

Studying Protein Higher-Order Structure and Immunogenicity Correlation

with Protein Conformational Array and Cytokine-Panel Analyses

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Immunogenicity refers to a biopharmaceutical's ability to generate an immune response upon administration. It is a major concern in biologics development because it can cause adverse events in patients or reduce treatment efficacy (1–9). Recent studies of clinical data report that antidrug antibodies (ADAs) have been found in 0.1% to 48% of patients treated with candidate monoclonal antibodies (mAbs) and related protein drugs (10–13). It also should be pointed out that many highly immunogenic biologics are eliminated during drug development. Thus, increased understanding of biologics immunogenicity would be of great value to drug developers.

Many known factors influence protein-drug immunogenicity and dictate its tolerance. For example, protein aggregates contribute significantly to immunogenicity, but due to the limitations of current biomanufacturing processes, a biologic with aggregate levels of 5–10% often is

PRODUCT FOCUS: Antibodies and other proteins

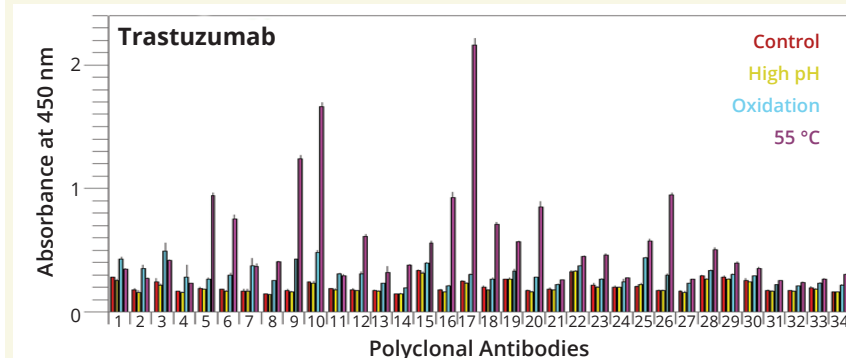
PROCESS FOCUS: Analytical

AUDIENCE: Process development and manufacturing

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LEVEL: Intermediate

Figure 1: Whole panel of protein conformational array (PCA) enzyme-linked immunosorbent assay (ELISA) analysis of trastuzumab higher-order structure (HOS) under different stress conditions; polyclonal antibodies 1–12 cover the variable regions of the light and heavy chains, and polyclonal antibodies 13–34 cover the constant regions. The low-pH (3.0) and urea-treated samples reached saturation levels of signal, so those factors are not shown in the graph. (See the Materials and Methods section.)

