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

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

Project Title:	ISPRI-HCP: CHO protein impurity immunogenicity risk prediction for improving biosimilar product development and assessing product interchangeability
Investigator:	Anne De Groot, M.D., Kirk Haltaufderhyde, Ph.D.
Organization:	EpiVax Inc.
Grant No. (if applicable)	U01FD007760
Project Objective:	To improve the ISPRI-HCP immunogenicity risk prediction model for biosimilar product development and assessing product interchangeability

Specific Aim(s)	Progress	Outcomes	Communication Timeline
1. Evaluate immunogenic similarity of variable antigen formats to stimulate de novo immune responses in vitro	100% completed	Obtained in-silico data with the immunogenicity assessment for over 140 commonly found CHO HCPs. Determined that PeptiCAD was the best HCP antigen format for AIM 2 T cell assays.	ISPRI-HCP Manuscript published September 2023 Progress report submitted -July 2023
2. Improve the ISPRI-HCP immunogenicity risk assessment model by training the model on data derived from in vitro studies of human immune response to CHO HCPs	15% completed	Performed in-vitro immunogenicity assessments for 25% of the HCPs impurities. Performed two studies to improve the in-vitro assay.	Progress report submitted -July 2024 Submit final report - July 2025 Publish manuscript -August 2025

2. PROGRESS SUMMARY

Overview. The identification and removal of host cell proteins (HCP) from biologic products is a critical quality attribute in biosimilar drug development. While the biosimilar sequence and mode of action may be identical to the innovator, the process and cell lines used to produce the biosimilar are highly likely to be different, which may lead to the introduction of HCPs that are not identical with the reference listed drug (RLD). HCPs have occasionally been linked to off-target effects and immunogenicity, raising concern about biosimilar drug safety especially if new HCPs are introduced into the final product. The significance is underscored by recent reports of unwanted

immunogenicity and loss of efficacy linked to HCPs in monoclonal antibody and recombinant protein products manufactured in the cells commonly used to produce biologics, Chinese hamster ovary (CHO) cells (Jones et al., 2021; Molden et al., 2021). Therefore, it is important to identify and quantify the potential for immunogenicity associated with HCPs that may have been introduced in each biosimilar product. Previous methods for identifying HCPs (such as two dimensional western blots using anti-HCP antibodies) do not quantify their potential for immunogenicity. New methods for assessing the potential immunogenicity of HCPs are needed to speed up the development of biosimilars.

Well-established methods for quantifying and identifying HCPs, such as ELISA assays and two-dimensional western blots using non-human species' anti-HCP antibodies, do not discriminate between non-immunogenic and immunogenic HCPs. More recently, drug developers have turned to identification and evaluation of individual impurities by LC-MS, with the goal of selectively removing the most immunogenic or biologically active impurities. To facilitate the immunogenicity risk assessment task, the EpiVax group has initiated the development of in silico tools. Eventually, developers and regulators may be able to use these tools as a means of assessing the risk of individual impurities. This method may eventually enable developers to focus on HCPs that need to be removed to lower immunogenicity risk, improving safety while speeding the process workflow. **EpiVax has proposed to improve the accuracy of the existing method for HCP immunogenicity to facilitate the assessment of clinically meaningful immunogenicity risk for biologics, enabling FDA regulators assess interchangeability between a biosimilar and an innovator product.**

The EpiVax immunogenicity risk assessment tool for HCPs is called ISPRI-HCP. It analyzes the sequence of CHO (and other cell line) HCP and compares the T cell epitopes that are present in the CHO sequence to similar epitopes in the human genome (using a tool called JanusMatrix). In addition to assessing similarity with the human genome, the tool evaluates the total foreign T cell epitope count and density (using EpiMatrix). By comparing this data to information obtained from previous studies of biologic protein immunogenicity, ISPRI-HCP is able to provide an initial assessment of overall immunogenicity risk. We hypothesize that the accuracy of the ISPRI-HCP tool can be improved using in vitro data obtained by performing T-cell immunogenicity assays. The EpiMatrix and JanusMatrix core algorithms that are integrated into ISPRI-HCP have been well established.

For this proposal, **Aim 1** was to: "Develop an ISPRI-HCP immunogenicity prediction model that is trained on the T cell immunogenicity of CHO HCPs", and **Aim 2** was to "Evaluate immunogenic similarity of variable antigen formats to stimulate de novo immune responses in vitro." In Aim 1, 87 HCP were to be evaluated. We have requested additional time to complete both tasks, since in the interim between proposal generation and receipt of funding, (1) we identified problems related to the sequences and purity of peptides being produced for overlapping peptide arrays and (2) the primary provider of our reformatted arrays filed for bankruptcy, and we have had to identify a replacement peptide provider for this project.
At the outset of the project, we also proposed to modify the project by re-ordering the aims so as to determine whether a computationally designed peptide array that reduced the repetition of epitopes, and properly centers the epitopes, would improve the accuracy of the results. The modified array format would also be less expensive and less wasteful of resources, leading to cost reductions and, potentially, the publication of a new and improved method for peptide arrays that could be used by other researchers. Once the array format was validated, then, the full complement of HCP could be evaluated in vitro. We anticipated that the PeptiCAD approach will be more accurate and less costly, which would be a significant advance for the field. **The re-ordered aims are provided along with an update on progress, below:**

SPECIFIC AIM 1: Evaluate immunogenic similarity of variable antigen formats to stimulate de novo immune responses in vitro. The suitability of overlapping peptides to model T cell responses of whole HCP proteins that would be encountered by patients in a biosimilar or innovator product was to be assessed. We would also explore the suitability of a focused set of peptides that is computationally selected (PeptiCAD) in comparison with recombinant protein and overlapping peptides. T cell immunogenicity equivalence would be evaluated by the method used Wullner et al. 2010 to stimulate de novo T cell responses for a subset of CHO HCPs. Six commonly found CHO protein impurities covering a wide range of immunogenicity risk were to be tested. Expected outcome: Methods would be made public about a more efficient way to screen for potential immunogenicity risk using peptide arrays.

