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NEXT GEN I-O APPROACHES: WHAT WILL FOLLOW THE PD-1S?

SPOTLIGHT

INNOVATOR INSIGHT

Optimizing antibody discovery strategy for clinical success of next-gen immuno-oncology therapeutics

Ilse Roodink, Patrick Ott & Nina Senutovitch

ImmunoPrecise Antibodies is a full-service therapeutic antibody discovery and development company with decades of experience and global coverage, with locations in Europe, Canada and the US. This article will present a case study utilizing robust, target-enrichment-based antibody discovery technologies that allow accelerated, high-throughput identification of diversified pools of lead antibody candidates, an essential first step for a successful therapeutic program.

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CASE STUDY DESIGN

The presented case study aimed at generating a diversified panel of antibodies – functional or non-functional – specifically directed against a transmembrane tumor target. Additionally, a subset of this panel would ideally show multi-species cross-reactivity to facilitate preclinical evaluation of leads. Due to the high homology between the human protein and its murine equivalent, and to circumvent tolerance issues that would hamper the identification of multi-species cross-reactive antibodies, two antibody sources were used: ready-to-use human phage display libraries and, based on genetic distance, immunized chicken.

Robust species-independent antibody discovery technologies were of great value in selecting the antibody panels of interest. The success rate of an antibody discovery phase also heavily depends on the ability to identify antibodies with the desired characteristics at an early stage. To this end, iQue® advanced flow cytometry-based high-throughput, multiplex cell screenings were integrated into the first output screenings in order to select clones for further characterization. The resulting program design is summarized in Figure 1.

To facilitate immunization, B cell and phage selection, and down-stream characterization, various recombinant extracellular domain (ECD)-proteins were produced. For screening purposes, recombinant cell lines expressing full-length target were also generated.

Following the production of recombinant ECD-proteins, subsequent immunization of chickens with these targets, and plasma reactivity analysis, one bird was selected for B cell selection. In parallel, in-house ready-to-use human phage display libraries were screened for binders towards the recombinant ECD-proteins.

Clones obtained after both target-specific phage and B-cell enrichment approaches were subsequently screened for binding towards the target of interest by ELISA and flow cytometry, and hits were subjected to sequence analysis. Currently, epitope and functional diversity of identified lead candidates is ongoing, after which final lead candidates will be subjected to appropriate optimization and engineering technologies.

TOOL GENERATION

Except for a deviation in the signal peptide, the human and cyno target are identical, and therefore generated human tools will represent both human and cyno protein. To allow alternating immunization and phage library selection approaches to minimize anti-fusion protein responses, different recombinant fusion-ECD proteins were produced for the human and mouse target. Following eukaryotic expression and purification, aliquots of Fc- and GST-fusion proteins were biotinylated and the various recombinant protein variants were subjected to quality

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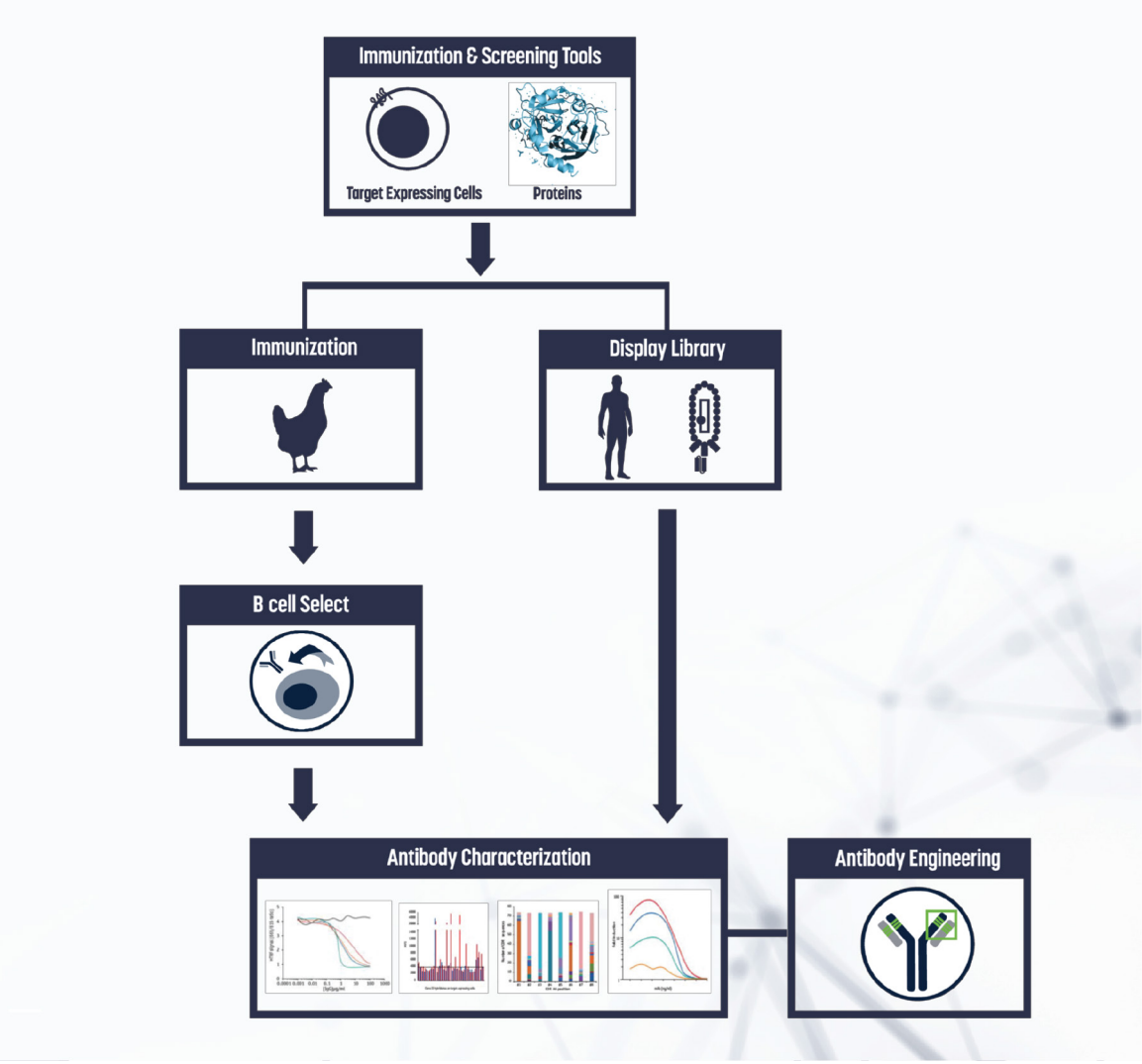
FIGURE 1

