Research-grade Biosimilars Enable the Study of Therapeutic Antibodies

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Abstract

Biosimilars for therapeutic antibodies contain identical variable sequences to FDA-approved antibodies. Recombinant BD research-grade biosimilars can be used to model several important aspects of major antibody therapies in use today. Our biosimilars with the huIgG1 (N297A) Fc mutation exhibit reduced background binding and clarify the identification and research of therapeutic antibody target cell subsets. These flow cytometry reagents are available with many fluorochrome options to facilitate deeper phenotyping using large panels. Secreted targets (e.g., TNF-α) of therapeutic antibodies can either be bound in solution or in a membrane/cell-associated state; biosimilars may also be useful in studying this context-dependent target engagement. Alternatively, Finally, wildtype huIgG1 Fc biosimilars in no-azide/endotoxin-free (NA/LE) formats permit comparative mechanism-of-action (MOA) studies. Biosimilars can be used in pre-blocking experiments to identify non-competing and competing clones used to study membrane target regulation, or gate on cell subsets defined by these targets in models of pre-exposure to therapeutic antibodies. In sum, our research-grade biosimilar antibodies can empower multiple avenues of research on clinically important therapeutic antibodies.

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.huIgG1N297A (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 hMAdCAM-1-Fc and blocked with BSA. HUT-78 cells were pre-treated with NA/LE Anti-Integrin $\alpha_4\beta_7$ 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 TNFα 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 BUV395 CD3 (BD Cat# 563546). 7-AAD or DAPI was added before flow cytometry.

Human TNFα 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 37C. BD GolgiStop™ Protein Transport Inhibitor (Cat. No. 554724) was added for intracellular staining conditions, and TAPI-1 (20µM) 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 Cytofix™ 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

N297A Biosimilars for flow cytometry

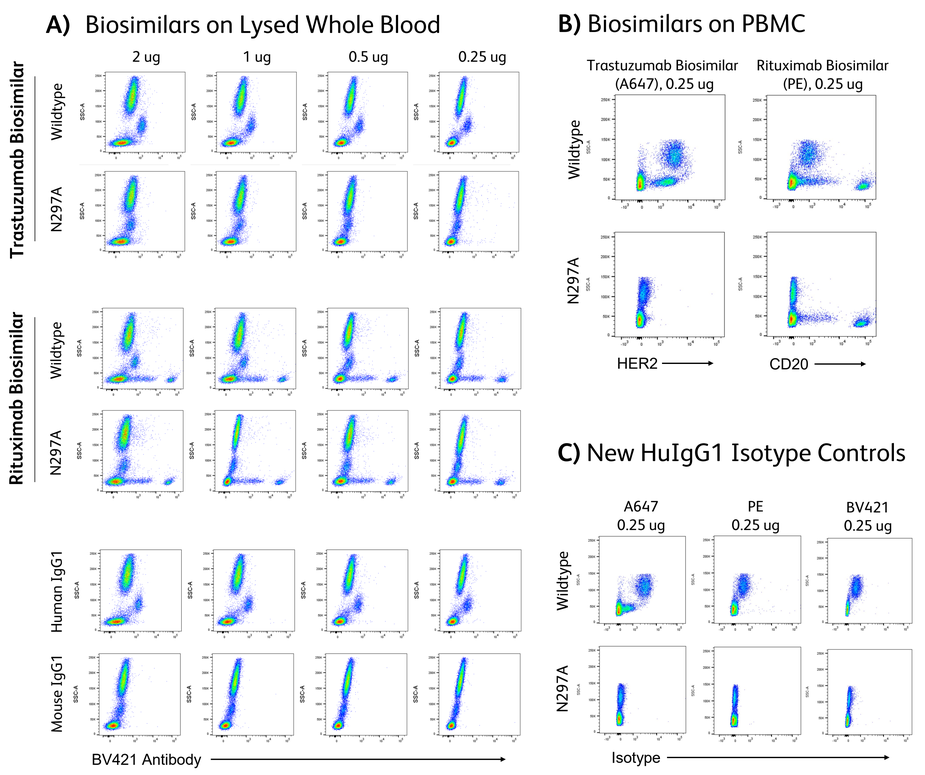

Figure 1. Human IgG1 Fc N297A mutant Biosimilars for flow cytometry. **(A)** Lysed Whole Blood (LWB) or **(B-C)** PBMC were stained with the indicated wildtype or N297A mutant huIgG1fluorochrome-conjugated Biosimilars or Isotype Controls. Dot plots display side scatter on the y-axis vs. antibody staining on the x-axis.

