Impact of Purification Process Impurities on Preclinical Assessment of Immunogenicity of Biotherapeutics

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Impact of Purification Process Impurities on Preclinical Assessment of Immunogenicity

of Biotherapeutics

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

     An immune response to biotherapeutics has the potential for a negative impact on patient safety and efficacy. Predicting biotherapeutic immunogenicity at the preclinical stage of drug discovery is important for the selection of the best biotherapeutic candidate. The early discovery protein production process for antibodies typically consists of protein expression in an human embryonic kidney (HEK) 293 cell line, followed by purification using protein A chromatography. Typically protein purification methods at the early discovery stages are not as stringent as during manufacturing stages of the process, so it is likely that biotherapeutic preparations might contain impurities such as host cell protein (HCP), residual DNA (rDNA), and protein A leaching that could impact immunogenicity predictions. Preclinical immunogenicity predictions can be based on an *in vitro* peripheral blood mononuclear cell (PBMC) assay, which measures the proliferation of CD4 T-cells in peripheral human PBMC cells when in contact with protein biologics. We used bevacizumab, an anti-VEGF monoclonal antibody which has been shown to have a low rate of immunogenicity clinically, as a control in our assay. While the commercial drug product gives a stable and low signal, we observed a batch to batch variation response in the PBMC assay with various preparations of the antibody. We hypothesized that HCP, rDNA, and protein A ligand contaminants introduced in the early discovery protein production process caused the elevated CD4 T-cell proliferation observed in the various protein batches of bevacizumab, and that a secondary purification step could remove a majority of those contaminants leading to a more reliable CD4 T-cell proliferation response. To test this hypothesis the levels of those impurities were

measured in the bevacizumab batches which had been previously produced and showed variability. No clear correlation was found between the levels of HEK HCP and *in vitro* T-cell proliferation response. There was some correlation where the sample with the lowest levels of rDNA led to the lowest percent donor response, and the sample with the highest levels of protein A led to the highest percent donor response, however these results require further testing to fully understand. We reproduced the bevacizumab mAb using either a one-step purification method consisting of protein A chromatography, or a two-step purification method consisting of protein A chromatography followed by Q-membrane chromatography run in flow through mode (Q Flow Through). These batches were tested for impurities (HEK HCP, rDNA, and protein A ligand) and were then analyzed in the PBMC assay. The results showed that using a secondary purification step (Q Flow Through) reduced the levels of impurities such as HEK HCP, rDNA and protein A ligand of bevacizumab. The process impurities were not a clear source of variability in percent donors responding with positive CD4 T-cell proliferation in the PBMC assay, but they appeared to impact the strength of the CD4 T-cell proliferation response of those donors.

Dedication

I would like to dedicate this thesis to my loving family, who has given me the strength, support, and encouragement to complete this work. I am extremely grateful for my husband, Ilya Chmykh, for providing me with unwavering support, always cheering me on, and encouraging me throughout the entire ALM thesis process. I am also wholeheartedly grateful for my parents, Sergiu and Veronica Musteata, and my sister, Anna Musteata, for inspiring me to continue my education, and for supporting and believing in me throughout the process.

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Chapter I:

Introduction

Biotherapeutics have been increasing in percentage of drug approvals for treating diseases like diabetes, cancer, autoimmune diseases, and enzyme deficiency replacement therapies (Groell, Jordan, & Borchard, 2018). A major class of protein biotherapeutics are monoclonal antibodies (mAbs) (Joubert et al., 2016), (Zhang et al., 2014). Monoclonal antibodies make effective therapeutics due to their high specificity which allows them to bind disease-specific targets, without affecting non-targeted cells (Trikha, Yan, & Nakada, 2002). However, as biotherapeutics, mAbs can elicit an immunogenic response in patients which can negatively impact the efficacy and safety of the therapeutic (Groell et al., 2018). In fact, immunogenicity has been reported in 89 percent of FDA approved biological products, where in half of those cases the efficacy of the drug was impacted (Schultz et al., 2017).

There are various unwanted effects that can be caused by an immunogenic drug. Immunogenicity is usually manifested by the development of T-cell dependent anti-drug antibodies (ADA), which can neutralize drug function by binding to its active site and decreasing efficacy. Immunogenicity can lead to hypersensitivity reactions such as injection site reactions (ISRs) or anaphylaxis, which could be life threatening (Groell et al., 2018). The risks depend on both the immunogenicity levels of the antibody as well as the health and treatment of the patient. The genetic background, immune status and disease state of the patient can change the risk profile of the drug (Jawa, Joubert, &

Zhang, 2016). With such severe adverse effects of immunogenicity, it is important to understand how the immune system works and what makes an antibody immunogenic.

Innate and Adaptive Immunity

Innate Immunity

The innate immune response is the first line of defense against non-self-pathogens, such as bacteria, which are entering the body. The surface receptors on phagocytic macrophages are able to recognize and bind a broad range of the pathogen surfaces, triggering the macrophage to engulf the bacteria and secrete cytokines and chemokines. The release of these cytokines and chemokines initiates inflammation, which is recognized by increased local blood flow leading to symptoms of heat, redness, and swelling. Inflammation leads to an influx of inflammatory cells such as macrophages and neutrophils, and later can involve the activation of lymphocytes which help initiate adaptive immunity (Janeway, Travers, Walport, & Sklochik, 2001).

Adaptive Immunity

Adaptive immunity is the secondary line of defense against non-self-pathogens, and is specific to the pathogen presented providing long lasting protective immunity. Adaptive immunity is initiated when the innate response is unable to eliminate a new pathogen, and antigen presenting cells are delivered to lymphoid tissues where a lymphocyte encounters the antigen (Janeway et al., 2001). Clonal expansion and differentiation of lymphocytes leads to the production of T-cell and antibody secreting B-cells. Naive B-cells can become either memory cells or effector B-cells.

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T-cells express T-cell receptors such as CD4 or CD8, which recognize antigens bound to membrane bound receptor molecules called Major Histocompatibility Complex class 1 (MHCI) and class 2 (MHCII). When the antigen presenting cells present these MHC molecules, the CD4 and CD8 T-cell receptors bind to the MHC activating T-cell recognition. Mature T-cells are divided into three types of cells: helper T-cells (express CD4), cytotoxic T-cells (express CD8), and T regulatory cells (express CD4 and CD25). Helper T-cells help activate B-cells and other immune cells, cytotoxic T-cells remove pathogens and infected host cells, and T regulatory cells distinguish between self and non-self-molecules (Hansson, Libby, Schonbeck, & Yan 2002).

Once T-cells recognize foreign antigens they initiate an adaptive immune response specifically against those antigens, directly attacking the antigen bearing cells by cytotoxic lymphocytes, stimulating B-cells to produce antibodies against the antigens, and induce inflammation to eliminate the foreign pathogen from the body. Overall, the adaptive immune system provides more specificity to a pathogen than the innate immune system, and also remembers previously introduced pathogens providing long lasting defense (Hannson et al., 2002).

Factors Leading to Antibody Immunogenicity

Physical Properties

An undesired adaptive immunogenic response to a therapeutic protein can be linked to a variety of factors. Physical properties of the product such as protein structure and peptide sequence can affect post-translational and chemical modifications of the protein, altering their immunogenicity profile. Aggregation, fragmentation, oxidation,

and glycosylation may also activate the adaptive immune system by generating an innate immune response which can all lead to higher levels of immunogenicity of a biotherapeutic (Joubert et al., 2016). In order to effectively control the product related factors, modifications need to be made to the peptide sequence of the protein itself to develop a more stable biotherapeutic (Groell et al., 2018).

Process Related Factors

There are also process related factors such as host cell protein (HCP), residual DNA (rDNA), raw material derived impurities, and contaminants such as viruses, bacteria and their toxic by-products, that can contribute to the higher immunogenicity response of a protein (Eon-Dubal et al, 2012).

Endotoxin Contaminants. Endotoxins are a common example of toxic by-products of bacteria. Endotoxins are lipopolysaccharides (LPS) found in the outer cell wall of Gram negative bacteria, and their toxicity can lead to severe adverse effects such as systemic inflammation and sepsis. Contaminants such as endotoxin can be introduced within the mAb production process or by non-sterile process conditions, and must be monitored and sufficiently removed prior to mAb administration *in vivo* (Ongkudon, Chew, Liu, & Danquah, 2012).

Host Cell Proteins (HCP). Host cell proteins are endogenous proteins derived from the host cell that can be released during cell culture during cell apoptosis (Eon-Duval, Broly, & Gleixner, 2012). HCPs in the drug product can induce an unwanted immunogenic response through biological activity, protease activity leading to fragmentation, and when acting as an enhancer causing an immune response to the biotherapeutic itself (Jawa et

al., 2016). HCP presence is likely to be unrecognized by the human body leading to hypersensitivity and adverse effects (Eon-Duval et al., 2012). However, the potential immunogenicity varies depending on the types of HCPs and their abundance. For example HCPs from mammalian expression systems share a high sequence homology with human proteins, resulting in a lower immunogenic response than seen from microbial HCP impurities (Duke & Mitra-Kaushik, 2019). The safety thresholds for some HCPs might be higher than others, but since the presence of any HCP could lead to an immunogenic reaction, it is required that the HCP limit in final biotherapeutic formulations be kept below 100 ng HCP/mg range (Levy, Valente, Choe, Lee & Lenhoff, 2013) (Chiverton et al., 2016).

Residual DNA (rDNA). Residual DNA (rDNA) in a biotherapeutic is another process related factor that poses the danger of an immunogenic response. Residual DNA is the DNA released by the host cell line during protein expression. It has been shown that different kinds of rDNA have the potential of developing an immune response in humans (Wang, Morgan, Wang, & Mozier, 2011). Bacterial rDNA is more likely to elicit an immune response, but it is possible for a response to occur due to mammalian host cell rDNA as well. Presence of mammalian rDNA in a biotherapeutic can also increase the potential of tumorigenic activity by direct integration of the DNA sequence into the patient's genome and expression of oncogenes, or by activation of a dominant proto-oncogene by integrating next to it. Although the risk for this tumorigenic activity is small, the maximum recommended level of rDNA in a biotherapeutic is 10ng per dose (Eon-Dubal et al., 2012).

Raw Material Derived Impurities. Raw material derived impurities such as components of cell culture media, feed solutions, components of purification buffers and resins, as well as excipients in the drug substance can lead to immunogenic activity by releasing toxic components that could alter the product. These factors can co-elute with the drug product and be toxic to humans. In an early discovery process the most monitored factor tends to be the protein A ligand of the protein A affinity chromatography which is used for mAb purification (Eon-Dubal et al., 2012). Protein A is a bacterial cell wall protein derived from *Staphylococcus aureus*. It is useful for binding a wide range of mammalian antibodies at the Fc domain, but has the potential risk of leaching into the final product with an eluted antibody, making it immunogenic (Singh, 2011). This study will focus on the effects of protein A resin on the CD4 T-cell proliferative response in our assay.

These process related factors could be introduced at various points in the protein production process, particularly during protein expression and purification. The cell culture process used for protein expression and the harvest clarification step done prior to purification are often a large source of HCP and rDNA variability due to the cell lysis that occurs during these steps (Zhang et al., 2014). Close attention needs to be paid to the condition of the cells during transfection, and cell viability and cell density at harvest, as the product of interest can often be secreted with HCP, nucleic acids, lipids, DNA, and other cellular material (Bracewell, Francis, & Smales, 2015).