SPECIFIC AIM 2: Improve the ISPRI-HCP immunogenicity risk assessment model by training the model on data derived from in vitro studies of human immune response to CHO HCPs. We would generate a T cell immunogenicity dataset for 87 commonly found CHO HCP impurities in licensed monoclonal antibodies that were

defined by a 26-company collaboration. Based on ISPRI-HCP sequence analysis, these proteins were known to span a wide range of immunogenic potential, from high- to low-risk. For each CHO protein, we would stimulate de novo immune responses in vitro for an HLA-diverse cohort and measure T cell immunogenicity of the HCP in dose ranging studies, by FluoroSpot assay. The experimental dataset generated for all the proteins would be used to evaluate performance of ISPRI-HCP risk classification by cross-validation methods to estimate the performance of the ISPRI-HCP machine learning model.

This research program is focused on improving the accuracy of the ISPRI-HCP platform, which would enable drug developers to use ISPRI, or, based on the data published in this project, develop other algorithms that would enable immunogenicity risk assessment of HCP, potentially reducing HCP-associated immunogenicity in biosimilar biologic products. In **future work**, we anticipate that we would use ISPRI-HCP to perform side-by-side predictions and in vitro assessments of the immunogenicity of CHO HCP impurities found in innovator versus biosimilar products.

MILESTONES AND TIMELINES

The overall goal of the research program is to assess and improve the predictive accuracy of ISPRI-HCP and demonstrate that immunoinformatics tools like ISPRI-HCP can be reliable means of assessing innovator versus biosimilar HCP immunogenicity risk. In Aim 1, proposed to first evaluate the immunogenic similarity of variable antigen formats using in vitro T cell assays (Year 1). This aim was accomplished. The PeptiCAD format that performed best was selected to go forward to Aim 2 studies that were scheduled to begin in Year 2 and continue to the end of the project.

Note: Percent completion values below reflect work completed from the award date, August 24, 2022, through the reporting date, July 1, 2024.

Blue Bars	show studies completed in Year 1	**Green Bars**	show studies completed in Year 2

AIM 1: Evaluate immunogenic similarity of variable antigen formats to stimulate de novo immune responses in vitro. (Year 1)

Objective 1.1: Select CHO HCP test articles for antigen format comparison.

- **Milestone 1.1:** Selection of six commonly found CHO HCP impurities (PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2) with ISPRI-HCP scores that cover a wide range of immunogenicity risk.
- **Timeline:** Year 1: start – end of Month 2
- **Percent completion:** 100%

Objective 1.2: Design PeptiCAD and peptide arrays for six CHO HCPs.

Milestone 1.2: Production of PeptiCAD and peptide array designs for PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2 CHO HCPs.

- **Timeline:** Year 1: end of Month 2 – start of Month 4
- **Percent completion:** 100%

Objective 1.3: Obtain PeptiCAD, peptide array, and whole protein test articles for: PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2 CHO proteins.

- **Milestone 1.3:** Production of PeptiCAD and peptide arrays for six CHO HCP test articles. Successful production of whole proteins for four out of the six HCPs.
- **Timeline:** Year 1: start of Month 4 – end of Month 8
- **Percent completion:** 100%

Objective 1.4: Determine the appropriate dose for Aim 1 T cell assays

- **Milestone 1.4:** Selection of the optimal dose for AIM 1 T cell assays. Complete a dose ranging study using PeptiCAD, peptide array and whole protein PLBL2 antigens.
- **Timeline:** Year 1: start of Month 5 – middle of month 8
- **Percent completion:** 100%

Objective 1.5: Complete AIM 1 T cell assays for PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2 CHO proteins and determine the best antigen format for AIM 2.

- **Milestone 1.5:** Determine the best HCP antigen format for AIM 2 T cell assays .
- **Timeline:** Year 1: end of Month 8 – end of Year 1
- **Percent completion:** 100%

AIM 2: Improve the ISPRI-HCP immunogenicity risk assessment model by training the model on data derived from in vitro studies of human immune response to CHO HCPs. (Years 1-2)

Objective 2.1: Design peptide arrays for commonly found CHO HCP impurities.

- **Milestone 2.1:** Design peptide arrays for 87 commonly found CHO HCP impurities
- **Timeline:** Year 1: start of Month 11 – Year 2: start of Month 1
- **Percent completion:** 100%

Objective 2.2: Generation of T cell immunogenicity dataset for 87 commonly found CHO HCP impurities.

- **Milestone 2.2:** Perform in-vitro immunogenicity assessment of 87 commonly found CHO HCP impurities, for 30-40 donors, based on production of IFNγ and IL-10 cytokines.
- **Timeline:** Year 2: start of Month 1 – end of Month 10
- **Percent completion:** 25%

Objective 2.3: ISPRI-HCP Classification model development.

- **Milestone 2.3:** Develop a classification model that will assign immunogenicity risk to CHO HCP impurities using ISPRI-HCP from the analysis of in-vitro experimental data.
- **Timeline:** Year 2: start of Month 2 – end of Year 2
- **Percent completion:** 0%

Project Objective:

The objective of this program is to improve existing in silico models that have been developed for assessing HCP immunogenicity risk, making the evaluation evaluate of HCPs more accurate, enabling FDA to determine whether biosimilar and an innovator product are interchangeable and without significant safety risk (due to HCP immunogenicity) for the patient.