Program overview.



control analysis. For multiplex cell-expressed target screening purposes, stable CHO cell lines expressing either the full-length human or full-length mouse target were generated.

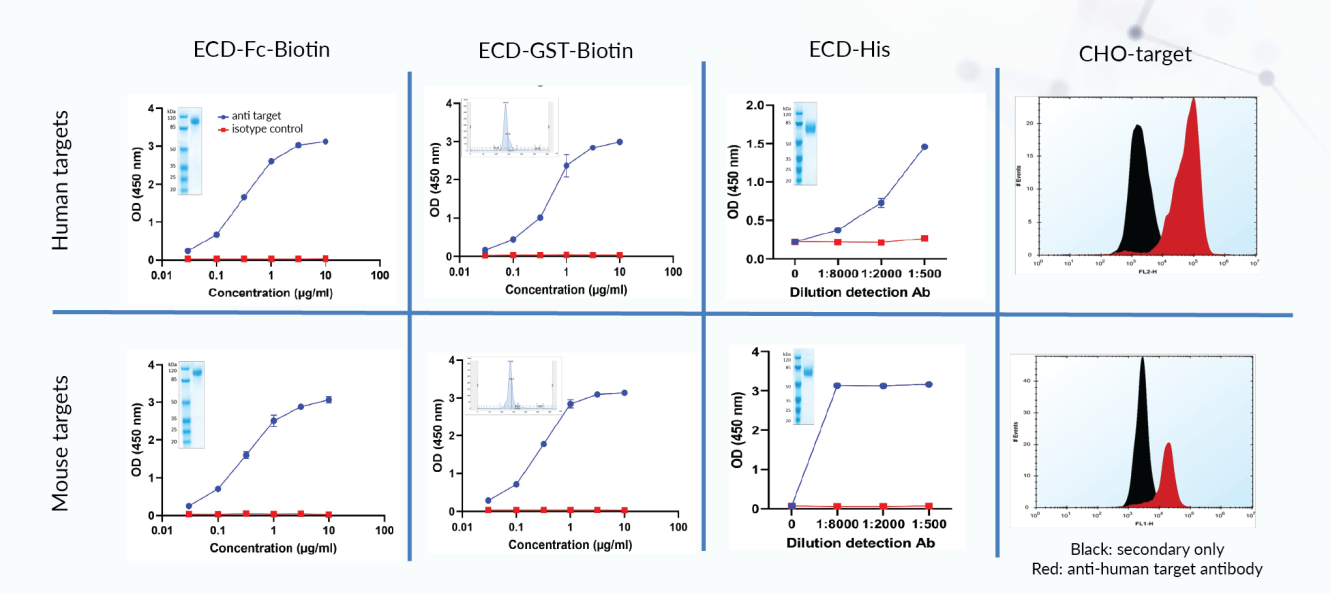
Protein batches of high purity and integrity were obtained, and the conformation of the recombinant ECDs were validated by ELISA using commercially available anti-target antibodies for detection. As indicated in Figure 2, dose-dependent reactivity was observed towards the various recombinant ECD-proteins, while the isotype control revealed flat liners. The in-parallel generation of stable CHO cell lines expressing human or mouse target was also successful.

ANTIBODY DISCOVERY UTILIZING CHICKENS

As discussed earlier, in addition to obtaining a variety of high affinity clones directed against the human target, the program aimed to obtain multi-species cross-reactive antibodies. Therefore, two different immunization strategies were applied. Whereas human recombinant ECD-protein was used as an immunogen in the primary immunization and first boost of chicken 1, with chicken 2, a strategy was applied to boost for shared epitopes early on. After the initial immunization with human target, bird 2 was boosted with mouse target. An alternating strategy

FIGURE 2

Tool generation.



Recombinant human and mouse target-ECD-Fc(biotin), -GST(biotin) and -His fusion proteins and target-expressing cell lines are successfully generated

with respect to fusion protein type was also applied to minimize responses towards the fusion partner.

Subsequently, blood was withdrawn for plasma reactivity analysis towards plate-immobilized and cell-associated target using ELISA and flow cytometry, respectively, to rationalize the next steps.

In Figure 3, the binding intensities plotted against plasma dilution series is shown. As anticipated, both birds showed dose-dependent target-specific reactivity towards both human and mouse target. The similar EC50 values seen towards recombinant human and mouse target support the choice to use chicken as a source for generating multi-species reactive antibodies for preclinical evaluation purposes.