Protein A affinity capture is unlikely to purify out all HCPs and rDNA. Protein A purification is effective in purifying out a majority of the HCPs found in the clarified bulk harvest, however it does not effectively purify out all HCPs (Aboulaich et al., 2014). Some HCPs may co-purify with the protein during protein A chromatography (Aboulaich

et al., 2014), and previous studies showed that HCP levels vary widely between eluates of different antibodies (Zhang et al., 2014) (Bracewell et al., 2015). Protein A chromatography can also lead to protein A ligand leaching, where the ligand co-elutes with the antibody. Protein A leaching can lead to an immunogenic response if not properly purified out (Carter-Franklin, Victa, McDonald, & Fahrner, 2007). Typically, other polishing steps such as size exclusion chromatography (SEC) or anion-exchange chromatography (IEX) are required to fully purify out the majority of host cell protein, rDNA, protein A ligands, and contaminants like endotoxin, however those steps are not always included in an early discovery protein production process.

Anion-exchange membranes are used in industry as polishing steps to bind impurities such as virus particles, endotoxin, host cell proteins, and DNA. Often they are run in "flow through" mode, meaning that the positively charged mAb flows through, while the membrane binds all of the negatively charged impurities. The membrane chromatography in flow through mode is advantageous over chromatography columns for contaminant reduction, as they are driven by throughput rather than mass, and can run at much higher flow rates (Fischer-Fruhholz, Zhou, & Hirai, 2010). An example of such an anion-exchange membrane is Sartobind Q membrane (Sartorius AG, Gottingen, Germany) which in previous studies has shown to reduce impurities such as viruses, Chinese Hamster Ovary (CHO) host cell protein, and DNA while also leading to good mAb recovery (Weaver, Husson, Murphy, & Wickramasinghe, 2013). Due to its ease of use, speed, and effective removal of process impurities, Sartobind Q membrane used in flow-through mode can be an effective secondary step in the early monoclonal antibody (mAb) discovery process.

Early Antibody Discovery Process

The goal of the early mAb discovery process is to identify a molecule that has activity for a specific target and can be a clinical candidate as a therapeutic for diseases related to that target. The early discovery process consists of target selection, followed by mAb candidate selection through High Throughput Screening (HTS) assays that measure binding to the target. Identifying a lead candidate that has good therapeutic activity, stability, developability and a low risk of immunogenicity requires production of hundreds of mAb candidates through DNA amplification, mAb expression in mammalian cells and purification using liquid chromatography (Hughes, Rees, Kalindjian, & Phipott, 2011).

mAb Expression

In our early discovery mAb production process, transiently transfected human embryonic kidney (HEK293) cells are used for protein expression. HEK293 cells are often used for high throughput recombinant protein expression due to their ease of handling and straight forward growth in culture. When transiently transfected, their titers are typically comparable to or higher than those of CHO cell lines, which are more frequently used during late stage development and manufacturing when the time needed to generated stable cell lines can be invested (Dumont, Euwart, Mei, Estes, & Kshirsagar, 2016). Production times are fifty percent faster when using HEK293 cells than stable CHO cell lines, and the transient transfection process is easily automatable which is

important for the high throughput nature of the early discovery process (Arena, Harms, & Wong, 2018).

mAb Purification

After the mAb is harvested, the conditioned media is purified using protein A affinity chromatography, where the protein A resin binds the Fc regions of the antibody. The protein is eluted using a high salt, low pH buffer, after which it is quickly neutralized. The protein is then analyzed by analytical size exclusion chromatography (aSEC) to determine the level of protein monomer and high molecular weight species (Mazzer, Perraud, Halley, O'Hara, & Bracewell, 2015), and sequence is confirmed using mass spectrometry. Samples are also tested for endotoxin using an Limulous amoebocyte lysate (LAL) assay. If the protein appears greater than 5 percent aggregated by aSEC or contains high endotoxin levels, it then undergoes further polishing purification steps. However, the product is not tested for HCP, rDNA, or protein A ligand leaching. The final product is submitted for a variety of screening assays, which will assist in lead biotherapeutic candidate selection.

Immunogenicity Predictions in Early Discovery Stages

Preclinical biotherapeutic candidate selection is aided by an immunogenicity prediction assay called the peripheral blood mononuclear cells (PBMC) based assay (Groell et al., 2018). This assay aims to replicate the immune response in humans through monocytes, dendritic cells, T- cells, and B-cells (Joubert et al., 2016). The PBMC assay measures the ability of the test biotherapeutic to induce antigen-specific T-cells in

PBMCs from healthy donors, leading to an antigen-specific response which is shown by T-cell proliferation as a way to predict immunogenicity in humans (Wullner et al., 2010). Various studies have shown correlation between the T-cell proliferation in the assay, and patient response to a biotherapeutic, finding it more predictive than *in vivo* animal studies which are not always predictive due to species differences (Baker, Reynolds, Lumicisi, & Bryson, 2010). Notably, the human system is very complex, and there are immunogenicity risks that could be impacted by the method of injection, dose, or the human disease state, which the PBMC assay might not predict. However, this *in vitro* assay can help identify a lead molecule at the discovery level based on the number of donors eliciting a CD4 T-cell proliferation response, where high number of donors eliciting a response can be a predictor of future immunogenicity (Schultz et al., 2017). Based on the CD4 T-cell proliferation results of a biotherapeutic at the discovery level, changes can still be made to the protein sequence to reduce this response prior to testing the biotherapeutic *in vivo* (Groell et al., 2018).

Improving Early Immunogenicity Risk Predictions by Identifying and Removing Impurities in mAbs

Predicting immunogenicity at an early stage of the discovery process is crucial for selecting and developing a successful biotherapeutic. In fact, early risk assessment is increasingly recommended by regulators to help with faster and more cost effective candidate selection (Duke & Mitra-Kaushik, 2019). Through the information gained by immunogenicity assays, we can improve the protein by altering it to be less immunogenic before it moves on to the clinic. To achieve a successful prediction about the protein's

immunogenicity, we need to ensure that we are not introducing immunogenic factors into the samples during the production process. Producing reproducibly clean and low-immunogenic material at such an early stage in drug discovery is challenging, as there are fewer polishing steps involved in cleaning up the impurities of the protein, creating a higher likelihood of a high immunogenicity assay result for a non-immunogenic protein. In our experience, we have seen multiple batches of the same non-immunogenic mAb show different levels of CD4 T-cell proliferation responses in the PBMC assay, however, the cause of this variability is still unclear.

In an effort to improve the accuracy of our CD4 T-cell proliferation assay, we analyzed multiple factors in the test material that could be impacting the PBMC assay signal. The three batches of the mAb showing variability in percent donors responding with CD4 T-cell proliferation were analyzed first. This mAb was an anti VEGF monoclonal antibody, bevacizumab (Avastin) made by Genentech, which showed variability in percent donors with CD4 T-cell proliferation levels when made using our early discovery production methods. Bevacizumab is a commercially available biotherapeutic developed by Genentech, used for anti-angiogenic therapy to stop tumor growth in patients with metastatic colorectal cancer (mCRC) (Kazazi-Hyseni, Beijnen, & Schellens, 2010). The drug has been shown to have low immunogenicity in the clinic (U.S. Food and Drug Administration, 2004) but when produced using our early discovery process, some batches showed a variable percent of donors with a positive CD4 T-cell proliferation response in PBMC assays.

In total, three batches of bevacizumab were generated separately and each batch was tested in a different cohort of the PBMC assay. Commercial bevacizumab was used

as a negative control and was tested in each of the three cohorts. The results showed

batch to batch variability in the percentage of significantly positive CD4 T-cell

proliferative response in forty healthy donors per cohort compared to the commercial

bevacizumab (12.9 ± 1.9 percent), which showed no significant variability between the

three assays. Bevacizumab batch 1 showed CD4 T-cell proliferation in 18.9 percent of

PBMC donors, bevacizumab batch 2 showed a response in 10 percent of PBMC donors

and bevacizumab batch 3 showed a response of 42.5 percent PBMC donors. This batch to

batch variability of a known non-immunogenic mAb suggests that our early discovery

process is inconsistent in reducing immunogenic contaminants leading to a positive CD4

T-cell proliferation response in the PBMC assay that is not drug related and impairs the

reliability of our immunogenicity predictions.

We hypothesized that rDNA, HCP, and protein A ligand contamination

introduced during the early discovery mAb production process could be causing the false

positive CD4 T-cell proliferation observed in the various batches of bevacizumab, and

that a secondary purification step could remove those impurities and lead to more

accurate immunogenicity predictions. This hypothesis was tested by first analyzing the

three previously generated bevacizumab batches for rDNA, HCP, and protein A ligand

contamination, to determine whether the presence of those factors influenced

immunogenicity. Second, the bevacizumab mAb was produced via two methods: one

with a one-step purification with protein A chromatography, and second with a two-step

purification method with protein A chromatography followed Q-membrane

chromatography run in flow-through mode (Q Flow Through). The newly generated

bevacizumab was analyzed for the same process impurities and was tested in the PBMC

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assay to see if the percent of donors with positive CD4 T-cell proliferation were reduced in samples that had undergone a secondary purification step. The goal was to optimize the early discovery mAb production process to successfully remove the immunogenic contaminants causing positive CD4 T-cell proliferation in PBMC donors and allow us to make more accurate early stage predictions about the future immunogenicity response of the biotherapeutic candidates.

Chapter II:

Research Methods

The purpose of this study was to determine if the HCP, rDNA or protein A ligand contaminants, present in our samples following mAb production using the early discovery process, caused variability in the CD4 T-cell proliferation response that was observed in our PBMC assays. Three batches of bevacizumab generated using the early discovery production process were tested in our PBMC assay, where a different PBMC donor cohort was used to test each bevacizumab batch. The bevacizumab samples were previously expressed at a 200ml scale using the Expi293 mammalian expression system, followed by protein A chromatography, and buffer exchange into 1X PBS. Commercial bevacizumab was used as a negative control in each PBMC assay, and showed a low CD4 T-cell proliferation response with 12.9 ± 1.9 percent donors responding. The early discovery generated bevacizumab batches showed varying levels of CD4 T-cell proliferation in PBMC assays compared to the commercial bevacizumab, where 18.9 percent of donors showed CD4 T-cell proliferation to bevacizumab batch 1, 10 percent responded to bevacizumab batch 2, and 42.5 percent responded to bevacizumab batch 3.

The existing information about the production of the three variable bevacizumab batches (batches 1-3), such as cell line and transfection reagents used, expression scale, purification methods, and available analytics was collected and compared for process differences. The bevacizumab mAb was then re-expressed and purified in three new batches (batches 4-6) to see if there was again batch to batch variability in the PBMC

assay and to determine if that variability correlated with the contaminant HCP, rDNA or protein A ligand levels in the sample. The new bevacizumab batches were purified using one step purification with protein A chromatography, and half of the eluted material was further purified using Q Flow Through chromatography to remove additional impurities. The new and old bevacizumab batches were analyzed for HEK HCP, protein A ligands, and residual DNA levels. Finally, the new bevacizumab batches purified by both one step protein A purification and two step purification (protein A followed by Q Flow Through) were tested in the PBMC assay to determine if there was batch to batch variability and a high percent of donors with CD4 T-cell proliferation after the protein A purification, and whether the variability and percent of donors with CD4 T-cell proliferation levels was reduced by the Q Flow Through purification step.

Expression of Bevacizumab

Bevacizumab was expressed in Expi293F cells which were cultured in Expi293F Expression Medium following the manufacturer's guidelines. The cells were split two days before transfection at a cell density of 0.8x10^6 cell/ml. On the day of transfection, when cells were at a cell density of 2.5-3.0x10^6 cells/ml, three 750 ml batches of Expi293 cells were transfected. Cell densities and viabilities at the time of transfection are listed in Table 1 below.

Table 1: Cell densities and viabilities at time of transfection.

Batch #	Cell Density (x10^6) cells/ml	Cell Viability %
Bevacizumab Batch 4	2.5	98.5
Bevacizumab Batch 5	2.9	98.6
Bevacizumab Batch 6	2.8	98.8

Table 1 shows cell densities and viabilities at time of transfection for the new batches of bevacizumab.

