

## Abstract

**Introduction:** Biosimilars for therapeutic antibodies contain identical variable sequences to those of FDA-approved antibodies. Produced as recombinant proteins, BD research-grade biosimilars are available as wildtype hulgG1 Fc biosimilars, in no-azide/endotoxin-free (NA/LE) format; and as reengineered molecules (N297A) to further reduce Fc receptor interactions, presented in fluorescent conjugated options. Both types can be used to model aspects of antibody therapies in use today. **Methods:** Different biosimilar formats (wildtype and N297A) of Rituximab, Trastuzumab, Adalimumab, Infliximab and Obinutuzumab were selected to exemplify their use in flow cytometry (FCM) staining protocols, and colorimetric functional assays. Human blood cells (PBMC and LWB) were used for surface and intracellular staining for FCM. Human cell lines (SK-BR-3, HUT-78 and L-929) were used for functional assays: proliferation, adhesion and viability to TNF-induced cytotoxicity (colorimetric, MTT). Cell killing assays by FCM used human Daudi cell line for Complement-Dependent Cytotoxicity (CDC), and PBMC for Direct Cell Death (DCD) and Antibody-Dependent Cell-mediated Cytotoxicity (ADCC). SK-BR-3 cells were used to identify non-competing antibody clones by FCM. **Results:** N297A biosimilars for direct fluorescent FCM exhibit reduced background binding and clarify the identification of therapeutic target cell subsets; N297A bound to their secreted target (TNF) in its soluble (intracellular) and cell-associated (membrane) states. Wildtype biosimilars in NA/LE format permit mechanism-of-action studies and functional assays through different methods. **Conclusion:** Research-grade biosimilar antibodies enable multiple avenues of research on therapeutic antibodies and empower translational medicine.

## Methods

### Lysed Whole Blood (LWB) and PBMC Staining

Whole blood from healthy donors was treated with BD Pharm Lyse™ Lysing Buffer (Cat# 555899) to remove erythrocytes. PBMC were isolated using Ficoll-Paque™ PLUS (GE Healthcare™) gradient centrifugation. Resulting samples were stained with the following Biosimilars: Rituximab, Rituximab297 (Cat# 570361), Trastuzumab, Trastuzumab297 (Cat# 756616/567979) or Isotype Controls: X40.hulgG1N297A (Cat# 756231/569961), Mouse IgG1, κ MOPC-21 (Cat# 554680), or human IgG1, κ (all conjugated to the indicated fluorochromes) for 20-30 min at RT in BD Pharmingen™ Staining Buffer (Cat # 55465), followed by washing, addition of 7-AAD or DAPI, and flow cytometric analysis.

### Functional Assays for NA/LE Biosimilars

For measuring Trastuzumab Biosimilar inhibition of tumor cell growth, Human SK-BR-3 cells were treated with either Purified NA/LE Human IgG1, κ Isotype Control (Cat# 569605) or Purified NA/LE Human Anti-Human ErbB2/HER-2 (CD340) Trastuzumab Biosimilar (Cat# 569599) for 1 week. Proliferation was measured by colorimetric MTT Assay (ATCC Cat # 30-1010K). For adhesion assays, ELISA plates were coated with hMAcAM-1-Fc and blocked with BSA. HUT-78 cells were pre-treated with NA/LE Anti-Integrin α<sub>v</sub>β<sub>3</sub>, Vedolizumab Biosimilar (BD Cat# 571605) or Purified NA/LE Human IgG1, κ Isotype Control (BD Cat# 569605) for 15 min. before adhesion at 37°C for 90 min., followed by gentle washing. Relative numbers of adhered cells were quantified by O.D. after addition of MTT. To measure Adalimumab and Infliximab Biosimilar neutralization of cytotoxicity induced by Human TNF, L-929 cells (ATCC® CCL-1™) were cultured with BD Pharmingen™ Recombinant Human TNF protein (BD Cat. No. 554618 at 0.3125 ng/ml), the metabolic inhibitor Actinomycin D (ThermoFisher™ Cat# A7592 at 0.5 μg/ml), and Purified NA/LE Human Anti-Human TNF (Adalimumab or Infliximab Biosimilar) antibodies for 24 hours and analyzed using the above MTT assay.

