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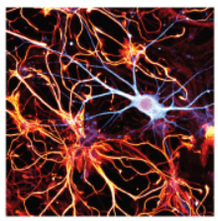

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REPORT

Heavy chain-only antibodies with a stabilized human VH in transgenic chickens for therapeutic antibody discovery

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ABSTRACT

Heavy chain-only antibodies have found many applications where conventional heavy-light heterodimeric antibodies are not favorable. Heavy chain-only antibodies with their single antigen-binding domain offer the advantage of a smaller size and higher stability relative to conventional antibodies, and thus, the potential for novel targeting modalities. Domain antibodies have commonly been sourced from camelids with *ex-vivo* humanization or transgenic rodents expressing heavy chains without light chains, but these host species are all mammalian, limiting their capacity to elicit robust immune responses to conserved mammalian targets. We have developed transgenic chickens expressing heavy chain-only antibodies with a human variable region to combine the superior target recognition advantages of a divergent, non-mammalian host with the ability to discover single-domain binders. These birds produce robust immune responses, consisting of antigen-specific antibodies targeting diverse epitopes with a range of affinities. Biophysical attributes are favorable, with good developability profiles and low predicted immunogenicity.

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Heavy chain-only; sdab; single-domain antibody; transgenic chicken; VHH

Introduction

Conventional antibodies consist of both heavy and light chains, creating a heterodimeric complex that utilizes both the heavy and light variable regions for antigen binding. Heavy chain-only antibodies (HcAbs), consisting of a single antigen-binding domain, were originally discovered in camelids in 1993,[1] and a more distantly related single-domain immunoglobulin known as Ig_{NAR} was discovered in sharks in 1995.[2] The viability of an autonomous heavy chain-only binding domain (VHH) has evolved by the camelid adopting a modified structure that improves stability and solubility, specifically by changes in the framework regions (FRs) and in particular the so-called hallmark 'tetrad mutations' in FR2 that compensate for the lack of light chain association.[3,4] Camelid HcAbs have also developed without a CH1 domain in the IgG_2 and IgG_3 isotypes.[5,6] This CH1 deletion of the camelid HcAb structure is accomplished by a splice-donor site mutation immediately downstream of the CH1 exon, causing expression of the constant domains to skip the CH1, splicing the VHH exon directly to the CH2 exon.[6] Interest in HcAbs has grown since their discovery, particularly in using this antibody format for human therapeutics.[7] Since this format does not use a light chain for binding, the antigen-binding variable domain on the HcAb is half the size of a conventional antibody wherein a heavy chain natively pairs with a light chain. This opens potential for binding antigens in different ways, including targeting epitopes that may be inaccessible to conventional antibodies. In addition, the isolated single variable domains (sdAbs) from HcAbs can have improved efficacy in terms of tissue and tumor penetration or quick (and tunable) clearance for imaging, diagnostic, and theranostic applications.

Initial work with heavy chain-only antibodies entailed immunization and collection of wild-type camelid tissues, with downstream *ex-vivo* humanization of antibody candidates.[4,8] Later, naïve libraries of camelid antibodies were introduced for *in vitro* selection of antibodies, followed by pre-humanized heavy chain-only libraries.[8,9] Additionally, transgenic rodents have been engineered to express human heavy chain-only antibodies.[10–13] The transgenes used in all these rodent platforms incorporate the fully human VH genes on human genomic constructs, so they lack the encoded stabilizing mutations found in camelid VHH that have evolved to mutate the exposed hydrophobic VH/VL interfacial contacts to hydrophilic ones, which enables the VHH unit to exist as a soluble, autonomous binding unit, the sdAb. This could lead to sdAb candidates with poor developability characteristics, such as low stability or propensity for aggregation. Each method of antibody development and selection has its own advantages and disadvantages. Immunization of wild-type camelids allows for *in vivo* affinity maturation and screening for stable heavy chain-only antibodies against the antigen target but also requires humanization of candidates. This process could

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diminish original binding affinity and stability, which may inadvertently introduce immunogenicity risks unless these attributes are co-optimized, which is challenging to accomplish by engineering.[14] *In vitro* selection methods avoid large animal handling but do not provide intrinsic screening for stability of constructs or affinity maturation of the antibodies. Transgenic rodents provide *in vivo* affinity maturation, but as mammals, they are still highly homologous to humans, lessening the immunogenic potential of conserved antigen targets.

The advantages of small size and improved tissue penetration by sdAbs provide the potential for better therapeutic uses in solid tumor targeting as well as applications in imaging.[15,16] Additionally, the modular nature of the single domain and the resulting ease of recombinant formatting also lend itself to the construction of bispecifics, multispecifics, and other non-conventional formats in which multiple sdAbs can be joined together with flexible linkers.[17,18] Currently, there are over 20 sdAb-based therapeutic antibodies either approved for human use or in clinical trials, and many more are in preclinical development.[19] These are being pursued for various indications mirroring the use of conventional antibodies mainly in oncology and autoimmune disease. The first regulatory approval was granted in 2018 to caplacizumab for the treatment of thrombocytopenia; subsequently, two more sdAb-based antibody therapeutics, envafolimab and ozoralizumab, received approval in Asia (https://www.antibodysociety.org/antibody-therapeutics-product-data/). The source of the current class of sdAb sequences is mainly camelid immunization.

Avian species naturally produce heterodimeric conventional antibodies, consisting of heavy and light chains, as in mammals.[20] The evolutionary distance between birds and humans means that for human therapeutic targets, in particular, mammalian-conserved targets, chickens offer a potentially superior immune recognition over mammalian immune hosts, such as rodents.[21,22] Highly conserved proteins or conserved epitopes on human proteins that may not be immunogenic in mice can elicit robust responses in chickens, resulting in successful antibody discovery campaigns. In addition, cross-reactivity to the mammalian homologs of the target (mouse, cynomolgus monkey, or other) is readily obtainable in chickens, obviating the need for parallel surrogate antibody discovery.

Chickens have been shown to be capable of producing HcAbs. Previous work to fully knock out the light chain caused the chicken to develop limited numbers of B cells and, thus, to produce limited amounts of chicken-sequence HcAb.[23] To improve the humoral immune development, a truncated light chain was included into the design, consisting only of the light chain constant domain, serving as a binding chaperone for wild-type chicken heavy-chain expression.[24] This adjustment to the transgene design significantly improved B-cell development, as well as antibody expression and stability, allowing this chicken to serve as a proof of concept for the viability of HcAb expression in an avian species. Interestingly, wild-type chicken VH was shown to be capable of functioning in a heavy chain-only format *in vivo* and as recombinant HcAb fused to human Fc. As those birds expressed chicken-sequence HcAbs, the antibodies would require humanization if they were to be used for therapeutic applications.

Here, we present a novel transgenic platform, Omni***dAb***®, expressing human variable region sequences in a HcAb format in an avian host for use in human therapeutic antibody discovery. The platform retains the advantages of the chicken host for recognition of human therapeutic targets, including the potential for targeting conserved proteins, as well as the potential for expanded epitope coverage. The Omni***dAb***® antibodies should require limited to no downstream engineering since they have been optimized in the animal, both by transgene design and by B-cell selection during the immune response.