The cells were transfected with expression vectors containing a codon optimized bevacizumab DNA sequence in 1.6 L Thomson Flasks, using the ExpiFectamine 293 Transfection Kit 10L (ThermoFisher Scientific, cat A14525), which is composed of the transfection reagent Expifectamine293, and transfection Enhancers 1 and 2 provided in the kit.

A DNA ratio of one part Heavy Chain (HC) to two part Light Chain (LC) at a concentration of 0.5 ug/ml of culture was used for this transfection. The LC and HC DNA starting concentrations were approximately 2 mg/ml for each batch of cells, 68 ul of HC and 138.5 ul LC was used. 2.7 ul ExpiFectamine293 (ThermoFisher Scientific, cat# 100014995) per ug DNA was then diluted in 10 percent of culture volume using OptiMEM (ThermoFisher Scientific, cat# 2085273). The Expifectamine and OptiMEM were left to incubate for 5 minutes. Following the incubation, DNA was added to the Expifectamine and OptiMEM mixture, and incubated for 15 minutes to allow the complex to form. Post incubation the complex was added to the 750 ml of Expi293 cells. The transfected cells were incubated at 37C, 8 percent CO_2, shaking at 125 RPM for 24 hours. After 24 hours the cells were fed with 0.5 percent working volume of Enhancer 1, a formulation used to support high-density transient transfections, and 5 percent working volume of Enhancer 2, a formulation used to enhance protein production. The cells were then incubated at the same conditions for 4 more days.

On day 5 after transfection we measured the cell density and viability, harvested and clarified the media. The day 5 cell densities and viabilities are listed below (Table 2).

Table 2: Cell densities and viabilities at time of harvest.

Batch #	Cell Density (x10^6) cells/ml	Cell Viability %
Bevacizumab Batch 4	4.17	79.5
Bevacizumab Batch 5	4.54	82.7
Bevacizumab Batch 6	4.32	85

Table 2 shows the cell densities and viabilities of the new bevacizumab batches at time of harvest.

The cells were removed from the media by spinning the cultures at 5,200 RPM for 20 minutes. The media containing the expressed and secreted antibody was then clarified and collected by filtering the supernatant over a 0.22um PES membrane vacuum filter. Bio-layer interferometry (using the Pall Blitz system and quantitative protein A sensors) (ForteBio, cat #18-5012) was used to estimate the protein titers in the filtered supernatant for each batch.

Protein A Purification of Bevacizumab

The three 750 ml batches of bevacizumab were first purified using protein A chromatography. The protein A chromatography was performed on the GE AKTA Avant 25 instrument, using a MabSelectSure 5ml prepacked column (GE Lifesciences, cat#11-0034-95). Prior to starting the method the system was cleaned using 0.2M Sodium Hydroxide, followed by a prime of all of the buffer lines in the buffers necessary for purification. The column was equilibrated with 1X DPBS (ThermoFisher, cat # 14190250). 750 ml of the clarified media was loaded on the column, and eluted with 100 mM Glycine, 150 mM Sodium Chloride pH 3.0. The mAb was eluted into 1M Tris pH 8 at 10 percent of elution volume, to neutralize the eluted protein. This process was repeated for each batch. 15 mls of protein eluate was collected for each batch, and the protein concentration was determined by measuring the A280 by nanodrop and

calculating the final concentration, accounting for the molar extinction coefficient of 0.6 mg/ml.

Sartobind Q Flow Through Chromatography

Half of the material eluted from the protein A purification was further purified using Sartobind Q Flow Through chromatography. The purification was done on an AKTA Avant 25 instrument, using a Sartobind Q SingleSep nano 1 ml column (Sartorius, lot # 426001003). The column was cleaned with 1M Sodium Hydroxide, then charged with 20 mM Tris pH 7.2, 1 M Sodium Chloride. The column was equilibrated with 20 mM Tris pH 7.2, and then half of the sample (7 ml) eluted from protein A chromatography was loaded through a loop chasing it down with 20 mM Tris pH 7.2. The mAb which does not bind the membrane was collected in the flow through separating it from impurities which bound the column. The bound impurities were then stripped using 20 mM Tris pH 7.2, 1 M Sodium Chloride. This process was repeated for each of the three batches. 15 mls of purified protein were collected for each batch, and the protein concentration was determined by measuring the A280 by nanodrop and calculating the final concentration by accounting for the molar extinction coefficient of 0.6 mg/ml.

Buffer Exchange

The six bevacizumab samples (three samples eluted from protein A purification only, and three samples eluted from protein A and Q Flow Through) were buffer exchanged using 5L of 1X DPBS. A Slide-a-Lyzer™ G2 Dialysis Cassettes 20kDa

(ThermoFisher) was used for dialysis. The 7 ml samples (protein A eluate) were dialyzed using the 15 ml capacity cassettes (ThermFisher, prod # 87736) and the 15 ml samples (Q Flow Through eluate) were dialyzed using the 30 ml capacity cassettes (ThermoFisher, prod # 87725). Dialysis was done overnight at 4C. After dialysis the protein concentration was determined by measuring the A280 by nanodrop and calculating the final concentration by accounting for the protein extinction coefficient of 0.6 mg/ml.

Endotoxin Measurement

Endotoxin levels were measured using the Endosafe MCS-100 (Charles River) Instrument. Each of the six protein samples was diluted in 1X DPBS at a 1:10 dilution in a 250 ul volume. The dilutions were performed in the tissue culture hood in 2 ml sterile Eppendorf tubes to prevent any additional endotoxin contamination. These samples were then used for the endotoxin test, using 5-0.05 EU/ml Endosafe cartridges (Charles River, lot # 9546140).

Analytics

All samples were submitted for analytical size exclusion chromatography (aSEC) and mass confirmation by liquid chromatography – mass spectrometry (LC-MS). The analytical SEC was run using a TOSOH TSKgel SuperSW3000 250A (4.6 mm ID x 30 cm, 4um) column, using 100 mM Sodium Phosphate, 150 mM Sodium Chloride, pH 7.2 as running buffer. LC-MS Intact Mass was run using the Zorbax C8 RRHD 2.1 x 50 mm, 1.8 um column, and 0.1 percent Formic Acid in Water as Buffer A and 0.1 percent Formic Acid in acetonitrile as Buffer B.

Host Cell Protein Assay

The HEK293 HCP ELISA kit (Cygnus Technologies, cat# F650R) was used to analyze all samples for HCP. The protocol provided with the kit was followed to perform the ELISA. A 96-well anti-HEK 293 coated microtiter plate (F652R) was first coated with 100 ul of anti-HEK 293:HRP (#F651R), then 50 ul of standards and samples were added, and the plate was incubated at room temperature for 2 hours while shaking at 400-600 RPM. The kit contained human HCP standards at the following concentrations: 0, 4, 10, 25, 75, and 200 ng/ml (cat# F653R). The samples were diluted to previously determined concentrations in which the HCP levels of the samples fell within the standard curve range. The samples were diluted using Cygnus Catalog # I094 preservative, which is the preservative used for the HCP standards. After 2 hours the plate was washed 4 times with Tris buffered saline wash concentrate (cat# F004), and 3,3',5,5' Tetramethylbenzidine (TMB) substrate (#F005) was added to the plate, which was then incubated for 30 minutes at room temperature without shaking. The reaction was then stopped with stop solution (#F006) and the absorbance was measured on an Envision plate reader at 450 nm. The data was analyzed using GraphPad software, which applied the sigmoidal/ 4PL/ least squares fit calculations.

Protein A Leaching Assay

The Cygnus Technologies Protein A ELISA Kit (cat #F400) was used to measure the protein A leaching in the purified samples. The assay was performed by using the protocol enclosed with the kit. The samples were first denatured by adding 100 ul of

samples to a 96-well sample treatment plate, and incubating with Sample Denaturing Buffer (cat# F054) at 80C for 15 minutes. The plate was heated at 80C for 15 minutes, and then cooled for 5 minutes. The plate was then centrifuged for 5-10 minutes at 3000 x g, and the supernatant was collected for analysis by ELISA. For the ELISA a microtiter plate (cat# F052) was coated with anti-Protein A: HRP (cat# F401) and 50 ul of the supernatant was added to the plate. The plate was covered and incubated on a shaker at 400-600 rpm for 2 hours at room temperature. Following this incubation the plate was washed four times with wash buffer, and TMB substrate (cat# F005) was added. The plate was incubated for 30 minutes without shaking at room temperature after which 0.5M sulfuric acid (Stop Solution) (cat# F006) was added and the absorbance was read at A450 nm on the Envision plate reader. The data was analyzed using GraphPad software, using the sigmoidal/ 4PL/ least squares fit calculations.

<center>Residual DNA Quantitation Assay</center>

The residual DNA quantitation assay was performed using the Cygnus Human Host Cell DNA Kit in 96 Well Plate "DNA Dye Binding Assay for the Measurement of Residual Human Host Cell DNA" (cat# D160W). The procedural notes were followed exactly as recommended by Cygnus Technologies. Samples were first diluted in Cygnus Tris-EDTA (TE) Buffer (cat# D001). 250 ul of diluted sample was transferred to the 96-well deep well plate (cat# D102) provided by the kit, and digested by incubating at 60C with Proteinase K (cat# D101). The standards (0, 1 , 2.5, 8, 30, 75, 200 ng/ml) (cat# D163) were then added to columns A-G rows 1-3 in the plate, which were previously left blank as the human standards should not be proteinase treated.

The DNA extraction was performed by incubating the samples with the DNA Extraction Buffer (cat# D105), followed by the DNA Precipitation Buffer (cat# D106). A series of wash steps using DNA Wash Buffer (cat# D104) was then performed to get rid of any contaminants. Finally the samples were dyed with Quant-iT PicoGreen dsDNA reagent Solution (cat# D003), and read on an Envision plate reader at Ex: 485 nm, Em: 525 nm. GraphPad was used for data analysis using the sigmoidal/ 4PL/ least squares fit calculations.

<div align="center">PBMC Proliferation Assay</div>

The PBMC proliferation assay was used to measure the proliferation of CD4 T-cells in peripheral human PBMC cells when in contact with protein biologics. First the PBMCs from healthy donors were isolated by Ficoll gradient centrifugation (GE Healthcare, Chicago, IL) or by MACSprep Miltenyi PBMC isolation kit (Miltenyi Biotech In, Auburn, CA), and were then frozen at -80C. A panel of 40 healthy PBMC donors composed of HLA class II types closely matching the world population frequencies was used for an assay run. Proliferation of CD4 T-cells was measured by loss of carboxyfluoroscein succinimidyl ester (CFSE) intensity. Commercial bevacizumab (Genentech, Inc, South San Francisco, CA) was used as a known negative control which provided an immunogenicity response of 0.63 percent in clinical trials (U.S. Food and Drug Administration, 2004), and was therefore expected to elicit a low CD4 T-cell proliferation response in donors in the PBMC assay. Keyhole limpet haemocyanin (KLH) (Millipore-Sigma, cas #9013-72-3) was used as a known positive control, which previously elicited a 92 percent immunogenic response in humans *in vitro* (Curtis, Hersh,

Butler & Rossen, 1971), and was therefore expected to elicit a high CD4 T-cell proliferation response in our assay.

First the PBMC cells were thawed at 37C, and mixed with 25ml RPMI (Lonza, cat # 12-115F) and Benzonase media. The cells were centrifuged at 1250 RPM at room temperature for 10 minutes, and the supernatant was then poured off and the cells were resuspended in 10 ml 1X PBS pre-warmed at 37C. The cells were then diluted 1:10 and counted on a Vi-Cell.

