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SHORT COMMUNICATION

Comparability of Antibody-Mediated Cell Killing Activity Between a Proposed Biosimilar RTXM83 and the Originator Rituximab

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Abstract

Background Biosimilars are described as biological products that resemble the structure of original biologic therapeutic products, with no clinically meaningful differences in terms of safety and effectiveness from the original. A wide range of biosimilars are under development or are already licensed in many countries. Biosimilars are earning acceptance and becoming a reality for immunotherapy treatments mainly based on the alternatives for the commercial anti-CD20 monoclonal antibody rituximab. The most important mechanism of action reported for this antibody is the induction of antibody-dependent cell cytotoxicity (ADCC), which is associated with the polymorphisms present at the 158 position in the IgG receptor FcγRIIIa.

Objective The aim of the study was to validate the functional comparability between the proposed rituximab biosimilar RTXM83 and the original product. To achieve this we assessed the binding capacity and ADCC induction of this biosimilar, taking into account the different FcγRIIIa-158 polymorphisms.

Methods Binding capacity was evaluated by flow cytometry using CD20 positive cells and a wide range of antibody concentrations. The FcγRIIIa-158 polymorphisms were analyzed by polymerase chain reaction (PCR) followed by allele-specific restriction enzyme digestion. ADCC was measured by a colorimetric lactate dehydrogenase-release assay, using effector cells from donors with different FcγRIIIa-158 polymorphisms.

Results Binding capacity assay showed no differences between both products. Regarding ADCC, a similar relative potency was obtained between both antibodies, showing a higher response for the FcγRIIIa-158 valine/valine (V/V) polymorphism compared to the phenylalanine/phenylalanine (F/F), for both rituximab and RTXM83.

Conclusion Our data strongly suggest the biocomparability between the proposed biosimilar and the originator rituximab, in antibody recognition and ADCC activity. Additionally, our results suggest that donors with the FcγRIIIa-158V/V polymorphism induce a higher ADCC response, as has been reported.

Key Points

Antibody-dependent cell cytotoxicity (ADCC) induction is rituximab's most relevant mechanism of action. The magnitude of this response is associated with the polymorphisms at the 158 position in the IgG receptor FcγRIIIa.

The biosimilar of rituximab, RTXM83, demonstrated a strong similarity with the originator in anti-CD20 binding and ADCC induction.

Both RTXM83 and rituximab develop a very similar anti-CD20 ADCC response in regard to each FcγRIIIa-158 polymorphism.

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1 Introduction

Rituximab (marketed as Rituxan® in the USA and MabThera® in the EU) is maybe the most known antibody-based treatment for cancer, being one of the biggest selling cancer drugs in 2014 [1, 2]. 2013 was the year of the patent expiration in Europe, while 2016 is the stipulated date for the USA [3]. This scenario offers the opportunity for the development of biosimilars of rituximab. Many companies around the world have accepted the challenge, and nowadays there are several rituximab biosimilars approved or under development [4]. According to the Food and Drug Administration (FDA) definition, biosimilars are those biological products highly similar to an approved one (innovator product, biological reference) with no clinically meaningful differences in terms of safety and effectiveness from the reference product. Also, a biosimilar must have the same mechanism of action as the reference product [5, 6].

Rituximab is a high affinity chimeric monoclonal IgG1 antibody that binds the CD20 antigen. It was approved for treatment of CD20+ B-cell non-Hodgkin's lymphoma (NHL), CD20+ follicular NHL, and chronic lymphocytic leukemia. It consists of light and heavy chain variable regions of a murine anti-human CD20 monoclonal antibody fused with human immunoglobulin light and heavy chain constant regions [7]. The CD20 molecule is a 33-kDA non-glycosylated polypeptide present on the microvilli of most mature B cells as part of supramolecular complexes with the B-cell receptor (BCR), CD40 and major histocompatibility complex (MHCs) [8–10]. Evidence supports that it functions as a calcium channel that modifies BCR signaling [11, 12]. However, no natural ligand of CD20 has been identified yet. Since its expression is confirmed in several hematological malignancies, CD20 has become an outstanding therapeutic target.