Aim 1: Evaluate immunogenic similarity of variable antigen formats to stimulate de novo immune responses in vitro

Objective 1.1: Select CHO HCP test articles for antigen format comparison.

To assist with our selection of HCPs for Aim 1, we first used ISPRI-HCP to evaluate the immunogenic potential of the 143 CHO HCPs that are frequently found to co-purify with mAbs. Shown in **Figure. 1** is a subset of these proteins plotted on the Y axis by their EpiMatrix Protein Score and on the X axis by their JanusMatrix Human Homology Score. Several of these commonly identified HCPs with their EpiMatrix and JanusMatrix Scores are illustrated on the accompanying Quadrant plot (**Fig. 1**). The bubble plot shows each of the HCP in a quadrant that is used for classifying their immunogenicity risk based on EpiMatrix (EMX) and JanusMatrix (JMX) thresholds. We find that the predicted immunogenic potential of CHO HCPs covers a wide range of scores, both in terms of epitope content and "human-ness" as defined by JanusMatrix. Using this data, we selected six HCPs that cover a wide range of immunogenicity, and they are identified by name in **Figure 1** (PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2) and their scores are listed in **Table 1**.

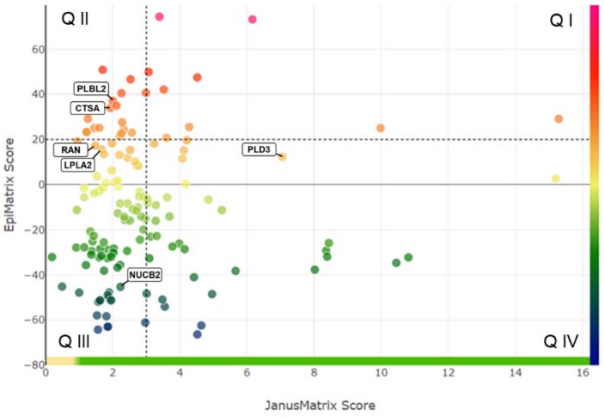

Fig. 1. ISPRI-HCP analysis of CHO HCP Landscape. Using a list of commonly found CHO protein impurities, we calculated the EpiMatrix (EMX) and JanusMatrix (JMX) scores for each protein. Shown here are 143 CHO HCP impurities and selected proteins. Proteins with high (orange), medium (yellow), and low (green) immunogenicity potential are shown. Proteins with EMX greater than 20 and JMX scores less than 3 are predicted to be high risk (Q II). Proteins with EMX less than 20 and JMX greater than 3 are predicted to be low risk (Q IV). The list of commonly found HCPs was sourced from: Jones, M. et al. "High-risk" host cell proteins (HCPs): A multi-company collaborative view. Biotechnology and Bioengineering vol. 118 2870–2885 (2021) and Molden et al. (2021), Host cell protein profiling of commercial therapeutic protein drugs as a benchmark for monoclonal antibody-based therapeutic protein development.

Table 1. CHO HCP test articles for antigen format comparison

Protein Name	Label	Risk Group	EpiMatrix Score	JanusMatrix Score
Phospholipas B-like 2	PLBL2	High	37.12	2
Lysosomal protective protein	CTSA	High	33.93	1.93
GTP-binding nuclear protein	RAN	Intermediate	13.46	1.74
Phospholipase D3	PLD3	Intermediate	12.23	7.05
Lysosomal Phospholipase A2	LPLA2	Intermediate	11.89	2.44
Nucleobindin-2	NUCB2	Low	-45.32	2.23

Objective 1.4: Determine the appropriate dose for Aim 1 T cell assays.

To determine the optimal dosage for evaluating CHO HCP immunogenicity, the immunogenic potential of PLBL2, a commonly found CHO HCP impurity was assessed using EpiVax's In Vitro Immunization Protocol (IVIP). IVIP is performed as follows: Peripheral blood mononuclear cell (PBMC) samples are isolated from de-identified whole blood filters obtained from the Rhode Island Blood Center. Each donor is screened for HCP immunogenicity. Based on a probabilistic model, we calculated that blood samples collected from 30 randomly selected individuals will cover each HLA class II DR supertype allele two times with a probability of 85%; the probability of covering each allele once is >99%. Male and female donors are equally weighted for consideration of sex as a biological variable. Cells are cultured in 96-well U-bottom plates at a density of 2.5 x 10^5 cells/well in complete human RPMI media (chRPMI) supplemented with IL-2 and IL-7 growth cytokines. Cells are stimulated with test articles (pooled peptides) over a range of concentrations from 1-20 µg/ml, an immunogenic protein positive control (KLH; 10 µg/ml), a non-immunogenic protein negative control (HSA; 10 µg/ml), an antigenic peptide pool positive control (CEFTA; 2 µg/ml), or media only. The test article concentration maximum is 20 µg/ml. Control concentrations are based on in-house assay development. All PBMCs are cultured for 14 days at 37 ºC with chRPMI media exchanges and growth cytokine supplementation on Days 4, 7, and 11. Per sample, 10 million PBMCs are taken for HLA typing to four-digit resolution by the sequence-specific oligonucleotide method at the American Red Cross. The dose response of the whole proteins, standard peptide arrays or peptide epitope Computer Assisted Design (PeptiCAD) PLBL2 antigen formats is compared. In this experiment PBMCs from normal healthy donors, with no known previous