CFSE (Molecular Probes, Cat# C34554) labeling was then performed. PBMC cells were brought to 1 million cells/ml with warm 1X PBS, and 0.5 mM CFSE was added per 1 ml PBMCs (1 million cells/ml in 1X PBS). The CFSE labeled cells were then incubated at 37C for 10 minutes, while being inverted 4-5 times throughout the incubation. 1 ml human serum was then added to each tube to stop the CFSE conjugation, and inverted 5-6 times. The conjugated PBMCs were centrifuged at 1250 RPM for 10 minutes, then the supernatant was slowly poured off and the PBMCs were resuspended with 10 ml of warm 1X PBS. The PBMCs were counted using the Vi-Cell, and 1X PBS was then added to the cells bringing the volume up to 40 mls. The cells were centrifuged again at 1250 RPM for 10 minutes. The supernatant was poured off and the PBMCs were resuspended with complete RPMI media which consists of 10 percent Human AB serum, 1 percent MEM NEAA (Gibco, cat# 11140-050), and 10 percent Pen-strep solution (Gibco, cat# 15140-122) in RPMI media, adjusting the cell concentration to 4 million cells/ml.

The protein samples were prepared by diluting each sample in 3.5 ml of warmed complete RPMI media. The PBMCs from each donor were plated into 96-well plates at

50ul/well (200k cells/well) with one donor cell line per plate, amounting in 40 96-well

plates total. The protein samples, as well as the controls, were dispensed at 50 ul/well in

each 96-well plate in 6 replicates per sample. The plates were then incubated at 37 C for

7 days.

After 7 days the cells were prepared for Fluorescence Activated Cell Sorting

(FACS). First, 100ul of FACS buffer (500 ml PBS + 5 g BSA) was added and

centrifuged at 1250 RPM for 5 minutes, at 4C. The supernatant was aspirated and 100 ul

of anit-CD4 APC (1:30 dilution) was added to the cell pellets. The plates were then

incubated at 4C for 20 minutes in the dark. 100 ul FACS buffer was added to each well

and the plates were centrifuged at 1250 RPM, 4C, 5 minutes again. The supernatant was

removed and the cells were fixed by adding 50ul fixation buffer (1X PBS with 3.7

percent formalin). The plate was then run on FACS Canto, and analyzed.

FACS analysis was based on creating four gates. The first gate was used to

identify all lymphocytes by looking at forward scatter (cell size) versus side scatter (cell

granularity). The second gate was used to narrow down the lymphocytes by looking at

CFSE on the X-axis and forward scatter on the Y-axis. The third gate was used to

identify all CD4 cells by looking at CD4 cells on the X-axis and forward scatter on the Y-

axis. The fourth gate was used to separate the resting CD4 cells and the proliferating CD4

cells, by looking at CD4 on the Y-axis and CFSE on the X-axis. The proliferating CD4 T-

cells would be CFSE negative, as they lose the CFSE label during proliferation. A donor

response was considered positive if it was above 2 standard deviations over the average

of the media proliferation from the 6 replicates on each donor plate. At least 2 out of 6

protein replicates should have elicited a positive donor response to be considered

positive, to prevent false positive assessments. The strength of the response was determined by measuring the sum of all the average percentages of CD4 T-cell proliferation above background per each donor in response to each protein batch.

Chapter III:

Results

Analysis of Bevacizumab Batches Showing Batch to Batch Variability in PBMC Assay

The previously expressed and purified bevacizumab batches yielded an

unexpected percent of donors with CD4 T-cell proliferation compared to the commercial

bevacizumab in our PBMC assays. The assay results are shown in Figure 1, where the

CD4 T-cell proliferation donor response to commercial bevacizumab (negative control)

between the three runs was 12.9 ± 1.96 percent, donor response to bevacizumab batch 1

was 18.9 percent in run 1, the donor response to bevacizumab batch 2 was the lowest at

10.3 percent in run 2, and the donor response bevacizumab batch 3 was the highest at

42.5 percent in run 3.
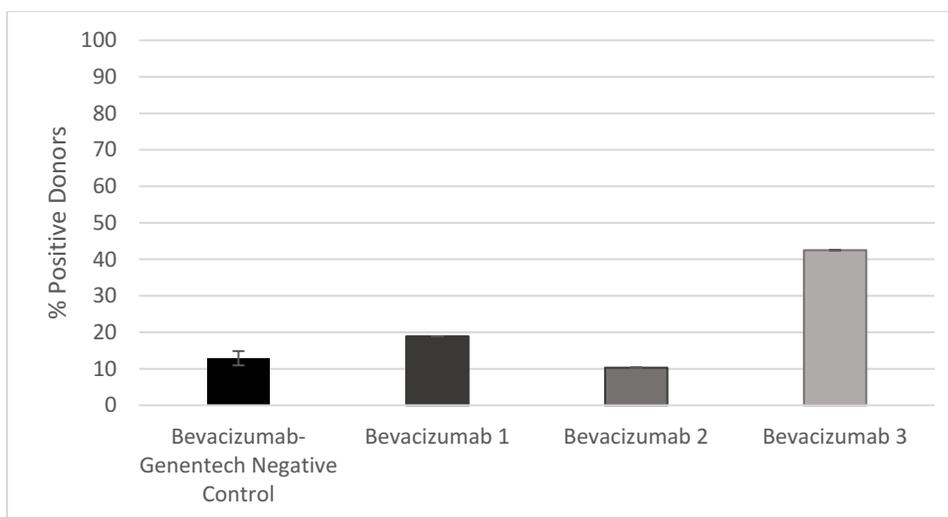
Figure 1: Percent positive donors in PBMC assay for original three batches of bevacizumab.

The bar graph depicts batch to batch variability in percent donors with positive CD4 T-cell proliferation to the original three batches of bevacizumab made using the early discovery process compared to the commercial bevacizumab control.

The three PBMC assays were run using different donor cohorts, and the donor response to the commercial bevacizumab was low and stable between the three cohorts meaning that the variability was likely not caused by the assay. Batch to batch variability was observed between the in-house bevacizumab where a positive response was observed in bevacizumab batch 1 and bevacizumab batch 3 but not in bevacizumab batch 2, making the immunogenicity prediction for this protein using these samples unreliable.

The expression titers, purified protein concentrations, endotoxin, aSEC, HEK HCP, residual protein A, and rDNA levels in the three batches were analyzed for any differences between the three bevacizumab batches that could explain the variable PBMC assay results. Table 3 below shows a summary of the results.

Table 3: Data summary of original three bevacizumab batches produced using the early discovery process.

Protein	Bevacizumab 1 Run 1	Bevacizumab 2 Run 2	Bevacizumab 3 Run 3
Protein Type	mAb	mAb	mAb
Expression System	Expi293	Expi293	Expi293
Expression volume (L)	0.2	0.2	0.2
Expression titer (mg)	13.9	NA	15.5
Purified Protein Conc. (mg/ml)	1.8	1.4	1.5
Purified Protein volume (ml)	15.0	16.5	17.0
Purified Protein delivered mass (mg)	27.0	23.0	26.0
EU/mg	< 0.055	< 0.072	< 0.065
SEC % monomer	96.6	96.7	97.1
Sequence Confirmed (intact LC-MS)	Yes	Yes	Yes
ng HCP/ mg protein	1006.0	31206.7	5917.8
ng Protein A/ mg protein	2.2	2.3	2.9
ng Residual DNA/ mg protein	7.1	3.8	6.1
PBMC Assay Cohort	49	50	51
PBMC Assay Results % positive donors	18.9	10.3	42.5

Table 3 shows the comparison of the expression and purification titers, analytics, process impurities, and PBMC assay results of the three original bevacizumab batches produced using the early discovery process.

Since the three batches were generated at three different times we also looked closely at any difference in the methods used for expression and purification. Each batch was expressed at a 0.2L scale in the Expi293 cell line, and was then purified using protein A purification followed by buffer exchange into 1X PBS. There were no differences observed in the production methods used for the three batches, however bevacizumab batch 1 and 3 were expressed by one user, and bevacizumab batch 2 was expressed by another user. All three batches were purified by the same user utilizing the same methods. No additional purification steps were performed.

All three batches had low EU/mg levels below 0.072 EU/mg which is below the required 1 EU/mg previously determined as the cutoff for our PBMC assay. All samples showed high monomericity (96-97 percent monomer) indicating there was little protein aggregation, and all clones sequence confirmed by LC-MS.

The HCP levels across the three batches were higher than the industry and regulatory authority recommended HCP amounts of being below100 ng HCP/mg protein (Levy et al., 2013) (Chiverton et al., 2016), ranging from 1006.04 ± 670 ng HCP/mg protein in bevacizumab batch 1, 5917.77 ± 1973 ng HCP/mg protein in bevacizumab batch 3, and as high as 31206.69 ± 7169 ng HCP/mg protein in bevacizumab batch 2 (Figure 2). Although batch 2 showed a higher level of HCP than the other two batches, it also had the lowest percent response in the PBMC assay (10.3 percent), which was consistent with the commercial bevacizumab (negative control) with a percent response of 12.9 ± 1.96 percent. This finding leads us to believe that it is unlikely that HEK HCP is the cause of unexpectedly high donor CD4 T-cell proliferation response observed in the PBMC assay.
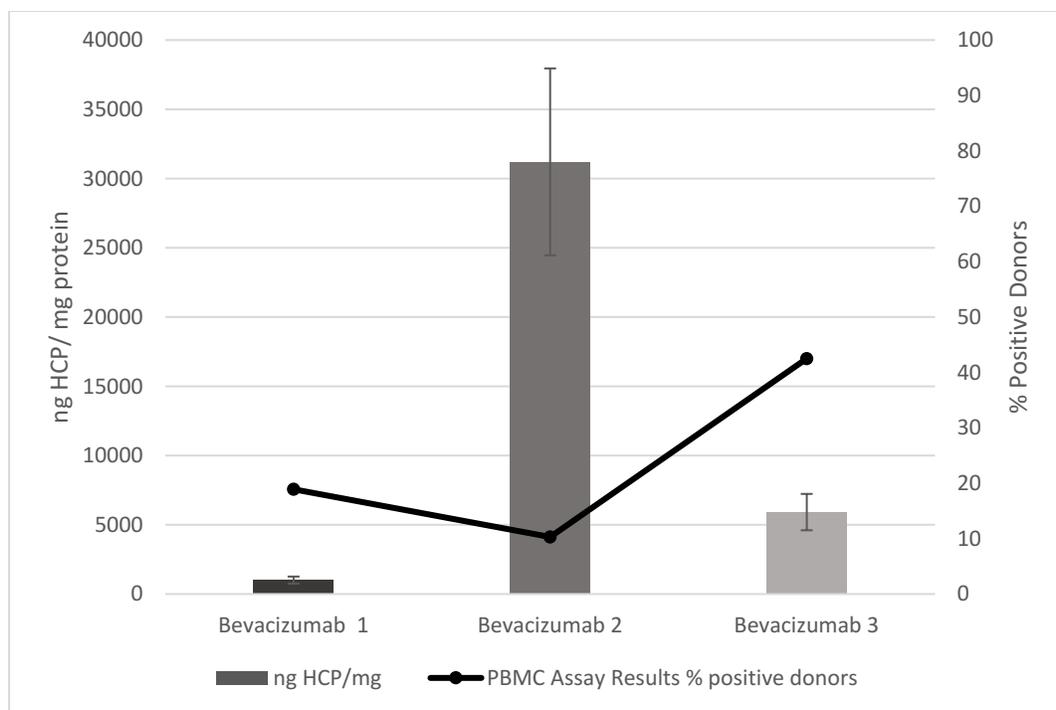
Figure 2: HCP levels vs percent positive donors in PBMC assay for original three batches of bevacizumab.

The grey bars show ng HCP/ mg bevacizumab, while the black line shows the percent positive donors in the PBMC assay for each of the three original bevacizumab batches produced by the early discovery process. Standard deviation is for n=2. There is no correlation observed between HCP levels and percent of donors with CD4 T-cell proliferation.