In vitro complement-dependent cytotoxicity (CDC) and induction of apoptosis on CD20 positive cells were reported as rituximab's mechanisms of action. Notoriously, antibody-dependent cell cytotoxicity (ADCC) is presumed to be the key effector function, and it is based on FcγR-bearing effector cell cytotoxicity [13]. This cellular function is regulated by several members of the FcγR family that are widely expressed on phagocytes and natural killer (NK) cells. NK cells mostly express the low-affinity receptor for IgG, FcγRIIIa (CD16) [14]. There has been an association between the FcγRIIIa-158 polymorphisms and increased rituximab binding and killing by ADCC. Most relevantly, clinical studies showed that patients with the valine/valine (V/V) polymorphism in the 158 position of FcγRIIIa have a better clinical outcome [15]. Cartron et al. showed for the first time that homozygous FcγRIIIa-158V/V genotype is beneficial in patients with follicular NHL [16]. These results were confirmed later in follicular lymphoma patients [17], and recently Veeramani et al. showed that rituximab induced NK activation and a significant decrease in circulating NK cell percentage in FcγRIIIa-158V/V and valine/phenylalanine (V/F) patients, but not in those with the FcγRIIIa-158F/F polymorphism [18].

In this work, we compare the biological activity of a proposed biosimilar of rituximab developed by PharmADN (Mabxience, Chemo Co.) with the originator's, regarding CD20 binding and the ability to develop ADCC in relation to the FcγRIIIa-158 polymorphisms.

2 Methods

2.1 Cell Lines

WIL2-S human B-lymphoblastoid cell line (CRL-8885), Jeko-1 mantle cell line (CRL-3006), and human small cell lung cancer cell line NCI-H82 (HTB-175) were obtained from the American Type Culture Collection. Tumor cells were grown in RPMI-1640 medium plus 10 % fetal bovine serum (FBS) (Gibco, 100091-148), in suspension culture, at 37 °C in a humidified atmosphere of 5 % CO_2.

2.2 Monoclonal Antibodies

Rituximab (MabThera®) 10 mg/ml was purchased from Roche and was used as the reference product. Biosimilar RTXM83 monoclonal antibody was obtained from PharmADN (Mabxience, Chemo Co.). Human IgG1κ was used as the isotype control (Sigma-Aldrich, I 5154). Polyclonal rabbit fluorescein isothiocyanate (FITC)-conjugated anti-human IgG antibody (Dako, F0185) was utilized as the secondary antibody in flow cytometry assays. For RTXM83 production, a Chinese hamster ovary (CHO) cell line was developed, harboring the genes of the light and heavy chains for rituximab, in order to obtain the biosimilar rituximab RTXM83. The production process was developed, established and validated at an industrial scale, under current good manufacturing practices (cGMP) conditions. This process includes an upstream stage, where a fed batch in disposable bioreactors is applied. The cells are harvested and the monoclonal antibody is captured using a protein A resin. After a low treatment, further purification is achieved using two ionic exchange steps (one in a cationic and one in an anionic column). Finally, a nanofiltration step is performed prior to product formulation using tangential flow filtration.

2.3 Cell-Based Binding Assay

CD20 positive cells (Jeko-1 or WIL2-S) were incubated with rituximab, biosimilar or isotype control (15 μg/ml) at different concentrations for 30 min on ice. Cells were washed with flow cytometry buffer [2 % volume in volume (v/v) FBS in phosphate buffered saline (PBS)], and incubated with a rabbit FITC-labeled anti-human IgG for 30 min on ice. Acquisition was achieved by a FACSCalibur flow cytometer (Becton Dickinson) and analyzed by the WinMDI 2.8 software. The binding 50 (B50) parameter was calculated by logarithmic transformation and linear regression of curves, representing the antibody concentration at which 50 % of cells were positive. CD20 negative NCI-H82 cells were used as a negative control.

2.4 Genotyping of the FcγRIIIa-158 Polymorphism

We analyzed the genotype of 12 healthy donors. DNA was extracted from peripheral blood using the Wizard Genomic DNA Purification kit (Promega, A1120). The analysis was performed by polymerase chain reaction (PCR) followed by allele-specific restriction enzyme digestion. Genotypification of the FcγRIIIa-158 polymorphism was confirmed by direct sequencing of the region of interest [19]. Synthetic 94-base pair (bp) FcγRIIIa-158V/V fragment was used as a digestion control (GenScript Company).

