U.S. FOOD & DRUG ADMINISTRATION

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

ANNUAL REPORT

CONTENTS

Check if this report is Progress or Final Report:

☒ **Progress report** ☐ **Final report**

1. REPORT OVERVIEW[1]

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

Specific Aim(s)	Progress	Outcomes	Communication Timeline
Aim 1: Adapt the IIRMI assay used to assess the risk of generic synthetic peptides to biosimilars	80% complete	- The IIRMI assays have been tested on multiple biologics and perform well with products manufactured on CHO cells, *E.coli* or yeast. In addition, we confirmed that pegylation does not mask IIRMI and have successfully tested products of various sizes. - Impact of protein parameters on assay performance (glycosylation, HCP levels, oxidation, aggregation, etc.) is ongoing.	- Interim Progress Report submitted July 2024 - 1st paper describing the studies in insulin glargine produced in *E.coli* and yeast platforms is in clearance. - A manuscript detailing the impact of various parameters on assay performance is planned for March 2025. - Final Report on Aim 1 will be ready July 2025

[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 2: Develop suitability standards for the IIRMI assay and coordinate manufacture, testing and distribution to interested sponsors for testing.	40% complete	- Contract established with NIST to develop standards (Contract IAA:#A2309-075-013-052172). - Candidate reference suitability standards from different suppliers were evaluated by FDA and provided to NIST. NIST has purchased some of the standards and chemical characterization is ongoing. - The request for testing & validation of the standards by Industry will be sent to sponsors by December 2024. Preliminary interest has been high.	- The development of standards was announced at European Immunogenicity Platform and Workshops on Recent Issues in Bioanalysis in 2024. - Interim Progress Report submitted July 2024 - A Final Progress Report on the standards is planned for December 2025 - A manuscript describing the standards and their testing by Industry partners is planned for end of 2026.

2. PROGRESS SUMMARY

Please note: The appendix and its references were redacted at the request of the awardee.

Overview

Biologics are produced in living cells and despite purification may have product and process related impurities that can impact on their risk of inducing an unwanted immune response. This is because even trace levels of impurities such as host cell remnants, product aggregates, and trace level contaminants, can induce parenchymal cells as well as immune cells embedded in the tissue to secrete cytokines and chemokines that recruit immune cells to the site of injection, enhance the ability of antigen presenting cells to process and present the product in the context of MHC, and foster the migration of loaded antigen presenting cells to draining lymph nodes where they can induce an adaptive immune response. While the sequence and mode of action of biosimilar products is highly similar to that of the innovator, the cell lines and manufacturing process used are different and can result in different product and process related impurities and uncertainty regarding their immunogenicity risk. This immunogenicity risk of biosimilars can be addressed using parallel arm clinical trials, however acquiring clinically informative data often requires lengthy and expensive trials that defy the intent of the abbreviated regulatory path. An approach that may reduce or replace the need for extensive clinical trials to assessing immunogenicity risk is to characterize and mitigate the factors that impact product immunogenicity risk, such as establishing that the impurities in the product do not act as adjuvants by inducing the activation of innate immune cells.

Our lab and others have established methods to assess innate immune response modulating impurities (IIRMI) (Haile et al, 2015; Holley et al, 2021). These assays use biomarkers of innate immune activation as a means of detecting and characterizing the IIRMI in drug products (Haile et al, 2015; Haile et al, 2017). Currently the assays to detect IIRMI use a variety of testing platforms and conditions including cell lines, whole blood, or purified primary cells such as dendritic cells. The biomarkers used to monitor the degree of inante immune activation also vary

and can include mRNA expression profiles, NFKB activation, cytokine/chemokine secretion or expression of activation markers on antigen presenting cells. Lastly, it is known that the product and its formulation can impact on cell viability and assay performance (Holley et al, 2021; Thacker et al, 2022). 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. Improved understanding of the product-related parameters that impact on assay performance and development of common reference standards that sponsors can use to benchmark their assays will help establish IIRMI assays that inform on the potential for the impurities in biosimilars to elicit an innate immune and/or inflammatory response reducing uncertainty regarding immunogenicity risk. Establishing and validating common suitability control standards and setting up identification thresholds for the assay using these standards will make it easy for the Sponsors to establish sensitivity of their assay across different assay platforms.