exposure to CHO HCPs were cultured in the presence or absence of PLBL2 antigens with cytokine support for 14 days alongside the appropriate controls. The interferon-gamma (IFNg) response to PLBL2 antigens was measured by Fluorospot (**Fig.2**). We find that the IFNg responses to the peptide array and PeptiCAD was greater than whole proteins. Note that the PLBL2 PeptiCAD design has half the number of peptides as the peptide array and produced similar IFNg responses. To determine the dose response to PLBL2 antigens, we exposed PBMCs to 1, 5, 10 and 20 µg/mL of each antigen format (**Fig. 3**). On average, 20 µg/mL produced the highest PLBL2 specific IFNγ responses. Unlike whole proteins, a dose dependant relationship was observed for the peptide array and PeptiCAD antigens. The responses to whole proteins was varied and is potentially due to incomplete antigen processing and presentation. Based on these results, we determined the optimal dose for testing the CHO HCPs to be 20 µg/mL.

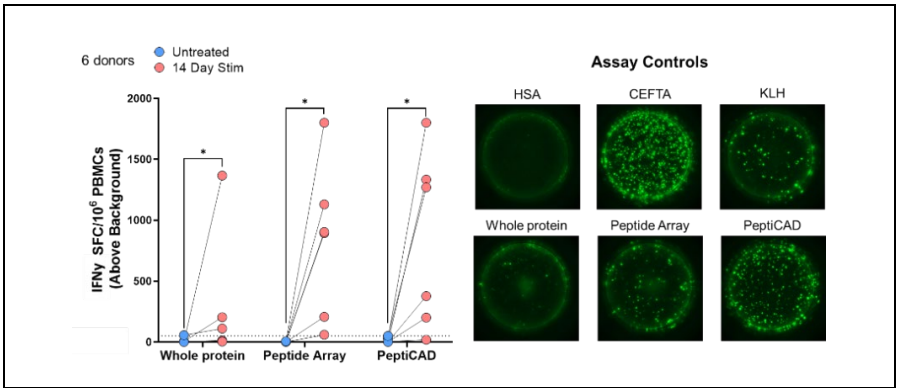

Fig. 2. CHO PLBL2 antigens induces INFγ in human PBMC . Shown is the INFγ induced after 14 day exposure (14 Day Stim) and without 14 day exposure (Untreated) to whole protein, peptide array, and PeptiCAD PLBL2 antigens. Representative figure with assay controls are shown: HSA (negative control), CEFTA and KLH (positive controls).

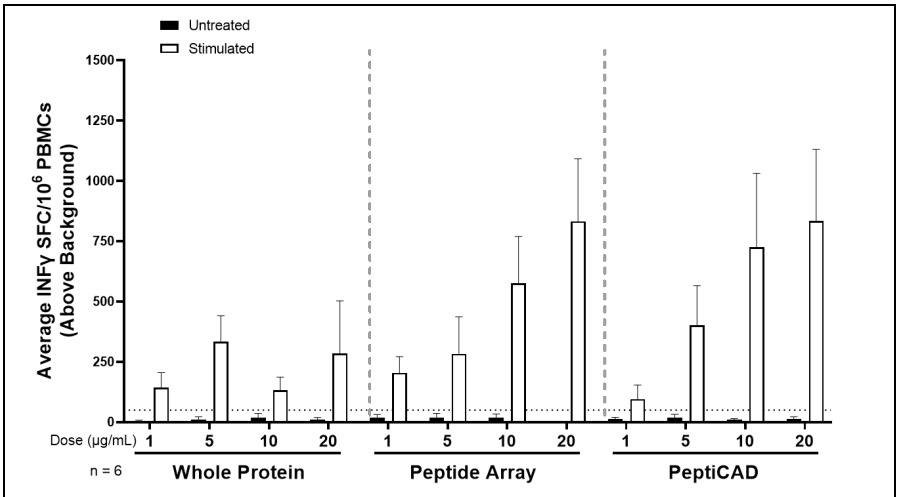

Fig. 3. Human PBMC dose response to PLBL2 antigens. Shown is the average INFγ SFC per million PBMC after 14 day exposure (Stimulated) with whole protein, peptide array, or PeptiCAD PLBL2 antigens. Without 14 day exposure (Untreated).

Objective 1.5: Complete AIM 1 T cell assays for PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2 CHO proteins and determine the best antigen format for AIM 2.

To determine the best antigen format for evaluating CHO HCP immunogenicity, the immunogenic potential of six HCPs (PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2) were tested using EpiVax's In Vitro Immunization Protocol (IVIP). The CHO proteins used for Objective 1.5 are identified by name in Figure 1 (PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2) and their scores are listed in Table 1. The percentage of positive responses for each HCP was the same or similar for PeptiCAD versus standard peptide arrays (**Fig.2B**).

We compared the distribution of the IFNγ SFC/million PBMCs with violin plots and performed a pair-wise Wilcoxon test for PeptiCAD versus standard peptide arrays HCP antigens (**Fig.3A**). Using the spearman's correlation test, we identified significant positive relationships between both formats for four out of six HCPs (**Fig.3B**). Altogether, PeptiCAD and Peptide Array formats showed a similar overall performance. Differences in responses between individual donors are evident and this may be a result of individual HLA-type. Since PeptiCAD performed similar to peptide arrays and required less peptides (production cost reduced by ~50%) we decided to move forward the PeptiCAD design for AIM-2.