2.5 ADCC Assay

ADCC was measured by a colorimetric lactate dehydrogenase (LDH)-release assay. For 30 min, 10^5 Jeko-1 cells (target) were incubated with rituximab, biosimilar or isotype control. Then 2.5×10^6 peripheral blood mononuclear cells (PBMCs) from healthy human donors (effector cells) were co-incubated (effector: test cell mix) in RPMI medium plus 10 % FBS for 16 h at 37 °C and 5 % CO_2. Then 50 μl of the supernatant was collected and lysis was detected by CytoTox 96 kit (Promega, G1780), according

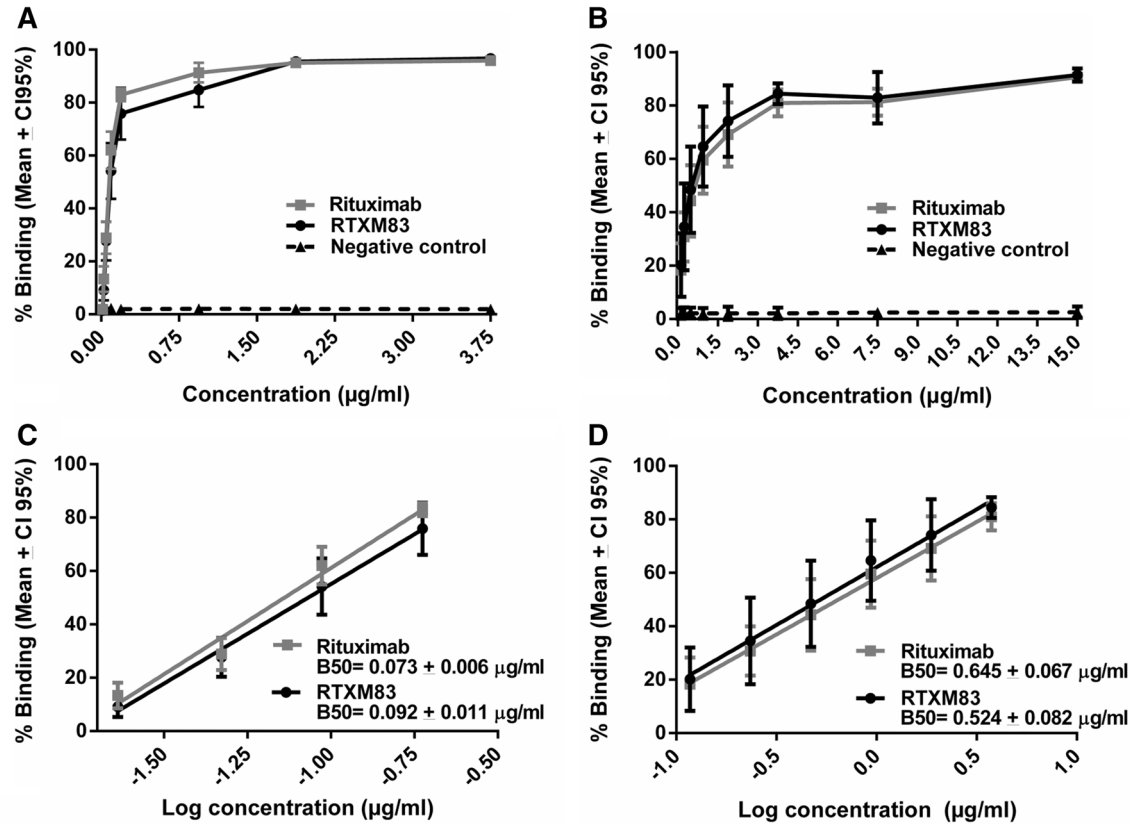

Fig. 1 Comparison of binding capacity between the reference product (rituximab) and the proposed biosimilar by quantitative flow cytometry assay. Jeko-1 and WIL2-S CD20 positive cell lines were incubated with a wide range of antibody concentrations followed by FITC-conjugated anti-human IgG antibody staining. The NCI-H82 cell line was used as a negative CD20 control (*dotted line*). **a** and **b** show binding curves for Jeko-1 and WIL2-S, respectively. No significant differences were observed for binding curves (two-way ANOVA followed by mean 95 % CI comparison post hoc test, $p > 0.05$). **c** and **d** show logarithmic transformation and linear regression for Jeko-1 and WIL2-S profiles, respectively. The slopes and elevations are not significantly different in either of the linear regressions ($p > 0.05$, *T* test). B50 represents the concentration at which 50 % of cells were positive. *B50* binding 50, *CI* confidence interval, *FITC* fluorescein isotiocyanate