Therefore, to enable the use of IIRMI assays in the biosimilar regulatory space we will (1) identify product quality attributes such as molecular weight or post-translational modifications that could modify the sensitivity of the assays, and (2) develop reference standards for sponsors to benchmark the different assays as this will aid in assay development and validation and help reviewers understand the sensitivity of the assays used. In addition, in future studies using these standards we will (a) characterize the innate immune response induced by a diverse array of reference products providing a reference point for sponsors who undertake this type of characterization. And (b), perform a metanalysis of the profiles of reference products and their licensed biosimilars to establish product-specific profiles of innate immune activation that are not associated with increased risk of product immunogenicity, as this will be helpful in evaluating the immunogenicity risk of emerging biosimilar products. **In summary, these studies focus on the characterization of critical elements of the IIRMI assay to enable their evaluation in the regulatory space, the development of suitability standards so that sponsors can benchmark their assays, and confirmation that the assay can be used to assess differences in IIRMI to inform the immunogenicity risk of biosimilars. The results will provide a roadmap for sponsors to adopt these assays to inform their immunogenicity risk assessments and inform the expectations of the Agency.**

Project Objective: Develop testing acceptance parameters and suitability standards to allow for the implementation of the IIRMI assay in the biosimilar space.

Specific Aim 1: Adapt the IIRMI assay for biosimilars.

- Determine whether the IIRMI assay can be used to screen HCP from bacteria, yeast, or CHO cells for immunomodulatory activity.
- Establish the impact of higher molecular weight proteins and complexity of biologics (vs smaller peptides) on the IIRMI assay. Suitability for different assay formats (reporter cell lines, PBMC, whole blood cells), as well as most frequently used readouts (mRNA, protein secretion, and flow cytometry), will be confirmed.
- Assess impact of impurities such as API oxidation or aggregation on assay performance and potential for IIRMI masking.

Significant progress was made on this aim. The ability of IIRMI assays using monocytic cell lines expressing reporters for NFKB activation (THP-1 and RAW) as well fresh primary peripheral blood monocytes (PPBMC) 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. Comparisons between the type of readouts (mRNA levels, protein secretion, cell surface markers) was initiated. Initial studies showed that the intraassay variability for flow cytometry was unacceptable for an assay aimed at detecting small changes due to impurities, so a spectral flow system was developed that significantly reduces intraassay variability, provides cell-specific activation data and identifies changes in key cell populations. Assessment of product-related parameters that could impact on assay sensitivity (pegylation, aggregation, glycosylation, etc.) is ongoing with testing expected to be finalized by December 2024. Expected outcome: An assessment of the impact of assay and product attributes on IIRMI assay sensitivity that will facilitate assay development by sponsors and assay review by assessors.

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

Candidate suitability standards from different suppliers will be tested by NIST (molecular characterization and stability) and FDA labs (immunomodulatory activity) (Year 1). Selected standards will be purchased by NIST, characterized as above by both labs, aliquoted, placed on stability, and distributed for testing by collaborating

sponsors (year 2). Initial discussions with HESI and APPS members regarding the testing by their Industry members indicates that they are very interested.

Significant progress has been made on Aim2: An inter agency agreement was put in place where our lab would identify types and sources of reference standards that performed well in the IIRMI assay and NIST would characterize, aliquot and distribute the reference standards to sponsors. Our lab tested over 30 PRR agonists that could serve as suitability controls for endotoxin content, PRR specificity and consistency of assay performance. We identified 4 sets of candidates that were characterized by NIST. Chemical characterization of the standards has been initiated. Some of the potential standards were found to have impurities leading to a reassessment of candidates for intracellular PRR agonists and our group is testing a new set to select alternative candidates. Expected outcome: A final well-characterized refence standard set is expected between December 2024 and March 2025. Once identified they will be distributed to 3-6 sponsors and contract facilities that volunteer to test them.