Results

*Production of Omni*dAb *transgenic chickens*

To express a heavy chain variable region without a light chain in a transgenic animal, two challenges associated with removal of the light chain need to be addressed: exposure of the VH/VL interface on the VH region and lack of a pairing partner for the CH1 domain of the heavy chain constant region that is normally paired with the CL. We designed a heavy chain variable region based on human VH3–23 and JH4 germline sequences, with 10 stabilizing mutations that enable its expression as an autonomous VH (Figure S1). These stabilizing mutations are found in camelid VHH and serve to stabilize the overall structure of the single unpaired domain by reshaping the former contact regions with VL and CH1 and increasing hydrophilicity of positions that become solvent-exposed when VL and CH1 are removed.[25] This variable region was inserted into the chicken heavy-chain locus in which the endogenous V, D, and J gene segments had been deleted (Figure 1). The construct contains the regulatory elements of the chicken IgH locus for proper temporal and tissue-specific expression. In wild-type chickens, single VH and JH genes and a small number of highly related D genes are utilized in VDJ recombination.[26] Because VDJ recombination provides minimal diversity to the antibody repertoire in chickens, we opted to use a pre-rearranged VH region for Omni***dAb*** antibodies, bypassing the recombination step. This germline variable region and the transgene that carries it is referred to as "VHH3". Repertoire diversity in chicken immunoglobulins is normally generated by gene conversion from upstream pseudogenes, and we have replicated this process by the inclusion of an array of designed human-based pseudogenes that are available in developing B cells for gene conversion of the single, expressed VHH3 gene (Figure S1). The stabilizing mutations are present in the FRs of the pseudogenes, serving to maintain their presence in the somatic repertoire. Any random mutations in the FRs would be reverted to the original sequence by gene conversion. In contrast, the complementarity-determining regions (CDRs) contain diverse sequences for the production of a diverse repertoire of antibody sequences. As for the CH1 domain in the constant region, which normally requires pairing with the light chain to displace BiP and enable proceeding through the secretory pathway, we have engineered a second transgene in the light chain: a truncated light chain (tLCi) consisting of only the constant region without a variable region.[24] This transgene

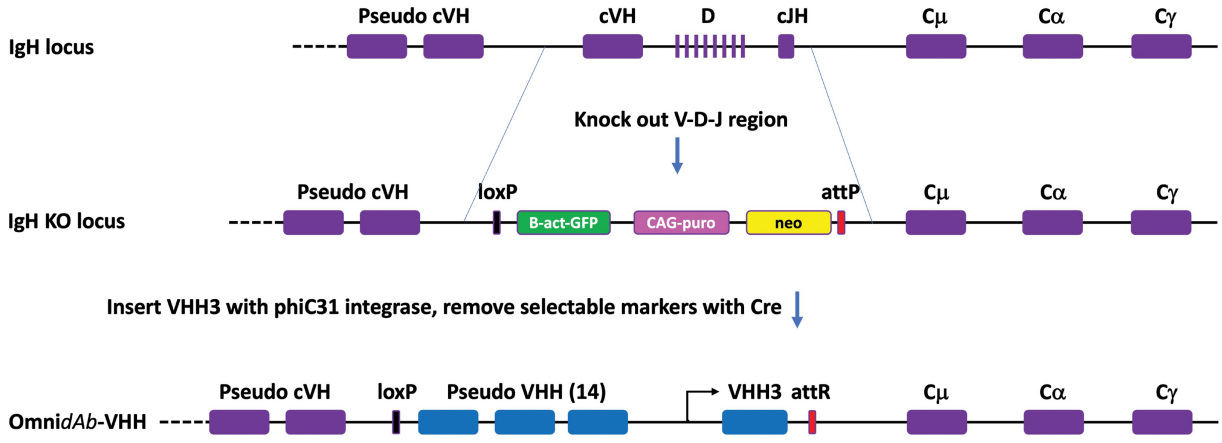

Figure 1. Schematic of VHH3 transgene design. The endogenous chicken heavy chain locus (top line) was genetically modified in a series of steps in chicken primordial germ cells (PGCs). First, the germline V-D-J genes were deleted by gene targeting in two steps.[34,35] In the targeting steps, loxP sites and selectable markers were inserted into the locus (middle line), including an attP site for insertion of an attB-bearing plasmid by phiC31 integrase, with a promoter-less neo gene (in reverse orientation relative to the locus). Upon insertion of the VHH3 transgene, a β-actin promoter on the incoming plasmid (not shown) activated the neo gene, allowing for G418-selection of transfectants. PGCs carrying the VHH3 insertion and selectable markers were injected into embryos to produce germline chimeras. Chimeric males producing germline progeny were bred to Cre-expressing females to loop out the selectable markers, resulting in the structure on the bottom line, with the VHH3 functional gene, 14 pseudogenes and intervening sequences properly positioned to express and splice to the downstream chicken constant regions. The chicken VH pseudogenes remain upstream. Schematics are not to scale. Abbreviations: cVH, chicken VH gene; D, chicken diversity gene segments; cJH, chicken JH gene; pseudo cVH, chicken VH pseudogenes; B-act-gfp, chicken β-actin promoter driving the EGFP gene; cag-puro, CAG promoter (chicken β-actin promoter with CMV enhancer) driving the puromycin resistance gene; neo, promoterless neomycin resistance gene.

was inserted into the light-chain locus, in which the endogenous V, J, and C regions had been deleted.

These transgenes were inserted into primordial germ cells, chimeras were produced, and the chimeras bred to establish fully transgenic lines. Birds with both the VHH3 and tLCi transgenes were produced, in addition to VHH3 birds with a homozygous IgL knock out (KO). At the heavy-chain locus, the genotype was heterozygous VHH3/IgH KO and at the light-chain locus: either heterozygous tLCi/IgL KO or homozygous IgL KO/IgL KO. To simplify references to these transgenic genotypes, these genotypes will be referred to here as VHH3-tLCi (for tLCi/IgL KO light chain alleles with a VHH3/IgH KO heavy chain) or VHH3-IgL KO (for homozygous IgL KO/IgL KO light chain alleles with a VHH3/IgH KO heavy chain).

B-cell development and antibody expression in OmnidAb chickens

Analysis of B-cell development in these heavy chain-only chickens showed similar titers of circulating IgM, but very limited isotype switching to IgY (Figure 2a), based on enzyme-linked immunosorbent assay (ELISA), correlating with the limited isotype switching observed in the previously developed heavy chain-only chicken-expressing wild-type chicken HcAbs.[24] Flow cytometric analysis (Figure 2b) of the VHH3-tLCi transgenic chicken peripheral blood mononuclear cells (PBMCs) exhibited proper development of B cells, based on Bu-1 staining, with the B-cell population averaging about 30–60% of normal (3% of total PBMC compared to 5–10% in wild-type chickens). Polyclonal staining for surface IgM also confirmed the expression of antibodies on these B cells, but monoclonal IgM staining specific to the CH1 domain did not detect a positive population in the VHH3-tLCi or VHH3-IgL KO transgenic genotypes. Monoclonal IgL staining was also negative for both transgenic genotypes, consistent with the lack of a CH1 domain for CL binding, but remained positive for the wild-type control. Additionally, polyclonal rabbit serum raised against the transgenic VHH3 domain was produced to serve as an additional VHH3 transgene-specific detection method during analysis of B-cell development. In flow cytometry, this anti-VHH3 antibody only detected cells in the transgenic birds and did not stain any cells in the wild-type chickens. Sample control staining to the T-cell receptors (TCRs) was comparable between the wild-type control and the transgenic birds. Reverse transcription (RT)-PCR of the PBMC samples for the detection of IgL or IgM messenger RNA (mRNA) was also performed (Figure 2c). For IgL transcription, the VHH3-IgL KO genotype contained no light chain, as expected, while the VHH3-tLCi genotype contained the truncated light chain, smaller than the full-sized light chain exhibited in the wild-type chicken. For IgM transcription, both transgenic genotypes contained heavy chains that were ~300 base pair (bp) smaller than the wild-type heavy chain, consistent with CH1 deletion in the heavy chain. Western blots of the plasma samples for the detection of IgM antibodies (Figure 2d) are consistent with the flow cytometry and RT-PCR data (Figure 2b–c), in that the heavy chain protein exhibited smaller bands compared to the wild-type, confirming the Omni***dAb*** transgenic chickens, both the VHH3-IgL KO and VHH3-tLCi versions of the genotypes, do not express the CH1 domain.