to the manufacturer's instructions. Finally, the absorbance was measured at 492 nm in an ELISA reader (Asys VVM 340). Maximum release of LDH was determined in target cells treated with 1 % Triton X-100 (high control), while minimum release (low control) was determined in target cells incubated with antibodies. PBMCs alone were included as control (effector cell control). The percentage of specific lysis was calculated according to the following formula:

$$\text{Cytotoxicity } (\%) = \frac{(\text{Effector: Test cell mix} - \text{Effector cell control}) - \text{Low control}}{\text{High control} - \text{Low control}} \times 100.$$

2.6 Statistical Analysis

The results presented in this study are expressed as mean values ± standard deviation. Statistical significance was evaluated using Prism 6 statistical software (GraphPad, Inc.). One-way ANOVA, followed by Tukey post hoc test or two-way ANOVA followed by mean 95 % confidence interval comparison post hoc test were performed for multiple group comparisons. Significant levels were defined as $p < 0.05$. The values from ADCC assays were also biostatistically analyzed by PLA 3.0 software. The parallelism between product performances and potency ratio of RTXM83 was evaluated according to the reference product (rituximab).

3 Results

RTXM83 has been proven to possess the exact same amino acid sequence as rituximab. It was extensively compared against rituximab in head to head studies for several physicochemical attributes, proving to be highly similar. Post-translational modifications such as *N*-glycan profile are known to have an impact on bioactivity. Non-fucosylated glycans, known to have an impact on ADCC activity, were found to represent 5 % of total glycans on both RTXM83 and the originator product (data not shown) [20].

Human CD20 molecule recognition by the biosimilar and rituximab was evaluated in a wide range of concentrations by flow cytometry assay. CD20 highly positive human cell lines were used. No significant differences were observed in the evaluation of the binding capability of the biosimilar compared with commercial rituximab. Figure 1a (Jeko-1), b (WIL2-S) show the recognition profile of the biosimilar and rituximab. To determine the concentration at which 50 % of the cells are positive, logarithmic regression of the data (Fig. 1c, d) and the B50 were calculated (inset in Fig. 1c, d).

The rituximab ADCC in vitro assay requires the co-culture of CD20 positive target cells and effector cells to produce, in the presence of the antibody, the cell cytotoxicity activity. As reported, rituximab-induced ADCC is sensitive to the F to V polymorphism at position 158 in FcγRIIIa, with V/V and V/F showing greater potency than F/F [16]. In this regard, we first evaluated the polymorphism of 12 healthy donors for the gene FCGR3A by sequencing and specific PCR amplification and further allele-specific DNA digestion, as is described in the Sect. 2. Six out of 12 donors were FcγRIIIa-158F/F, three were FcγRIIIa-158V/F and the remaining three were homozygous for FcγRIIIa-158V/V. Figure 2 shows representative results of the detection of the three different polymorphisms.

In order to evaluate the similarity in the ADCC response between the biosimilar and rituximab, we first evaluated the ADCC response using FcγRIIIa-158V/V effector cells, expecting to reach the maximum cytotoxicity response as it has been published. The CD20 positive Jeko-1 cell line was used as target. As shown in Fig. 3a, in this setting, both antibodies developed a dose-dependent response with no significant differences between them. Statistical evaluation at maximum concentration tested between biosimilar and rituximab showed no differences, whereas highly significant differences were observed for both antibodies compared with the low cytotoxic activity developed by the isotype control antibody (Fig. 3b). In this setting, the analysis of the parallelism between curves and the relative