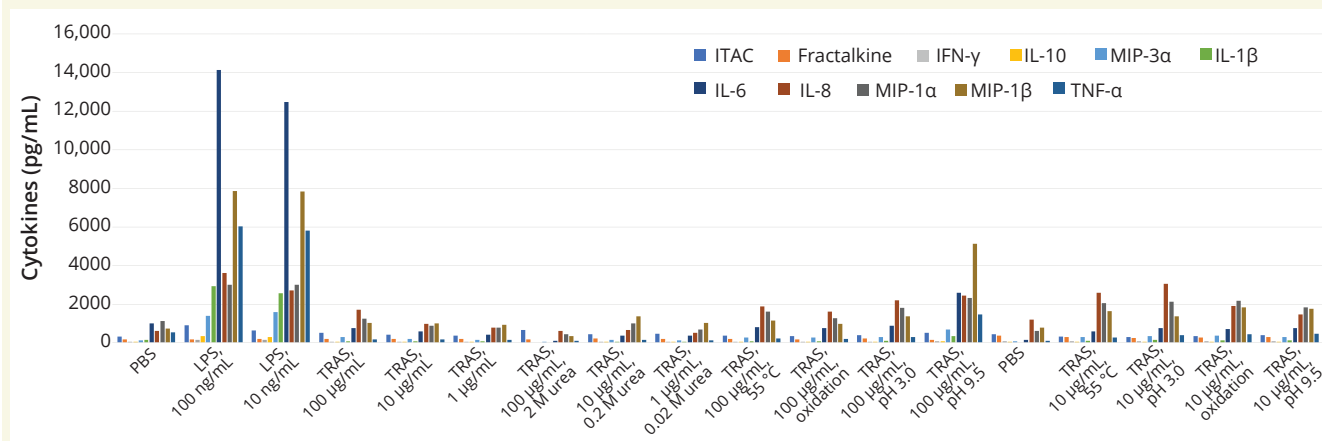


deemed acceptable (14–16). As bioprocessing improves over time, the allowance of protein aggregates is likely to tighten further. Besides protein aggregates, other factors that relate closely to immunogenicity include protein sequences, post-translational modifications (PTMs), target-population traits, dosing frequency, and route of administration. Our report will focus on three antibodies as examples (17–20).

Great progress has been made in the past decade toward understanding biologics immunogenicity. Among the multiple mechanisms involved, antigen-presenting cells (APCs) play a key role (21–23). Upon entering an APC, an immunogenic molecule is processed by multiple proteases into peptides of various lengths. Those peptides then form complexes with major histone complex II (MHC-II) and are transported to the outside of the APC for antigen

presentation. T cells will recognize the MHC-II complex and trigger an immune response that includes production of cytokines and antibodies. Recently, technology has been developed to characterize the details of the MHC-II presentation process (21–23). One interesting finding from such studies was that peptides complexed with a MHC-II protein have a wide range of lengths, from 10 to 35 amino-acid residues (21–23). The majority of peptides identified were in the range of 14 and 18 amino acids. Another interesting finding was that peptide length was target-protein specific. For example, keyhole limpet hemocyanin (KLH) and Remicade (infliximab) were processed into peptides of different lengths for APC presentation. Further study in this area will help to provide more insights regarding immunogenicity mechanisms.

Figure 2: HSTC384-28K kit analysis of cytokine release from human whole blood after treatment with trastuzumab (TRAS); shown here are data from 11 cytokines (from 21 total) with relatively high responses to TRAS treatment. (IFN = interferon, IL = interleukin, ITAC = interferon-inducible T-cell alpha chemoattractant, LPS = lipopolysaccharide, MIP = macrophage inflammatory protein, PBS = phosphate-buffered saline, TNF = tumor necrosis factor)



Protein conformational array (PCA) technology was developed for comparing the higher-order structures (HOSs) of biosimilar and reference proteins. Since then, it has been used in bioprocess development for biosimilars and novel protein products alike as well as in protein characterization (24–28). PCA enzyme-linked immunosorbent assays (ELISAs) are based on arrays of polyclonal antibodies that primarily target linear epitopes on the surfaces of test molecules. Studies have shown that PCA results correlate well with those from traditional methods for biologics HOS analysis, including hydrogen–deuterium exchange mass spectrometry (HDX-MS), X-ray crystallography, size-exclusion chromatography (SEC), and bioassays (29, 30).

We used PCA in combination with a (human) whole-blood assay and 23-cytokine panel analysis to study possible correlations between HOS and immunogenicity. Our approach is complementary to the (human) immune-response–mechanism study described above. Together, such studies will provide a more comprehensive picture of biologics immunogenicity.

MATERIALS AND METHODS

Reagents: All chemicals used in our study were purchased from MilliporeSigma, and 96-well microplates were purchased from Corning Corp. We obtained streptavidin–horseradish peroxidase (HRP) conjugate and biotin-labeling kits from Thermo Fisher Scientific.

PCA Antibodies and ELISA Kits: All antibodies and ELISA kits used in our study were products of Array Bridge Inc. Polyclonal antibodies against mAb peptides were produced in New Zealand white rabbits. For the full-panel ELISA analysis, antibodies against each region of a mAb (three types tested) were coated on the 96-well plate, with each antibody coating one column (eight wells) in rows A through H. Test antibodies and stress-treated samples were diluted to a standard concentration of 5 µg/mL in a blocking solution of 1% bovine serum albumin (BSA) in phosphate-buffered saline T (PBS-T).