The titration data obtained also suggest that the mouse target expression level is significantly higher compared to the human target. It was not possible to fully compare expression levels during cell line generation, as no cell-associated target recognizing antibodies with human/mouse cross-reactivity were available for direct comparison of expression levels. As plasma screening did not reveal a significant difference in immune response between the 2 birds, bird 2 was nominated for additional boosting with a mixture of both human and mouse target, and subsequent B cell selection. To anticipate downstream multiplex screenings of the output clones from both antibody discovery approaches in a high-throughput way, thereby minimizing the amount of antibody sample required for obtaining full cell reactivity profiles, each screening well was filled with a mixture of 3 cell lines. Each line was encoded with different fluorescent intensities using an encoder dye to facilitate gating after data acquisition. As shown in Figure 3, 3 different cell populations could clearly be identified in the viable cell gate, representing mouse target-expressing cells, human target-expressing cells and parental CHO cells.

Following a pre-harvest boost, one bird was euthanized, and B cells were isolated for subsequent incubation of single B cells with bead-captured target. Using a fluorescent dye-labelled secondary antibody to result in a fluorescent signal due to antibody clustering on the target-loaded beads, B cells expressing anti-target antibodies were selected. This approach allowed the identification

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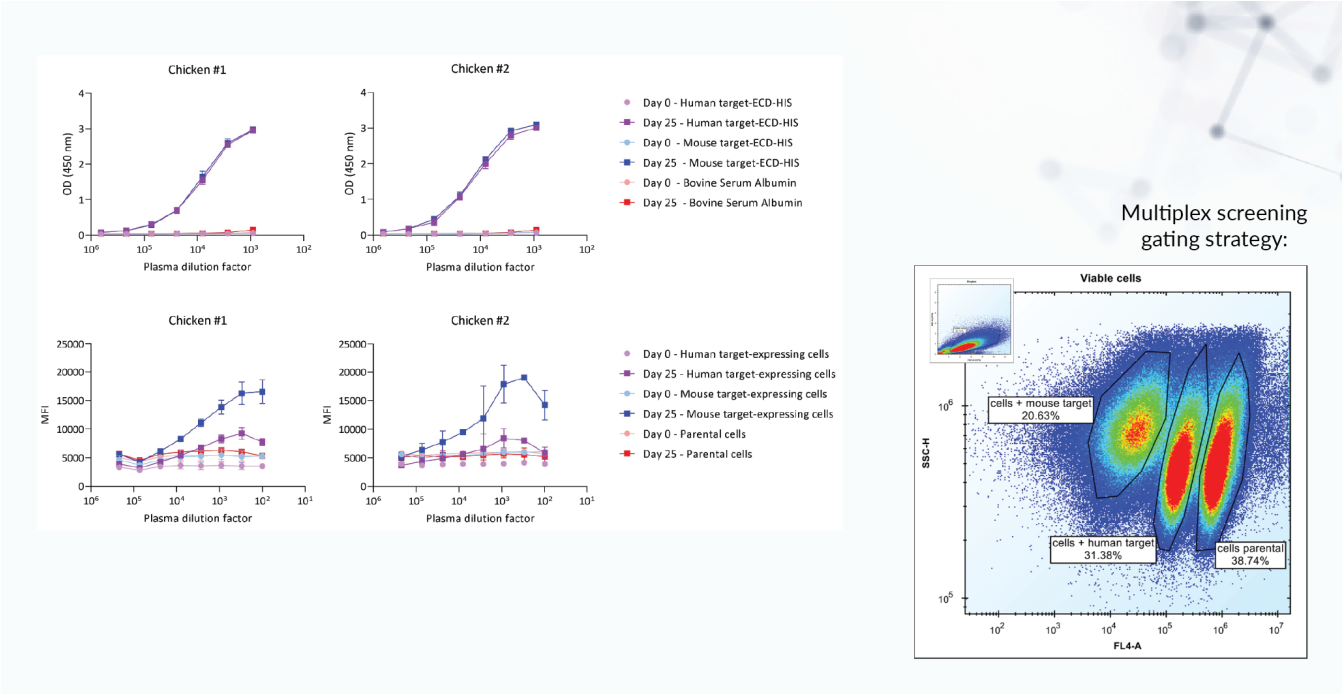
FIGURE 3

Chicken – immunization. Plasma reactivity analysis.



Both chickens show dose-dependent, target-specific immune plasma reactivity towards plate-immobilized and cell-expressed human and mouse target.

of B cells which express fully matured antibodies, thereby representing plasma reactivity responses. Subsequently, selected B cells were subjected to V-domain amplification and cloning in a human IgG eukaryotic expression vector set for subsequent, high-throughput, small-scale production of corresponding recombinant antibodies for high-throughput, multiplex screenings. Using this approach, both V-domain sequences and reactivity profiles of recombinantly expressed antibodies are available within approximately 4 weeks of initial organ/cell harvest.

Following recombinant expression of selected human target-reactive chicken antibodies, high-throughput multiplex cell-based screenings using Sartorius' iQue® Advanced Flow Cytometry Platform was performed.

As reactivity towards cell-associated target is, in this case, the most therapeutically relevant read-out, this was used as a discriminator to identify lead candidates. Approximately 65% of the chicken B cell-derived chimeric antibodies showed target-specific binding towards cell-associated target. High-throughput multiplex screening of crude small-scale production supernatants allowed the identification of a panel of target-specific chimeric chicken antibodies, of which 75% showed cross-reactivity towards the human and mouse protein. The data obtained also supported the prediction that the mouse target expression level was anticipated to be significantly higher. Although crude production supernatants were used and antibody expression level was not corrected for at this stage, the differences in reactivity profiles suggested that a diversified set of lead candidates was identified using chicken B cell selection.