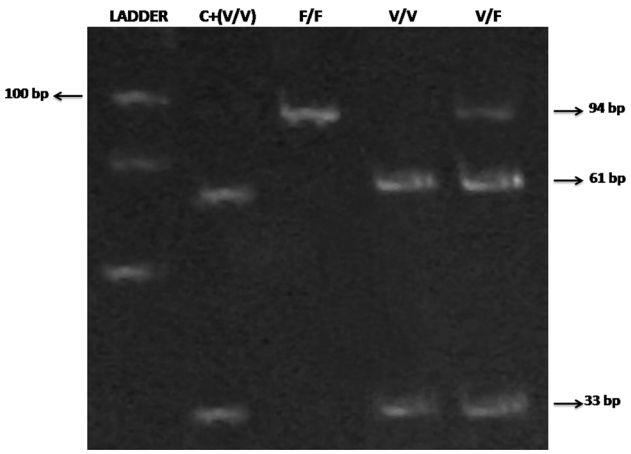

Fig. 2 Genotyping of FcγRIIIa-158 polymorphisms by specific DNA digestion in healthy donors. A 94-bp specific fragment, was amplified from genomic DNA by nested PCR. A mismatch introduced creates a *NlaIII* restriction site only in the FcγRIIIa-158 Valine (V) allele. The enzyme digestion generates a donor polymorphism-specific band pattern. C+: digestion control, synthetic sequence FcγRIIIA-158 V allele, the fragment was completely digested and two bands with 61 bp and 33 bp were visible. Phenylalanine/Phenylalanine (*F/F*): homozygous FcγRIIIa-158F/F 94-bp fragments were not digested. Valine/Valine (*V/V*): homozygous FcγRIIIa-158V/V fragments were completely digested. Valine/Phenylalanine (*V/F*): heterozygous fragments present three bands (94 bp, 61 bp, and 33 bp). *bp* base pair