We used a biotin-labeled, rabbit-derived anti-human-immunoglobulin (IgG) developed by Array Bridge Inc. to detect captured antibody complexes and applied streptavidin–HRP to detect complexes formed by the antibody-capturing antibody. TMB (3,3',5,5'-tetramethylbenzidine) served as a substrate for the HRP enzyme activity assay. After a 15-minute period to allow color formation from HRP enzymatic activity, we added an equal volume of 1 M sulfuric acid to stop the reaction. A SpectraMax Plus spectrophotometer (from Molecular Devices) was applied to measure the color change at 450 nm.

Our sandwich ELISA's signal strength depended on the relative epitope exposure of each test-mAb region. If more mAb epitopes were recognized by the peptide-derived antibody, then stronger signals were produced; fewer recognized epitopes resulted in weaker signals.

Human Whole-Blood Cytokine-Release Testing: We quantified cytokines generated in whole human blood (from six donors) with three antibodies under different stress conditions. The antibodies tested were human-serum–derived IgG (HlgG), trastuzumab, and rituximab. HOS analysis was performed on the same set of samples.

Testing subjected samples to one of six chemical- or physical-stress conditions:

- no treatment
- overnight treatment with 8 M urea (to induce unfolded antibodies)
- overnight heat treatment at 55 °C
- overnight oxidation using H₂O₂ (desalting performed before analysis)
- overnight exposure to high pH (9.5, desalting performed before analysis)
- overnight exposure to low pH (3.0, desalting performed before analysis).

Blood samples of 20 mL were drawn from each donor into heparin collection tubes, ultimately for testing in a 96-well microplate format. Samples were incubated with one of the three testing antibodies at 37 °C with 5% CO₂. Three antibody levels were assessed to mimic levels that are present in human blood during patient treatment: 100, 10, and 1 µg/mL. On each plate, up to 40 samples were loaded in duplicate (totaling 80 wells), plus positive and negative controls. Samples underwent overnight incubation under one of the six stress conditions. Then, we assayed those samples for cytokine expression.

Controls: PBS served as a negative control to determine the assay

Figure 3: Whole panel of protein conformational array (PCA) enzyme-linked immunosorbent assay (ELISA) analysis of rituximab higher-order structure (HOS) under different stress conditions; polyclonal antibodies 1–12 cover the variable regions of the light and heavy chains, and polyclonal antibodies 13–34 cover the constant regions. Urea-treated samples reached the maximum detectable level of signal, so that condition is not shown in the graph. (See the Materials and Methods section.)

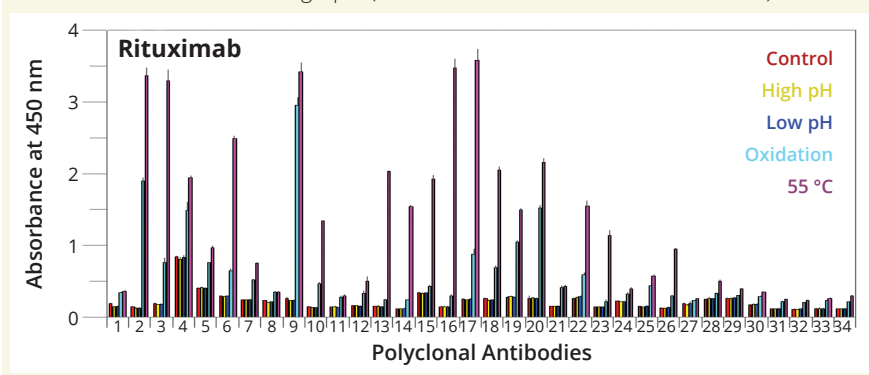
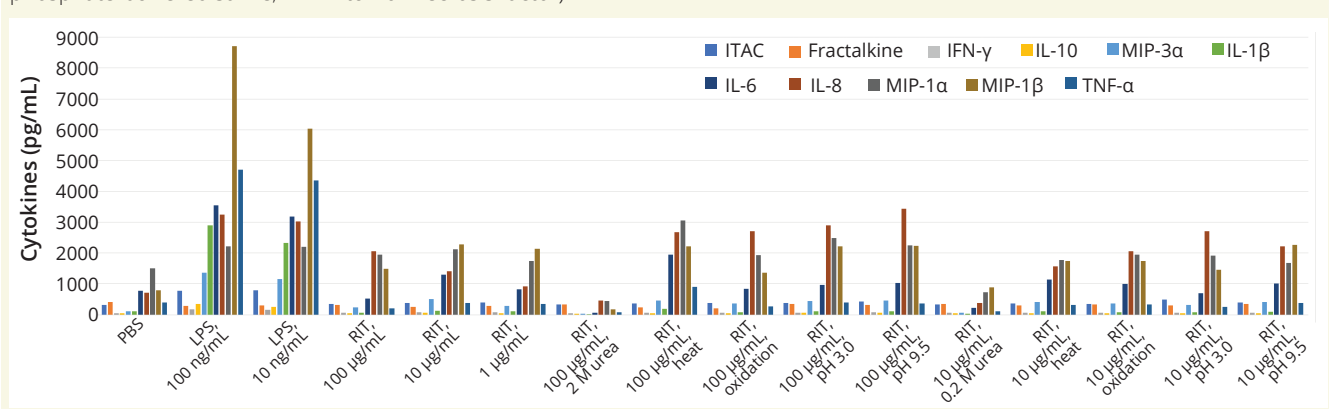


