situation when CDC markedly increases with available complement, i.e. given mAb uses it efficiently.

Our first, overall observation was that cells characterized by high ratio of CD20 to membrane-bound complement inhibitors were killed equally efficient by both antibodies, even at low serum concentration (Fig. 3, example of Daudi cells), thus having high *b* and low *a* values. As described earlier (Fig.2), four such cases were identified within NHL cell lines and no case within CLL cells (Fig. 4A,B). Similarily to Fig.2, analysis of trendlines confirmed that OFA utilizes complement more efficiently than RTX in CLL cells as revealed by paired T-test ($p=0.0016$, Fig. 4D) and in some of NHL cells (Fig. 4C, cluster 1). Initial sensitivity to complement at low serum concentration described by *b* coefficient value was significantly higher ($p=0.035$ in paired T test, Fig. 4F) for OFA in CLL cells but not in whole group of NHL cells (Fig.4E).

Given that application of OFA may exert superior CDC in certain cell types, we analyzed whether ratio of target (CD20) to complement inhibitor (CD46, CD55 or CD59) may distinguish the cells potentially capable to complement-dose dependent response from those highly-sensitive. Importantly, expression of CD20 alone was not significantly different in these two groups of cells ($p=0.11$ according to T-test). Two-way ANOVA performed for matched values of all CD20:complement inhibitor ratios indicated significant effects only for CD20:CD55 and CD20:CD59 but not for CD20:CD46 (Fig. 5A). Next, we asked whether these two parameters influence the slope values in the entire group of CD20 positive cells, which may gradually respond to complement upon CD20 mAb sensitization. One premise was that NHL cells grouped in cluster 1 (Fig. 4C) differed in these ratios from other ¨low b¨ cells (cluster 2, blue points). Therefore, we correlated CD20:CD59 and CD20:CD55 ratios with slope values for OFA and RTX of all CD20 positive cells. Four cell lines highly sensitive to both OFA and RTX (red points, Fig. 2AC, 4C,E) were excluded not to confound with cells poorly responding to given mAb and thus also having low slope values. Spearman's analysis showed significant correlation of CD20:CD55 and CD20:CD59 with slope values obtained for cells treated with OFA but not RTX, as the latter utilized complement less efficiently (Fig.5B,C). Interestingly, b coefficient values correlated with CD20:CD55 for both OFA and RTX ($r>0.8$, $p<0.001$) and with CD20:CD59 for RTX only ($r=0.51$, $p=0.03$).

Discussion

Our results remain in agreement with previous observations that OFA is superior to RTX in CDC activation, when effective mAbs concentrations are compared [10]. To this end, others observed that cells resistant to RTX may be sensitive to OFA [15]. Also, there are reports

showing increased CDC of CLL cultures treated with OFA upon blockade of CD55 / CD59 [10] or CD59 alone [16]. Importance of CD20, CD55 and CD59 in RTX –mediated CDC was shown in [17] [8]. Nonetheless, to our best knowledge no one has modeled the complement availability as a factor limiting the efficiency of rituximab and ofatumumab. Our study shows that at saturating concentrations of mAbs, which caused maximal attainable lysis, OFA was more efficient in utilizing available complement to generate CDC in target cells. This finding may relate to the *in vivo* situation when availability of active complement due to previous exhaustion or host factors is a significant parameter limiting an effective therapy. Also, our results suggest that ratios of CD20:CD59 and CD20:CD55 expression in target cells should be taken under consideration as parameters helpful in selecting an optimal immunotherapy adjusted to individual demands. This concept extends the premise reported by *Takei et al*. [18], who described acquired resistance of Ramos cells to RTX, which was attributed to loss of CD20 and selection of the subpopulation highly expressing CD55 and CD59. Yet performed on single cell line, results indicate the potential importance of target (CD20) to complement inhibitor (CD55, CD59) ratio rather than expression of single molecules. Also, Manches *et al*. reported correlation between rituximab –induced CDC and CD20 to complement inhibitor ratio [19]. However, they used non-saturating concentration of mAb accompanied with 30% serum and did not distinguish between particular complement inhibitor as combined MFI readout of CD46, CD55 and CD59 was taken for calculations. We found that CD20:CD55 and CD20:CD59 ratio can distinguish cell lines highly sensitive to both OFA and RTX and found correlation with CDC trendlines in other CD20 –expressing cells. Thus, these ratios emerge as significant parameters for complement - mediated killing of tumor B cells and as such may be potentially important for selection of adequate immunotherapy.