Antibody discovery in OmnidAb transgenic chickens

Omni***dAb*** transgenic chickens were immunized with either the human progranulin (PGRN) protein or the extracellular domain of the human natural killer (NK) cell receptor, NKp46. PGRN is a multi-domain protein that has served as an antigen target for multiple transgenic chicken evaluations,[27–29] with well-established reference controls for

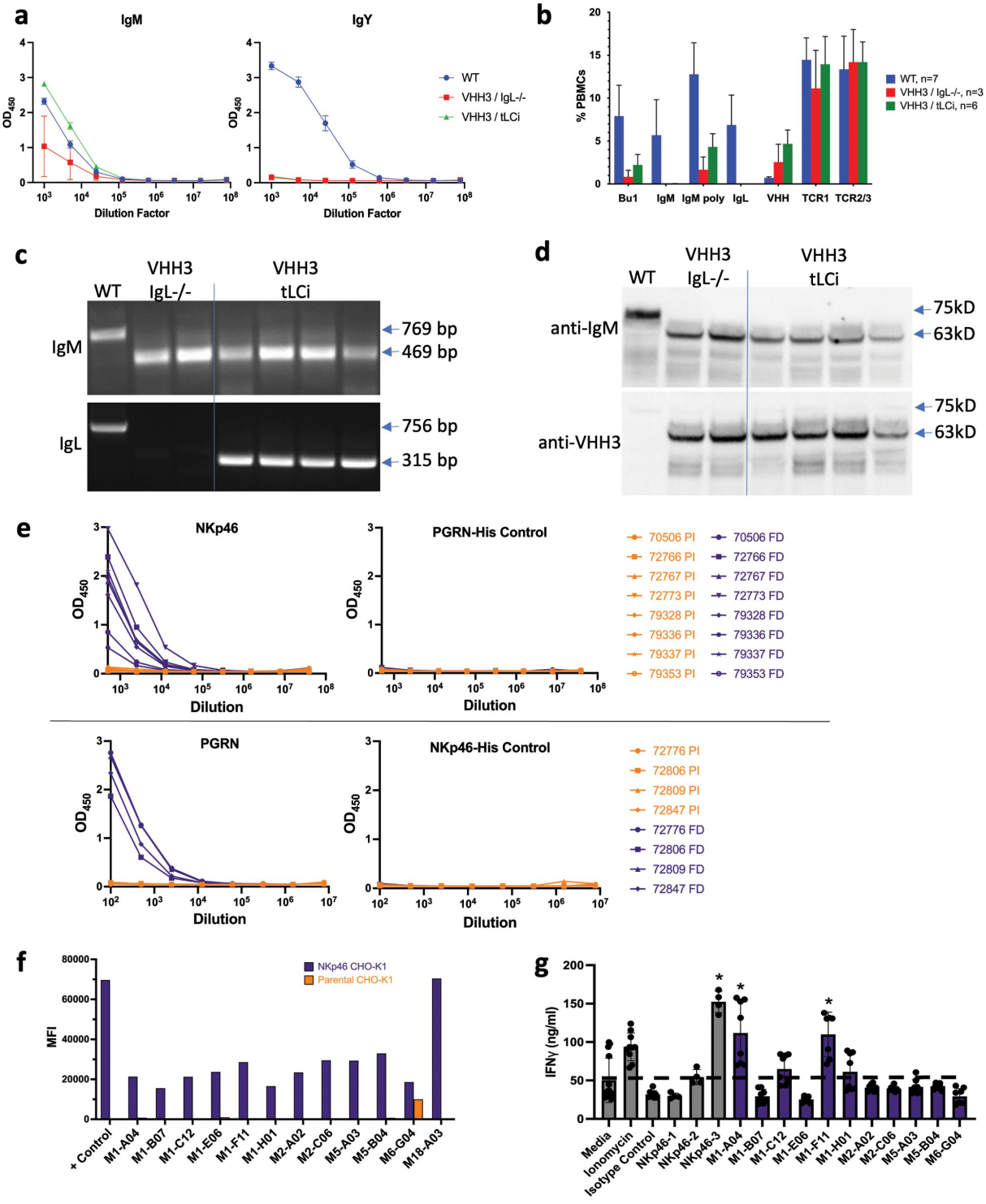

Figure 2. Transgenic bird evaluations for B-cell development and subsequent antibody discovery. Total circulating IgM or IgY titer (a) of different versions of the transgenes (VHH3/IgL-/- or VHH/tLCi) was compared to wild-type (WT) chicken based on plasma ELISA. Transgenic chickens exhibited only IgM antibodies, with no IgY isotype switching/expression. Flow cytometric analysis (b) of PBMCs with immunostaining for chicken B-cells (Bu1), CH1-domain-specific IgM (IgM), polyclonal IgM (IgM poly), light chain (IgL), the VHH3 transgene (VHH), and T-cell receptor staining controls (TCR1 and TCR2/3). Transgenic chickens presented with a reduced population of B-cells, IgM antibody expressed without CH1 domain, no light chain, but positively expressing the VHH3 transgene. Gel electrophoresis image (c) of PBMC RT-PCR product showed a reduced size IgM after amplification, as compared to the wild-type chicken. Light chain mRNA was not observed in the VHH3/IgL -/- genotype, while a reduced-sized light chain was observed in the VHH3/tLCi genotype. Western blot (d) of plasma samples for verification of IgM or VHH3 transgene protein expression exhibited a reduced-sized IgM (no CH1) and positive expression for the VHH3 transgene protein, as compared to the protein weight of the wild-type which expressed full-size IgM and no VHH3. A subset of Omni*dAb* chickens were immunized with either NKp46 extracellular domain protein or progranulin (PGRN) protein to verify immuno-responsiveness to immunization and development of candidate monoclonal antibodies. Antigen-specific immune responses (e) were evaluated from plasma samples by ELISA. The pre-immune (PI) titer and final draw (FD) titers are presented against their respective antigen target used in immunizations or a His-tag control target. No background binding to the target antigen was observed in the PI samples. FD samples exhibited strong binding to their respective target antigen and no binding to the His-tag control antigens. A select group of NKp46-binding clones were screened for binding to native protein (transfected CHO-K1 cells) via flow cytometry (f), showing mean fluorescence intensity (MFI) binding to the NKp46-expressing CHO cells and minimal binding to the non-transfected parental CHO cells. A commercially purchased mouse anti-NKp46 antibody was used as a biological positive control. A select group of NKp46-binding clones was also screened for functional ability (g) to induce IFNγ release from primary nk-cells in cell culture, with two clones exhibiting a significant ability to induce IFNγ as compared to the media-control threshold. Ionomycin served as an ionophore positive control. The isotype control antibody was a non-binding IgG_1 antibody, matching the experimental samples' antibody isotype. Reference antibodies NKp46–1, −2 and − 3 were published NKp46 antibodies.

each of the granulin domains in epitope binning.[21] NKp46 was also chosen as a target because of the potential for the development of bispecific, NK-cell engager antibodies as therapeutics. Strong antigen-specific plasma titers were observed in these birds, with no background binding to the nonspecific protein detected (Figure 2e). Upon reaching sufficient titer, splenocytes were collected and screened for the secretion of antigen-specific antibodies using GEM and xPloration technologies. GEM and xPloration are both single B-cell screening technologies, the former utilizing agarose gel microenvironments for screening on a microscope with manual removal of selected cells and the latter utilizing a microcapillary chip with laser extraction of selected wells. Following single B-cell screening for antigen-specific binding, variable regions were amplified and cloned into an expression vector carrying a human Fc. A recombinant sdAb-Fc antibody was the format tested in all downstream assays. A total of 38 anti-PGRN clones and 128 anti-NKp46 clones were identified and confirmed in ELISA. Binding was specific as no binding to an unrelated protein or His-tag was observed.

A randomly selected subset of 12 antibodies was screened for their ability to bind native NKp46 protein expressed on the surface of Chinese hamster ovary (CHO) cells by flow cytometry (Figure 2f) and all exhibited binding to the NKp46-expressing cells with little to no background binding to the parental CHO cells. Thus, binding to the immobilized protein on ELISA (data not shown) correlated well with binding to the native protein for the NKp46 target. Most of this subset was also tested for the ability to activate primary human NK cells to release interferon gamma (IFNγ) in cell culture. Of the 11 clones tested, two clones were able to significantly induce the release of IFNγ, as compared to the media-only negative control, and were comparable to the positive reference control antibody included in the assay (Figure 2g). As expected in antibody discovery, not all of the antigen-binding antibodies, in this case NKp46, have the ability to activate the target receptor, but functional activity can be easily included in the pipeline during antibody discovery should this be a desired characteristic for the antibody.