Figure 4: HSTC384-28K kit analysis of cytokine release from human whole blood after treatment with rituximab (RIT); shown here are data from 11 cytokines (from 21 total) with relatively high responses to RIT treatment. (IFN = interferon, IL = interleukin, ITAC = interferon-inducible T-cell alpha chemoattractant, LPS = lipopolysaccharide, MIP = macrophage inflammatory protein, PBS = phosphate-buffered saline, TNF = tumor necrosis factor)



background and baseline cytokine levels. Lipopolysaccharide (LPS) stimulation was used as a positive control for measuring cytokine release. Urea (screened at three concentrations) was tested as the vehicle control for urea-induced unfolded antibody. Untreated trastuzumab (a marketed mAb with low immunogenicity levels reported during clinical testing) served as a low-immunogenicity mAb control.

Detailed Protocol: Necessary reagents for this protocol include heparin tubes (e.g., 68099-104, VWR International), LPS (L2630, MilliporeSigma), PBS without Ca_2^+ and Mg_2^+ (14190-136, Invitrogen, Thermo Fisher), HSTC384-28K cytokine-analysis kits (MilliporeSigma), and PCA kits for trastuzumab (HercBridge), rituximab (RituBridge), and HlgG (InnoBridge) from Array Bridge.

First, donors are identified for blood collection. Donors must be healthy and

medication free, especially from antiinflammatory drugs for at least one week. From each donor, 20 mL of blood are collected into tubes with heparin. If blood from one donor is collected into several tubes, then material should be pooled before setting up incubation. Care should be taken to minimize the time between blood collection and incubation setup (<2 hours).

Whole-blood samples of 180 μL are dispensed into each well of a round-bottom 96-well plate. Outline wells should be avoided to prevent “edge effects.” Then, 20 μL of 10 $\times$ concentrated stock solution is added to achieve a final volume of 200 μL per well and final concentrations of

- LPS: 1000 $\mu\text{g/mL}$
- testing antibody: 100 $\mu\text{g/mL}$
- testing antibody: 10 $\mu\text{g/mL}$
- testing antibody: 1 $\mu\text{g/mL}$.

Duplicate wells are set up for each overnight-incubation condition.

Samples are incubated in the 96-well microplates at 37 °C with 5% CO_2 . Then, centrifuge microplates at 1800 rpm for 10 minutes to recover plasma. Carefully transfer plasma from each well to a cytokine-testing 96-well plate.

After performing overnight incubation, analyze plasma samples with a human-cytokine HSTC384-28K kit. The associated Luminex assay (MilliporeSigma) should be performed the same day that incubation is completed. Freeze–thaw of plasma samples is not recommended.