NA/LE wildtype Biosimilars for functional investigations

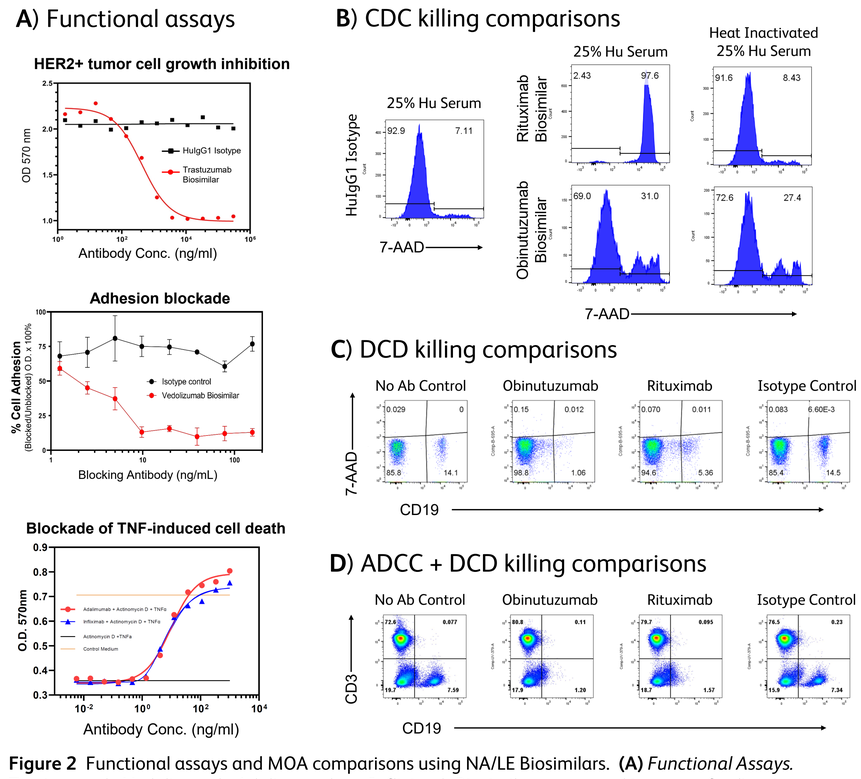

Figure 2 Functional assays and MOA comparisons using NA/LE Biosimilars. **(A)** *Functional Assays.* Trastuzumab, Vedolizumab, Adalimumab, or Infliximab Biosimilars were used in assays for (top to bottom): SK-BR-3 growth inhibition, HUT-78 adhesion blockade, or inhibition of L929 TNF-induced cell death. **(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

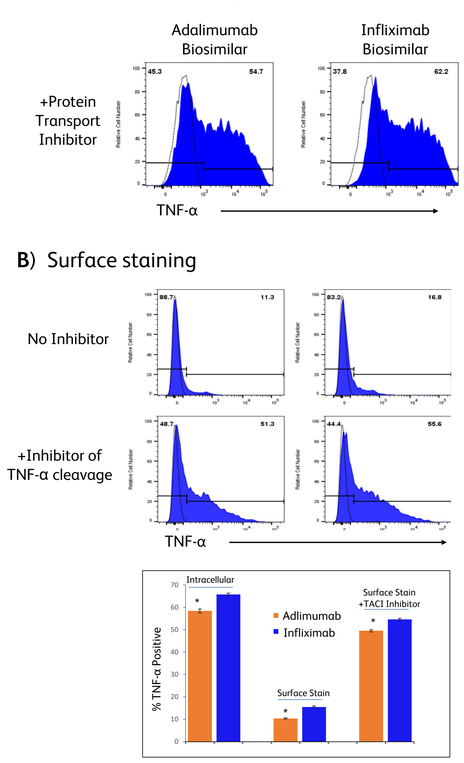

Figure 3. PBMC were stimulated with PMA/Ionomycin in the presence **(A)** or absence **(B)** of the protein transport inhibitor Monensin for intracellular or surface staining, respectively. For surface TNF staining, some samples were treated with an inhibitor of surface TNF-α cleavage (TAPI-1). For both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

Analyzing target modulation

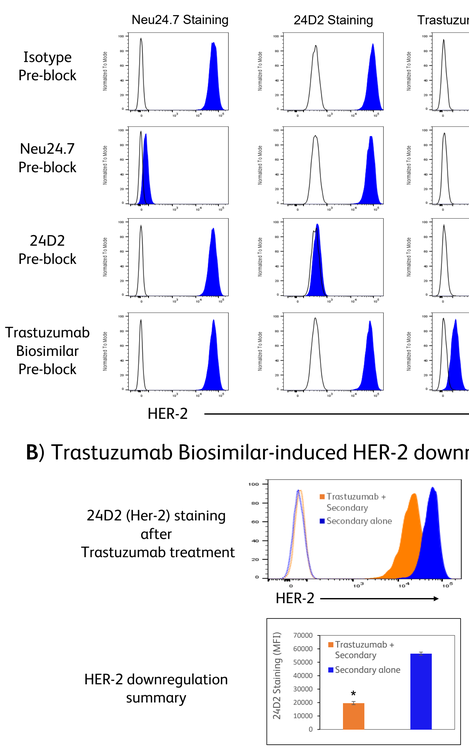

Figure 4. (A) SK-BR-3 cells were pre-blocked with NA/LE Trastuzumab Biosimilar (filled) or Isotype (open lines) to identify non-competing HER2 clones; neither 24D2 nor Neu24.7 clones appeared grossly affected by Trastuzumab Biosimilar binding. **(B)** Potential modulation of HER2 by Trastuzumab was investigated 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 and Further Uses

- N297A mutants permit Biosimilar FCM
- Wt NA/LE allow Biosimilar MOA studies
  - Perform in multiple functional assays
  - CD20 Abs have expected MOA differences
- Non-competing clones enable study of Biosimilar target down-regulation
- Additional possible uses of Biosimilars:
  - Receptor-occupancy assays
  - Combination treatment modeling
  - Deep phenotyping/RNAseq on target cells
  - Target cell purification/sorting
  - Antigens in anti-drug antibody research
  - Antigens for anti-idiotype Ab development