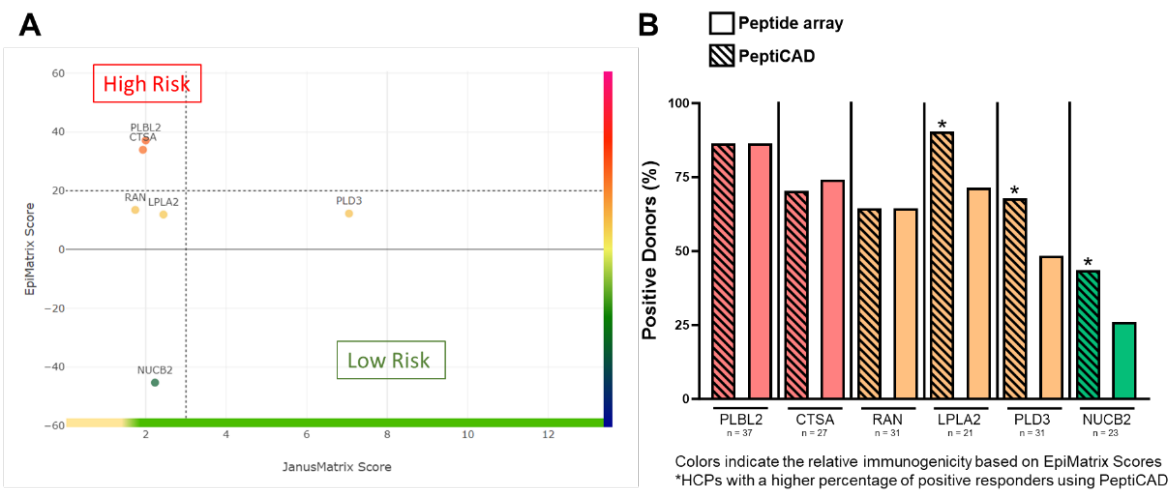

Fig. 2. Percentage of positive responders for Peptide array versus PeptiCAD HCP antigen formats. ISPRI-HCP analysis of immunogenicity risk: High (PLBL2, CTSA), moderate (RAN, LPLA2, PLD3) and low (NUCB2) risk HCPs are shown (A). The percentage of positive responders after exposure to PLBL2, CTSA, RAN, LPLA2, PLD3, and NUCB2 PeptiCAD versus Peptide array antigens. Asterisks indicate HCPs with a higher percentage of positive donors for the PeptiCAD format. A response is considered positive when there are 50 or greater spot forming cells per well the number of spots is at minimum twice the background (B).

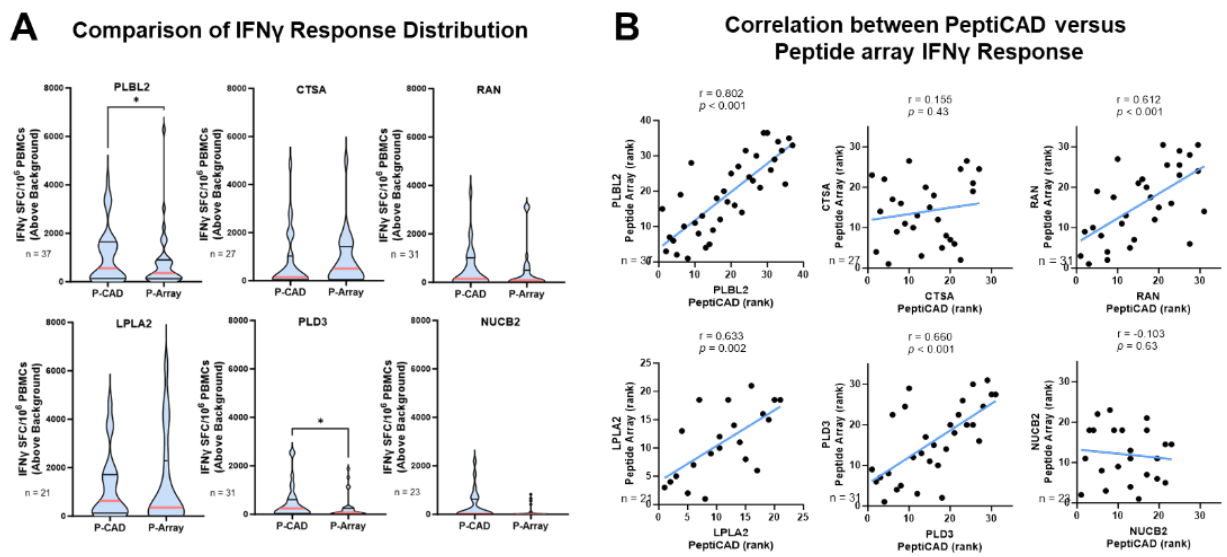

Fig. 3. IFNγ response to Peptide array versus PeptiCAD HCP antigen formats. Distribution (violin plots) of IFNγ SFC/million PBMCs after exposure to PeptiCAD (P-CAD) versus Peptide array (P-Array) CHO HCP antigens. The median values (red line) and quartiles (black lines) are shown. The Wilcoxon signed-rank test was used to compare groups; * $p < 0.05$. (A). The correlation between PeptiCAD IFNγ response versus Peptide array IFNγ response (SFC/million PBMCs). The Spearman's rank correlation coefficient is shown (r) ; $p < 0.05$ was considered significant (B).

Aim 2: Improve the ISPRI-HCP immunogenicity risk assessment model by training the model on data derived from in vitro studies of human immune response to CHO HCPs

Objective 2.2: Generation of T cell immunogenicity dataset for 87 commonly found CHO HCP impurities.