RESULTS

Previous studies have shown that a protein drug’s immunogenicity relates to its amino-acid sequence, aggregation level, and PTMs as well as other factors (12, 15). However, researchers have yet to investigate a close correlation between a biologic’s structural features and immunogenicity. In the realm of

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small-molecule pharmaceuticals, study of structure–activity relations (SARs) is a driving force for the development of successful products. Similarly, study of biologics’ structure–immunogenicity relations (SIRs) could reduce safety risks significantly during development and increase the rate of successfully marketed products.

Previous studies using PCA technology have demonstrated that protein aggregates display significantly higher epitope exposure as compared with mAb monomers (24). Moreover, candidate mAbs that failed during clinical development showed higher levels of linear epitope exposure as compared with mAbs that are currently on the market (data not shown). In a sense, we selected trastuzumab and rituximab in our study to represent polar opposites of immunogenicity for mAbs on the market. Trastuzumab has shown low levels of immunogenicity in the clinic, whereas rituximab has demonstrated high levels (31, 32).

In our PCA study, trastuzumab presented a low level of linear epitope exposure in its native form and was relatively stable in its HOS when treated with different stress conditions (Figure 1). Only high-temperature treatment induced a mild increase in linear epitope exposure.

In the whole-blood cytokine-release assay, trastuzumab displayed close to baseline levels of cytokine induction, similar to that of PBS, which was used as negative control (Figure 2). That result correlated well with a recent study that found significantly fewer

MHC-II peptides for trastuzumab during APC presentation compared with other tested biologics (11). On the other hand, rituximab — even in its native form — displayed relatively higher linear epitope exposure, especially in its variable region (Figure 3, Ab1 to Ab12).

Upon stress treatment, rituximab exposed more linear epitopes than trastuzumab did under the same conditions. (In Figure 3, see the y-axis difference between trastuzumab and rituximab due to the significantly higher signal in the rituximab assay). That result suggests that rituximab is not as stable as trastuzumab in its HOS.

Previous studies have reported on the immunogenicity of rituximab (31, 32). Note that, in our study, rituximab did not demonstrate dose-dependent cytokine-release activity. In the three levels tested (100, 10, and 1 µg/mL), cytokine-release activity remained at

the same elevated level (Figure 4), suggesting that the factors eliciting an immune response already had reached saturation at 1 µg/mL. (Such a level is still 1000× higher than that of the LPS control: 1 ng/mL.) Urea treatment diminished the cytokine-induction activity from the rituximab-bearing samples, suggesting that the property of cytokine induction depends on certain structural features.

Human serum and HlgG have been used to enhance immunity for multiple indications. For instance, clinicians used such means to treat SARS-CoV-2 infection before vaccines had become widely available (32, 33). Because the constant regions of HlgG antibodies are highly conserved, our study evaluated the antibodies’ HOS stability and cytokine-release properties. Note that stress-treated HlgGs induced significant increases in linear epitope exposure,

Figure 5: Whole-panel protein conformational array (PCA) enzyme-linked immunosorbent assay (ELISA) analysis of human immunoglobulin G (HlgG) higher-order structure (HOS) under different stress conditions; polyclonal antibodies 1–12 cover the variable regions of the light and heavy chains, and polyclonal antibodies 13–34 cover the constant regions.