### Cell Killing Assays

To measure Complement-Dependent Cytotoxicity (CDC), Daudi cells (ATCC® CRL-213™) were treated with 1.25 μg/ml of Purified NA/LE Human IgG1, κ Isotype Control (Cat# 569605), Rituximab Biosimilar (Cat# 569496), or Obinutuzumab Biosimilar (Cat# 570921), in the presence of 25% normal or heat-inactivated Human serum as indicated at 37°C for 5 hours. Harvested cells were pelleted and resuspended in BD Pharmingen™ Stain Buffer (Cat# 554656). BD Via-Probe™ Cell Viability 7-AAD Solution (Cat# 555815/555816) was added to cells before flow cytometric analysis. To measure Direct Cell Death (DCD) or Antibody-dependent Cell-mediated Cytotoxicity (ADCC)+DCD, Human PBMC from healthy donors were treated with 1.25 μg/ml of Purified NA/LE Human IgG1, κ Isotype Control (Cat# 569605), Rituximab Biosimilar (Cat# 569496), or Obinutuzumab Biosimilar (Cat# 570921) at 37°C for 2 hours (DCD) or 72 hours (DCD+ADCC). Cells were stained with FITC CD19 (BD Cat# 555413), PE CD19 (BD Cat# 555412), or BVU395 CD3 (BD Cat# 563546). 7-AAD or DAPI was added before flow cytometry.

### Human TNF Localization

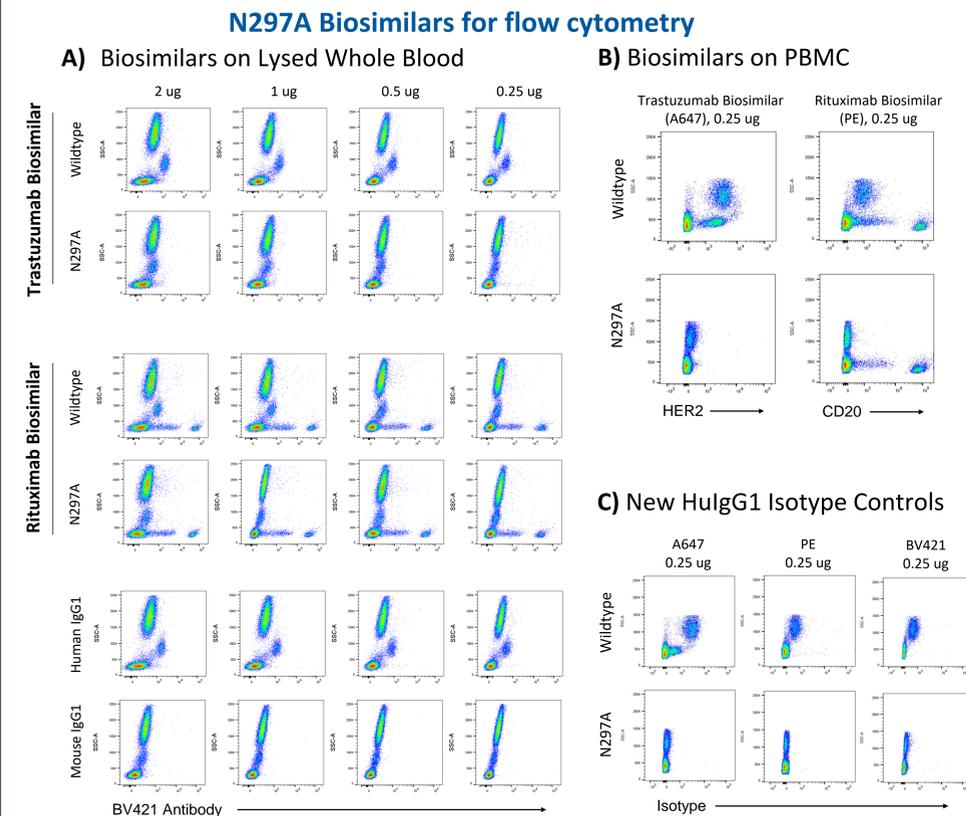
Human PBMC from healthy donors were stimulated with Phorbol 12-Myristate 13-Acetate (PMA; Sigma P8139; 50 ng/ml) and ionomycin (Sigma I9657; 1 μg/ml) for 5 hours at 37°C. BD GolgiStop™ Protein Transport Inhibitor (Cat. No. 554724) was added for intracellular staining conditions, and TAPI-1 (20 μM) acetate salt (Sigma SM0739-1MG) was added to indicated surface staining samples. After stimulation for intracellular staining, PBMC were stained with FVS 510 (BD Cat. No. 564406), fixed with BD Cytotfix™ Fixation Buffer (Cat. No. 554655), and permeabilized with BD Perm/Wash™ Buffer (Cat. No. 554723). Surface and intracellular staining conditions were stained with BD Pharmingen™ Alexa Fluor™ A647 Adalimumab297 Biosimilar (Cat# 569793) or Infliximab297 Biosimilar (Cat# 570532), or Isotype Control Alexa Fluor™ 647 Human IgG1 (N297A), κ (Cat No. 569794). DAPI solution was added before flow cytometry.

