U.S. FOOD & DRUG ADMINISTRATION

Biosimilar User Fee Act (BsUFA) III Regulatory Science Pilot Program

ANNUAL REPORT

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Contents

Report Overview[1]

Table 1: High-level overview of the project objective, aim(s) progress, outcomes, and timelines for communication and regulatory impact.

Project Title:	**Develop acceptance parameters and standards for the Innate Immune Response Modulating Impurities (IIRMI) assays in the Biosimilar space**
Investigator:	Verthelyi
Organization:	OPQR-Division IV
Grant No. (if applicable)	N/A
Project Objective:	Develop in vitro assays that can be used to reduce/replace clinical trials to assess immunogenicity risk of biosimilars

[1] This section will be used by program for broader research portfolio and regulatory impact analysis by the BsUFA III steering committee.

Specific Aim(s)	Progress	Outcomes	Communication Timeline
Aim 1: Adapt the IIRMI assay used to assess the risk of generic synthetic peptides to biosimilars	95% complete	• We demonstrated that cell-based assays can be deployed to assess IIRMI in proteins manufactured using different platforms (bacterial, mammalian or yeast). • We demonstrated that cell-based assays that involve both cell lines and primary human cells can be deployed to assess IIRMI in proteins of different sizes and structures. • We established that pegylation does not mask IIRMI detection. A technical report was filed with OPQ and a manuscript is in preparation to describe the finding. • We developed a novel flow cytometry assay to characterize the impact of trace levels of IIRMI on specific cell populations that may affect immunogenicity (dendritic cells and B cells). We used it to compare the profile of a reference product and its biosimilar. A manuscript paper describing the use of spectral flow cytometry to assess the impact of IIRMI on these specific cell populations is currently under review at AAPSJ.	• A paper describing the application of the IIRMI assay to insulin glargine produced in *E.coli* and yeast platforms was published in doi:https://doi.org/10.1208/s12248-024-00983-x • A manuscript paper describing the use of spectral flow cytometry to assess the impact of IIRMI on these specific cell populations is currently (manuscript has been submitted and is under review). • A technical OPQR report was filed describing the impact pf pegylation on IIRMI assay performance. A manuscript describing the studies is in preparation.
Aim 2: Develop suitability standards for the IIRMI assay and coordinate manufacture, testing and distribution to interested sponsors for testing.	70% complete	• Contract established with NIST to develop standards (Contract IAA: #A2309-075-013-052172). • Over 30 candidate reference suitability standards from different suppliers were evaluated and 5 were selected by FDA and characterized by NIST. • A group of collaborators from industry was selected to test the standards in their assays. Currently they are reviewing the legal terms of the CRADA agreement produced by NIST. • Standards have been aliquoted and will be shipped by NIST to Industry collaborators to validate them. • Testing by collaborators is expected to take place in August-October 2025.	• Analysis and manuscript preparation will take place in November -December 2025. • A manuscript describing the standards and their testing by Industry partners is planned for early of 2026.

Specific Aim(s)	Progress	Outcomes	Communication Timeline
Aim 3: Characterize the IIRMI response profile of 8-10 different reference biologics	20% complete	• Studies assessing the impact of the product on reporter cell lines cells and primary cells and minimal required dilution (MRD) to detect IIRMI are complete. • The assessment of the product's profile using mRNA and cytokine secretion were scheduled to take place in 2025, once the reference standards were available.	• Manuscript detailing the mRNA and cytokine profiles for refence products and the similarities/differences seen with their corresponding biosimilars was expected by end of 2026

Progress Summary

Project Objective:

Biologics have the inherent potential to be immunogenic and to trigger immune responses directed against themselves (or endogenous counterparts), with consequences ranging from no effect to potentially diminishing treatment effectiveness and/or impacting patient safety. These may involve innate, cellular, and humoral immune reactions including acute or delayed hypersensitivity, infusion- related reactions (IRRs), injection site reactions (ISRs) and/or development of anti-drug antibodies (ADAs). To date, the immunogenicity risk of biosimilars has been addressed primarily by using parallel arm clinical trials, however obtaining clinically informative data often requires large, lengthy, and expensive trials that defy the intent of the abbreviated regulatory path. Developing an alternative strategy that mitigates the root causes of residual uncertainty, thus reducing the risk that a given biosimilar will pose a higher risk of unwanted immune or reactogenic responses compared to the reference product would facilitate the licensing of biosimilars. A key risk factor that can be mitigated, is potential differences in innate immune response modulating impurities (IIRMIs) including host cell proteins and other cell remnants resulting from differences in manufacturing process used to produce the biosimilar product (Panikulam et al., 2024; Panikulam, Morgan, Gutknecht, Karle, et al., 2025; Panikulam, Morgan, Gutknecht, Villiger, et al., 2025; Rane et al., 2019; Zenatti et al., 2018). Previous studies have shown that IIRMIs can increase local inflammation and act as adjuvants increasing the immunogenicity risk of the drug product, particularly in the presence of product aggregates(Haile et al., 2017; Polumuri et al., 2018; Ratanji et al., 2017). Importantly, while routine bioanalytical testing can be used to characterize the similarity of the active ingredient, IIRMIs activate immune receptors at very low concentrations that frequently cannot be detected using these tests. Therefore, our lab and others have established different in vitro methods to detect and characterize the IIRMI in drug products (Haile et al., 2015; Holley et al., 2021; Jarvi & Balu-Iyer, 2023; Siegel et al., 2024). While these have been useful, the diversity of assays, testing modalities, and biomarkers in the absence of common refence standards have precluded comparisons between products and hindered interpretation of the results. The current studies focus on the development and characterization of standards that can be used by any sponsor to benchmark

their assays to assess (IIRMI). In addition, we examine whether certain product quality attributes such as molecular weight or post-translational modifications modify the sensitivity of the assays. Together, this will inform the expectations of the Agency and provide a roadmap for sponsors to integrate these assays into their immunogenicity risk assessments. Moreover, the use of common standards will allow for metanalysis of the innate immune activation profile across reference products and their licensed biosimilars to establish product-specific profiles of innate immune activation that are not associated in clinical trials or commercial use with increased risk of product immunogenicity. The aggregate data will be helpful in establishing a safe margin of in vitro innate immune activation that can aid in assessing the risk for new biosimilar products.

Aim 1: Adapt the IIRMI assay for biosimilars:

- Demonstrate the IIRMI assay can detect impurities in biologics manufactured in bacteria, yeast, and CHO cells. **Status:** Completed and published (AAPS J 2024).
- Established the impact of molecular size and complexity of biologics on the IIRMI assay in primary cells and cell lines. **Status:** Completed.
- Demonstrate the impact of peglyation and PEG in the formulation of biologics on the ability to detect IIRMI in primary cells and cell lines. **Status:** Completed; manuscript in preparation.
- Developed a novel flow cytometry assay to characterize the impact of trace levels of IIRMI on specific cell populations that may affect immunogenicity using cell activation markers instead of gene induction or cytokine secretion. **Status:** Completed; manuscript in preparation.
- Assess impact of impurities such as API oxidation or aggregation on assay performance and potential for IIRMI masking. **Status:** Ongoing.
- Assess whether IIRMI assay detects immunomodulatory host cell proteins derived from CHO cells, bacteria, and yeast. **Status:** Ongoing this is a lingering concern for biosimilars.

Aim 2: Develop suitability standards for the IIRMI assay and coordinate manufacture, testing and distribution to interested sponsors for testing.

- Establish contract established with NIST to develop standards (Contract IAA:#A2309-075-013-052172). **Status:** Completed.
- Screen and select suitability standard to be used in PBMC IIRMI assays. Over 30 candidate reference suitability standards from multiple suppliers were evaluated and 5 were selected by FDA and characterized for impurities by NIST. **Status:** Completed.
- Convene a group of collaborators from industry to test the selected standards in their assays. Currently they are reviewing the legal terms of the CRADA agreement produced by NIST. **Status:** Team is formed, legal agreements are in progress.