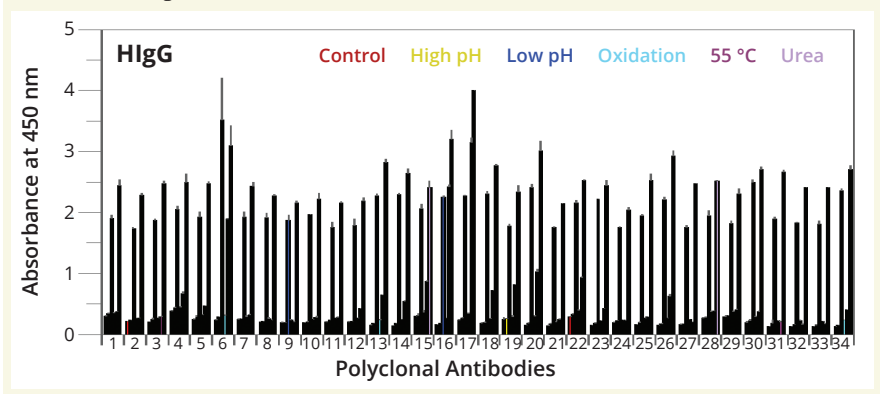


Figure 6: HSTC384-28K kit analysis of cytokine release from human whole blood after treatment with human immunoglobulin G (HlgG); shown here are data from 11 cytokines (from 21 total) with relatively high responses to HlgG treatment. (IFN = interferon, IL = interleukin, ITAC = interferon-inducible T-cell alpha chemoattractant, LPS = lipopolysaccharide, MIP = macrophage inflammatory protein, PBS = phosphate-buffered saline, TNF = tumor necrosis factor)

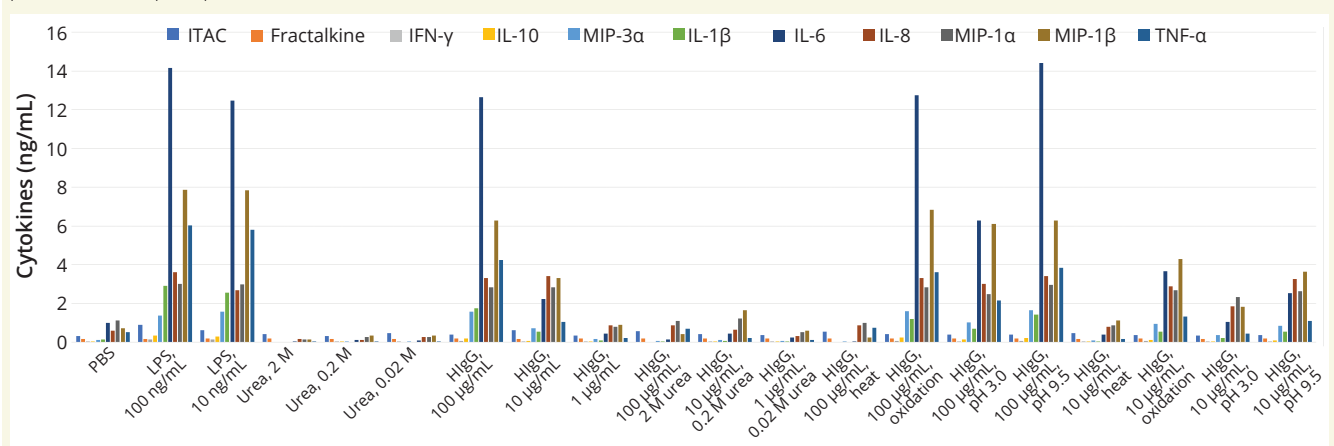


Figure 7: Pairwise alignment of amino-acid sequences for trastuzumab and rituximab light and heavy chains; sequences were obtained from the US National Center for Biotechnology Information (NCBI) protein database. Antibody constant regions are marked with red rectangles.



especially in the low-pH and urea conditions (Figure 5; see the y-axis scale differences for the trastuzumab and rituximab results).

In the whole-blood assay, HIgGs exhibited dose-dependent cytokine induction — a result observed with neither trastuzumab or rituximab. Urea treatment diminished cytokine induction, suggesting that some structural features are responsible for that property. HIgG samples also showed sensitivity to heat treatment. Unlike rituximab, heat-treated HIgG showed significantly reduced cytokine-induction activity at 10 and 100 µg/mL, (Figure 6). Note that we assume biologics doses of 10 mg/kg of patient weight, with an average patient weighing 60 kg and holding about 5 L of blood; thus, the initial blood level for an administered biologic is ~120 µg/mL.