### HER2 Levels after Trastuzumab Biosimilar Treatment

To identify HER2 mAb clones that do not compete for binding with Trastuzumab, SK-BR-3 cells (ATCC® HTB-30™) were harvested with TrypLE™ Express Enzyme (Gibco® LifeTech Cat#12604021) and pre-treated with Purified NA/LE anti-Human HER2 Trastuzumab Biosimilar clone Trastuzumab (BD Cat# 569599) or Purified NA/LE Human IgG1, κ Isotype Control (BD Cat# 569605) for 15 min at RT, before staining by addition of BV421 X40 Isotype (BD Cat# 562438), BV421 24D2 (BD Cat# 566458), PE MOPC-21 (Cat# 554680), or PE NEU 24.7 (BD# 340879). Pre-treatment antibodies were used at 20x concentration of staining antibodies. To measure HER2 surface levels post-treatment, SK-BR-3 cells were treated with 10 μg Purified NA/LE anti-Human HER2 Trastuzumab Biosimilar clone Trastuzumab (BD Cat# 569599) or Purified NA/LE Human IgG1, κ Isotype Control (BD Cat# 569605) +/- anti-mouse secondary antibody at 37°C for 44 h. Cells were harvested and stained as above for HER2 using 24D2 or Isotype antibodies and analyzed by flow cytometry for possible HER2 modulation.

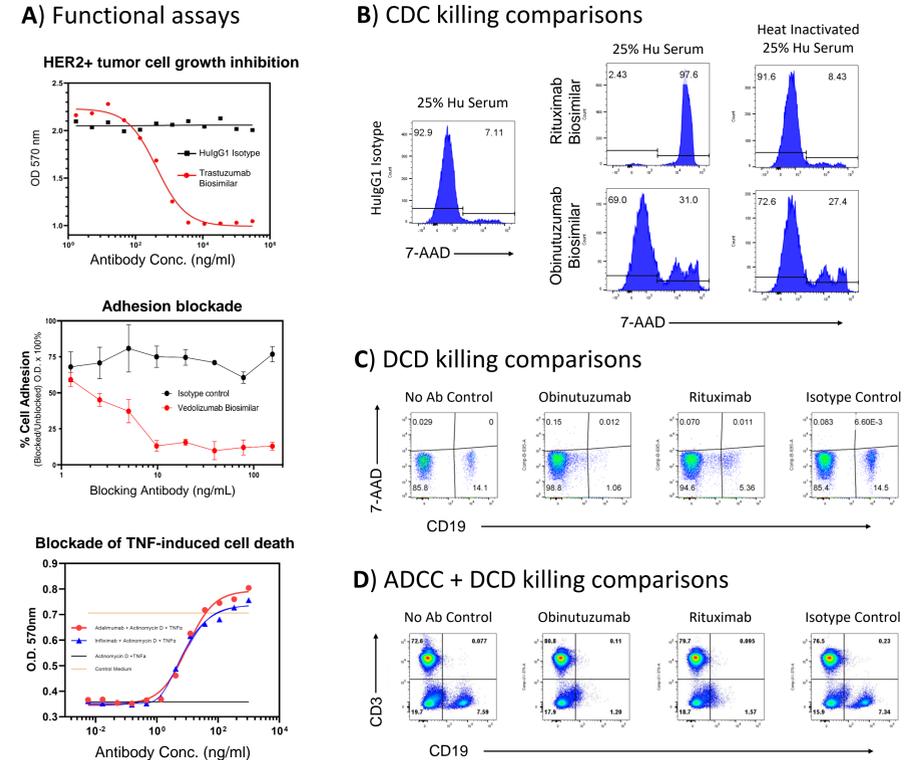
Flow Cytometry and data analysis were performed by BD LSRFortessa™ X-20 Cell Analyzer and BD FlowJo™ Software; further data analysis was performed by GraphPad Prism™ software.

## Results:



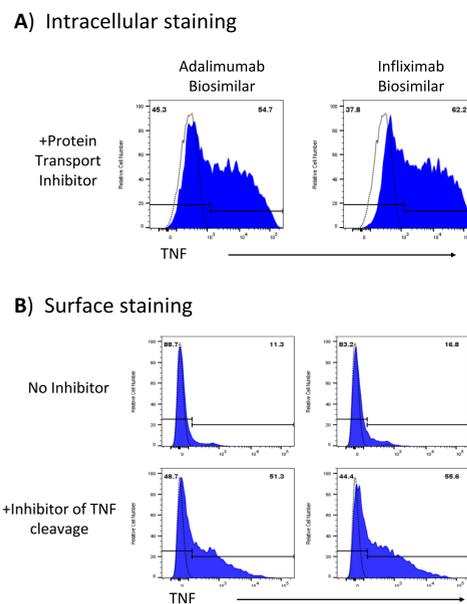
**Figure 1.** Human IgG1 Fc N297A mutant Biosimilars for flow cytometry. **(A)** Lysed Whole Blood (LWB) or **(B-C)** PBMC were stained with the indicated wildtype or N297A mutant hulgG1fluorochrome-conjugated Biosimilars or Isotype Controls. Dot plots display side scatter on the y-axis vs. antibody staining on the x-axis.