- Aliquot standards and distribute to collaborators for testing. Distribution of the standards will be done by NIST. **Status:** Ongoing; pending receipt of signed CRADA agreements between NIST and collaborators.
- Analyze data from study conducted by the team of sponsors and CROs to determine if proposed controls are appropriate for use as assay controls with different assay formats. **Status:** Not started. Expected timeframe: Fall 2025. Manuscript Expected timeframe: Spring 2026.

Aim 3: Characterize the IIRMI response profile of 8-10 different reference biologics to understand what level of innate immune activation might be linked to increased immunogenicity risk.

- Determine the minimal required dilution (MRD) for the selection of reference biologics in both primary and cell lines. **Status:** Completed.
- The assessment of the 8-10 product's profile using mRNA and cytokine secretion were scheduled to take place in 2025, once the reference standards were available, but the ORISE fellow's contract was not renewed. **Status:** Halted.

Research Outcomes

Multiple studies indicate that trace levels of impurities including HCP can foster immunogenicity particularly in the presence of protein aggregates so control of IIRMI can mitigate the uncertainty regarding immunogenicity risk (Panikulam, Morgan, Gutknecht, Villiger, et al., 2025; Polumuri et al., 2018; Rane et al., 2019). Our studies show that the IIRMI assay can be used to assess the presence of immunomodulatory impurities in drug products of different molecular weights and complexity, ranging from simple peptides to monoclonal antibodies. Testing included over 20 proteins of different size, charge, and structure, including monoclonal antibodies, glycosylated and pegylated products as well as products with direct immunomodulatory activity. The ability of different in vitro IIRMI assays using monocytic cell lines expressing reporters for NFKB activation (THP-1 and RAW) as well changes in mRNA or protein expression using fresh primary peripheral blood monocytes (PBMC) to detect low levels of impurities capable of triggering pattern recognition receptors despite differing size, complexity, and manufacturing platform (mammalian, bacterial or yeast cells) was confirmed. Proof of concept studies using insulin glargine (produced in *E. coli*) and its interchangeable biosimilar insulin (produced in *P. pastoris*) were recently published (Her et al., 2024). Studies assessing different types of impurities showed that mRNA and protein production yielded consistent detection of most microbial contaminants, however detection of immunomodulatory nucleic acid impurities was negligible with on monocytic cell lines since they often lack the appropriate receptors and not consistent with PBMC. We reasoned that the inconsistent results were tied to the low frequency of conventional dendritic cells (cDC) and plasmacytoid DC (pDC) in PBMC. Since these are key cells in mounting an immune response we developed a novel highly sensitive spectral flow cytometry-based testing system to detect the

immunomodulatory activity of different nucleic acid structures on cDC and pDC. Using this assay, we demonstrate the absence of immunomodulatory differences affecting specific cell types in biosimilar mAb products (manuscript undergoing internal review).

To develop standards that could be used across a spectrum of different assays measuring IIRMIs, we set up an Inter-agency Agreement (IAA) with NIST. Our FDA lab and NIST tested over 30 commercially available PRR agonists in an iterative scheme to select suitability controls that demonstrated PRR specificity, low levels of impurities, and consistent assay performance. A final set of suitability standards were selected, acquired, and characterized by NIST. The standards have been aliquoted and are ready for distribution to external collaborators in Industry for validation. A testing plan was structured with the collaborating team where each sponsor or contracting research facility will test the standards using own assays, which should confirm that the standards can be used across testing platforms. FDA and NIST prepared legal agreements for a team of external collaborators that have expertise in these assays and volunteer to test them. Testing will take place in the Fall of 2025 and a manuscript describing the standards is expected in the spring of 2026.