Because trastuzumab and rituximab have significantly different HOSs and cytokine-induction profiles, we closely compared their amino-acid sequences. Figure 7 depicts the pairwise alignment of their constant-region amino-acid sequences. As we can tell from the matching sequences, the mAbs have identical constant regions in both the light and heavy chains.

In our PCA assay, the trastuzumab and rituximab constant regions showed significant differences between their native and posttreatment states. The suggestion, then, is that factors other than protein amino-acid sequence play important roles in protein HOS and

stability. Another interesting observation from our study relates to the diversity of cytokine-induction patterns across blood donors. The average readouts in Figures 2, 4, and 6 enable us to assess trends in cytokine induction. When looking at individual responses, we find a polymorphism in cytokine induction toward the same biologics (Figure 8). Thus, some of the important cytokine-induction signals from biologics can be averaged out. Results from individual donors indicate that cytokine responses toward the same biologics can differ by several folds. Figure 8 uses trastuzumab as an example, but samples with the other two testing antibodies demonstrated similar polymorphism in their cytokine induction (data not shown).

DISCUSSION

Protein immunogenicity is a major concern in biologics development. Severe immunogenicity can prevent a promising candidate from advancing through clinical development or even fail a marketed product. It has been reported that as many as 15% of antibody–drug conjugates (ADCs) have shown significant immunogenicity (34–38). Discontinuation during clinical development is extremely costly. Therefore, implementing a quality-control process for assessing a biologic's immunogenicity early in development would be of great value. In small-molecule drug development, SAR

evaluations are essential. Likewise, studies of SIR can provide valuable insights during biologics development.

For SIR assessment, two essential capabilities need to be in place. The first is technology that can analyze a biologic's structure at high resolution. Currently, two analytical approaches address that criterion. One is HDX-MS, which uses amine-group exchange in proteins to derive their HOS. HDX-MS has a sensitivity of ~20% as a quantitative method. The second available technology is PCA testing. It uses polyclonal antibodies raised toward peptide linear epitopes (4–12 amino acids are found to form linear epitopes) and, to a less extent, some HOS features formed from the peptides to investigate biologics surface-epitope exposure. That, in turn, derives the HOS status of a target molecule. In an ELISA format, PCA can quantify as low as 0.1% epitope change for a biologic, thus providing the sensitivity necessary to study SIR (26). The second integral capability for SIR assessment is the ability to measure an immune response systematically at a sufficiently sensitive level. Assays based on induction of 23 cytokines in human whole blood meet that need.

Over the years, both PCA and cytokine-release assays have been used separately in many studies, yielding interesting findings for biologics development. To our knowledge, the study reported herein represents the

