Rheumatoid Factors (RFs) Do Not Bind Fc-Free Certolizumab Pegol, but Do Bind to Fc-Containing Biological DMARDs, Leading to Immune Complex Formation which Induces Cytokine Release from Peripheral Blood Mononuclear Cells (PBMC) In Vitro

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Susanna R. Bidgood,[1] Sophie Hopkin,[1] Jacqueline O'Neill,[1] Joana Sousa,[1] Kathryn R. Malpas,[1] David M. Kallenberg,[1] Geofrey Odede,[1] Kerry Tyson,[1] Sue Cross,[1] Tatiana Sokolova,[2] Bernard Lauwerys,[3] Lena D'Hooghe,[1] Baran Ufuktepe,[3] Patrick Durez,[2] **David P. Humphreys**[1]

[1]UCB, Slough, UK , [2]Université Catholique de Louvain, Brussels, Belgium, [3]UCB, Brussels, Belgium

Objective

To evaluate whether immune complexes formed between rheumatoid factor (RF), Fc-containing adalimumab (ADA) and tumour necrosis factor alpha (TNFα), can induce pro-inflammatory cytokine release from human PBMC in vitro, in comparison with Fc-free certolizumab pegol (CZP).

Background

- Human RFs are naturally occurring polyclonal autoantibodies which bind the Fc domain of IgGs.
- In RA, patients with high RF levels often show reduced serum drug concentrations and poorer disease control when treated with Fc-containing biologic Disease-Modifying Anti-Rheumatic Drugs (bDMARD).
- In contrast, the Fc-free TNFα inhibitor CZP maintains stable serum concentrations and efficacy regardless of RF levels[1–4].
- Recombinant monoclonal RF IgMs and RF in RA patient sera bind Fc-containing bDMARDs, forming immune complexes that are cleared by human macrophages. Fc-free CZP is not bound by RF and avoids this clearance mechanism[5–7].
- This study examined whether immune complexes formed by RF, TNFα and the Fc-containing bDMARD ADA, can stimulate pro-inflammatory cytokine release from human PBMC.

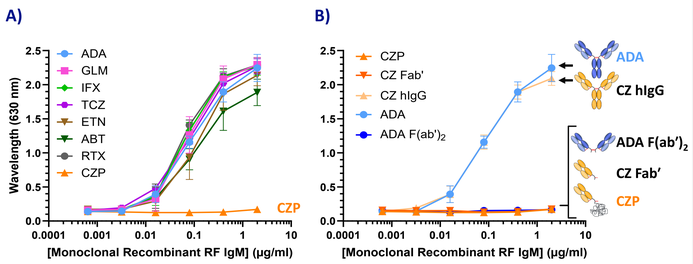
Results

- RF IgMs bound to all Fc-containing bDMARDs tested but not to CZP or other Fc-free molecules (**Figure 1**).
- RF IgM, TNFα and Fc-containing ADA formed immune complexes that were detectable by negative-stain transmission electron microscopy (TEM), whereas Fc-free CZP did not (**Figure 2**).
- High RF patient sera formed immune complexes with ADA and TNFα but not with CZP. No immune complexes were detected in RF negative sera (**Figure 3**).
- An IgG Fc domain is required for immune complex formation in high RF patient sera (**Figure 4**). CZ hIgG is bound by RF but Fc-free CZP is not.
- Protein mixtures composed of Fc-containing ADA, TNFα, and high RF RA patient sera induced significantly higher levels of pro-inflammatory cytokines from human PBMC in vitro compared with equivalent mixtures containing Fc-free CZP (**Figure 5**).

Conclusion

Fc-free CZP is not bound by RFs and does not form immune complexes with RFs. CZP does not induce pro-inflammatory cytokine release and is not subject to RF-dependent clearance by macrophages in vitro[5–7]. These findings provide a further mechanistic basis for why CZP is equally efficacious in patients independent of their RF status [1–7,9].

Figure 1 Monoclonal RF IgM binds to Fc-containing bDMARDs in vitro but not Fc-free CZP

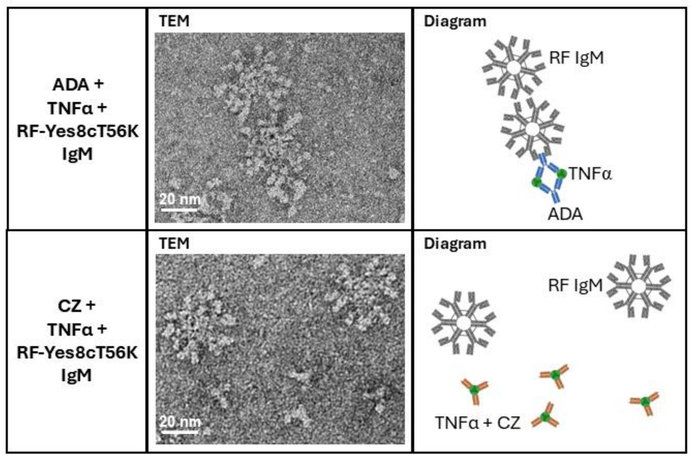


Direct-binding ELISAs using the monoclonal recombinant RF-Yes8cT56K[8] IgM demonstrate that:

A) All Fc-containing bDMARDs were bound by RF but not Fc-free CZP

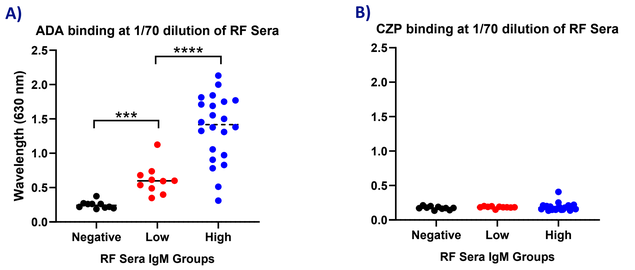
B) An IgG Fc domain is required for RF binding

Figure 2 Negative-stain transmission electron microscopy (TEM) shows that RF IgM binds ADA in the presence of TNFα to form complexes, whereas CZP is not bound by RF IgM and does not form immune complexes



Diagrams created with BioRender.com.

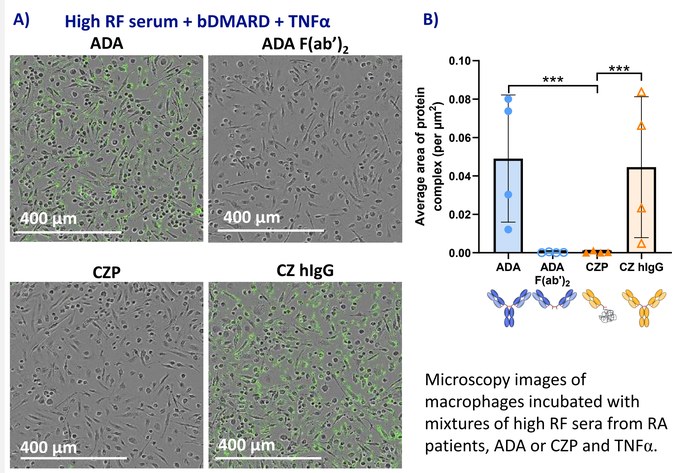
Figure 3 RFs in RA patient sera bind to Fc-containing ADA but not Fc-free CZP



Direct binding ELISAs with sera from patients with RA:

- RF negative (<14 IU/mL) patient sera has no binding to ADA or CZP. Low RF (14-50 IU/mL) and high RF (>180 IU/mL) patient sera bind to ADA but not to CZP.
- Significantly higher binding to ADA was observed in the high RF sera cohort compared to the low RF sera cohort and the RF negative cohort. No binding was observed to CZP in any RA patient samples.

Figure 4 IgG Fc domain is required for immune complex formation in high RF patient sera

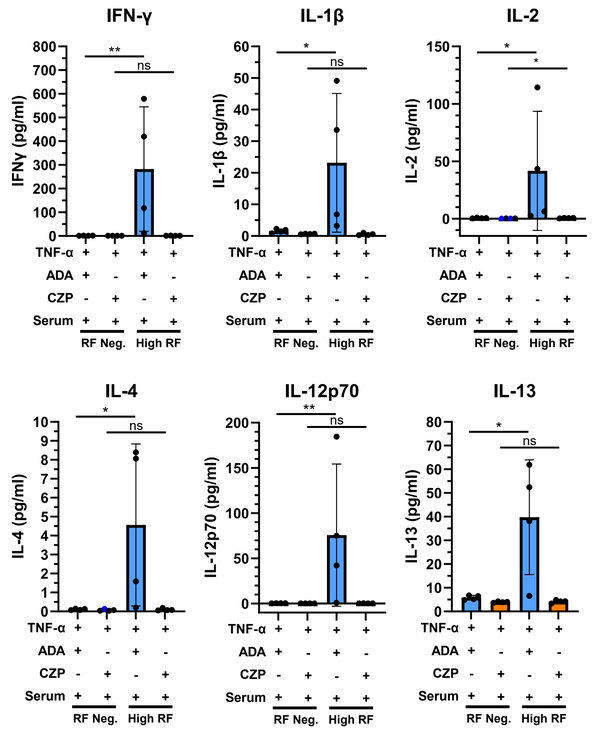


Microscopy images of macrophages incubated with mixtures of high RF sera from RA patients, ADA or CZP and TNFα.

A) Fc-containing ADA and CZ hIgG formed immune complexes (green) in the high RF patient serum, Fc-free CZP and ADA $F(ab')_2$ did not.