Although screening for epitope and functional diversity of the current set is ongoing, sequencing data shows that this set consists of 19 sequence-unique clones, which were categorized into 13 different families based on shared VH-CDR3. 60% of the cell-expressed target-recognizing antibodies which were human/mouse target cross-reactive had a sequence unique V-domain pair.

ANTIBODY DISCOVERING UTILIZING HUMAN PHAGE DISPLAY LIBRARIES

The second antibody source included in this study was in-house human phage display libraries. Following phage rescue of these ready-to-use scFv libraries, 3 different selection strategies were used to increase the chance of obtaining multi-species cross-reactive scFvs:

- A human target only panning strategy alternatingly applying 2 recombinant human ECD-fusion proteins (strategy A)
- A human-mouse target alternating panning strategy in which human ECD-fusion protein was used as initial bait (strategy B)
- A human-mouse target alternating panning strategy in which mouse ECD-fusion was used as target in the first panning round (strategy C)

For strategies B and C, the two different fusions proteins were alternatingly used to avoid selection of Fc- or GST-binders.

The workflow used for phage display-orientated antibody discovery is efficient and species-agnostic. After finalizing the consecutive rounds of panning, re-amplified polyclonal phage pools obtained after each selection round are screened for target reactivity to select outputs for monoclonal analysis. Subsequently, picked clones are induced to express soluble antibody fragments followed by isolation of periplasmic fractions. These so-called 'peripreps' containing soluble antibody fragments are used for target reactivity screenings, and hits are then subjected to sequence analysis. This workflow facilitates the delivery of reactivity profiles of monovalent antibody fragments and their corresponding V-domain sequences within approximately 4 weeks after phage library rescue.

Following 4 consecutive rounds of selections according to the 3 different panning strategies described above, amplified polyclonal phage outputs obtained from 2 human scFv libraries were analyzed for reactivity towards plate-immobilized His-tagged recombinant human and mouse target, and off-targets streptavidin and BSA. Panning a human repertoire obtained from autoimmune-diseased patients resulted in the polyclonal phage reactivity profiles seen in Figure 4. Reactivities were plotted against phage dilution series and in general, no clear differences in reactivity profiles were obtained by applying different panning strategies. For all strategies, a clear target-specific reactivity can be observed after the second round of panning. Regarding the phage pools obtained after the 3rd and 4th round, substrate precipitation due to over-reaction was observed, which caused clear hook effects. It might have been expected that reactivity increased during the consecutive rounds of selection – however some increase in reactivity towards off-target streptavidin can also be observed.

No significant differences in reactivity profiles of the various polyclonal phage pools were observed using the human repertoire obtained from healthy donors as panning input. However, a slightly different outcome was obtained with full dose-response curves already observed after the second round of panning.

To maximize output diversity, a decision was made to nominate output round 2 of the different strategies for clone picking and subsequent reactivity and sequence analysis, thereby accepting that the hit rate might be lower compared to picking clones from later outputs.

Picked clones were induced to express scFv followed by isolation of the periplasmic fractions which were used for reactivity screenings. Similar to the B cell antibody discovery branch of this case study, reactivity towards cell-associated target was used as a read-out to nominate lead candidates. The ability to perform multiplex cell-based screenings of crude periplasmic fractions with the iQue platform significantly accelerated the lead identification process.

Approximately 20% of the picked clones showed target-specific binding towards

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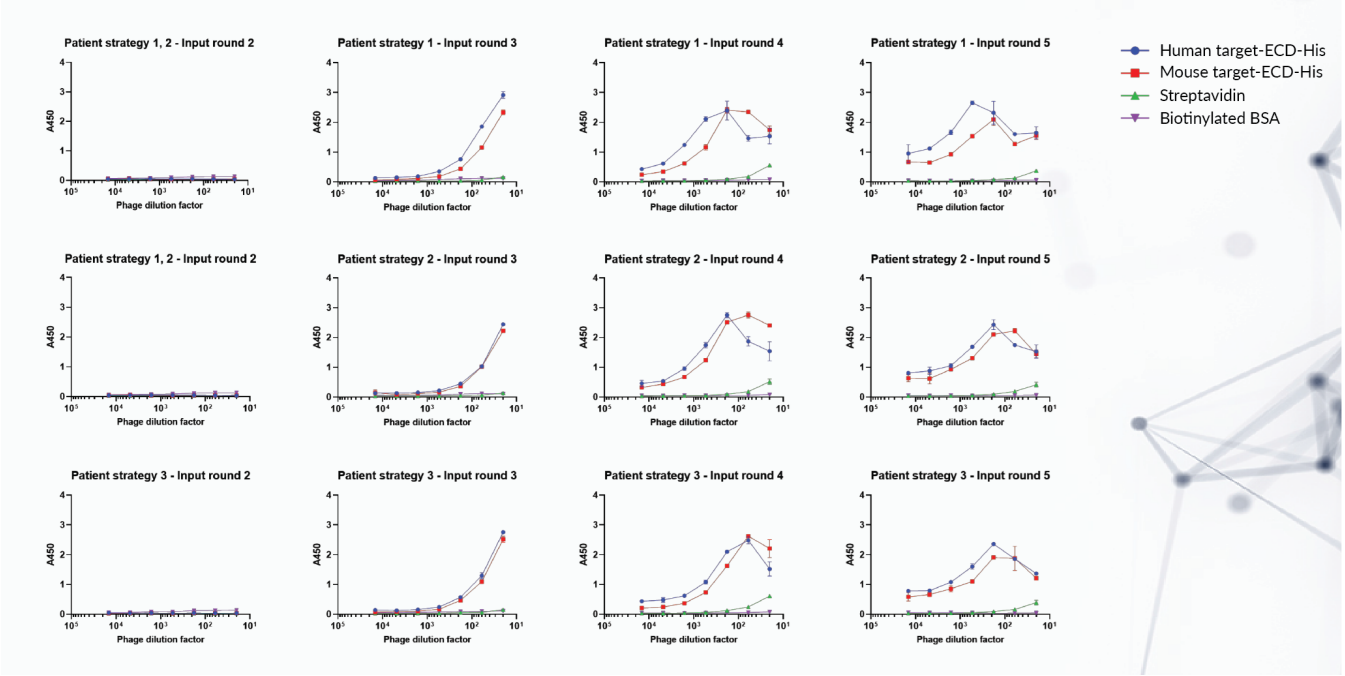
FIGURE 4

Human – phage display.