Milestones Achieved and Timeline by Sub-Aim:

Aim 1: Adapt the IIRMI assay for biosimilars:

1.1. **Establish whether the higher molecular weight and complexity of biologics (vs smaller peptides) impacts on the IIRMI assay.**

1.1.1. Milestone: Confirmed sensitivity to IIRMI in proteins of different MW using >10 products of different complexities including several mAbs.
1.1.2. Completion: 100%

1.2. **Determine whether the IIRMI assay can be used to detect IIRMI in products produced in bacteria, yeast, or CHO cells.**

1.2.1. Milestone: Confirmed that material made in different substrates can be tested
1.2.2. Completion: 100%

1.3. **Assess impact of impurities such as aggregation on assay performance and potential for IIRMI masking.**

1.3.1. Milestone: Confirmed that assay can detect spiked selected IIRMI in highly aggregated drug product. Impact on assay sensitivity (LOD) and breadth of IIRMI detected will be completed in year 2.
1.3.2. Completion: 80%

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

1.1 **Establish contract with NIST to characterize and distribute suitability standards for sponsors to benchmark their assays.**

1.1.1 Milestone: A 2-year interagency agreement was established with NIST. (Contract IAA:#A2309-075-013-052172) *for a service agreement been U.S. FDA and NIST for the development and distribution of consistent and validated standards for innate immune assays that are used to test the immunogenicity of generic and biosimilar biological products as part of the development of abbreviated paths of regulatory approval under 505(b)(2) path for peptides and oligonucleotides and 351(k) path for biosimilar proteins of biological products.*
1.1.2 Completion: 100%

1.2 **Select suitability standards from different suppliers and provide information to NIST**

1.2.1 Milestone: Over 30 candidates from different suppliers were tested for endotoxin content, selectivity using cell lines expressing defined receptor arrays, and IIRMI assay performance. Agonists for 4 pattern recognition receptors (PRR) were identified and provided to NIST.
1.2.2 Completion: 90%. A set of reference product candidates was identified by FDA however, during NIST's characterization of these agonist impurities were discovered that will require a new round of selection for a non-TLR binding agonist. This work is underway and will be completed by October 2024.

1.3 **Characterization of suitability control candidates: NIST will perform the chemical characterization and stability studies and FDA labs will characterize their immunomodulatory activity.**

1.3.1 Milestone: Products were acquired by NIST and chromatographic assays were set up for characterization. Bioactivity of each candidate was confirmed. A complete profile for each suitability control using Nanostring and Luminex will be performed once the final set of reference controls is selected.

1.3.2 Completion: 30%. Estimated timeline: The characterization of the final reference suitability standards will be performed in year 2.

1.4 **Selected standards will be purchased by NIST, characterized as above by both labs, aliquoted, placed on stability, and distributed for testing by collaborating sponsors.**

1.4.1 Milestone: Initial freeze-thaw studies have been completed for several candidates.

1.4.2 Completion: 10%. Estimated timeline: Final selection and characterization of the suitability controls is expected by December 2024-March 2025.

3. RESEARCH OUTCOMES

Reference biologics and biosimilars may differ in their impurity content. Potential process-related-impurities including host cell proteins, remnants of adventitious agents, product related variants, and contaminants could act as adjuvants increasing the immunogenicity risk of the product. It is not possible to identify, isolate, or synthesize these impurities however, our studies suggest that it is possible to use an impurity-agnostic assay to assess if they could increase innate immune activation relative to the corresponding reference biologic.

Our studies show that IIRMI assays can detect changes in immunomodulatory activity for most products, regardless of their molecular weight, or manufacturing cell platform. Further, minor sequence changes, side chains or even pegylation did not modify the LOD for the PAARag tested. Interestingly, subsets of proteins commonly found among HCP have immunomodulatory activity suggesting that it is important for IIRMI assays to detect these.

Since there are many different assays being used to test products of different immunomodulatory activity and measuring a diverse array of readouts, it will be critical for these assays to be benchmarked and validated using broadly availably and well characterized reference standards. To that effect we identified candidate standards that were receptor specific, free of detectable endotoxin, and performed consistently in IIRMI assays that use NFKB activation in reporter cell lines, cytokine expression (mRNA or protein) by PBMC or activation markers expression (flow cytometry) as readouts for innate immune activation. Through an interagency agreement with NIST, these are being characterized and will be made available to sponsors so they can benchmark their assays to a common reference standard. Establishing and validating common suitability control standards and setting up identification thresholds for the assay using these standards will make it easy for the Sponsors to establish sensitivity of their assay across different assay platforms.