Sequence analysis of the anti-PGRN and anti-NKp46 Omni***dAb*** clones showed that FRs 1, 3, and 4 were largely unchanged, with the stabilizing mutations still present in most of the clones (Figure 3a). This was expected since the VHH3 pseudogenes contained FR sequences identical to those in the functional gene, so gene conversion in the FRs would serve to maintain the original sequence rather than mutate it. The one exception was FR2, in which substantial levels of mutation were observed, even though the pseudogenes contained identical sequences. These mutations must have occurred through random somatic hypermutation (SHM) and were not reverted or erased by gene conversion (GC). These changes were mainly in the hallmark FR2 stabilizing residues that are defined as framework by the international ImMunoGeneTics information system (IMGT),[30] but the other residues also affected are considered CDR1 or 2 in the Kabat definition, suggesting that they could be antigen-contact residues. One of the stabilizing changes is in Vernier residue W52 (IMGT) and shows diverse mutations, suggesting it may affect the CDR structure in those clones. In a few clones, W118 (the first residue of FR4) was mutated to arginine, as is observed in some camelid VHHs.[31] These FR2 and FR4 changes are likely undergoing positive selection, considering the other FRs did not show similar levels of mutation. The overall hydrophobicity of FR2 as measured by GRAVY remained in the hydrophilic range, with some clones having a higher score (tending toward hydrophobic) than the

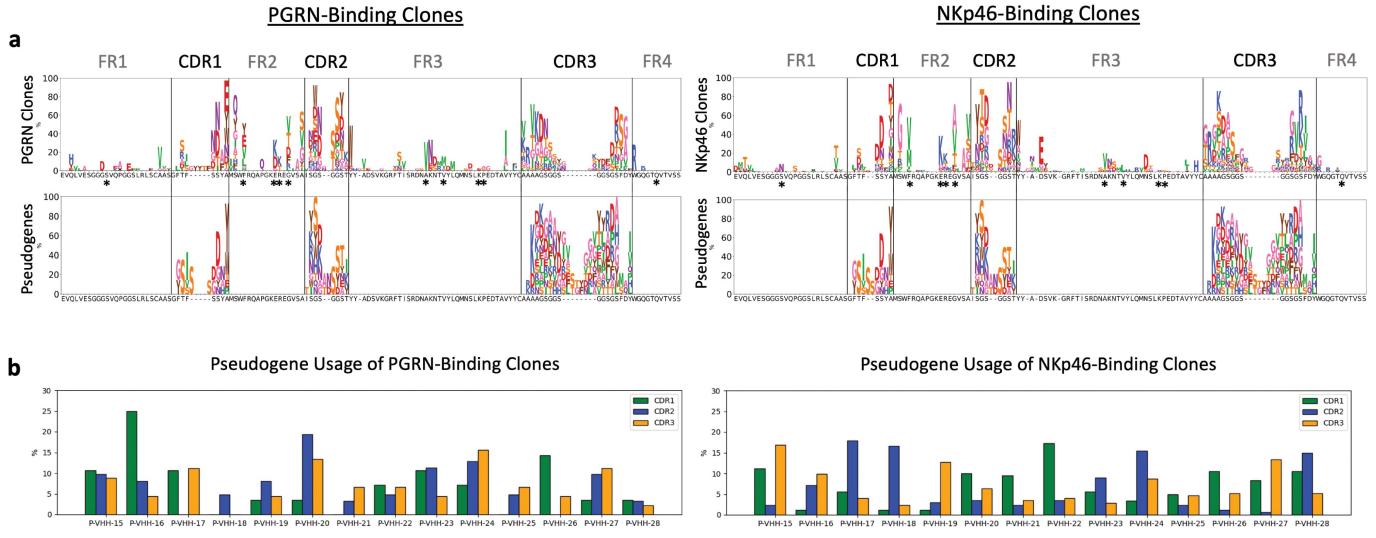

Figure 3. Sequence analysis of antigen-specific HcAbs. Mutational frequencies in the anti-pgrn and anti-NKp46 VH regions were calculated and compared to the frequencies of potential donor residues in the pseudogene pool. VH sequences to PGRN (38 clones) or NKp46 (128 clones) were aligned to the germline VHH3 sequence (a) and mutational frequencies at each position are shown in a WebLogo plot. The top plot in both panels shows the results for the antibody clones, and the lower plot shows the same analysis for the pseudogene sequences in the VHH3 construct for comparison. The location of the stabilizing framework changes in the VHH3 functional gene (relative to the human VH3–23 gene) are indicated by asterisks under the top logo plot. The usage frequency of gene conversion (b) in the anti-pgrn and anti-NKp46 HcAb clones by each pseudogene in the VHH3 construct is shown. Identification of gene conversion and its pseudogene source was first performed. For each pseudogene, the percentage of HcAb clones in which the pseudogene CDRs were utilized to mutate the clones was then calculated. Clones and CDRs in which gene conversion was not identified or where gene conversion was identified but the pseudogene was ambiguous were not included. Gene conversion by the three CDRs is shown in a different color as indicated. Examples of gene conversion by each individual pseudogene CDR were found.

germline and others lower (more hydrophilic) (Figure S2). Thus, the mutations in FR2 were not the result of a consistent selection for increased hydrophilicity, although selection for other biophysical attributes such as stability could be occurring. The CDRs also showed high levels of variation in the set of clones. The CDR3 lengths for the set of PGRN and NKp46 clones were centered around a length of 17, which is the length in the germline VHH3 gene (Figure S3). However, for both targets, some shorter or longer CDR3s were observed, indicating that gene conversion and/or SHM can lead to insertions or deletions in the repertoire in birds. For the PGRN clones, a prominent lineage with a CDR3 length of only nine residues was apparent.

Gene conversion by the designed pseudogenes was analyzed for the set of PGRN and NKp46 clones using an algorithm that detects stretches of sequences that can be traced to a pseudogene. Gene conversion was detected in most of the sequences (Table 1), whereas some clones contained mutations such as point mutations or stretches shorter than four base pairs that could not be assigned to gene conversion with the algorithm. Most of the gene conversion events could be assigned to a specific pseudogene, while some of them were ambiguous and had sequence matches in more than one pseudogene. All pseudogenes were found to participate in gene conversion and contribute sequence diversity to the clones (Figure 3b). Mapping of the gene conversion events showed that up to 6 events within a CDR could be involved in mutating the sequence (Figure 4). CDRs showed a range in terms of the number of gene conversion events and lengths of sequence donated by pseudogenes in each case.

Omni***dAb*** antibody sequences were evaluated *in silico* for potential immunogenicity and were compared with benchmark controls as well as clinical-stage VHH molecules.

Table 1. Gene conversion frequencies.

Target	CDR	# Total CDR	# CDR w/GC not found	# CDR w/GC	# CDR w/GC defined	# CDR w/GC undefined
NKp46	CDR1	128	13	115	98	17
	CDR2	128	1	127	104	23
	CDR3	128	16	112	90	22
PGRN	CDR1	38	6	32	24	8
	CDR2	38	5	33	29	4
	CDR3	38	4	34	23	11

HcAb VH sequences specific for NKp46 (128 clones) or PGRN (38 clones) were analyzed for evidence of gene conversion (GC) by the upstream-designed pseudogenes in the VHH3 construct. For most of the CDRs, gene conversion could be assigned, with only a few sequences showing mutations that could not be traced to a pseudogene (GC not found). None of the CDRs showed the germline VHH3 sequence. For the GC events, most were traced to a specific pseudogene (GC defined), whereas in some cases, it was ambiguous as more than one pseudogene could have donated the sequence (GC undefined).