B) The average area of immune complexes in microscopy images. Mean ±SD, n=4 for RA patient sera, *** p <0.001.

Figure 5 RA patient serum + ADA + TNFα significantly induced cytokine release in high RF patient sera compared to RF negative patient sera. RA patient serum + CZP + TNFα did not induce elevated cytokine levels in patient sera from either cohort



Cell culture supernatant cytokines levels were measured after ex vivo treatment of PBMC with RA patient sera (RF negative or high RF) and ADA or CZP plus TNFα. Cytokines were quantified by MSD. Mean ±SD, n=4. Blue circles indicate values below the lower limit of detection. Significance: ns ≥ 0.05, * $p \leq 0.05$, ** $p \leq 0.01$.

Abbreviations: ABT: abatacept; **ADA:** adalimumab; **ADA F(ab')$_2$**: Fc-free ADA di-Fab; **bDMARD:** biologic disease-modifying anti-rheumatic drug; **CZ**: PEG-free certolizumab Fab'; **CZ hIgG**: Certolizumab Fab' reformatted as human IgG1; **CZP:** certolizumab pegol; **ELISA:** enzyme-linked immunosorbent assay; **ETN:** etanercept; **Fc:** fragment crystallizable; **GLM:** golimumab; **IFN-γ:** interferon gamma, **IFX:** infliximab; **IgG:** immunoglobulin G; **IgM:** immunoglobulin M; **IL**: interleukin; **IU:** international units; **min:** minutes; **ml:** milliliters; **MSD**: meso scale discovery; **nm:** nanometers; **ns:** non-significant; **PBMC:** peripheral blood mononuclear cells; **pg:** picogram; **RA:** rheumatoid arthritis; **RF:** rheumatoid factor; **RTX:** rituximab; **SD:** standard deviation; **TCZ:** tocilizumab; **TEM**: transmission electron microscopy; **TNFα:** tumour necrosis factor-α; **µg:** microgram, **µm**: micrometre.

Methods: Transmission electron microscopy (TEM): RF-Yes8cT56K IgM, TNFα and ADA or CZ were mixed, fixed on grids with 1% uranyl acetate stain and imaged in a JEOL JEM-1400 TEM at a magnification of 120000x for ADA+TNFα+RF-Yes8cT56K IgM and 50000x for CZ+TNFα+RF-Yes8cT56K IgM. **Direct binding ELISAs:** Plates were coated with bDMARDs, challenged with a titration of monoclonal RF-Yes8cT56K IgM or serum from a biologic-naïve patient with RA, detected with HRP-conjugated anti-human IgM and read using a Synergy2 microplate reader (BioTek). Statistical analysis: an unpaired t-test with Welch's correction. **Live cell imaging:** Sera from biologic-naïve patients with RA, bDMARDs and TNF-α were mixed and incubated with primary human macrophages at 4°C. Cells were washed and bound immune complexes detected with Fab anti-human IgM FITC. Cells were incubated at 37°C and imaged for 4 hours (Incucyte, Sartorius). Overlayed phase contrast and green channel representative images are shown. The average area of immune complexes in the images were quantified using Fiji ImageJ. Statistical analysis: a paired one-way ANOVA with Dunnett's multiple comparison test on log-transformed data (compared to CZP group). **Cytokine release assays:** PBMC were isolated from healthy volunteers, plated, and incubated with mixtures of bDMARD+TNFα ,or RA serum+bDMARD+TNFα, or control mixtures for 4 hours at 37°C. The levels of 9 pro-inflammatory cytokines (interferon gamma [IFNγ], interleukin- [IL-]1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, and IL-13) in the cell supernatants were quantified by multiplex meso scale discovery (MSD). An unpaired two-tailed t-test was performed on log-transformed data (negative RF RA patient serum against high RF RA patient serum for each bDMARD).

References: [1]Martínez-Feito A. Clin Exp Rheumatol. 2024;42(5):999–1005; [2]Smolen J S. Rheumatol. 2024;63(11):3015–24; [3]Smolen J S. Arthritis Rheumatol. 2024;76(suppl 9); [4]Miyazaki Y. Mod Rheumatol. 2025;35:S56; [5]Bidgood S. Ann Rheum Dis. 2024; 83(1):727; [6]Bidgood S. Arthritis Rheumatol. 2024;76(suppl 9); [7]Hopkin S. Arthritis Rheumatol. 2025;77(suppl 9); [8]Shiroshi M. J Biol Chem. 2018;293(18):7008–16; [9]UCB Pharma. Cimzia 200 mg solution for injection: Summary of Product Characteristics. European Medicines Agency; 2026. Available from: https://www.ema.europa.eu/en/documents/product-information/cimzia-epar-product-information_en.pdf

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