The observed level of protein A leaching was similar between the three batches, ranging from 2.22 ± 0.15 ng protein A/mg protein in bevacizumab batch 1, 2.31 ± 0.15 ng protein A/mg protein in bevacizumab batch 2, and 2.97 ± 0.03 ng protein A/ mg protein in bevacizumab batch 3 (Figure 3). There was some correlation between protein A and immunogenicity with the highest levels of protein A leaching and the highest percent positive donors with CD4 T-cell proliferation in bevacizumab batch 3, but more data would need to be generated to determine if that observation is reproducible.
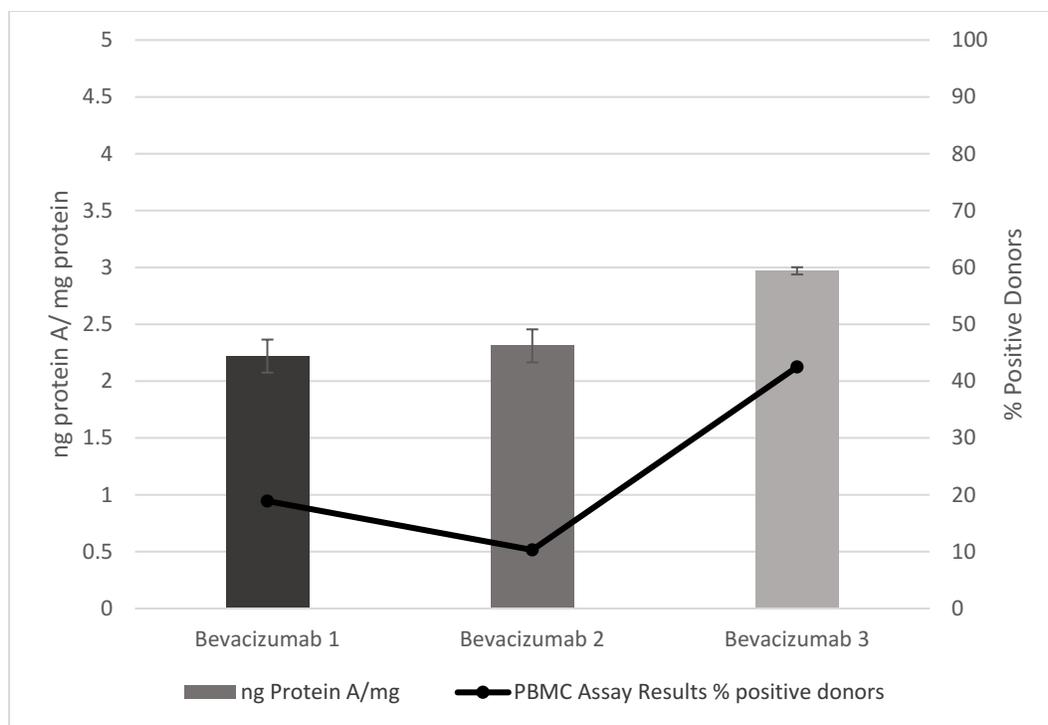
Figure 3: Protein A ligand levels vs percent positive donors in PBMC assay for original three bevacizumab batches.

The grey bars ng protein A ligand/ mg bevacizumab, while the black line shows the percent positive donors in the PBMC assay for each of the three original bevacizumab batches produced by the early discovery process. Standard deviation for n=2. Increased levels of protein A in bevacizumab 3 also show an increase of percent donors with CD4 T-cell proliferation in the PBMC assay, however the correlation for bevacizumab 1 and 2 is not strong.

Residual DNA concentrations in the three batches of bevacizumab ranged from 7.06 ng ± 0.76 rDNA/mg in bevacizumab 1, 3.8 ± 0.88 ng rDNA/mg in bevacizumab batch 2, and 6.05 ± 1.15 ng rDNA/mg in bevacizumab batch 3. There was a correlation between the lowest amount of rDNA (3.8 ng rDNA/mg) in bevacizumab batch 2 and the lowest percent of donors responding in the PBMC assay (10.3 percent) found in bevacizumab batch 2. Bevacizumab batches 1 and 3 had a similar amount of rDNA, with 7 ng/mg in bevacizumab batch 1, and 6 ng/mg in bevacizumab batch 3, however there was no correlation between the highest amount of rDNA and percent donors with a

positive response in the PBMC assay (Figure 4). These results show that rDNA could

have impacted CD4 T-cell proliferation, however further investigation is necessary.
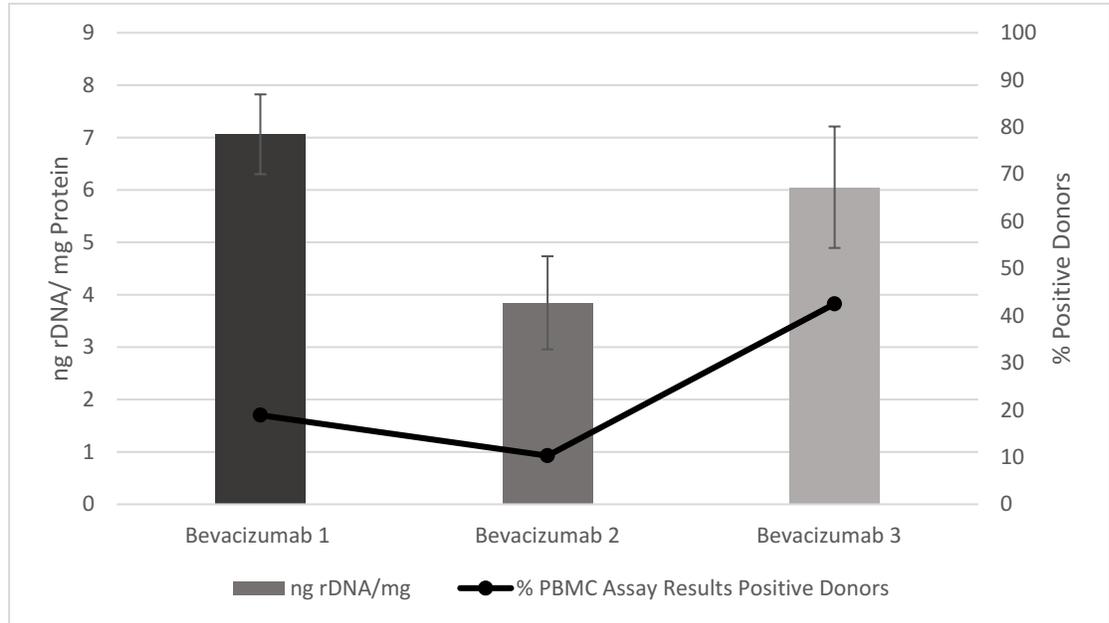
Figure 4: rDNA levels vs percent positive donors in PBMC assay for original three bevacizumab batches.

The grey bars show ng rDNA/ mg bevacizumab, while the black line shows the percent positive donors in the PBMC assay for each of the three original bevacizumab batches produced by the early discovery process. Standard deviation is for n=2. The lowest levels of rDNA in bevacizumab 2 also correlate with the lowest percent of positive donors that showed CD4 T-cell proliferation in the PBMC assay, however the correlation in bevacizumab 1 and 3 is not strong.

Analysis of Bevacizumab Batches Purified by One-Step vs Two-Step Purification

Since analysis of the original bevacizumab batches was inconclusive, we chose to

generate three new batches of the bevacizumab mAb and introduce an additional

purification step to further reduce process contaminants. The three batches of

bevacizumab protein (bevacizumab batches 4, 5 and 6) were generated using the same

early discovery production methods used for the production of the previous batches:

expression in Expi293 cell line, protein A purification, and buffer exchange into 1X PBS.

As an additional polishing step, half of the material eluted by protein A purification was also purified by Q Flow Through which is designed to reduce residual impurities. This process generated three batches of protein with two samples per batch, one purified by protein A purification alone, and the other purified by protein A purification and Q Flow Through. All samples were analyzed for HCP, protein A, and rDNA levels, and were then tested in the PBMC assay. Table 4 below summarizes the analysis results following one step purification with protein A chromatography and two step purification with protein A and Q Flow Through. The proteins were first assessed for protein concentrations, percent monomericity by aSEC, and endotoxin levels. After protein A purification the protein concentrations averaged 5.4 mg/ml with a total yield of 37 ± 1 mg for each batch of protein. After further processing the samples using Q Flow Through the sample concentrations averaged 2 mg/ml with a total yield of 31 ± 3 mg. There was an 18 percent protein loss during the Q Flow Through step.

Table 4: Analytics summary of newly generated bevacizumab batches using one step vs two step purification in the early discovery process.

Purification Method	Protein and Batch #	mg/ml in 1X PBS	ml	mg total in 1X PBS	% Monomericity	Mass Confirmed	EU/mg
Protein A	Bevacizumab 4	5.44	7.00	38.11	94.11	Yes	0.08
	Bevacizumab 5	5.27	7.00	36.88	93.96	Yes	0.08
	Bevacizumab 6	5.46	7.00	38.21	93.72	Yes	0.07
Protein A and Q Flow Through	Bevacizumab 4	1.79	15.00	26.89	96.78	Yes	0.41
	Bevacizumab 5	2.03	15.00	30.45	96.54	Yes	0.28
	Bevacizumab 6	2.33	15.00	35.03	96.23	Yes	0.21

Table 4 summarizes the analytics for the newly generated batches of bevacizumab purified by protein A vs protein A and Q Flow Through purification. Protein yields show that there was some protein loss after the Q Flow Through step. Percent monomericity improved post the Q Flow Through purification step. Endotoxin levels were below the 1EU/mg cutoff necessary for assays.

The analytical SEC results showed that post protein A purification the protein is at an average of 93.93 ± 0.16 percent monomer. Post Q Flow Through the monomericity increased to 96.5 ± 0.22 percent monomer on average, indicating that the Q Flow Through removed of some of the aggregation present post protein A purification. There was no significant batch to batch variability in yields or percent monomericity, and all of the samples mass confirmed by LC-MS.

Endotoxin levels were higher after the protein A and Q Flow Through purification than after protein A purification alone. The Q Flow Through is expected to bind and remove the endotoxin, so this was an unexpected result. It is likely that the endotoxin was introduced in the buffers used during purification. However, in all samples the endotoxin levels were between 0.07-0.41 EU/mg which is well below the level shown previously to affect CD4 T-cell proliferation levels in our PBMC assay (1 EU/mg).

Testing the HCP levels of the three new batches of bevacizumab processed using the two different purification methods showed that the Q Flow Through was successful in reducing the HCP in the samples, as seen in the Figure 5 below. The results show that after protein A purification the HCP levels were high with an average of 17,406 ± 2,236 ng HCP/mg protein with high variability between the three batches. However, post Q Flow Through purification the HCP levels were reduced by 99.7 percent to 44 ± 22 ng HCP/ mg protein, and there was much less variability in HCP levels between the three batches. These results confirmed that the Q Flow Through is a good secondary step for reducing HCP levels.
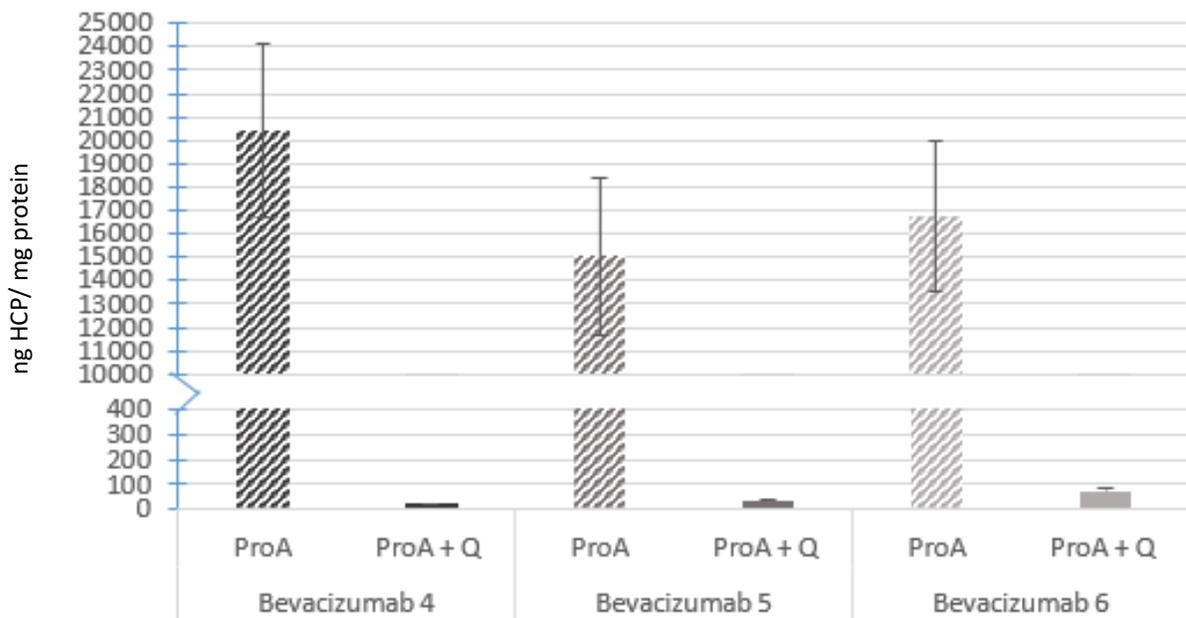
Figure 5: Comparison of HCP levels in bevacizumab batches purified by protein A vs protein A and Q Flow Through chromatography.