Regulatory Impact

Biosimilar products are used for the same indication, in the same target patient population, and have a highly similar active product ingredient as the reference product (RP), however the manufacturing process is expected to be different from that of the reference product and consequently there is no expectation that the product and process related impurities will be the same. Thus, a key residual uncertainty regarding the immunogenicity risk of biosimilars is the presence of impurities such as remnants of the host cell or microbial bioburden. Since host cell proteins and other remnants could act as adjuvants by activating local antigen presenting cells, establishing that the biosimilars don't contain excessive immunomodulatory impurities can aid in mitigating the immunogenicity risk and reduce the need for clinical trials and facilitate the availability of biosimilars. Importantly, the tests usually used to assess host cell proteins and other remnants or contaminants in a biologic product such as ELISA or Mass spectrometry, have limited coverage (usually limited to impurities higher than 0.1% of product) and are not suitable for assessing whether they have immunomodulatory activity. To address this gap, several different assays have been adopted to assess by sponsors products for immunomodulatory impurities. While these assays allow for direct comparisons of biomarkers of immune activation, the absence of standards makes it difficult to understand whether the assays have sufficient sensitivity and hinders comparisons across studies that would allow sponsors and reviewers to gain understanding, so thus far they have not been used to support regulatory submissions. To enable the development, validation and implementation these studies are: 1) exploring the different product -related parameters that could impact on assay performance, 2) establishing reference standards that can be used by sponsors to benchmark their assays, which will improve comparisons across products, and 3) provide profile of innate immune activation induced by a variety of reference products as well as their biosimilars. Together, this information will facilitate the development and validation of sensitive IIRMI assays that are host and manufacturing process independent. This will aid sponsors to generate interpretable data as well as the assessment by

reviewers, with the expectation that the resulting data will complement, reduce, or replace clinical data depending on the inherent immunogenicity risk of the product.

The characterization of the assay, the standards, and as the innate immune profile developed for frequently used RP can inform the expectations of the Agency, inform FDA guidances, and provide sponsors with a roadmap to adopt these assays to inform their immunogenicity risk assessments. Successful implementation of the IIRMI assay for biosimilars will reduce costs, improve public health and is in-line with the Biologics Price Competition and Innovation Act (BPCIA).

Communication and Dissemination

Table 2: Summary of communications and dissemination of information, results, outcomes, etc. related to this study.

Title	Type of Communication (e.g., poster, manuscript, presentation)	Source	Link (if available)
Cell-Based Assays to Detect Innate Immune Response Modulating Impurities: Application to Biosimilar Insulin	Journal article	The AAPS Journal AAPS J. 2024 Dec 20;27(1):20. doi: 10.1208/s12248-024-00983-x. PMID: 39707070.	Cell-Based Assays to Detect Innate Immune Response Modulating Impurities: Application to Biosimilar Insulin
Proceedings of the 14th European immunogenicity platform open symposium on immunogenicity of biopharmaceuticals	Journal article	MAbs. 2024 Jan-Dec;16(1):2324801. doi: 10.1080/19420862.2024.2324801.	Proceedings of the 14th European immunogenicity platform open symposium on immunogenicity of biopharmaceuticals
Proceedings of the 15th European immunogenicity platform open symposium on immunogenicity of biopharmaceuticals	Journal article	MAbs. 2025 Dec;17(1):2487604. doi: 10.1080/19420862.2025.2487604.	Proceedings of the 15th European immunogenicity platform open symposium on immunogenicity of biopharmaceuticals
New approaches to assess immunogenicity risk: Regulatory considerations	Invited presentation	EIP Lisbon, April, 2024.	New approaches to assess immunogenicity risk: Regulatory considerations

Title	Type of Communication (e.g., poster, manuscript, presentation)	Source	Link (if available)
Assays supporting immunogenicity risk assessments: The road ahead	Invited presentation	WRIB, San Antonio April, 2024.	N/A
Fit for Purpose assays to assess innate immune response modulating impurities	Invited presentation	WRIB, San Antonio April, 2024.	N/A
Innate Immune Response Modulating Impurities Testing as a component of immunogenicity risk assessments	Invited presentation	Boulder Peptide Foundation. May, 2024 (virtual)	Innate Immune Response Modulating Impurities Testing as a component of immunogenicity risk assessments
Testing process and product related impurities to inform immunogenicity risk	Invited presentation	American College of Toxicology Annual meeting 2024	Testing process and product related impurities to inform immunogenicity risk
Innate immune response modulating impurities testing for immunogenicity risk assessments	Invited presentation	Immunogenicity and Bioassay Summit, Washington DC, 2024	Innate immune response modulating impurities testing for immunogenicity risk assessments
Immunogenicity Risk Evaluations of follow on Peptide Products	Internal oral communication	OPQR All hands	N/A
OPQR technical report in the impact of pegylation on IIRMI assay performance	Internal written communication	N/A	N/A
Innate Immune Response Modulating Impurities Testing as a component of immunogenicity risk assessments	Internal oral communication	OPQR Scientific Forum March, 2025	N/A
Spectral flow cytometry captures single cell responses to trace levels of nucleic acid innate immune response modulating impurities in therapeutics.	Poster Presentation	FDA Science Fair 2025	N/A