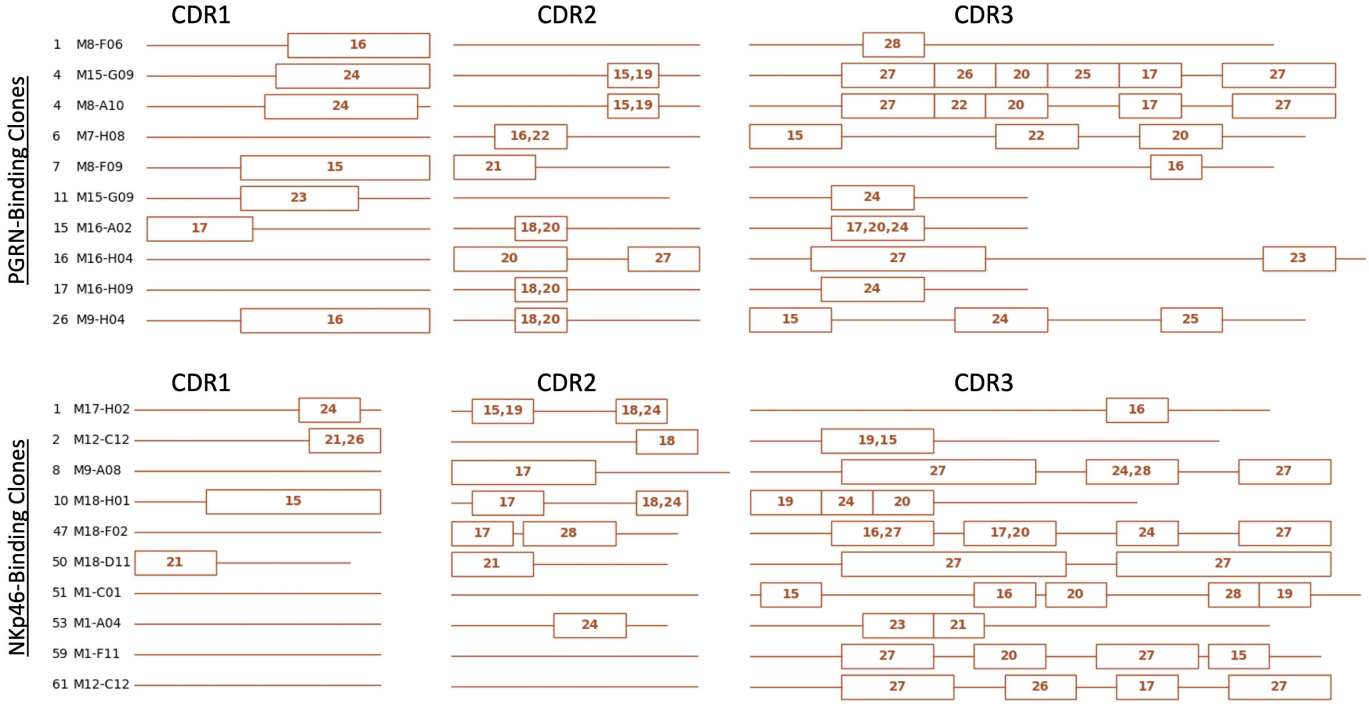

Figure 4. Gene conversion in CDRs. Gene conversion tracts in the CDRs of antigen-specific HcAb clones were mapped to their pseudogene source. For each clone shown, the CDRs are represented by lines, with a box to indicate position and approximate length of each gene conversion tract. The numbers in the boxes indicate the identifier number of the pseudogene that donated sequence (figure S1). Tracts with two or more numbers indicate that the pseudogene could not be unambiguously assigned and any of the listed pseudogenes could have donated the sequence. Only the CDRs are shown since the VHH3 construct pseudogenes contain no diversity in the FRs and thus gene conversion is only observable in the CDRs. CDRs with no boxes did not have recognizable gene conversion, but all CDRs did show mutation from the germline VHH3 sequence. Somatic hypermutation and point mutations are not shown in the diagrams. Anti-pgrn clones are shown in the upper panel and anti-NKp46 clones are shown in the lower panel. Representative clones from different lineages are shown.

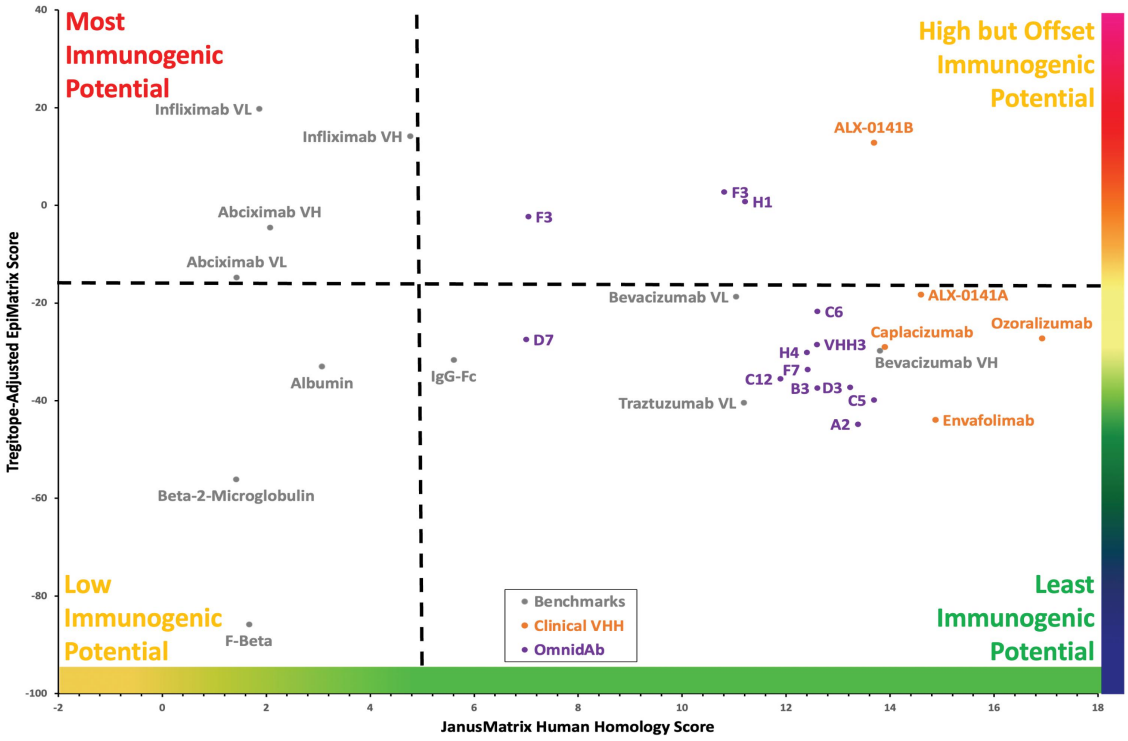

Figure 5. Immunogenicity plot. *In silico* evaluation of immunogenicity was performed on a random subset of Omni*dAb* molecules with antibody sequences analyzed as overlapping 9-mer segments and screened with EpiVax software for the presence of class ii-restricted HLA ligands and putative T-cell epitopes to calculate binding potential. Tregitope-adjusted EpiMatrix scores <-15 are considered to have low predicted immunogenicity and scores <-30 are considered minimal immunogenicity. A JanusMatrix human homology score for putative T-cell epitopes > 5 suggests reduced immunogenic potential.

Tregitope-Adjusted EpiMatrix Scores were calculated and plotted relative to the JanusMatrix Human Homology Score (Figure 5). The plot can be divided into quadrants based upon high/low T-cell epitope density (EpiMatrix score) and high/low human homology (JanusMatrix score). The sequences with the lowest immunogenic potential have low T-cell epitope density and the putative epitopes that are present have high homology with the human proteome. Conversely, high T-cell epitope density and low homology with human proteins predict a high risk of immunogenic response. All Omni*dAb* sequences had a high JanusMatrix human homology score. Tregitope-adjusted EpiMatrix scores were variable but primarily fell into the low to minimal risk categories. Consequently, the majority of the sequences analyzed fell within the lowest risk quadrant and no Omni*dAb* sequences were observed in the highest risk quadrant. A small number of the sequences had higher (>−15) EpiMatrix scores, but this may be partially offset by high human homology. The immunogenic potential of Omni*dAb* sequences was comparable to other clinical-stage VHH molecules.

*Omni*dAb *antibodies show broad epitope coverage*

To assess the epitope coverage of antibodies produced from the immunization of Omni*dAb* engineered animals, we performed detailed epitope binning studies on panels of Omni*dAb* clones. The results in Figure 6a,b summarize the epitope coverage produced by 38-unique-sequence anti-PGRN clones as judged by epitope mapping and binning experiments. Figure S4 shows the design of the PGRN chimeras used for epitope mapping, a heat map of the chimera mapping data, and a summary of the epitope binning (antibody competition) results visualized as a network plot. Epitope binning studies served to corroborate and expand upon the results determined by our chimeric-swap epitope mapping strategy, which was restricted (by design) to only human-specific, and not mouse cross reactive, clones. Despite the small sample size of only 38 Omni*dAb* antibodies, their epitope coverage not only overlapped with, but extended beyond, that of our standards, which represented a curated set of clones from previous PGRN campaigns in wild-type,[21] OmniChicken,[27,29] and OmniClic[28] chickens. Figure 6a shows a pie chart of the results obtained from a merged analysis of the epitope mapping and binning experiments on PGRN. A total of 11 blocking profiles or 'bins' were identified, covering all 7 granulin subdomains (A, B, C, D, E, F, and G) including the small N-terminal paragranulin (p). Some newly identified epitopes not represented by our standards included a CD epitope that bridged two non-overlapping epitopes represented by our standards (CD + CD'), an E bin epitope that did not block our E bin standard (E'), an E bin epitope that bridged E+E', a new G bin (G'), and a B epitope that blocked our B standards, but showed no binding to any of the chimeras, thereby deviating from the mapping result characteristic of B bin clones (chimera-2