Polyclonal phage reactivity analysis; AI patient repertoire.

cell-associated target. Reactivities of these lead candidates against human target-expressing cells, mouse target-expressing cells, and binding signals towards the parental CHO cells, are shown in Figure 5. Although crude periplasmic samples were used, which does not allow for scFv concentration correction, the diverse reactivity profiles suggest the isolation of a diversified pool of target-specific human antibody fragments. Over 90% showed human/mouse target cross-reactivity. Identified lead candidates were subsequently subjected to sequence analysis.

Through screening of in-house human phage libraries, 68 sequence unique, cell-associated target recognizing clones were identified in the picked population, which were categorized in 44 different families based on shared VH-CDR3. 60% of the cell-expressed target-recognizing scFvs which are human/mouse target cross-reactive had a sequence unique V-domain pair.

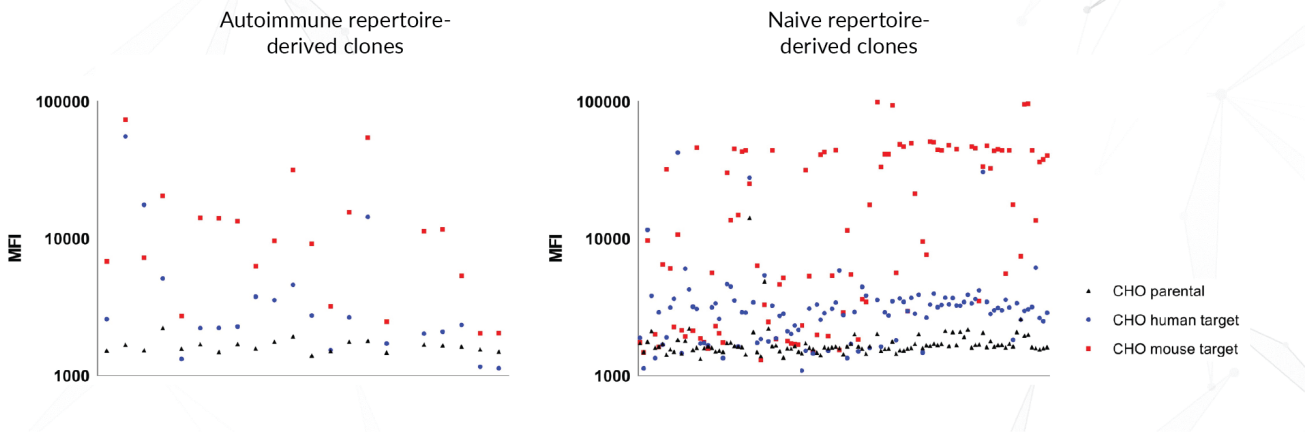
The sequence diversity of the lead antibody pool generated, as shown on a phylogenetic tree (Figure 6), displays heavy and light chain sequence diversity of lead candidates obtained from both the chicken and human library antibody discovery approaches. A clustering of clones derived from the chicken B cell procedure is seen (visualized by the blue oval shaped line), although diversity is present within this set. For the human library-derived clones, related clusters are visible, but overall a large sequence diversity is obtained.

CONCLUSION

Epitope and functional diversity of sequence unique library-derived clones, expressed as full human IgG1 antibodies, is currently ongoing in parallel to evaluation of the chimeric antibodies from the chicken B cell branch. The outcome will assist with nominating final leads for further development. The current data available demonstrate that leveraging the high-throughput, robust antibody discovery technologies used in this case study accelerated the selection of a diversified panel of genetically distinct lead antibody candidates.