To evaluate the immunogenic potential of 87 CHO HCPs frequently found to co-purify with mAbs, we used the PeptiCAD antigen format in our IVIP assay. The criterion for a positive response is 50 or greater spot forming PBMCs per well and the number of spots being a minimum of twice the background. Unexpectedly, the majority of the 21 HCPs tested had a high percentage of positive responders (**Fig.4**). Four of the moderate/low risk HCPs had a percentage of positive responders greater than or equal to 90%. This finding indicated that additional steps were needed to troubleshoot and improve the sensitivity of the assay. To improve the in-vitro assay we performed two studies:

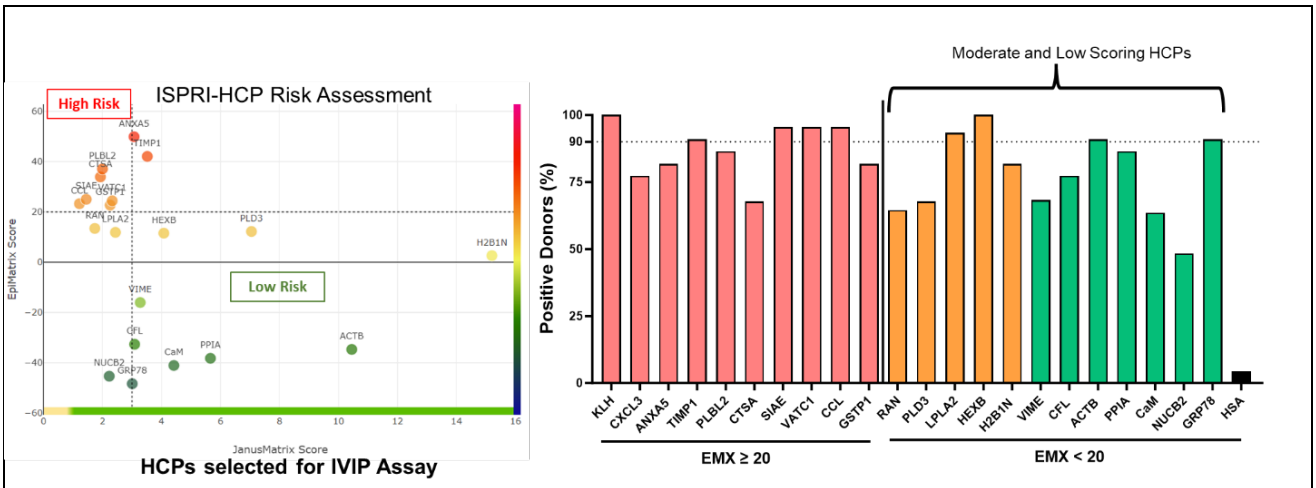

Fig. 4. Percentage of positive responders to CHO HCP antigens. ISPRI-HCP immunogenicity risk assessment of 21 commonly found CHO HCPs impurities (A). The percentage of positive responders to 21 commonly found CHO HCPs. Colors indicate the in-silico immunogenicity assessment: high risk *(red)*, moderate risk *(orange)*, low risk *(green)*. A response is considered positive when there are 50 or greater spot forming cells per well the number of spots is at minimum twice the background (B).

Study#1 - Compare shorter 7-day primary culture to 14-day culture:

In this study, we compared reducing the primary incubation to seven versus fourteen days. During primary incubation, antigen specific T cells are activated and can undergo rapid proliferation and expansion. If the primary stimulation is too long, this could explain why donors exposed to moderate and low risk HCPs are also exhibiting positive responses. We hypothesized that reducing the primary stimulation to 7-days will reduce the number of positive responses to moderate and low risk HCPs and allow us to better differentiate between the HCPs assessed as high versus low immunogenicity risk. Unexpectedly, the 7-day stimulation did not reduce the number of positive responders and may have increased the percentage of positive responses (**Fig.5**). This finding suggests that other immune cells besides T cells are contributing to the production of IFNγ.

Study#2 - Test alternative peptide synthesis methods.

We hypothesized that the peptide method of synthesis and purity contributed to the high percentage of positive responses. In this study, we used ACTB, a low risk HCP to compare the Diffusion versus Liberty Blue method. Since the Diffusion and Liberty Blue method both produce crude peptides at 50-70% purity, we also tested the Liberty Blue method when purified to 95% purity. We found that all 8 donors had a positive response for both peptide synthesis methods and increasing the purity to 95% did not reduce the number of positive responders. There was also no significant difference in median IFNγ