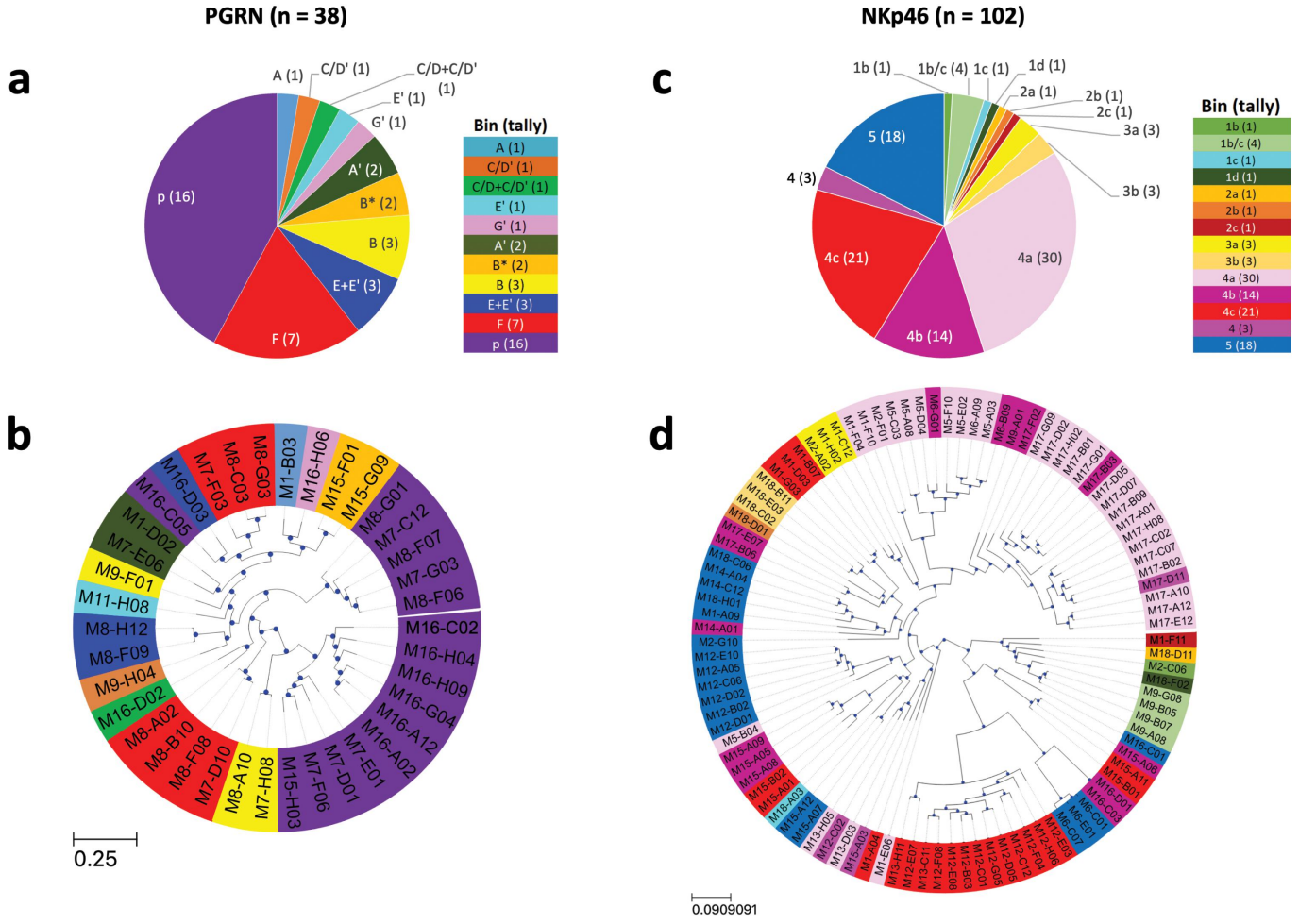

Figure 6. Epitope coverage of HcAbs raised against model targets PGRN and NKp46. Pie chart (a) showing the distribution of 38 unique-sequence anti-pgrn clones to epitope bins named by their specific granulin subdomain (A, B, C, D, E, F, G, and p) and sub-bin nuance within those granulins (A′, B*, CD′, CD+CD′, E′, E+E′ and G′) using a merged analysis of epitope mapping and binning data. Eleven epitope bins were identified. Sequence tree (b) merged with bin assignments for the anti-pgrn VH sequences. Pie chart (c) showing the distribution of 102 unique-sequence anti-NKp46 clones to different blocking profiles or 'bins' as determined by epitope binning assays. Five non-overlapping bins (1, 2, 3, 4, and 5) were identified, with nuanced blocking behaviors fracturing them into sub-bins (1b/c/d, 2a/b/c, 3a/b, and 4a/b/c) giving a total of 12 observed profiles. The blocking profiles of the benchmark clones were defined by bins 1a (NKp46–1), 1e (NKp46–4), 2c (NKp46–2), and 3b (NKp46–3). Sequence tree merged with bin assignments for the NKp46 VH sequences (d). The scales in the trees represent relative distance (based on amino acid differences in the sequence).

binders). The most populated bins were p and F with 16 and 7 members each, with other bins each being populated by one to three clones. Figure 6b shows how the epitope bin assignments correlated with the antibody sequence for anti-PGRN antibodies. As expected, clones with highly similar sequences targeted the same bin, but we also observed cases of dissimilar clonal lineages converging on the same bin, as shown for p and F binders, which were each represented by three distinct clonal lineages.

The pie chart in Figure 6c summarizes the epitope coverage produced for a panel of 102 unique-sequence anti-NKp46 antibodies. Bin assignments were deduced from a merged analysis of three heat maps generated from a series of epitope binning experiments that used an intersecting but non-identical set of clones (Figure S5). A total of 12 blocking profiles were identified, representing five non-overlapping bins (1, 2, 3, 4, and 5) with nuanced blocking behaviors fracturing bin 1 through bin 4 into sub-bins; 1b/c/d, 2a/b/c, 3a/b, and 4a/b/c. In comparison, the four literature benchmarks used (NKp46-1, NKp46-2, NKp46-3, and NKp46-4) were assigned to bins 1a, 2c, 3b, and 1e, respectively. Therefore, the Omni***dAb*** antibodies not only closely recapitulated the blocking profiles of all the benchmarks used but showed expanded epitope coverage that produced new and distinct epitopes, bin 4 (comprising 4a/b/c sub-bins) and bin 5.

Figure 6d shows how the epitope bins deduced for anti-NKp46 clones clustered by their antibody sequences. As observed for our PGRN example, clones with highly similar sequences targeted the same bin, while, for some bins, multiple disparate clonal lineages converged on the same bin. Fine epitope differences, as defined by sub-bins, were also highly correlated with antibody sequences, showing the exquisite discriminatory power of our binning method.

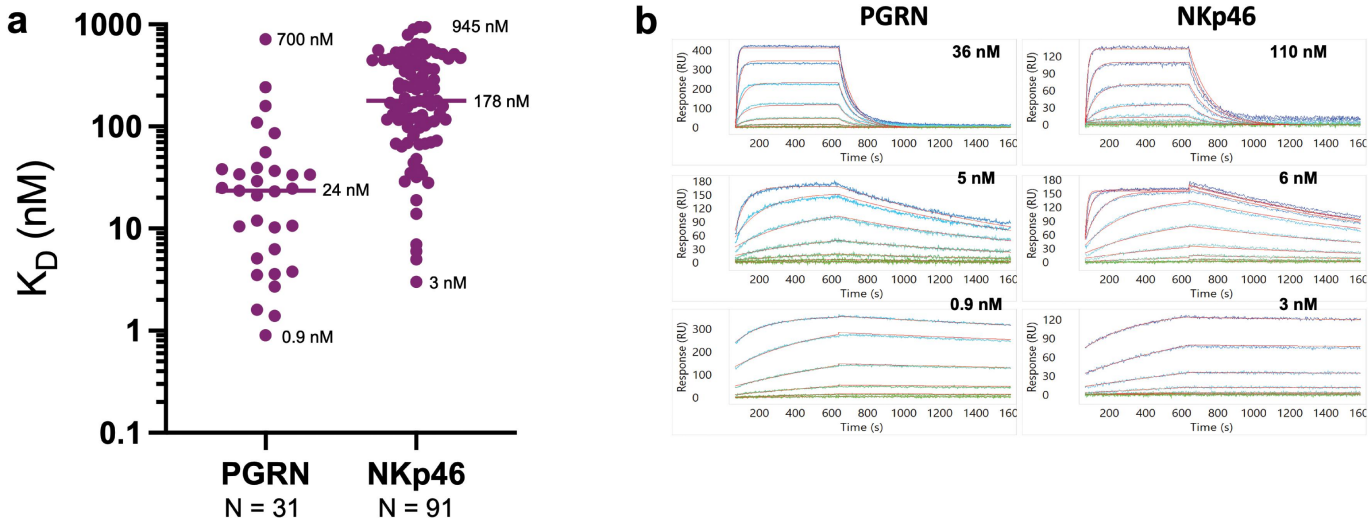

Figure 7. Kinetic and affinity determinations for anti-pgrn and anti-NKp46 Omni*dAb* clones. Scatter plot showing affinity distribution (a) with highest, median, and lowest affinities indicated. Global fits showing diverse kinetic profiles (b) for high, medium, and weak affinities (K_D values reported on plots) of select PGRN and NKp46 clones. Measured data are shown in blue–green with global fits overlaid in red.