FIGURE 5

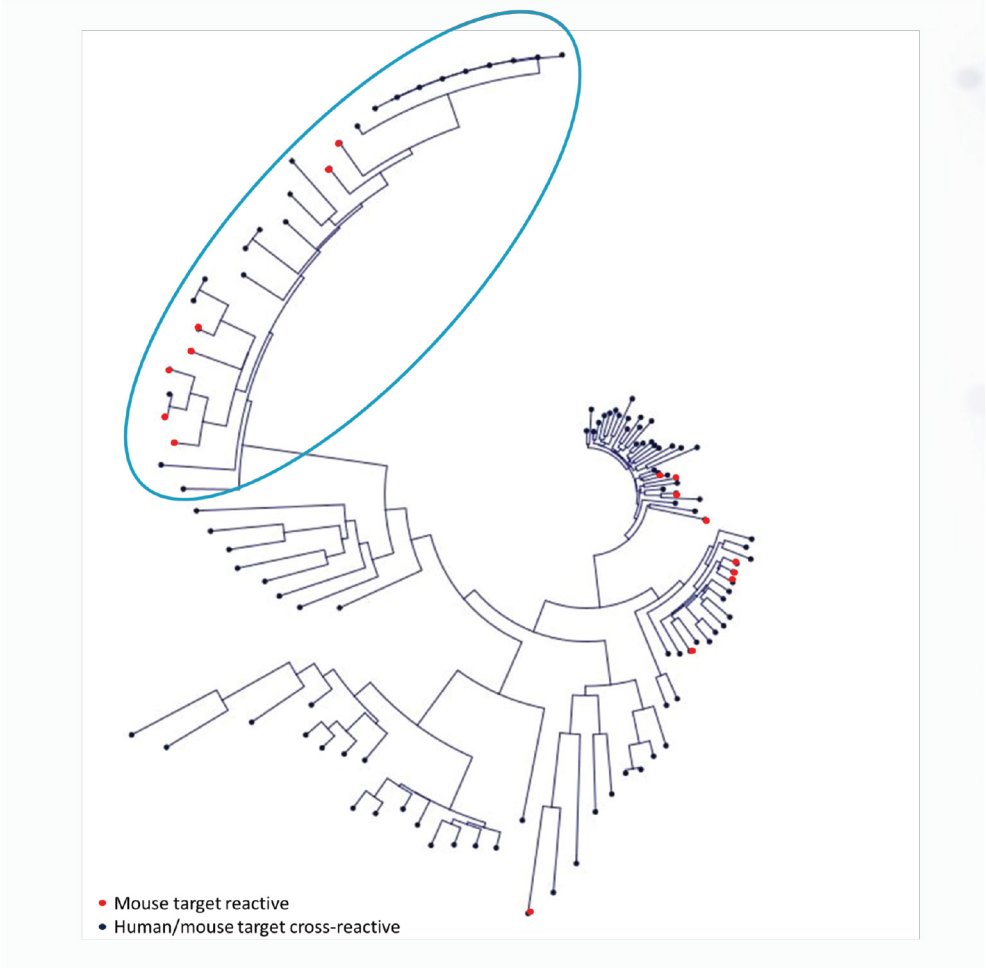
Human–phage display. Monoclonal scFv reactivity analysis.



- ~20% of the picked clones show reactivity towards cell-expressed target.
- >90% of target-reactive scFvs are cross-reactive towards cell-expressed human and mouse target.

FIGURE 6

Phylogenetic tree.



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Q & A

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Q What affinity do you usually obtain from the immunization and display approaches?

IR: With respect to the libraries, our repertoire is a naïve one, so there is no affinity maturation in the repertoire itself. However, if you perform a phage display selection feature, you have the affinity pressure during selections. Due to competition, if you have a given amount of target, and keep the amount of phages the same, you get selection based on affinity. This is how you force the outcome to be of a certain tolerate affinity.

Of course, we anticipate that most often our library-derived clones are of a lower affinity than the ones derived from the immunization campaign. Nevertheless, the desired affinity of your lead molecule depends on the final clinical application. If you have a highly defined molecule, this does not mean it is doing what you want in the clinic – other characteristics are also important.

In general, we feel that we often obtain at least nanomolar antibodies from our libraries. From immunization approaches, it will be evident more in the picomolar range.

Q Regarding the considerations for using chickens in the study, are there any issues regarding immunogenicity in humans if you are looking to use this in the clinic? Have you encountered problems with chicken B cell antibodies?

IR: Chicken may indeed seem a bit of a strange source, but I predict that it will be seen more in the future for generating therapeutics.

In this case study, we were looking for the discovery of diversified antibodies. Next to binders we were also looking for functional antibodies, antagonistic and agonistic antibodies, and so on. The homology of the target between human and mouse is very high, and we prefer to use our lead molecules in preclinical studies, instead of surrogates. We wanted to overcome tolerance issues and felt chicken might represent a very good source due to the genetic distance.

Additionally, chickens use gene conversion instead of the better known affinity maturation seen in mice and humans. They use pseudogenes using donor sequences for B cell repertoire formation. This might result in quite a different repertoire compared to what is obtained from our human libraries, for instance. We know that immunizing birds will probably yield responses to different epitopes compared to immunizing mice, for example. There were various different reasons for going to the chicken, and taken together, we decided that birds were a good source for generating a significantly different set of antibodies against this target.

This also partly addresses the affinity question – if you immunize birds, the resulting antibodies are known to be of quite high affinity. That also matches with our goal to generate antibodies which have binding capacities next to ones that are functional. If you want to use an antibody only as a binder, affinity might be an important discriminator.

With respect to the immunogenicity, of course we might have more work to do in optimizing the sequence compared to our human library-derived clones. They are fully human V-domains, so you would expect more immunogenicity with chicken derived antibodies. But with advanced software and the corresponding analysis, you can select clones for the next phase, and for optimization. For the ones with less viability, you can subject them to humanization and optimization. If you do your work properly, I think that using chicken-derived clones in the clinic is a real option.

Q What types of approaches, tools, or technologies do you think are most useful to support a robust antibody discovery campaign?

NS: It is really helpful in your antibody discovery process to have the ability to perform high-throughput, multiplex, and functional screens early on in your discovery phase.

This means using tools such as advanced flow cytometry, which combines high-throughput screening, as well as plate-based data analytics, for example. This lets you multiplex assays and rapidly screen hits based on a large number of desired properties: affinity as well as cross species reactivity and target specificity. Using cell-based assays that can distinguish between your target antigen binding versus a irrelevant antigen binding is important.

Harnessing technologies that allow for cell-based screens is critical, because you want the antigen target to be displayed in a native conformation, similar to how you would find it *in vivo*. Cell-based native screens can be quite an advantage to reduce attrition later on in the lead

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selection process, versus technologies that require you to immobilize your target antigen, and very likely disrupt the confirmation of that antigen target.