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Conflict of interest

Marcin Okroj, Ingrid Eriksson and Anna Blom declare no competing interests. Anders Österborg has received grant support and honoraria for scientific lectures from Glaxo-SmithKline.

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Figure legends

Fig.1 Saturation of CDC by mAb concentration.

OFA and RTX were used at 50 or 500 □g/ml in chromium release assay performed on cell lines low sensitive (A), moderately sensitive (B) and highly sensitive (C) to CDC. Experiments were carried out in 10% or 50% of normal human serum. Increasing mAbs concentration ten times does not result in increased CDC in any of the cell line tested. Effect of 10% serum and 50% serum without mAbs is indicated by small green and violet bars, respectively. Data were collected from 3 independent experiments and compared by one-way ANOVA, error bars represent S.D.

Fig.2 Differences in CDC exerted by OFA and RTX between low and high serum concentration.

Top panels show differences in CDC measured at 5% and 50% serum in NHL cells (A, circles) and CLL cells (B, stars). Bottom panels show differences in CDC measured at 10% and 50% serum in NHL cells (C, circles) and CLL cells (D, stars). Red color depicts cells, which showed very high CDC already at 5% serum.

Fig. 3 CDC exerted by OFA and RTX in association with expression of CD20 and membrane bound complement inhibitors.

Top panels show CDC expressed as % of full lysis (cells lysed with water + 1% Tritox X-100) exerted by OFA and RTX in four representative cell lines (one highly –sensitive to CDC, two showing complement dose –dependent response and one low sensitive to CDC) as well as trendlines calculated as linear function $y=ax+b$ by linear regression. Bottom panels show expression of CD20, CD46, CD55 and CD59 in each cell line. Expression is presented in relative expression units, which is the ratio of mean fluorescence intensity (MFI) obtained for given surface antigen to MFI of corresponding isotype control. Data were collected from 3 independent experiments, error bars represent S.D.

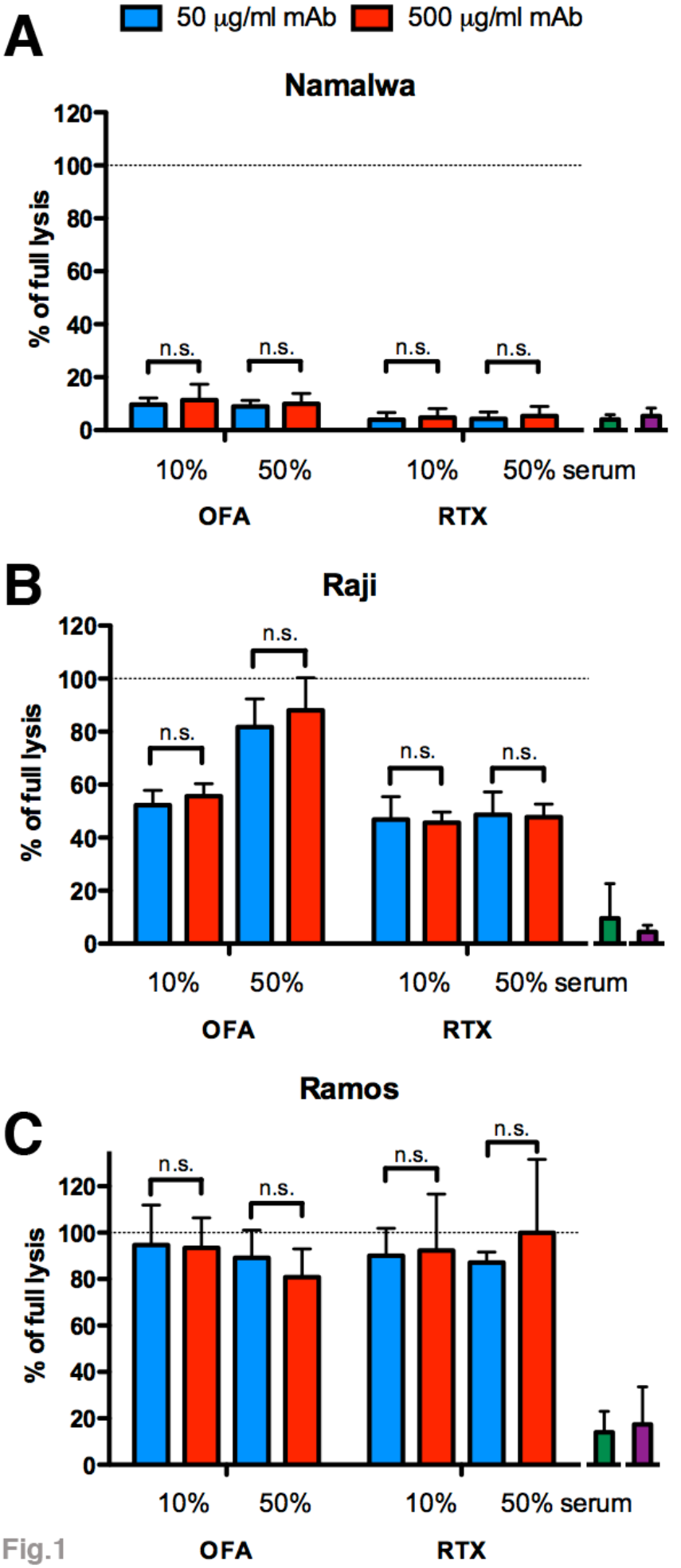
Fig. 4 Differences in slope and *b* coefficient values obtained for OFA and RTX in NHL and CLL cells.