Dark grey bars depict bevacizumab batch 4, grey bars depict bevacizumab batch 5, and light grey bars depict bevacizumab batch 6. Diagonal stripes show protein A purification, and solid fill shows protein A and Q Flow Through chromatography. Standard deviation is for n=2. Q Flow Through significantly reduced the HCPs that were not reduced by protein A chromatography alone.

Testing the protein A levels between the three batches and two purifications methods showed that there was a decrease in residual protein A levels from samples purified by protein A purification alone versus the samples that were also polished by Q Flow Through after protein A purification. The samples purified by protein A chromatography alone had an average of 4.1 ± 0.4 ng protein A/ mg protein, while samples purified by protein A and Q Flow Through had an average of 2.5 ± 0.3 ng protein A/ mg protein. The Q Flow Through step reduced the residual protein A levels by 39 percent, which confirms that it is a good method for cleaning up protein A ligand post protein A purification (Figure 6).
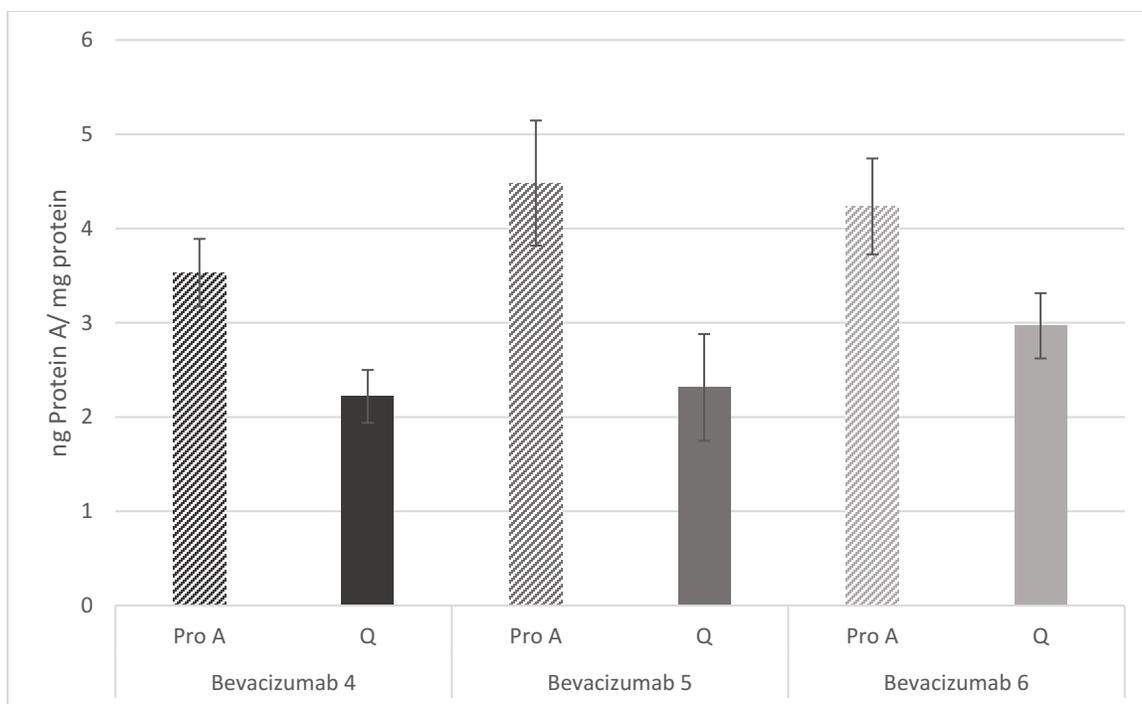
Figure 6: Comparison of protein A ligand levels in bevacizumab batches purified by protein A vs protein A and Q Flow Through chromatography.

Dark grey bars depict bevacizumab batch 4, grey bars depict bevacizumab batch 5, and light grey bars depict bevacizumab batch 6. Diagonal stripes show protein A purification, and solid fill shows protein A and Q Flow Through chromatography. Standard deviation is for n=2. Q Flow Through reduced the protein A ligand that was found post protein A purification.

The residual DNA levels were also reduced with the two step protein A and Q Flow Through purification compared to protein A chromatography alone. The material purified with protein A chromatography alone had an average of 19.7 ± 4.5 ng rDNA/ mg protein, but post Q Flow Through the rDNA levels were significantly reduced to an average of 4.5 ± 2.1 ng rDNA/mg protein. Q Flow Through purification reduced the residual DNA levels by an average of 77 percent, which was especially noticeable in bevacizumab batch 6 which had the highest level of rDNA at 41.2 ng rDNA/ mg protein after protein A purification, and was reduced to 6.0 ng rDNA/mg protein after Q Flow Through. Even in the sample with the lowest starting rDNA concentration of 7.7 ng

rDNA/mg, Q Flow Through purification reduced the rDNA levels below the limit of

detection of 1.5 ng rDNA/mg protein for bevacizumab batch 4 as shown in Figure 7

below.
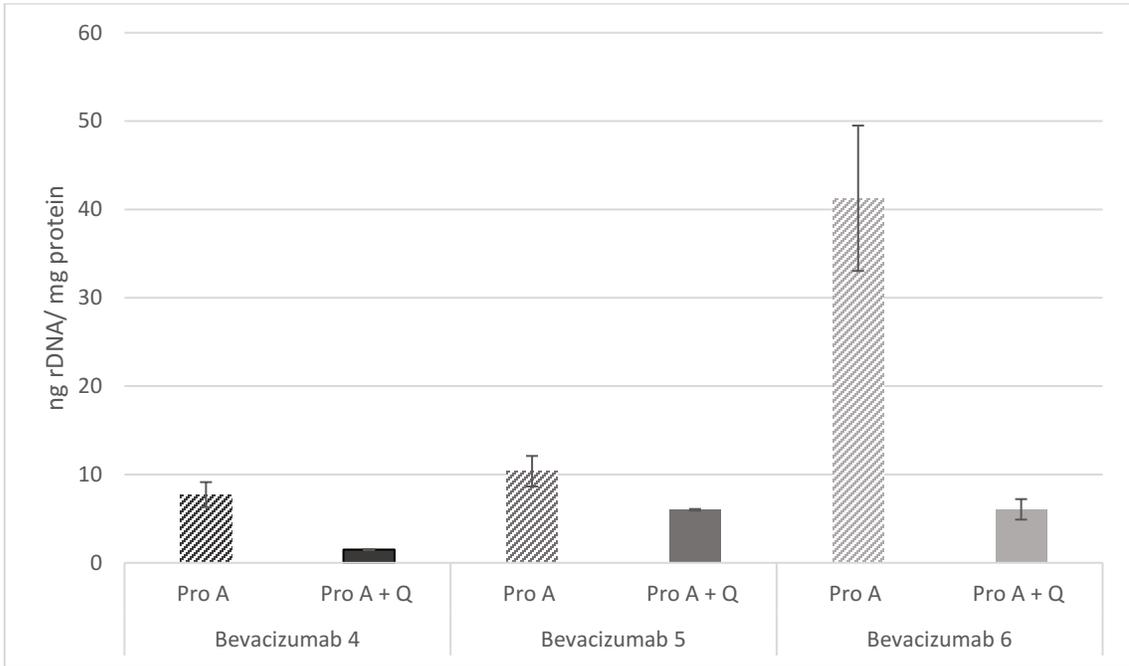
Figure 7: Comparison of rDNA levels in bevacizumab batches purified by protein A vs protein A and Q chromatography.

Dark grey bars depict bevacizumab batch 4, grey bars depict bevacizumab batch 5, and light grey bars depict bevacizumab batch 6. Diagonal stripes show protein A purification, and solid fill shows protein A and Q Flow Through chromatography. Standard deviation is for n=2. Q Flow Through significantly reduced the rDNA that was not reduced by protein A chromatography alone.

To determine whether or not these differences in HCP, protein A, and rDNA

levels, observed as a result of the differing purification processes, had an effect on CD4

T-cell proliferation, the PBMC assay was run twice. Figure 8 below shows the average

percentage of positive responders (out of two cohorts of forty donors each) for the two

assays. The commercially available version of bevacizumab, routinely used in this assay

as the negative control, yielded a low percentage of positive responders (10 ± 3 percent),

while the positive control (KLH) showed a high percentage of positive responders (92.45
± 5 percent). The control results were consistent with historical data.
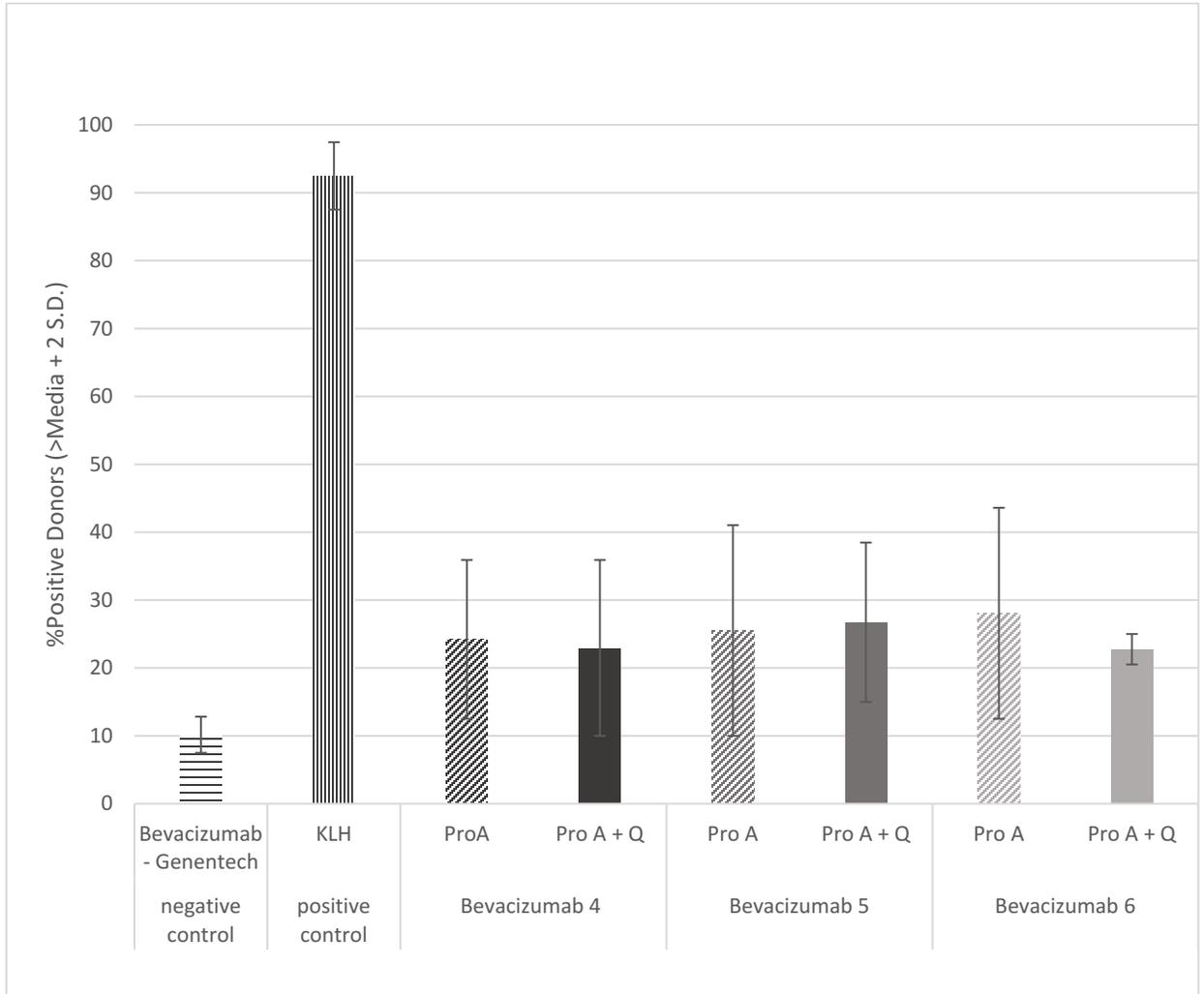
Figure 8: Average percentage of donors with CD4 T-cell proliferation in response to bevacizumab in
PBMC assay.