## NA/LE wildtype Biosimilars for functional investigations



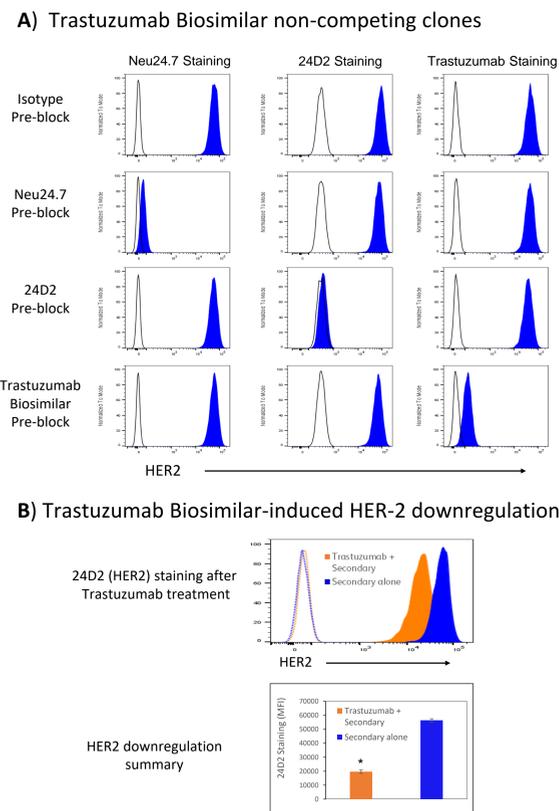
**Figure 2** Functional assays and MOA comparisons using NA/LE Biosimilars. **(A) Functional Assays.** Trastuzumab, Vedolizumab, Adalimumab, or Infliximab Biosimilars were used in assays for (top to bottom): SK-BR-3 growth inhibition, HUT-78 adhesion blockade, or inhibition of L929 TNF-induced cell death. **(B-D) MOA Comparisons.** The MOAs of Rituximab and Obinutuzumab CD20 Biosimilars were compared in 5h complement-mediated lysis (CDC), 2h depletion (Direct Cell Death, DCD), or 72h B cell depletion (ADCC+DCD, gated on 7-AAD-negative) assays analyzing fresh PBL.

## Investigating target localization



**Figure 3.** PBMC were stimulated with PMA/Ionomycin in the presence **(A)** or absence **(B)** of the protein transport inhibitor Monensin for intracellular or surface staining, respectively. For surface TNF staining, some samples were treated with an inhibitor of surface TNF cleavage (TAPI-1). For both intracellular and surface TNF staining, Adalimumab or Infliximab Biosimilar or Isotype control was used. \**p*-value < 0.0003

## Analyzing target modulation



**Figure 4.** **(A)** SK-BR-3 cells were pre-blocked with NA/LE Trastuzumab Biosimilar (filled) or Isotype (open lines) to identify non-competing HER2 clones; neither 24D2 nor Neu24.7 clones appeared grossly affected by Trastuzumab Biosimilar binding. **(B)** Potential modulation of HER2 by Trastuzumab was investigated by treating with Biosimilar and secondary antibody for 44h, followed by staining for HER2 with a non-competing fluorochrome-conjugated clone. \**p*-value < 0.00005

## Conclusions

In this work, we have shown examples of applications for research-grade biosimilar antibodies produced as recombinant proteins and available in no-azide/endotoxin-free (NA/LE) format for functional investigations and as reengineered molecules (N297A) for direct fluorescent flow cytometry. In summary, our results showed:

- N297A formats of Rituximab and Trastuzumab biosimilars exhibited reduced background binding and clarified the identification of target cell subsets in lysed whole blood and PBMC by FCM.
- N297A formats of Adalimumab and Infliximab bound to their secreted target (TNF) in its soluble (intracellular) and cell-associated (membrane) states in PBMC using FCM.
- Different wildtype biosimilars in NA/LE format were used for mechanism-of-action studies and functional assays through different methods using cell lines and PBMC.
- NA/LE Trastuzumab biosimilars was used to identify non-competing antibody clones suitable for assessing targeted modulation of HER2 in SK-BR-3 cells using FCM.

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