Panels A and B shows scatter plots of slope (X axis) and b coefficient (Y axis) values obtained for OFA (A) and RTX (B). Red color depicts cells highly sensitive to CDC induced by both mAbs (high b values) whereas blue color depicts cells potentially capable of complement availability dependent response. Circles refer to NHL cell lines whereas stars refer to CLL cells / cell lines. Panels C and D compare slope values whereas panels E and F compare b coefficient values obtained for OFA and RTX. Paired points (these corresponding to the same cell lines)

were connected with dotted lines. * and ** symbols refer to $p<0.05$ and $p<0.01$ according to paired T test.

Fig.5 Ratios of target (CD20) to complement inhibitor as critical factors for CDC efficiency.

Panel A shows comparison of CD20:CD46, CD20:CD55 and CD20:CD59 ratios for cells with high b values and low/moderate b values. Ratios were calculated from values of relative expression of given antigen (explained in Fig. 2). Error bars represent SD, * = $p<0.05$ and ** = $p<0.01$ calculated by two-way ANOVA for matched values. Panels B and C show correlation of slope values with CD20:CD55 and CD20:CD59, respectively.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RTX

Fig.1

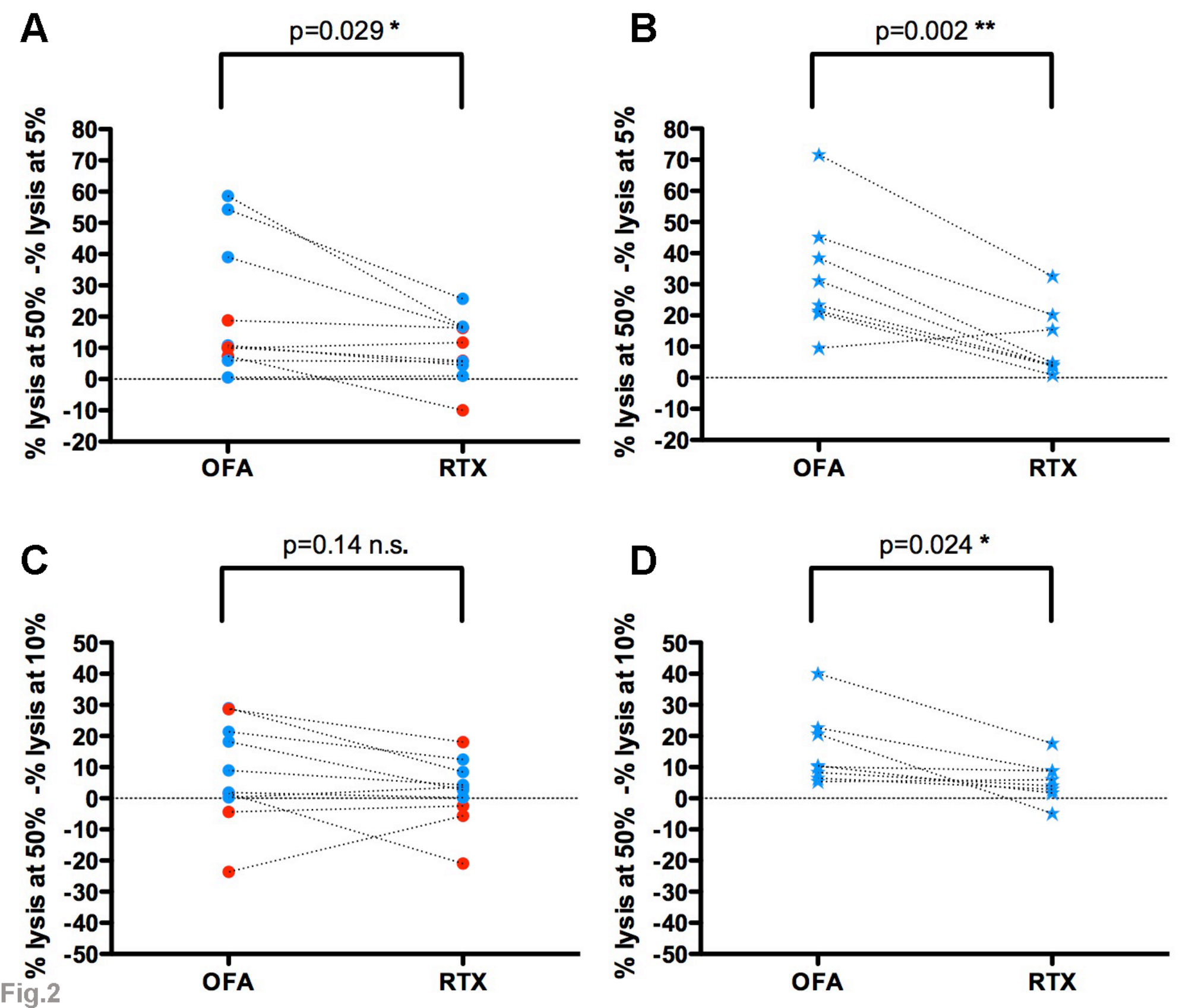