*The horizontal striped bar depicts the negative control- commercial bevacizumab made by Genentech. The
vertical striped bar depicts the positive control –KLH. Dark grey bars depict bevacizumab batch 4, grey
bars depict bevacizumab batch 5, and light grey bars depict bevacizumab batch 6. Diagonal stripes show
purification by protein A chromatography, and solid fill shows protein A and Q Flow Through
chromatography. Standard deviation is for n=2. No significant difference is observed between the percent
of donors with CD4 T-cell proliferation between the two purification methods in each batch.*

There was no significant difference between the donor response to the three new batches of bevacizumab, and no significant difference in percent of responders between the bevacizumab purified using the one step purification method (25.9 ± 14.4 percent) versus the two step purification method (24.1 ± 10.3 percent). All six samples still showed higher percent of donor with positive CD4 T-cell proliferation than the commercial bevacizumab (negative control), and there was no statistically significant difference in response between the samples with reduced levels of the tested process impurities.

There was a slight difference in the strength of the CD4 T-cell proliferation response between the two purification methods. Figure 9 below represents the cumulative strength of CD4 T-cell proliferation response from eighty donors, where the strength was measured by the sum of the average percent CD4 T-cell proliferation over background for each donor. Consistent with historical results, the strength of the donor CD4 T-cell proliferation response to the negative control, commercial bevacizumab, was very low while the strength of the response to the positive control, KLH, was significantly higher. The strength of the donor CD4 T-cell proliferation response in the bevacizumab samples purified by one step protein A purification was slightly stronger than the response to the bevacizumab samples purified by the two step protein A and Q Flow Through chromatography. The CD4 T-cell proliferation response is the strongest in bevacizumab batch 6 purified by protein A alone, which also correlates with the highest levels of rDNA (41.2 ng rDNA/mg). This finding could mean that rDNA may have an impact on the strength of the response, however further investigation is necessary. Although the total number of responders was similar for the two purification methods, the strength of the response was slightly reduced as a result of the secondary purification step.
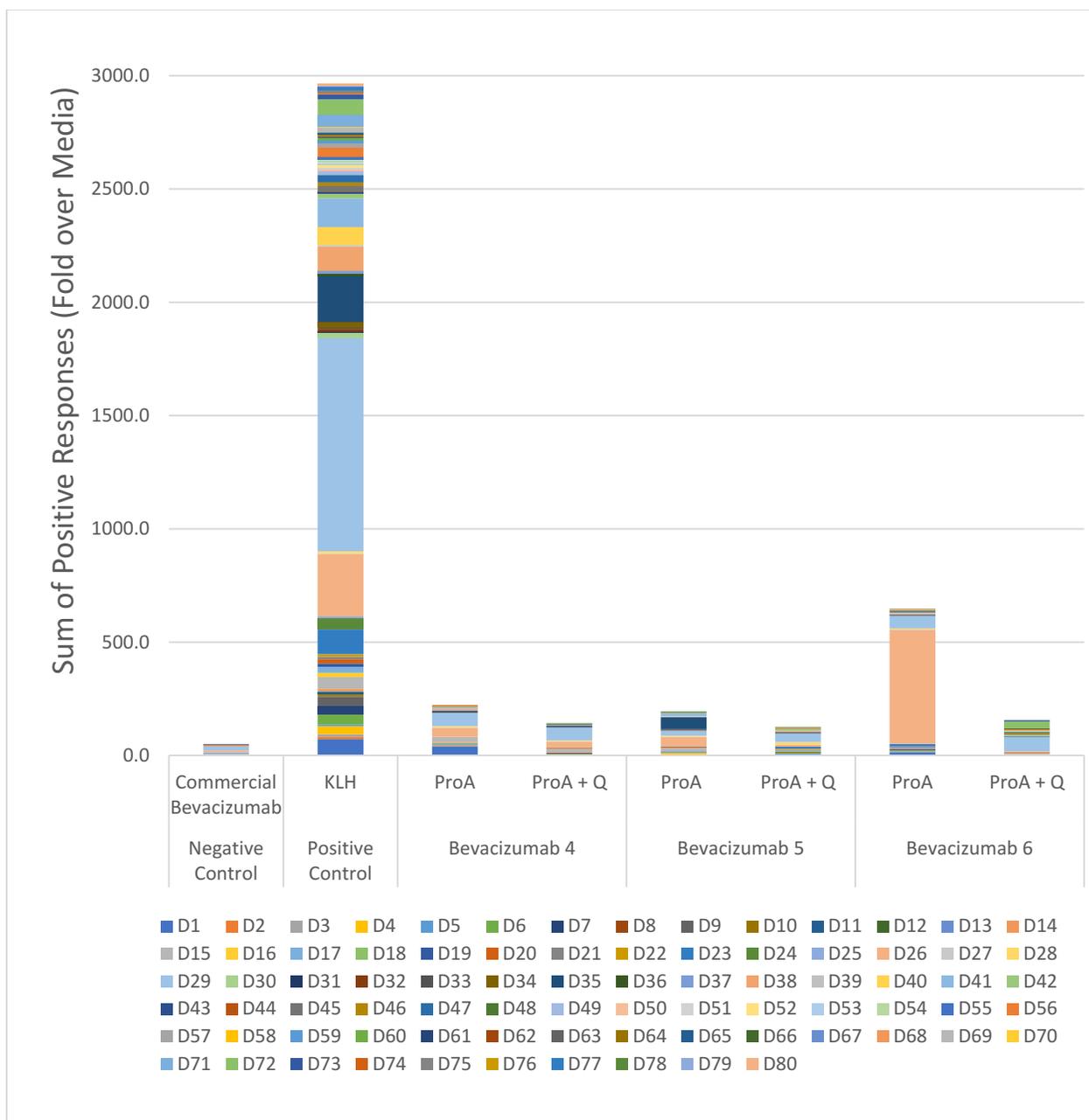
Figure 9: Strength of donor response to bevacizumab in PBMC assay.

This bar graph shows the sum of positive CD4 T-cell proliferation responses from 80 donors from 2 different cohorts. The negative control, commercial bevacizumab from Genentech shows a very low response, while the positive control, KLH, shows a very high level of response. The strength of the response is lower in the samples purified by both protein A and Q Flow Through chromatography, compared to the samples purified just by protein A chromatography, especially noticeable in bevacizumab 6.

Chapter IV:

Discussion

In the early discovery stages of biotherapeutic protein production, it is critical to produce the highest quality material, in order to make accurate measurements of the protein's immunogenicity response *in vitro* based on CD4 T-cell proliferation, before testing the protein *in vivo* and moving on to the manufacturing stages (Jawa et al., 2016). The PBMC assay results from early discovery process production of bevacizumab, showed a variable and unexpectedly high percent of donors with positive CD4 T-cell proliferation response compared to the commercial bevacizumab. The source of variability and high CD4 T-cell proliferation of this response was unclear, and we hypothesized that it was due to the process impurities that were not removed by protein A chromatography alone in the early discovery protein production process. We hypothesized that using a two-step purification consisting of protein A chromatography followed by Sartobind Q Flow Through purification would reduce the known immunogenic process impurities such as HEK HCP, protein A ligand, and rDNA in the sample, and would allow for more predictive immunogenicity assessments through CD4 T-cell proliferation assays.

First, the production processes for bevacizumab batches 1 through 3, which previously showed batch to batch variability between three different PBMC assay cohorts compared to commercial bevacizumab, were analyzed for any differences that could have resulted in variable levels of contaminants. However, the only difference identified was

in the users, with one user expressing bevacizumab batches 1 and 3, and a different user

expressing bevacizumab batch 2. Although a difference in user handling during protein

expression could play a role in variability, we have not identified any inconsistencies in

their methods and the variability in the response did not appear user specific. In addition,

all three batches of bevacizumab were purified by the same user using the same methods

of protein A purification, followed by buffer exchange in 1X PBS. The concentrations,

percent monomericity, and endotoxin levels of all three batches were also comparable,

showing that the quality of protein was comparable between the three batches.

The levels of process impurities such as HEK HCP, HEK residual DNA, and

protein A leaching were measured in each batch. There was no observed correlation

between levels of HEK HCP and positive CD4 T-cell proliferation response. In fact, the

batch that had the highest HCP levels (bevacizumab batch 2) produced the lowest CD4 T-

cell proliferation response. There was a slight correlation between rDNA and

immunogenicity in bevacizumab batch 2 having both the lowest levels of rDNA and

percent CD4 T-cell proliferation response, but there was no correlation between CD4 T-

cell proliferation response and levels of rDNA in the other two samples (bevacizumab

batch 1 and 3). It is possible that rDNA has an effect on the immunogenic response but it

is likely not the only contributing factor.

Similarly, there was a slight correlation between protein A levels and high CD4

T-cell proliferation response, with bevacizumab batch 3 having the highest protein A

levels as well as the highest CD4 T-cell proliferation donor response. Notably, the

difference in protein A levels in bevacizumab batch 3 compared to bevacizumab batch 1

and 2 is very slim (~0.75 ng protein A/mg), and therefore this result would require further

investigation. It is possible that there was a stronger effect from protein A leaching than from the HEK HCP and residual DNA impurities, as protein A is bacterial protein and may be more immunogenic in human cells (Singh, 2011) than HEK HCP and DNA which are derived from a human cell line. However, considering that the original three batches of bevacizumab were produced three years prior to the current study, it is possible that analyzing them now may not have provided us with the most accurate results as the samples have been handled by multiple users over time which could have affected the levels of impurities by potentially introducing contaminants from the production environment.

As a follow up step, three new batches of bevacizumab (bevacizumab batches 4-6) were generated using the same HEK293 expression system and purification process. Each batch was purified using one-step purification with protein A chromatography which was the only purification step used in the original three batches. In an attempt to reduce the level of impurities and evaluate the effect of impurities on the donor response in the CD4 T-cell proliferation assay, half of the protein A eluate material was further purified using Q Flow Through chromatography. Samples from both one-step and two-step purification were saved and tested for impurities.