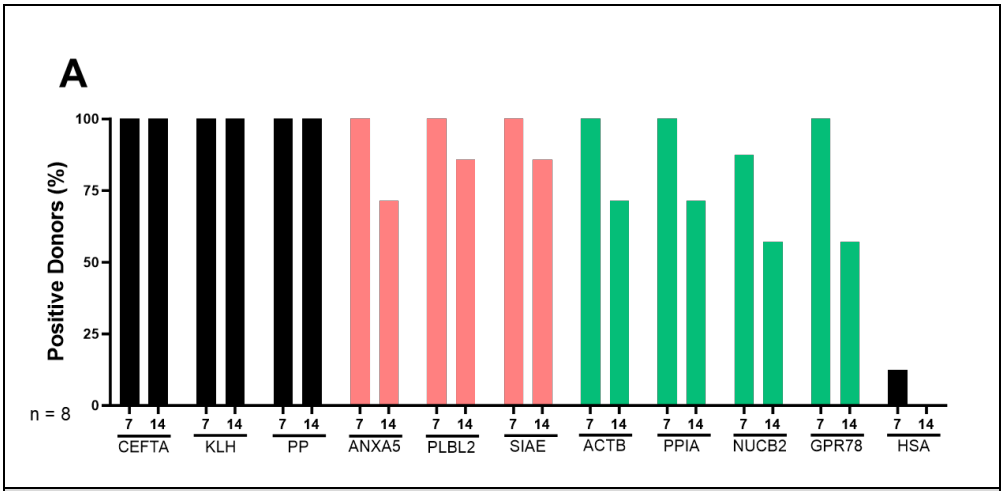

Fig. 5. Comparison of 7-day versus 14-day primary stimulation with HCP antigens. 8 donors were evaluated, and the percentage of positive responders are shown for seven versus 14-day primary stimulation (A). A response is considered positive when there are 50 or greater IFNγ spot forming cells per well the number of spots is at minimum twice the background. Black bars show positive (CEFTA, KLH, PP) and negative (HSA) controls. ANXA5, PLBL2, and SIAE are classified as high immunogenicity risk (red bars). ACTB, PPIA, NUCB2, and GRP78 are classified as low immunogenicity risk (green bars

produced (SFC/million PBMCs) for the Diffusion, Liberty Blue, and Liberty Blue 95% purity peptide pools (**Fig.6**).

From both studies, we concluded that further development of the assay to characterize the cell types responding to the peptide pools is a needed improvement. ISPRI-HCP is designed to assess immunogenicity risk based on HLA-DR class II binding potential and ability to induce a CD4 T cell response. Therefore, we are developing a flow cytometry assay to determine the frequency of proliferating CD4 T cells after exposure to CHO HCP antigens. We expect that this will improve our ability to detect differences in immunogenicity risk and provide quality data for model training.

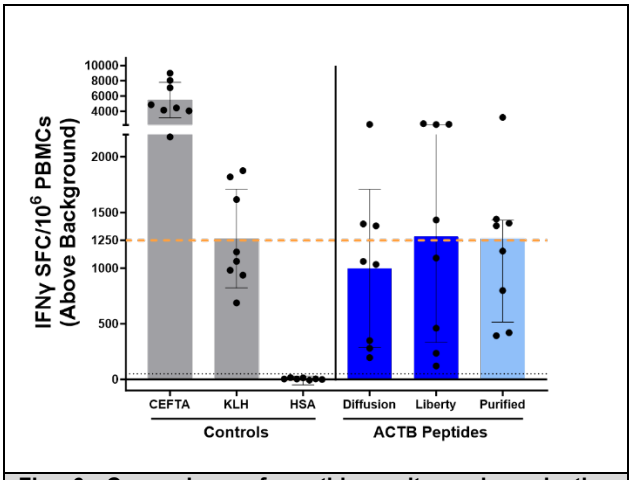

Fig. 6. Comparison of peptide purity and production methods. The median IFNγ SFC/million PBMCs after exposure to ACTB peptide pools produced using the Diffusion, Liberty Blue (Liberty), and Liberty Blue 95% purity (Purified) methods are shown. Positive (CEFTA, KLH) and negative (HSA) controls (*grey bars*).

3. RESEARCH OUTCOMES

AIM 1 and AIM 2.1 objectives have been completed. For AIM 2 objective 2.2, 21 of 87 CHO HCPs have been tested using the proposed in vitro T cell assay (IVIP, or In Vitro Immunogenicity Protocol). We also evaluated improvements to the assay, such as shorter primary culture and alternative peptide production methods.

4. REGULATORY IMPACT

In silico immunogenicity assessment of host cell protein (HCP) impurities could impact biosimilar development by streamlining the clinical data needed to support the designation of a proposed biosimilar product to be interchangeable with the reference listed drug (RLD). In 2017, EpiVax programmers developed a toolkit comprised of several integrated algorithms for immunogenicity screening of host cell proteins, known as ISPRI-HCP (Interactive Screening and Protein Reengineering Interface for Host Cell Proteins). In this project, in vitro T cell immunogenicity data can be generated by screening commonly found CHO HCP impurities in in vitro "IVIP" assays. These results will be used to further validate and refine the immunogenicity assessments made by ISPRI-HCP, providing a valuable resource for biosimilar development.

5. COMMUNICATION AND DISSEMINATION

Publications:

Haltaufderhyde K, Roberts BJ, Khan S, Terry F, Boyle CM, McAllister M, Martin W, Rosenberg A, De Groot AS. Immunoinformatic Risk Assessment of Host Cell Proteins During Process Development for Biologic Therapeutics. AAPS J. 2023 Sep 11;25(5):87. doi: 10.1208/s12248-023-00852-z. Erratum in: AAPS J. 2023 Dec 19;26(1):6. doi: 10.1208/s12248-023-00868-5. PMID: 37697150.

Conference Presentations:

Haltaufderhyde K, McAllister M, Roberts B., Gutierrez A, Tivin J, Ardito M, Martin W, De Groot A (2024) Assessing Immunogenicity Risk of Host Cell Proteins using In Silico and In Vitro Methods. *Artificial Intelligence / Human Intelligence & Immunoinformatics Workshop*. Oral Presentation. Providence, RI.

Roberts B, Haltaufderhyde K, McAllister M, Gutierrez A, Tivin J, Ardito M, Martin W, De Groot A (June-2023) Assessing Immunogenicity Risk of Host Cell Proteins using In Silico and In Vitro Methods. *AAPS Community*. Oral Presentation. Virtual.

Roberts B, Haltaufderhyde K, McAllister M, Gutierrez A, Tivin J, Ardito M, Martin W, De Groot A (Sept-2023) Assessing Immunogenicity Risk Associated with Host Cell Proteins using In Silico and In Vitro Methods. *AAPS Community*. Oral Presentation. Virtual.