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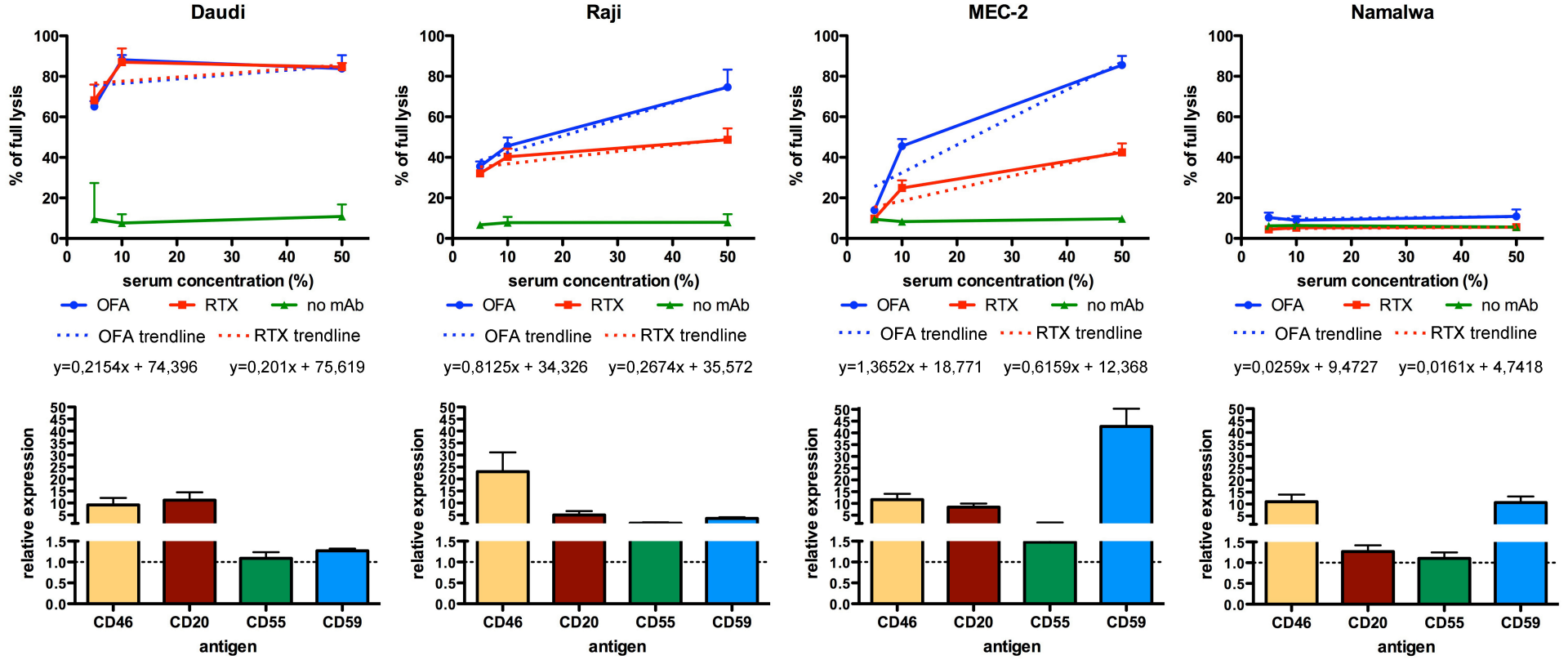
Fig.2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CD20
CD55
CD59
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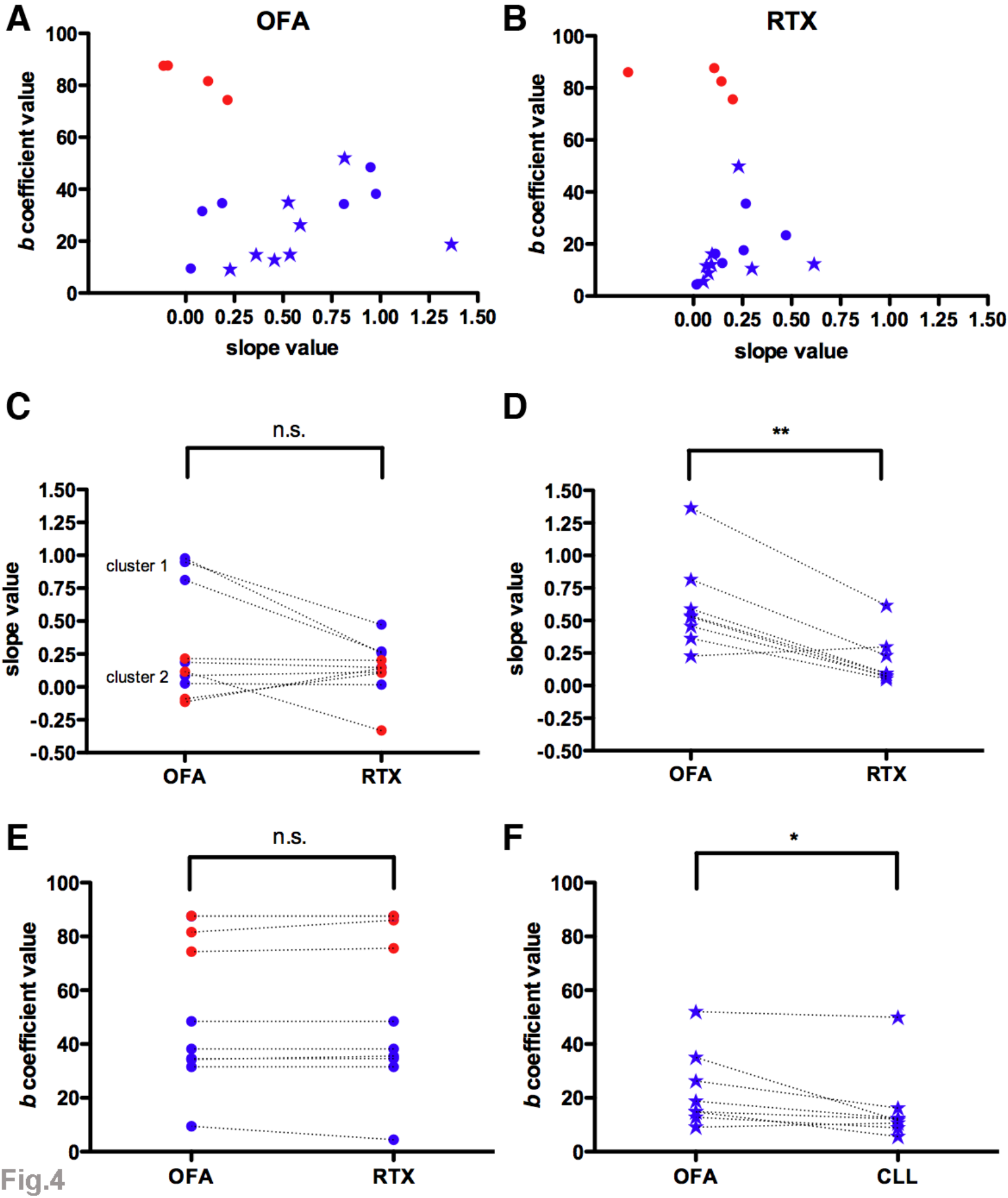
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b coefficient value
slope value
B
RTX
b coefficient value
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C
n.s.
slope value
cluster 1
cluster 2
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RTX
D
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slope value
OFA
RTX
E
n.s.
b coefficient value
OFA
RTX
F
*
b coefficient value
OFA
CLL