The secondary purification step (Q Flow Through) was successful in reducing levels of the impurities tested. The samples purified by both protein A and Q Flow Through had lower levels of HCP, protein A ligand, and rDNA compared to the samples that were purified by protein A chromatography alone. Q Flow Through reduced HCP levels by an average of 99.7 percent, reduced protein A ligand levels by an average of 38.5 percent, and reduced rDNA levels by an average of 69.4 percent. In addition to

impurity reduction, the Q Flow Through step took about an hour to run per sample, and did not negatively impact protein monomericity and endotoxin levels, making it a quick and easy step to permanently incorporate in the early discovery purification process. This data confirms that Q Flow Through is a successful secondary polishing step for reducing sample impurities quickly and effectively.

The newly generated bevacizumab batches (bevacizumab batches 4-6) were tested for their effect on CD4 T-cell proliferation using the PBMC assay. There was no significant batch to batch variability observed, however the percent of donors with CD4 T-cell proliferation was still higher for these batches than for the commercial bevacizumab (negative control). There was also no difference in percent donors with CD4 T-cell response between the samples purified using one-step purification or the two-step purification methods, showing that reducing the process contaminants did not impact the percent donors responding. There was a difference in CD4 T-cell proliferation levels between the two PBMC assays that were run, but in both cases the response was higher than the 10 percent of donors that responded to the commercial bevacizumab, and no difference in response between the purification methods was observed.

These results suggest that the impurities tested did not impact the percent of donors that had a CD4 T-cell proliferation response. The percent of responders was similar for samples that had high levels of process impurities and low levels of process impurities. It is possible that the levels of HCP, rDNA, and protein A in these samples were not high enough to elicit a response. In addition, since the HCP and rDNA were derived from the HEK293 cell line, which is a human cell line, it is likely that even at higher levels these impurities would not elicit a CD4 T-cell proliferation response in

humans as the impurities are homologous to human proteins, and are not perceived as foreign pathogens by the human immune system. A similar observation was made in a study by (Jawa et al., 2016) showing that samples manufactured in CHO cells and containing CHO HCP levels as high or higher than 4000 ng HCP/mg did not increase the potential risk of immunogenicity in human cells compared to highly purified samples with lower HCP levels. Jawa et al. (2016) saw no immunogenicity impact from high levels of CHO HCPs, so it is not surprising that we are seeing no effects of HCPs from HEK HCPs on human CD4 T-cell proliferation.

This data also showed that the reduction of protein A ligands did not lead to a difference in the percent of donors with a positive CD4 T-cell proliferation response. In the original three batches of bevacizumab (batches 1-3) there was a slight correlation of an increased level of protein A ligand in bevacizumab batch 3 and the highest percent of donors that responded to bevacizumab batch 3. This correlation was not observed in the newly generated batches 4-6 of bevacizumab. The data from bevacizumab batches 4-6 showed no impact of levels of protein A ligands on the percent of donors with CD4 T-cell proliferation. This leads to the conclusion that protein A ligands may not be found in large enough quantities to elicit an immunogenicity response in these samples.

Although there was no observed difference in percent of donors with CD4 T-cell proliferation response based on the levels of impurities, there was significant variability between the two PBMC assays that were run. In both assays the commercial bevacizumab (negative control) elicited a low donor response (10 ± 3 percent) and KLH (positive control) elicited a high donor response (92.4 ± 4.9 percent), while the in-house bevacizumab elicited an average donor response of 14.2 ± 5.1 percent in the first assay

and an average donor response of 35.9 ± 7.4 percent in the second assay, leading to an inter assay average of 25.0 ± 12.5 percent. This data is consistent with the data from the original three batches of bevacizumab, where the inter assay donor response to the commercial bevacizumab showed low variability with an average of 12.9 ± 1.9 percent, while the inter assay response to the in-house bevacizumab batches was variable with a response average of 23.9 ± 13.6 percent. It is notable that the negative and positive controls do not show as much variability as the sample proteins, so it is possible that there are sample components such as the sample buffers that may impact proliferation. It is also possible that the higher donor response of the second assay was due to poorer stability of the bevacizumab formulated in-house compared to the commercial bevacizumab, however, the effects of protein stability on *in vitro* CD4 T-cell response needs to be further investigated.

The PBMC assay is only used for predictive measures, and the cohorts of donors are different every time, so there is also inherent variability between the donor responses. As different cohorts of donors were used in these assays, it is possible that one cohort had more donors that were pre-disposed to elicit a response to the samples. Although there was some variability between the two assays, neither assay showed a significant difference in percent of donors with positive CD4 T-cell proliferation levels between the samples with higher versus lower impurities. Both assays showed that the in-house bevacizumab elicited a higher immunogenic response than the commercial bevacizumab, and there was no significant intra-assay variability observed between the three batches of in-house bevacizumab. Both assays supported the findings that lower levels of impurities did not lead to a lower percentage of donors responding, demonstrating that HEK HCP,

rDNA, and protein A at the levels found in these samples may have less of an impact on preclinical *in vitro* immunogenicity predictions than originally hypothesized.

The levels of impurities did, however, have an impact on the strength of the response. The strength of the response is measured by the amount of CD4 T-cell proliferation elicited by each donor in response to the protein. Out of the donors that had a positive CD4 T-cell response to the protein, the strength of the response was lower in the samples with lower levels of impurities, and higher in the samples with higher levels of impurities. The strength of the response was the highest in bevacizumab batch 6 which was purified only by protein A purification, and was the only sample with residual DNA levels as high as 41 ng rDNA/mg of protein. It is notable that in bevacizumab batch 6 the high strength of the response was effected by one donor (donor 26), an outlier who elicited more CD4 T-cell proliferation than the rest of the donors combined, potentially skewing the actual strength of the response. The effects of residual DNA on the strength of the response would need to be confirmed with further experimentation.

Not enough research has been done on how the strength of the response in the PBMC *in vitro* assay co-relates to the strength of the response *in vivo*, so we should be cautious in using the strength of the response as a predictive measure of the impact of impurities on immunogenicity. It is possible that the higher levels of impurities could have activated the innate immune system, and increased the levels and types of cytokines released which would increase the strength of the response. This is especially likely in a low immunogenic protein like bevacizumab, which has fewer T-cell epitopes, and might be more likely to show a difference in the strength of the response than in the percent of donors responding. Further investigation of the cytokines released through cytokine

release monitoring assays, which help identify which MHC mediated a T-cell response and innate response, would be necessary to support this theory. In addition, more clinical data correlating strength of response is necessary to better understand the correlation of strength of the response *in vitro* and *in vivo*. If further evidence shows that this correlation is accurate, it would be important to identify which impurity is causing the increase in response strength.

The PBMC assay is a tool that can be used in early discovery to help predict the potential immunogenicity of a therapeutic, however in some cases a biotherapeutic might still have a different effect *in vitro* than it would *in vivo*. Looking at CD4 T-cell proliferation in donors that change from assay to assay introduces inherent variability. The PBMC assay described in this study uses cells from healthy donors, which are good for providing an immunogenic response as they are antigen inexperienced and do not have a pre-existing therapeutic-specific memory T and B cells. Even though immune responses from healthy donors can be predictive of a protein's immunogenicity when used as a therapeutic, they might also inaccurately predict a response from a diseased individual. Ideally the assay should include both cells from healthy donors and from donors with a relevant disease. However, obtaining cells from donors with the relevant disease is very challenging, and can contribute their own complications due to the effects of other treatments that the donor is already using (Duke & Mitra-Kaushik, 2019).

In addition to assay limitations, it is also important to keep sample size in mind. This study only assessed the immunogenicity of one mAb produced using our early discovery process, which is just one example of variability in CD4 T-cell proliferation response between batches. Bevacizumab is a relevant protein for this initial analysis since

there is clinical data available showing that it is not significantly immunogenic (U.S. Food and Drug Administration, 2004), while our *in vitro* PBMC assay has yielded different immunogenicity predictions from the bevacizumab batches produced using the early discovery process than would be expected based on human studies. However, to complete a more thorough investigation it is recommended to use a larger portfolio of biotherapeutics which have available clinical immunogenicity data but can also be produced using the early discovery process. This approach would provide a larger data set to analyze the impact of process impurities on immunogenicity.

It is also important to keep in mind the limitations of our impurity quantitation assays. The limitation of the ELISA method is that it might not quantitate all of the HCPs, and it cannot be used to characterize their identity (Hogwood, Tait, Koloteva-Levine, Bracewell, & Smales, 2012). For the purpose of this study the HCP identities were not important, however future studies could benefit from using mass spectrometry (Reiter, Suzuki, Olano, & Narum, 2019) to identify which HCPs are harder to purify and more prone to elicit an immunogenic response.

There are also some limitations with using the Quant-iT PicoGreen Assay for quantitating rDNA, which is a quick and easy method to quantify upstream bioprocess samples with higher rDNA concentrations, but is not sensitive enough to accurately quantitate the samples with rDNA levels below 0.20 ng/ml. For more sensitive measurements in samples with low rDNA, it is recommended to use real-time polymerase chain reaction (RT-PCR) which can detect rDNA at levels as low as 0.01 pg/ml (Wang et al., 2001). The Quant-iT PicoGreen Assay was sufficient for measuring rDNA in the samples for this study, as they were generated by the early discovery process and were

likely to have higher levels of rDNA, however for future studies quantitation by both Quant-iT PicoGreen Assay and RT-PCR would be recommended.

It would also be useful to assess the impact of HCPs and rDNA from different cell lines commonly used in the early discovery process, such as CHO cell lines. Since HEK cell lines are derived from human cells, it is likely that the HEK HCP and rDNA might not impact immunogenicity as much as HCP and rDNA from non-human cell lines. For more accurate evaluations of process impurity impact, it is recommended to use a larger sample size, as it would provide more information on whether the HCPs and rDNA differ between different cell lines and biotherapeutics, and whether the level of purification of the impurities is dependent on the biotherapeutic itself as well (Hogwood et al, 2012).

The analysis of the bevacizumab batches produced using the early discovery process did not provide clear evidence that impurities such HEK HCP, HEK rDNA, and protein A leaching have an impact on the percentage of CD4 T-cell proliferation responders. However, they appeared to have some impact on the strength of the response, which should be studied further. It is likely that because our study used a HEK cell line, the HEK HCP and rDNA were homologous enough to human DNA and protein sequences, and therefore did not elicit a response in human T-cells. However, there was some evidence that rDNA might have played more of a role in the strength of the T-cell proliferation response than other impurities. Notably, this finding could have been exaggerated by an outlier with an unusually strong T-cell proliferation response by one donor, so further experimentation is necessary to confirm this finding.

Although there was no clear correlation between HCP, protein A ligand, rDNA impurities and CD4 T-cell proliferation, it has been determined that Q Flow Through

successfully removed impurities that are not removed by protein A purification alone. As it is important to generate high quality and pure material for the best biotherapeutic production and analysis, it is recommended to use the two-step purification process when generating samples where ensuring low immunogenicity of the protein is critical. Even though this study did not show a clear impact of impurities on CD4 T-cell proliferation, having pure biotherapeutic material will provide more confidence in the response observed in future assays.

The true source of variability in percent donors with a positive CD4 T-cell proliferation in the original three batches of bevacizumab was not found in this study, but further investigations could provide more insight. As future steps, we plan to generate a larger data set by measuring protein quality and process impurities in all biotherapeutic samples tested for immunogenicity in the early discovery process. Collecting data on levels of HCP, rDNA, and protein A leaching, along with aggregation levels, and glycosylation patterns, which have not been assessed in this study, could be useful in determining if specific impurities have a higher impact on CD4 T-cell proliferation. Further assessments of the effect of the stability of proteins over time on CD4 T-cell proliferation would be useful for understanding inter-assay variability. In addition, obtaining more clinical data on the immunogenicity and strength of a response to a biotherapeutic would be helpful in further understanding the data obtained from the PBMC assay. A combination of these and future studies will help increase the confidence levels in making accurate immunogenic predictions of biotherapeutics at the early discovery stages.

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