Title	Type of Communication (e.g., poster, manuscript, presentation)	Source	Link (if available)
Identification and Characterization of Suitability Controls for Innate Immune Activation Assays	Poster Presentation	FDA Science Fair 2025	N/A
Evaluating the impact of free or conjugated PEG on the performance of the innate immune response modulating impurities (IIRMI) assay	Poster Presentation	FDA Science Forum 2025	N/A
Innate Immune Response Modulating Impurities Testing for Immunogenicity Risk Assessments.	Featured talk. Immunogenicity Bioassay Summit.	Washington DC, October 2024	N/A
Assay review tool and training	Internal communication for reviewers	2024	N/A

Scientific and Technical Challenges

No scientific or technical challenges were reported for this past year.

Next Steps

1. Perform the validation of the standards chosen: Five standards have been characterized by NIST and FDA and will be distributed to 6 collaborating companies. The collaborators have already received the CRADA agreement from NIST and FDA. Each group will test the controls in their assays at 3 different concentrations as well as 10-20 blinded testing samples spiked with different unidentified impurities using at blood cells from at least 10 different donors. The results of the tests will be shared with FDA and the analysis of the data will be performed here. A manuscript describing the performance of the standards when using the different methods will be drafted and published.

2. Complete Aim3: Characterize the IIRMI response profile of reference biologics and biosimilars to understand what level of innate immune activation might be linked to increased immunogenicity risk. Using the reference suitability standards developed for Aim2, we will perform a survey of the innate immune profile of 8-10 different reference product mAbs and their biosimilars to aid in understanding of the levels of innate immune activation in vitro that are routinely induced by products of known immunogenicity risk. We

will then correlate the levels of innate immune activation induced with the immunogenicity levels from the parallel arm clinical studies and use this information to establish a level of innate immune activation observed in vitro that is not associated with increased immunogenicity risk. The data from this study will support the establishment of "relative safety margins" that will aid in risk assessment of future products. Gaining this understanding is important because biologics often will have an immunomodulatory effect in vitro, so it will be critical to establish what are meaningful differences in in vitro signals. Since the use of the validated standards allows for comparison across testing platforms and can thus be extended to the data from sponsors provided the same standards are used. (*Example: The innate immune profile elicited by Adalimumab and at least 5 biosimilars that did not lead to an increase in product immunogenicity in clinical studies should yield a "level of activation" for new biosimilar of adalimumab that is not expected to be associated with increased risk. Performing this type of assessment on multiple sets of products should yield an "acceptable margin" of innate immune activation for a broad range of mAbs*). The original intent of Aim 3 was to create a profile of a broad array of biologics to aid in the assessment of biosimilars. Given the clinical experience attained with the parallel arm studies to date, our current plan is to narrow the focus of this Aim to monoclonal antibodies (mAbs), which constitute the majority of the biosimilar products approved to date.

3. Complete Manuscripts:

    a. A manuscript describing the suitability standards selected and their performance in different assays used to assess IIRMIs.

    b. A description of a spectral flow-based assay that allows for a highly granular assessment of the impact of impurities on specific cell types. This new assay increases the sensitivity of the assay by 10-100-fold and provides an assessment of the cell types that could be affected by the impurities in the product, allowing for a better risk assessment.

    c. A manuscript describing the use of the IIRMI assay in pegylated products. Pegylation is a common modification used to reduce immunogenicity risk and extend bioactivity, but it could alter the activity of the cells used to sense the presence of IIRMI. The study assessing the impact of pegylation on comparative assessment of IIRMIs in biosimilar products is close to completion (85%).

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