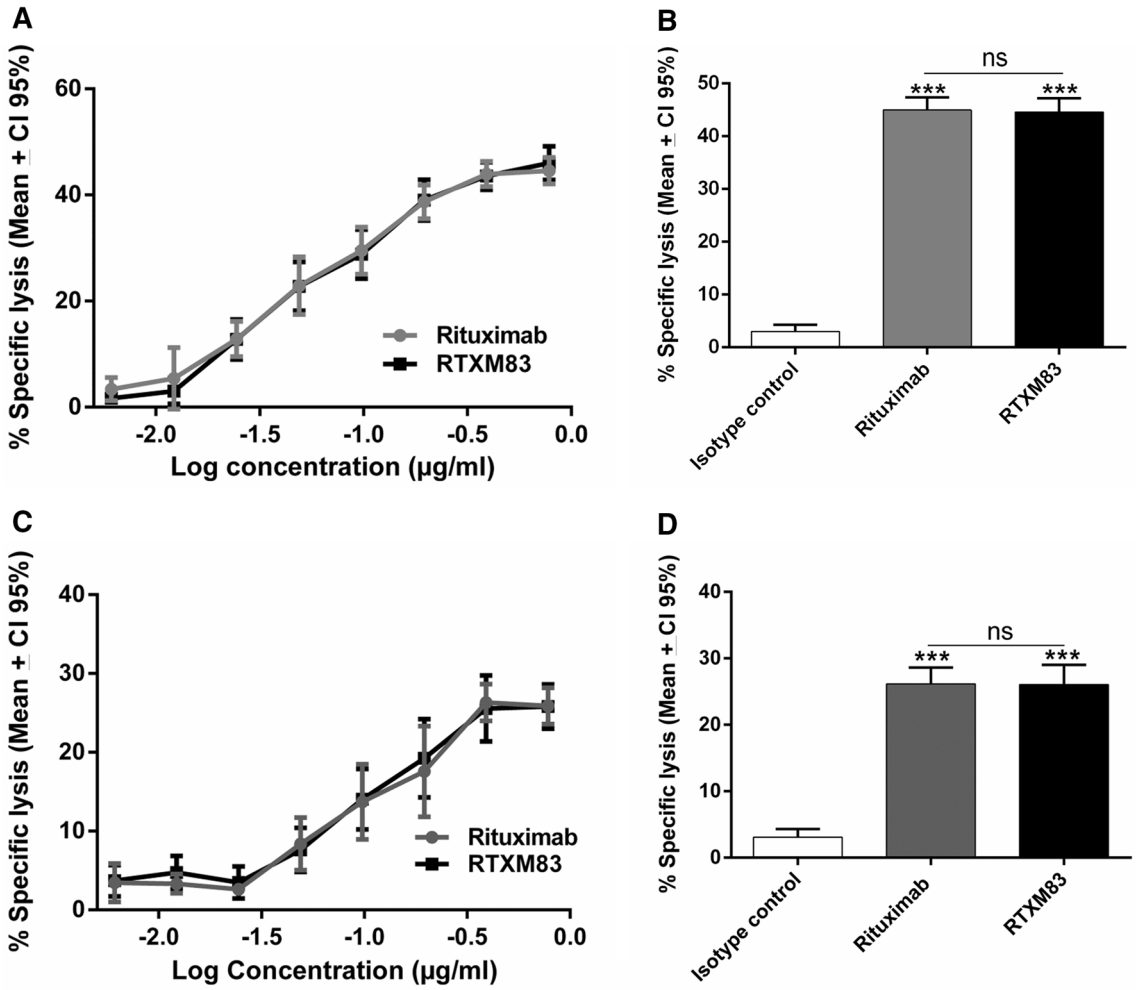

Fig. 3 Induction of ADCC. Jeko-1 cells were treated with a wide range of antibody concentrations (rituximab and RTXM83) followed by co-incubation with effector cells from healthy genotyped donors. Cytotoxicity was measured by an LDH-release assay. *Numbers* represent the percentage of specific lysis calculated (as described in the Sect. 2). **a** and **c** show dose-dependence for ADCC induction using FcγRIIIa-158V/V and F/F effector cells, respectively. No significant (ns) differences for dose-dependence ADCC were observed between biosimilar and rituximab (two-way ANOVA followed by mean 95 % CI comparison post hoc test, $p > 0.05$). **b** and **d** show ADCC response at 0.782 µg/ml (maximum concentration) of each anti-CD20 monoclonal antibody compared with isotype control, using FcγRIIIa-158V/V and F/F effector cells, respectively (one-way ANOVA, followed by Tukey post hoc test, $***p < 0.001$). *ADCC* antibody-dependent cell cytotoxicity, *CI* confidence interval, *F* phenylalanine, LDH lactate dehydrogenase, *V* valine

potency obtained by PLA 3.0 bioassay software have demonstrated the biocomparability between the biosimilar and the originator rituximab in the in vitro ADCC assay for all the batches analyzed (Table 1). Evaluation of in vitro ADCC assay using FcγRIIIa-158F/F effector cells demonstrated a positive dose-dependent cytotoxicity response for the biosimilar as well as for rituximab (Fig. 3c). Cytotoxic activity showed no differences at the maximum concentration tested between both antibodies, but it was significantly different comparing any of them against the low response developed by the isotype control (Fig. 3d). Our results strongly suggest the biosimilarity of

Table 1 Potency ratio evaluation of RTXM83 batches

Biosimilar batch	Potency ratio + CI 95 %
#001	0.949 + 0.191
#002	0.916 + 0.238
#003	1.061 + 0.213
#004	0.929 + 0.189

Potency ratio was calculated using the dose-dependence ADCC results with FcγRIIIa-158V/V effector cells. Reference product used was rituximab (MabThera), as cited in the Sect. 2. Calculation of potency ratio was performed by PLA 3.0 software

ADCC antibody-dependent cell cytotoxicity, *CI* confidence interval, *V* valine

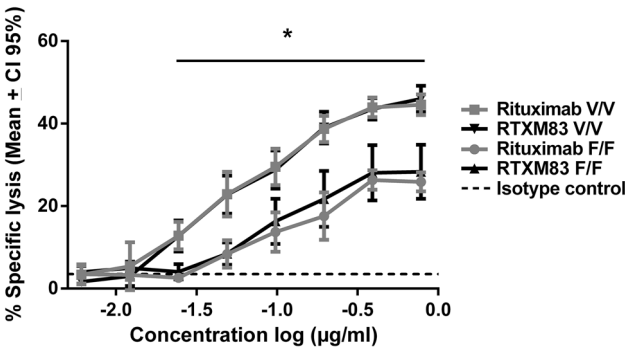

Fig. 4 Comparison of ADCC response between rituximab and biosimilar using FcγRIIIa-158V/V and F/F effectors cells. Significant differences were observed among FcγRIIIa-158V/V and FcγRIIIa-158F/F ADCC responses (two-way ANOVA followed by mean 95 % CI comparison post hoc test, $*p < 0.05$). *Dotted line* represents the isotype control response. *ADCC* antibody-dependent cell cytotoxicity, *CI* confidence interval, *F* phenylalanine, *V* valine