We also determined the affinities of the antibodies produced against both model targets and found that they exhibited a range of kinetic profiles, with some of the highest affinity clones attaining single-digit nM K_D values (Figure 7). Full kinetics and bin assignments for PGRN and NKp46 antibodies are provided in Tables S1 and S2.

Developability assessment

Omni***dAb*** antibodies specific to PGRN and NKp46 were evaluated in sdAb-Fc format for various development metrics, such as thermal stability, analytical size-exclusion chromatography (aSEC), and self-interaction using affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS). All of the antibodies specific for PGRN and NKp46 exhibited high T_m and T_{agg}, as determined by thermal stability analysis (Figure 8a,b). The aSEC analysis confirmed that all the antibody hits showed a homogeneous monomeric peak corresponding to a size of approximately 70 kDa based on elution time (Figure S6), the expected size for this format, indicating no aggregation of the antibody preparations.

AC-SINS is an assay developed to monitor the propensity for antibody self-association. Self-association at high concentrations can result in developability challenges such as poor solubility, aggregation, and high viscosity. We defined $\Delta\lambda max < 5$ nm as a fairly stringent limit for the selection of only well-behaved monoclonal antibodies (mAbs) for downstream development.[32] The AC-SINS studies revealed that most identified Omni***dAb*** antibodies displayed minimal plasmon shifts ($\Delta\lambda max < 5$ nm), supporting their potential for developability. As references, we included eight human and humanized antibodies in late-stage clinical trials and one FDA-approved humanized VHH-Fc (caplacizumab) (Figure 8c). All of these clinical antibodies have $\Delta\lambda max$ values of less than 5 nm, confirming their good developability. We also ran a set of antibodies confirmed in the previous literature to have poor AC-SINS scores[32,33] and all showed $\Delta\lambda max$ values greater than 5 nm, suggesting strong self-association in phosphate-buffered saline (PBS) at pH7.4.

Discussion

Here, we report the development of a heavy chain-only chicken expressing human variable regions, Omni***dAb***. Expression of HcAbs in the intact immune system requires engineering of the variable region, which lacks a VL partner, and dealing with the CH1 domain, which lacks a CL partner. Our engineered single domain variable region framework in the VHH3 transgene was able to express well as an autonomous VH region, and for the CH1 domain, we provided a light chain consisting of only the constant region, the tLCi transgene. However, upon expression of the VHH3 transgene in Omni***dAb***, we discovered that the tLCi chaperone light chain was no longer absolutely required for B-cell development or antibody expression in these birds. The tLCi transgene did have some effect however, as somewhat higher antibody expression was observed in the tLCi birds (Figure 2a), but the homozygous IgL knock-out birds were still able to produce adequate levels of functioning B cells and antibodies. The mechanism remains unknown, but this suggests that tLCi aided in B-cell development or expression of the heavy chain; yet, this was not required when the variable region was engineered for single-domain expression. Previous work with heavy chain-only wild-type chickens showed that the expression of tLCi was essential for proper B-cell development and heavy chain expression.[24] It was somewhat surprising that the wild-type, non-engineered chicken VH region, which normally pairs with the VL, could readily express as a HcAb both *in vivo* and as a recombinant antibody. In contrast, when a non-engineered fully human VH transgene was combined with the tLCi transgene in birds, no expression or B-cell development was observed. Thus, the engineered framework

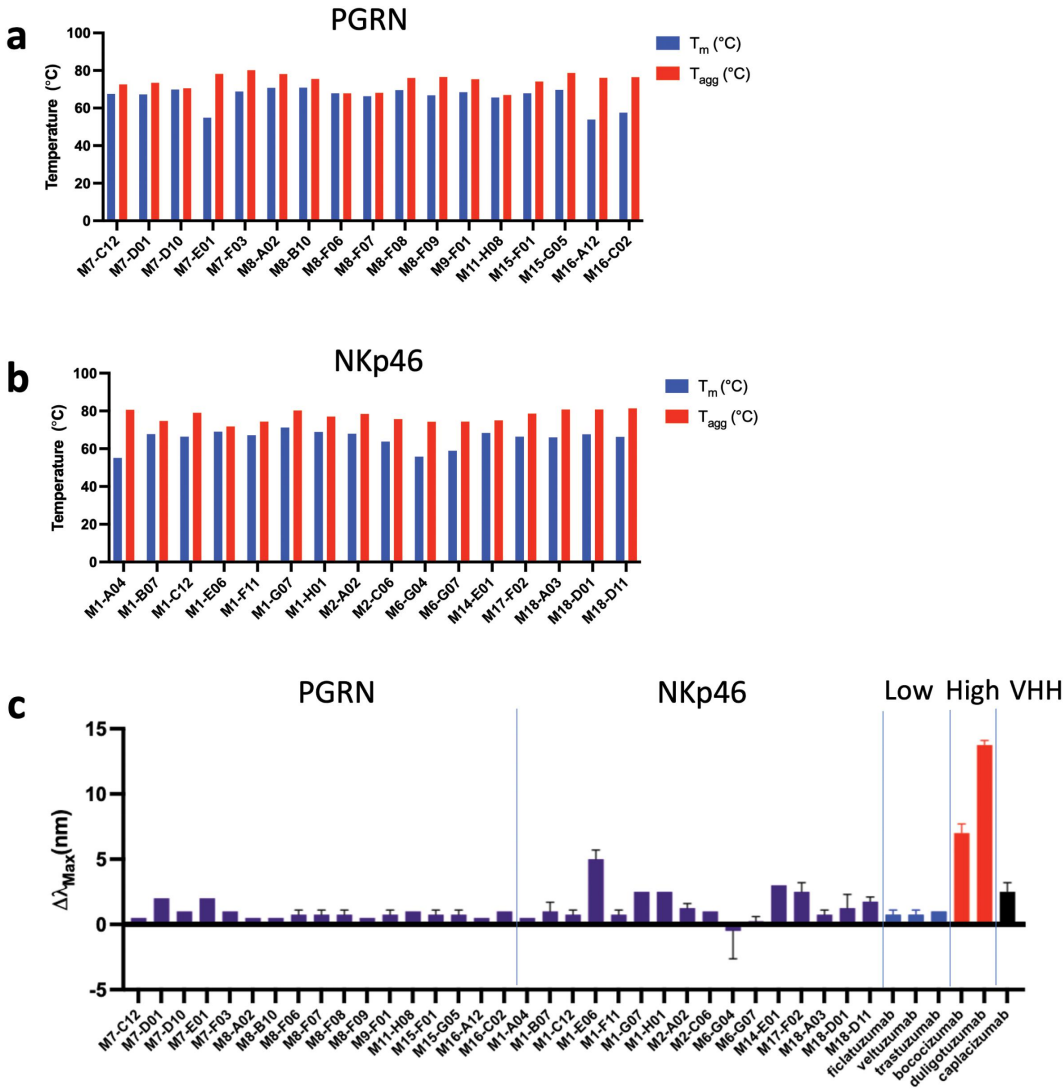

Figure 8. Developability assessment of antibody clones. Differential scanning fluorimetry analysis was performed on the selected anti-pgrn (a) and anti-NKp46 (b) Omni***dAb*** antibodies in sdAb-Fc format that showed high melting temperature (T_m) and aggregation temperature (T_{agg}). AC-SINS was performed to assess self-association for the selected anti-pgrn and anti-NKp46 clones (c), with the positive reference (ficlatuzumab, veltuzumab, and trastuzumab) and negative reference (bococizumab and duligotuzumab) IgG antibodies with known low and high AC-SINS values, respectively.[32,33] The FDA-approved VHH-Fc antibody caplacizumab was also included as a reference for the sdAb-Fc format.

in the Omni***dAb*** birds was essential for expression, support of B-cell development, and subsequent antibody discovery of human HcAbs in transgenic chickens. The introduction of VHH3 did not resolve the birds' inability to class switch to IgY, but it was observed that the antigen-specific IgM antibodies produced were affinity matured with diverse epitope coverage (Figure 6).