Having high-throughput screening systems that also allow you to rapidly screen for hits using different display technologies, for example using immunizations with peptide targets as well as cell display targets, and additionally from display libraries, can be critical.

Part of the importance of having a high-throughput tool is not only having an instrument, but also having software that can couple with the large amount of data coming out of these types of screens. Ideally you want software that is specifically built for plate-level data analysis, in order to avoid exporting individual files or individual sample wells, so you can quickly move on to interpretation of your results. This lets you streamline your entire discovery process, as you are able to analyze and quickly visualize all those hits that will be the best for the lead selection process.

Q How can you make sure your antibodies are specific and not cross-reactive with other proteins? Could you go into more detail on off-target screening for lead candidates?

IR: In initial reactivity screens we always include off-targets, in case we do not have a related family member or something else available for counter screening, we use more common proteins. But once we have confirmed our lead candidate pool, meaning it is target specific and not binding to off-targets, we will focus on our lead also based on specificity. You can consider multiple different protein arrays, and also binding characteristics such as binding based on charge, for example.

There are many downstream assays that you need to perform before you can decide an antibody has good behavior and is suitable for moving forward for clinical application. So, including protein arrays is not part of the early antibody discovery phase, but it is important to include appropriate off-targets during your antibody discovery.

Profiling towards different protein types or charge based assays is important to do before you choose a lead molecule – this is why we have a lead candidate pool. They need further characterization downstream, in a developability profiling setting.

Q Patrick, could you share your perspective on where you think we have seen the most clinical success when it comes to next generation antibody therapeutics in oncology?

PO: To make an introductory comment, listening to this discussion is very educational for me. I am coming from the other end of the spectrum of the clinical development process.

As an immuno-oncologist, and someone interested in making immunotherapies better, we are all thinking about targets, and we don't think as much about the different antibodies. Not in the sense of not thinking of BiTEs and different ways to engage the immune system, but in

not thinking as much about the pharmacology. It is actually a complicated process to come up with even one antibody against a certain target.

Anti-PD-L1 antibodies are amazing examples that have shown over the last couple of years that an antibody by itself can actually induce immune responses and be effective for solid cancers. It is very much an outlier, unfortunately, but it is proof of principle that targeting one inhibitor receptor as expressed on T-cells and tumor cells can be effective. It is great for immuno-oncology, and in some ways has jumpstarted the field.

Now we are looking for different ways to engage other targets and finding innovative ways to do that. BiTEs are a good example for conceptually doing more than just blocking an inhibitory receptor: actually bringing T-cells to where they should be, engaging antigen on a tumor, and bringing the T-cell together.

The question is, is that going to be enough to have clinical effect? There is a whole layer of complexity in the preclinical biology part of this. It is not enough to have a good antibody; you also need that antibody to direct the right target in the right context. You need the right tumor type or right tumor microenvironment where that antibody can have a role. Another layer is whether we have good mouse models to test this, and I would say that right now we don't.

An example is the co-stimulatory antibodies that have already been tested fairly widely in the clinic, CD137, CD40, and so on. There are mouse models where they show good efficacy, but it has not been translated into the clinic. From a translational perspective, I think we need better models before things move into the clinic.

Q Could you describe how you have overcome bottlenecks in the hit generation and lead selection process?

NS: I see the main bottleneck in hit generation and lead generation as time. You want to save time in your early phase of discovery, and there are several ways to do that.

Having an excellent strategy in order to screen for hits is crucial. Using a combination of display libraries as well as animal immunizations allows you to set the stage to have a very excellent quality pool to select your hits from, so you avoid a junk in, junk out scenario. You don't want to have to rescreen if you don't find suitable targets.

Assuming you have gone through this careful strategic process of library and display technologies, as well as immunization, and you have a diverse starting material, you can also save a lot of time later on by beginning to profile a variety of hits based on your desired function in a rapid manner and at an early stage

You can do high-throughput cell-based screens on a relatively large selection of your antibody candidates from your hits, thereby improving your chances of finding the best possible candidates to move forward.

For example, for many immuno-oncology targets you want to identify antibodies that are able to induce antibody-dependent cell cytotoxicity. Building in an assay screen early on can be very helpful to reduce attrition down the road.

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Another thing that has implications for studies further towards the clinical phase is being able to get early insight into your bioprocessing or manufacturing capabilities. For example, you want to make sure that the antibody candidates that you are most interested in are the ones that are going to be druggable; and if they going to be successful candidates you want to produce a lot of, you need to make sure that they don't aggregate.

One way you can do that is to simultaneously evaluate antibody titer, as well as cell count of cell lines producing your antibodies of interest. In this way, you can identify the best antibody producing cell line from your pool of hits, and then carry this forward to lead selection.

IR: I agree with Nina – you already want to have a very fast strategy. Prior to the initial screening, which allows you to have a high hit rate, you want to have a very diverse pool. If you can combine that with downstream high-throughput multiplex screenings, that is beneficial.

If you have a big pool, you can narrow down your clones based on more biophysical characteristics, because there will be dropouts. This helps you to select the best molecule for moving forward to the clinic.

Q What role do you see automation playing, from discovery and through to the antibody developability phase? How do you deal with the vast amount of data generated along the way?

NS: Automation can play a really important role in allowing you to save time. If you have a high-throughput screening system, for example, that allows you to harness automation and reduce hands-on experiment time.

The only way to overcome the overwhelming amount of data generated is to have a system incorporating software that allows you to use plate-based data analytics, because otherwise you will be spending a lot of time managing and analyzing single bits of data. You want something that is plate-based so you can screen through a variety of your library's target display strategies or immunization strategies in a rapid manner. If your software is specifically designed to handle huge amounts of data from screens, you can manage, save and visualize all of that data quickly.