the tested antibody and the reference product regarding ADCC response. Interestingly, comparison between the responses obtained with FcγRIIIa-158V/V or FcγRIIIa-158F/F effector cells clearly showed that cell-killing activity is stronger when using the FcγRIIIa-158V/V phenotype (Fig. 4).

4 Discussion

Rituximab's patent expiration opens a broad scenario of opportunities for biosimilar development of one of the best-selling cancer drugs globally [1, 2]. The first biosimilar of this product was presented by Dr. Reddy's Laboratories (Hyderabad, India) in 2007 [21]. Nowadays, the development of rituximab's biosimilars is the main goal of several pharmaceutics worldwide.

Three mechanisms of action are accepted for rituximab in B-cell killing: induction of apoptosis, complement activation and ADCC. All these activities depend on the recognition of the CD20 antigen by the antibody and the consequent stimulation of the effector component [22]. The biosimilarity between RTXM83 and the originator regarding apoptosis and CDC has been presented previously by Seigelchifer et al. [20].

In this work, we present a new biosimilar of rituximab and evaluate its in vitro biological behavior. Binding of anti-CD20 antibodies was evaluated by quantitative flow cytometry assay using the CD20 positive cell lines WIL2-S and Jeko-1. Results show that the binding of the biosimilar was equal to rituximab's in the recognition of these two cell lines. Accordingly, the high-CD20 B-lymphoblastoid cells, WIL2-S, showed a tenfold increase in the recognition compared with Jeko-1 for both antibodies [23]. Regarding the ADCC response, we have shown that, under these experimental settings and having evaluated four different batches, the RTXM83 antibody has an anti-CD20 cell-killing response similar to the originator product. Additionally, in order to evaluate the response achieved in relation to the Fc-polymorphism in the amino acid position 158 of the FcγRIIIa, we carried out an ADCC evaluation using effector cells from genetically different donors. We observed no differences between the biosimilar and rituximab, even with different FcγRIIIa polymorphisms.

As mentioned before, the maximum ADCC response triggered by rituximab is related to the presence of at least a valine (V) in the amino acid position 158 of FcγRIIIa. Accordingly, our results show that a better cytotoxic response was achieved using FcγRIIIa-158V/V rather than FcγRIIIa-158F/F effector cells. In these experimental settings, we observed that both biosimilar and rituximab develop the same cell-killing activity in accordance with the Fc-polymorphism on effector cells, with no significant differences between them.

Biosimilars are a growing commercial and therapeutic opportunity nowadays. The patent expiration of such an important anti-cancer drug as rituximab is a big challenge for several pharmaceutics. In addition to the chemical and biophysical characterization, the evaluation of biological responses, such as ADCC activity, is a key feature to support the biosimilarity of these new molecules.

5 Conclusion

Our results suggest that RTXM83 presents a high similarity to the originator rituximab, regarding its biological function. Binding to CD20 positive cells measured by flow cytometry and induction of ADCC measured by LDH release showed non-significant differences between antibodies. These results were also confirmed using effector cells expressing different FcγRIIIa-158 polymorphisms.

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Author contributions All authors made substantial contributions to the conception or design of the work and/or the acquisition, analysis or interpretation of data. All authors were involved in developing and critically revising the content of the manuscript, and all provided final approval of the version submitted for publication. Mariano R. Gabri is the guarantor for the overall content.

Compliance with Ethical Standards

Conflict of interest Hector A. Cuello, Valeria I. Segatori, Marina Alberto, Daniel F. Alonso and Mariano R. Gabri have no conflicts of interest that are directly relevant to the content of this study. Analía

Pesce is a full-time employee of PharmADN. The authors have no other conflicts of interest related to this work.

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