Another important observation is that most products have some inherent immunomodulatory activity. Developing a baseline profile of the reference biologic for every product class will provide critical in informing assessors and sponsors on the patterns of innate immune activation that could be expected and inform on whether the magnitude of the innate immune response elicited by a biosimilar product would suggest a potential clinical concern. In summary, our studies are progressing as planned and laying the foundation for implementation of the IIRMI assay in immunogenicity risk assessment in the biosimilar space.

4. REGULATORY IMPACT

Biosimilar products can reduce the cost and increase the availability (fewer shortages) of life-saving drugs, however residual risk regarding their potential to induce an unwanted immune response can hinder development and licensing. While this risk could be addressed using parallel arm clinical trials, study designs that can detect clinically relevant differences in immunogenicity require lengthy and expensive trials that defy the intent of the abbreviated regulatory path. An alternative approach to assessing immunogenicity risk is to characterize and mitigate the factors that impact product immunogenicity risk. For biosimilar products that will be used for the

same indication, in the same target patient population, and have a highly similar active pharmaceutical ingredient (API) as the reference product (RP), residual risk can be primarily attributable to impurities. Thus, establishing that there are no innate immune response modulating impurities (IIRMI) that could act as adjuvants can inform the immunogenicity risk assessment reducing uncertainty and the need for clinical trials.

Side-by-side studies of IIRMI have been used to assess the immunogenicity risk of generic synthetic peptides (FDA 2021), however, for biologics, which are produced in living cells, the expectation is that the IIRMI content will be product and process specific and thus not amenable to side-by-side comparison of biomarkers. Instead, to assess and mitigate the risk of potential IIRMI acting as adjuvants, sponsors can deploy well-controlled assays capable of detecting changes to the innate immune response caused by a broad array or combination of impurities in the product. To support the development of this approach, these studies are (1) exploring the different product-related parameters that could impact on assay performance, and (2) establishing reference standards that can be used by sponsors to benchmark their assays, which will improve comparisons across products. Together, these studies will facilitate the development and validation of sensitive IIRMI assays that are cell host and manufacturing process agnostic. This will aid the sponsors to generate interpretable data as well as inform 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.

Importantly, as part of the Biosimilar User Fee Amendments of 2022 (BsUFA III), FDA recently requested comments on the development of product class specific or product specific guidance documents to help biosimilar product development (Docket No. FDA-2024-N-3228). The characterization of the assay as well as the innate immune profile developed for frequently used RP can inform the expectations of the Agency for both types of 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).

5. COMMUNICATION AND DISSEMINATION

Publications:

1. Cell-based assays to detect innate immune response modulating impurities: Application to biosimilar insulin. Cheng Her1#, Seth Thacker1#*, Joseph Balsamo1, Logan Kelley Baker1, Derek DM Ireland, Eric Pang2, Daniela Verthelyi1*. In clearance.
2. European Immunogenicity Platform Open Symposium on Immunogenicity of Biopharmaceuticals. Tourdot et al. Bioanalysis 2024. S. Proceedings of the 14th EIP symposium.

Internal dissemination:

1. Immunogenicity Risk Evaluations of follow on Peptide Products. OPQR All hands, April, 2024.
2. Internal technical report on the impact of pegylation on IIRMI assay performance
3. The information obtained from these studies aided in the development of a review tool for assessing IIRMI assays; twelve reviewers were trained to use the tool.

External dissemination:

1. New approaches to assess immunogenicity risk: Regulatory considerations. EIP Lisbon, April, 2024.
2. Assays supporting immunogenicity risk assessments: The road ahead. WRIB, San Antonio April, 2024.
3. Fit for Purpose assays to assess innate immune response modulating impurities. WRIB, San Antonio April, 2024.
4. Innate Immune Response Modulating Impurities Testing as a component of immunogenicity risk assessments. Boulder Peptide Foundation. May, 2024 (virtual)
5. Enhancing the Efficiency of Bioequivalence Approaches for Generic Products with Complex Active Ingredients Workshop. SBIA, Maryland, May, 2023.