Fig.4

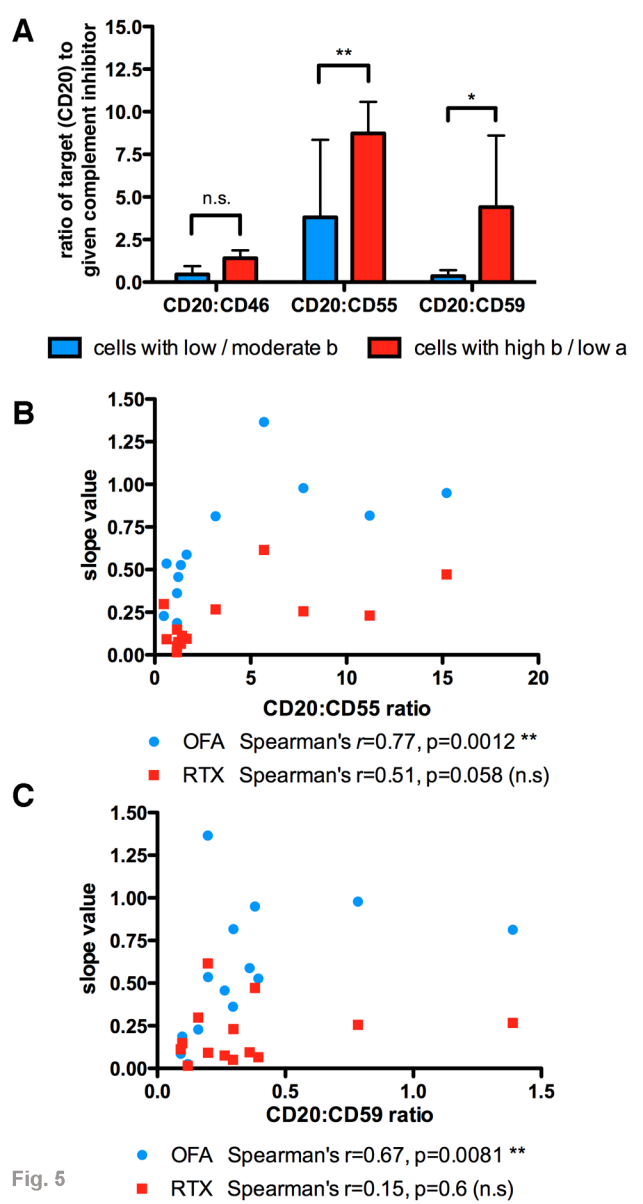
A
ratio of target (CD20) to given complement inhibitor
15.0
12.5
10.0
7.5
5.0
2.5
0.0
n.s.
**
*
CD20:CD46
CD20:CD55
CD20:CD59
cells with low / moderate b
cells with high b / low a
B
slope value
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
CD20:CD55 ratio
OFA Spearman's r=0.77, p=0.0012 **
RTX Spearman's r=0.51, p=0.058 (n.s)
C
slope value
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0.0
0.5
1.0
1.5
CD20:CD59 ratio
OFA Spearman's r=0.67, p=0.0081 **
RTX Spearman's r=0.15, p=0.6 (n.s)

Fig. 5