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AUTHORSHIP & CONFLICT OF INTEREST

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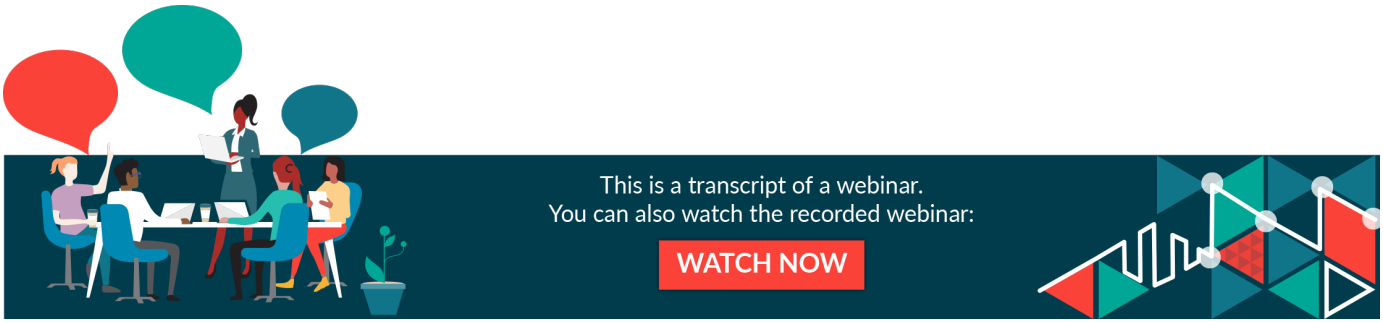

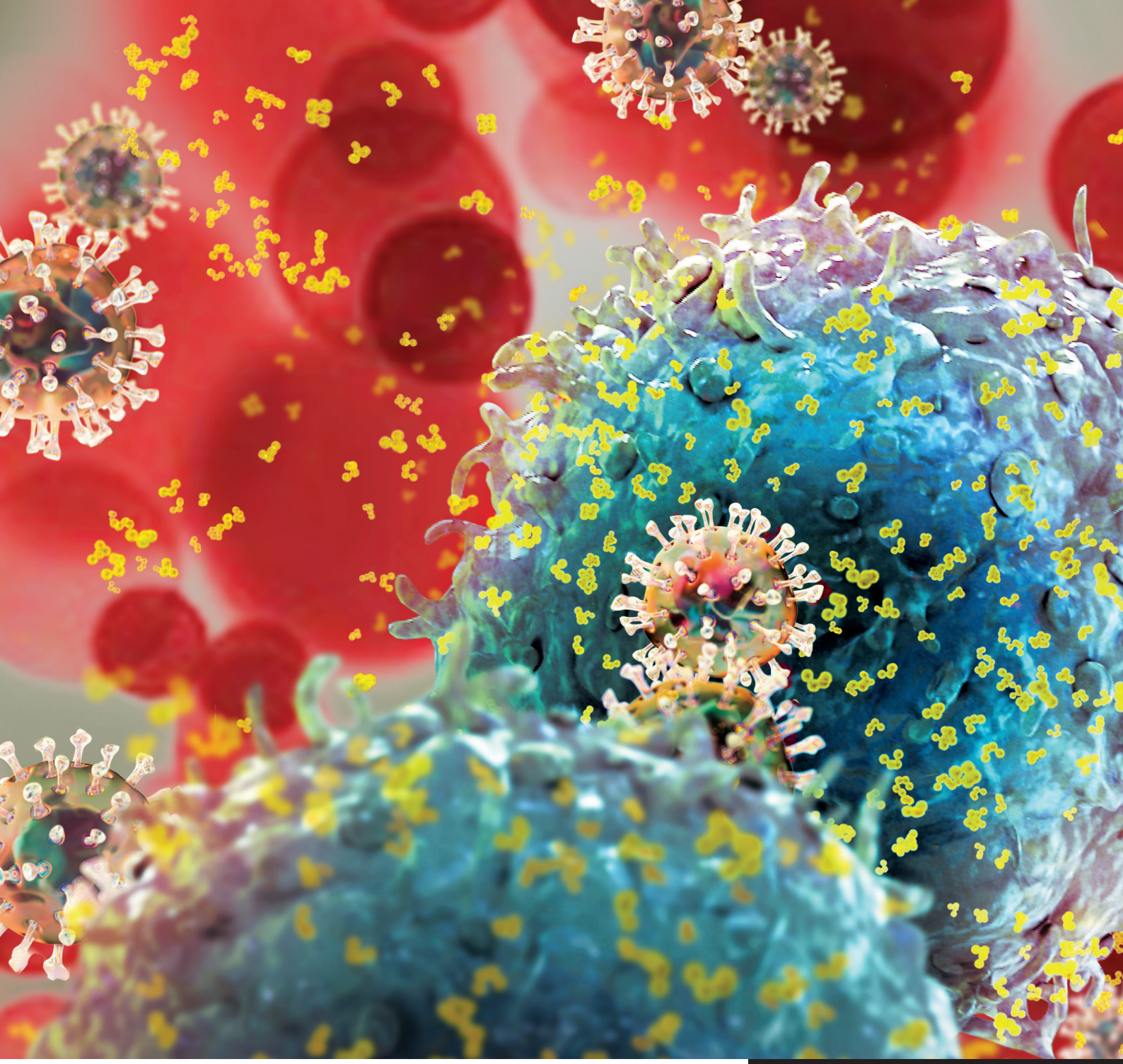

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