Porter K, Haltaufderhyde K, Roberts B, Terry F, Boyle C, William W, De Groot A (2023) Comprehensive assessment of immunogenicity risk of host cell proteins in biologics using in silico and in vitro methods. *Biopharmaceutical Emerging Best Practices Association (BEBPA) HCP Conference.*
Oral Presentation. Dubrovnik, Croatia.

Websites

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Communication Timeline	2024					2025						
Aim 2: Improve the ISPRI-HCP immunogenicity risk assessment model by training the model on data derived from in vitro studies of human immune response to CHO HCPs	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	April	May	Jun	July
										Manuscript & Final Report Production		

6. CHALLENGES

Multiple studies were needed to troubleshoot/optimize the in-vitro assay for CHO HCP peptide pools.
To improve the data acquired, we are switching from FluoroSpot to flow cytometry. If needed, we will test other variables such as the media conditions and dosage.

While peptides were in production, our peptide vender filed for Chapter 7 bankruptcy on May 7th, 2024. Recovery of the peptides and finding an alternative vender may impact the funds available for future peptide orders. Since ISPRI-HCP is not designed for CHO proteins only, sourcing commercially available peptide arrays not derived from CHO may be an option.

7. NEXT STEPS

The results from our first round of T cell assays indicated that additional steps were needed to improve the assay sensitivity. We therefore tested changes to the timing of the assay and alternative peptide synthesis methods. We are currently conducting flow cytometry studies to phenotype the CD4 T cell response. We expect that this will improve the assay and provide quality data to inform ISPRI-HCP. During the no cost extension period, we will have an improved T cell assay designed for immunogenicity screening of CHO proteins and complete Aim 2 objectives. The research effort will go towards the generation of T cell immunogenicity dataset for 40-60 commonly found CHO HCP impurities (Objective 2.2), and the ISPRI-HCP risk classification model development (Objective 2.3).

Objective 2.2: Generation of T cell immunogenicity dataset for 87 commonly found CHO HCP impurities. We will perform in vitro immunogenicity assessments of over 40-60 commonly found CHO HCPs for 16-24 donors. Proliferation of CD4 T cells will be used as a biomarker of immune response and potential to induce anti-HCP antibodies. The experimental data generated will be used in Objective 2.3 to inform the current ISPRI-HCP four-quadrant classification model by fine-tuning the thresholds set for EpiMatrix and JanusMatrix scores.

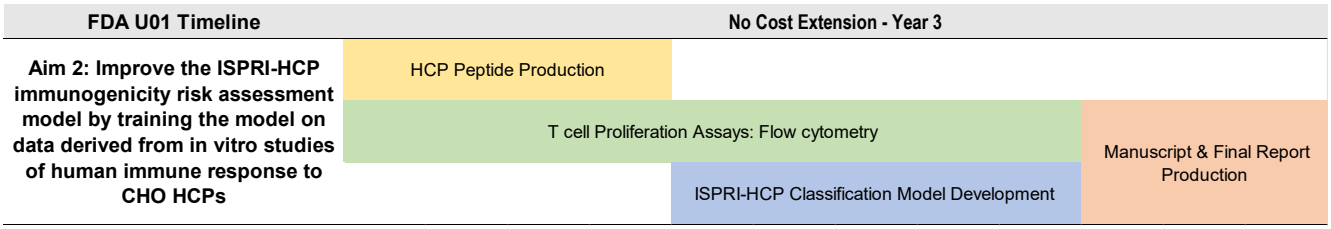
Objective 2.3: ISPRI-HCP Classification model development. We will develop a classification model to assign immunogenicity risk to CHO HCP impurities. Our task is to approximate a mapping function from predictive scores (EpiMatrix, JanusMatrix, or a combination thereof) to flow cytometry CD4 T cell proliferation data. Predictive scores will be mapped to flow cytometry data using machine learning techniques with flow cytometry data split into training and validation sets.

We will begin modeling with the simplest approach and implement mapping methods with increasing complexity to increase predictive accuracy, if needed. Immunogenicity risk will be initially modeled as a binary variable (immunogenic or non-immunogenic HCPs) using EpiMatrix and JanusMatrix scores as predictor variables. Various HCP immunogenicity risk definitions will be explored, including frequency of positive donor responses (responses above media control) and mean, median, or inter-quartile range proliferating CD4 T cell frequencies. A logistic regression model with k-fold cross-validation using a typical k = 5 value will be used as it yields test error rate estimates that do not suffer from high bias and high variance.

If modeling by the initial approach does not generalize well and/or overfits the training data, we will try more complex mapping algorithms for classification (i.e., random forest, stochastic gradient descent, etc.), try different regularization techniques to reduce overfitting, and/or try different predictor variables (epitope cluster count, or ratio of predicted immunogenic to tolerogenic clusters, for example). We will also consider multi-class classification with HCPs assigned as low, intermediate, or high risk for immunogenicity over a range of proliferating CD4 T cell frequencies.

We expect this Aim will yield an accurate CHO HCP immunogenicity predictor that is ready for prospective testing for biosimilar (and innovator) development and evaluation of interchangeability of reference and biosimilar products.

Timeline



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9. APPENDIX A: ADDITIONAL MATERIAL

No additional material.

10. APPENDIX B: ABBREVIATIONS

This section includes all acronyms used in this document along with a corresponding definition.

ABBREVIATION	DEFINITION
IVIP	In Vitro Immunogenicity Protocol
PBMC	Peripheral blood mononuclear cell
HCP	Host cell protein
PeptiCAD	Peptide epitope Computer Assisted Design
CHO	Chinese hamster ovary
RLD	Reference listed drug
SFC	Spot-forming cells