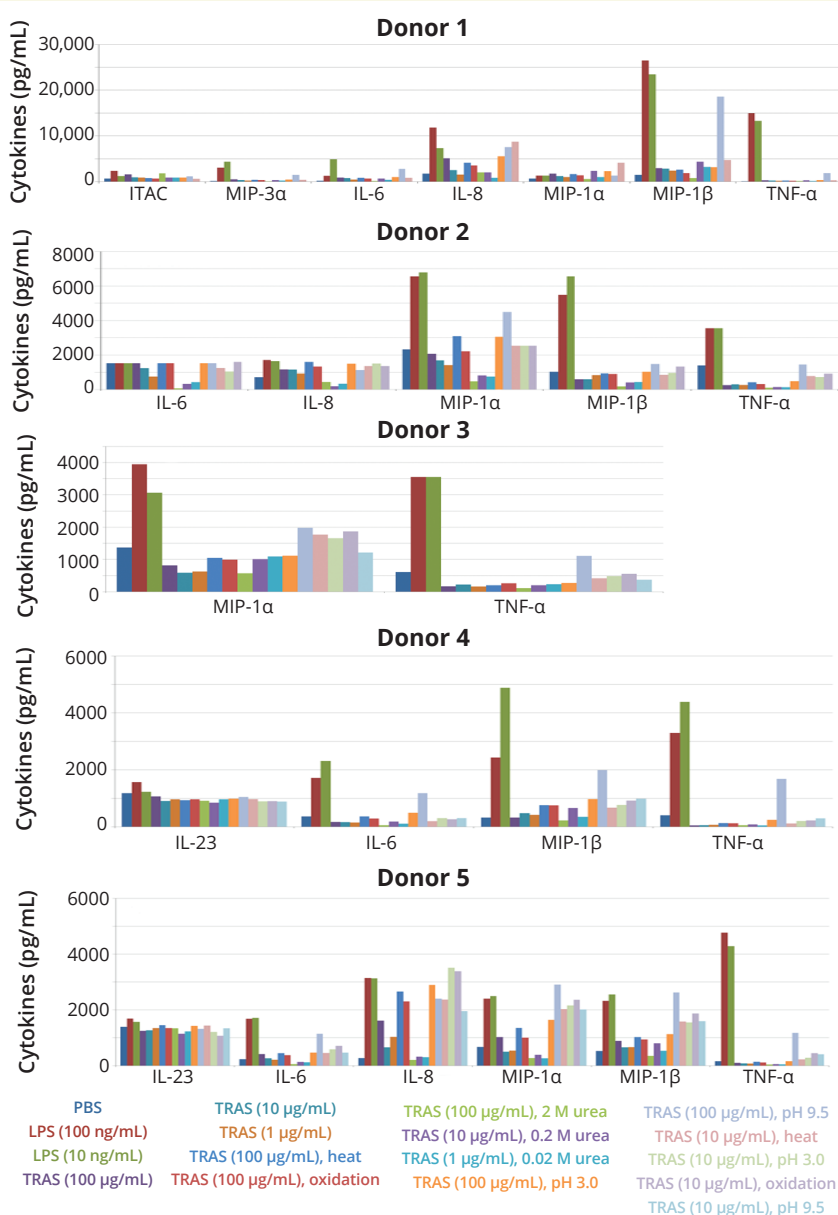
Given the pending wave of antibody–drug conjugate (ADC) development and previous findings showing high rates of immunogenicity among such products, studies of structure–immunogenicity relation may prove to be **INVALUABLE** in ADC development and biologics development more generally.

first effort to combine those assays. Our results reveal several interesting findings. First, a biologic's HOS is not solely determined by its primary amino-acid sequence. As can be seen in the HOSs of trastuzumab and rituximab, both mAbs share the same constant regions, with matching amino-acid sequences. Those sequences constitute about two-thirds of each protein (Figure 7). However, their HOS profiles and responses to multiple stress conditions are extremely different (Figures 1 and 3). It will be interesting to study the cause of such differences further. Possible influences include the proteins' variable regions, the locations of gene insertion into the Chinese hamster ovary (CHO)–cell genome, and bioprocessing and formulation conditions.

Also of interest is that immunogenicity manifested before we applied any stress conditions. Rituximab exhibited no dose-dependent responses in cytokine release. Cytokine responses were high across the three antibody levels of tested (1, 10, and 100 µg/mL), indicating the whole-blood assay was extremely sensitive to rituximab's immunogenic properties (Figures 2 and 4).

The third observation to emerge from our study relates to the polymorphism of immune responses from individuals.

Figure 8: Cytokine-release polymorphism from HSTC384-28K kit analysis; only the most significant cytokine-response differences are shown here. Please note the y-axis scale differences across the five donors.



Blood samples from different donors generated a broad array of immune responses toward the same sample (Figure 8). Those results suggest that group, average, and individual cytokine-release results need to be analyzed closely during immunogenicity evaluation using a whole-blood system to recognize important immune-response signals.

In recent years, scientists have made great progress in understanding the antigen-presenting process using MS-based technologies (11–13). Our study focused on the initial stage after biologics administration by measuring

cytokine release from a whole-blood system. Taken together, approaches such as MS-based epitope identification, HDX-MS, and PCA for HOS characterization will provide the field with a better understanding of biologics immunogenicity and thus improve the success rate of biologics development. Given the pending wave of ADC development and previous findings showing high rates of immunogenicity among such products, studies of SIR may prove to be invaluable in ADC development and biologics development more generally.

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