Results from these studies will be presented at:

1. Invited talk at the American College of Toxicology Annual meeting 2024

2. Immunogenicity Summit (CHI conference) in October 2024.
3. Invited for a talk at WCBP in January 2025
4. Invited for a talk in EIP symposium in February, 2025.
5. Invited for a talk at WRIB in May 2025
6. Planned Manuscripts:
7. A manuscript describing the application of the IIRMI assay to different insulin products is under review. Internal reports describing assay performance for pegylated products have been finalized.
8. A manuscript describing the comparison of assay platform and biomarker performance will be generated in year 2.
9. A white paper on the critical attributes of IIRMI assays to assess biosimilar products year 2.

Guidance:

1. Knowledge attained from these studies ill be used to inform FDA Guidance for Industry on Immunogenicity Risk Assessments.

6. CHALLENGES

A potential challenge to the time-line is the implementation of Research Agreements and Material Transfer Agreements with the Industry partners who will be invited to test the standards. Moreover, the completion of the testing exercise will likely depend on their priorities and thus this timeline may need to change.

Minor challenges we encountered include:

1. The presence of unanticipated impurities in some of the commercial reference standard candidates. This necessitated our lab to acquire and test new reference standard candidates.
2. Inability to order from suppliers of oligonucleotides that were previously used. This will require us to identify and assess products from a new vendor.

Despite these challenges, provided continued support, we anticipate keeping our timelines as projected.

7. REFERENCES

- FDA (2021). Guidance for Industry: ANDAs for certain highly purified synthetic peptides drug products that reference peptide drug products of rDNA origin. F. a. D. Adminsitration;, U.S. Department of Health and Human Services.
- Haile, L. A., S. K. Polumuri, R. Rao, L. Kelley-Baker, D. Kryndushkin, R. Rajaiah, T. Israely, V. A. Rao and D. Verthelyi (2017). "Cell based assay identifies TLR2 and TLR4 stimulating impurities in Interferon beta." Sci Rep **7**(1): 10490.
- Haile, L. A., M. Puig, L. Kelley-Baker and D. Verthelyi (2015). "Detection of Innate Immune Response Modulating Impurities in Therapeutic Proteins." Plos One **10**(4).
- Holley, C. K., E. Cedrone, D. Donohue, B. W. Neun, D. Verthelyi, E. S. Pang and M. A. Dobrovolskaia (2021). "An In Vitro Assessment of Immunostimulatory Responses to Ten Model Innate Immune Response Modulating Impurities (IIRMIs) and Peptide Drug Product, Teriparatide." Molecules **26**(24).
- Moussa, E. M., J. Kotarek, J. S. Blum, E. Marszal and E. M. Topp (2016). "Physical Characterization and Innate Immunogenicity of Aggregated Intravenous Immunoglobulin (IGIV) in an In Vitro Cell-Based Model." Pharmaceutical Research **33**(7): 1736-1751.
- Polumuri, S. K., L. A. Haile, D. D. C. Ireland and D. Verthelyi (2018). "Aggregates of IVIG or Avastin, but not HSA, modify the response to model innate immune response modulating impurities." Sci Rep **8**(1): 11477.
- Thacker, S. G., C. Her, L. Kelley-Baker, D. D. C. Ireland, M. Manangeeswaran, E. S. Pang and D. Verthelyi (2022). "Detection of innate immune response modulating impurities (IIRMI) in therapeutic peptides and proteins: Impact of excipients." Front Immunol **13**: 970499.

8.APPENDIX: ABBREVIATIONS

ABBREVIATION	DEFINITION
API	Active pharmaceutical ingredient
CHO	Chinese hamster ovary
CRO	Contract research organization
D35	CpG ODN D35 (TLR9 agonist)
EIP	European Immunogenicity Platform
FSL-1	Fibroblast-stimulating Lipopeptide (TLR2/6 agonist)
HCP	Host cell protein
hIl-6	Human Interleukin 6
hMX1	Human gene that encodes a guanosine triphosphate (GTP)-metabolizing protein
IIRMI	Innate immune modulating impurity
LOD	Limit of Detection
LPS	Lipopolysaccharide/ endotoxin (TLR4 agonist)
MDP	Muramyl dipeptide (NOD2 agonist)
NIST	National institute of standards and technology
PBMC	Peripheral blood monocytes
R484	Imidazoquinoline (TLR7/8 agonist)
WRIB	Workshops on Recent Issues in Bioanalysis
Zymo	Polysaccharide Zymosan (Dectin 1 – TLR2 agonist)