The optimized single VH scaffold in the Omni***dAb*** birds was designed to be maintained by the process of gene conversion, in which the pseudogenes upstream of the single functional gene in the transgene contain the same stabilizing changes. For the most part, the stabilizing changes engineered into the VH scaffold were maintained, and the biophysical characterization of the antibodies suggests that these changes provide important stability and solubility to the antibodies. The one notable exception was in the hallmark "tetrad" in FR2, where random mutation occurred despite the propensity of gene conversion to erase it. We speculate that the high level of mutation to a variety of different amino acids (AA), not just of one type, suggests that those residues are involved in antigen contact. Another possible scenario is that the elongated CDR3 in HcAbs may fold down and contact FR2, with variation in these contacts. These FR2 mutations would thus be selected for improving biophysical characteristics of the variable region such as stability or lack of aggregation rather than participating in antigen-binding. Structural studies will be necessary to investigate these ideas further. High levels of diversity were introduced by gene conversion in the CDRs, as designed. CDR3 lengths were centered around the germline length of 17 residues, even though most of the pseudogenes have longer CDR3s, consistent with most gene conversion events involving partial sequence replacement rather than complete CDR replacement. Although Omni***dAb*** antibodies showed a high level of sequence variation, from both gene conversion and somatic hypermutation, they had low predicted immunogenicity. The natural *in vivo* B-cell selection process for these diverse

sequences may underscore the viability of these changes as they favor good biophysical properties, such as high-level expression, stability, and solubility.

A subset of the NKp46-specific antibodies activated IFNγ release. For multispecifics, if the aim is to use the receptor as a tether for targeting of an NK or T cell to a tumor, receptor activation might be disadvantageous. Therefore, a cohort of antibodies displaying functional diversity could be an advantage during an antibody discovery campaign to give options compatible with custom molecular formats and geometries designed to achieve the intended mechanism of action. In self-interaction studies, the anti-NKp46 antibodies seemed to have slightly elevated self-association as compared to the anti-PGRN clones, although they were still within an acceptable range for developability. The different target specificity could have resulted in these differences based on the paratopes that evolved for the NKp46 clones. No correlation with GRAVY was observed.

As with the other transgenic chicken platforms OmniChicken[27,29] and OmniClic,[28] the Omni***dAb*** chickens recognized a broad range of epitopes, consistent with the superior recognition of human targets by the chicken as a host immunization species, due to its divergent phylogenetic distance.[22] Omni***dAb*** chickens also produced clones with a range of affinities, including high affinities showing K_D values in the single digit nM range. When designing a bispecific, kinetic diversity is an advantage since moderate affinity may be sufficient for therapeutic effect, since avidity effects with 2 + 2 bispecifics could produce unwanted or excessive activation if the binding affinity were too high. For PGRN, we observed a relatively high number of clones that targeted the small paragranulin (p) domain, many of which had a short CDR3 of only nine residues. PGRN is composed of seven ~55 AA highly disulfide-bridged granulin domains (GRN) separated by linker regions (P1-P7) of 10–26 AA. At the N-terminus is the 25 AA p domain with a linker of 15 AA to the first granulin domain, GRN-G. Based on our chimera experiments, the p binders could possibly bind the linker as well. There is no structural information for PGRN, so it is unknown whether the linkers would be accessible to antibody binding. For NKp46, we observed a broad epitope coverage that overlapped with and extended beyond that of four literature benchmark clones, each targeting non-overlapping epitopes. We identified five distinct, non-overlapping epitope bins, of which three (bins 1, 2, and 3) overlapped with the benchmarks (which were assigned to bins 1a, 1e, 2c, and 3b) and two (bins 4 and 5) were not represented by the benchmarks. It is intriguing to speculate that the HcAb format in Omni***dAb*** chickens enabled targeting of epitopes inaccessible to the other chicken strains expressing conventional heavy and light-paired antibodies, but it will require comparison of larger sets of antibodies from those strains to address this question.

Taken together, our two case studies on unrelated model antigens demonstrate that Omni***dAb*** chickens can produce clones with broad epitope coverage, a range of affinities, including high affinities in the low nM range, and favorable developability metrics. When building multispecifics, it is attractive in early-stage drug discovery to identify a diverse set of high-quality clones as building blocks for constructing into custom molecular formats for specific application needs.

Materials and methods

Transgene design and development

The design and development of the truncated light chain (tLCi) was previously published.[24] The Omni***dAb*** transgene (called VHH3) was designed with a pre-rearranged V region consisting of a human VH3-23 and JH4, with an artificial CDR3 of 17 residues in length, consisting mainly of Gly and Ser residues (Figure 1). This germline-encoded CDR3 is expected to undergo somatic mutation in the chicken B cell to produce novel repertoires. The variable region frameworks (FRs) contain 10 stabilizing 'back' mutations inspired by camelid VHH sequences and commonly engineered into clinical and approved VHH-based therapeutics. The VHH3 transgene contains this single functional V region and non-coding sequences (V gene promoter, leader intron, and 5' and 3' untranslated regions) from the chicken heavy chain locus. Upstream of the single-expressed VHH3 germline gene, an array of designed pseudogenes (P-VHH) with diverse CDR sequences was cloned, for use in gene conversion to mutate the expressed functional V gene in B cells. CDRs 1 and 2 in the pseudogenes were derived from germline human VH3 family members, whereas CDR3s were sourced from expressed human repertoires and selected for a range of lengths and sequence diversity. These pseudogenes contain FRs that are identical to the functional VHH3 gene to preserve the embedded stabilizing mutations. In the case of FR4, sequences encoding the last 5 residues were omitted from the pseudogenes to remove potential primer-binding sites from the genome. For insertion into the genome of chicken primordial germ cells and production of transgenic birds, a β-actin promoter and attB site were included on the vector for integration by phiC31 integrase into an attP site previously targeted to the chicken heavy-chain locus.[34,35] A loxP site was also included for removal of all selectable markers and plasmid backbone elements by Cre recombination.[36]

Chicken primordial germ cells (PGCs) were derived and cultured as described,[36–38] in KO-DMEM (ThermoFisher 0,829,018) containing 40% buffalo rat liver (BRL)-conditioned medium, 7.5% fetal calf serum (FCS) (Hyclone, SH30088.03), 2.5% chicken serum (ThermoFisher 16,110,082), 2 mm Glutamax (ThermoFisher 35,050–061), 1 mm pyruvate (ThermoFisher 11,360-070), 1X non-essential amino acids (ThermoFisher 11,140–050), 0.1 mm β-mercapto-ethanol (ThermoFisher 21,985–023), 6 ng/ml recombinant murine stem cell factor (R&D Systems, 455-MC), and 4 ng/ml recombinant human FGF basic (R&D Systems, 234-FSE) on irradiated BRL feeder cells.[36] Cells were passaged every 2–3 days. The PGC line VDJ10–9 was rederived from the germinal crescent of a transgenic bird carrying the IgH knockout and loxP sites flanking the VDJ region,[34] and these cells were used for transfection. 5×10^6 cells were resuspended in Nucleofector

V buffer (Lonza, Walkersville, MD, VCA-1003) with 15 µg of linearized VHH3 insertion vector and 15 µg of a CMV-phiC31 integrase vector[39] in a total volume of 100 µl and transfected in a 2 mm cuvette using a BMX ECM830 square wave pulse electroporator (BTX, Holliston, MA) at 350 V, 100 µsec, and 8 pulses. Cells were resuspended in complete growth medium and transferred to a 48-well plate with G418-resistant irradiated BRL feeder cells.[36] G418 (Teknova, Hollister, CA, G5005) was added at 350 µg/ml 3 days after transfection and the medium changed every 2–3 days. G418-resistant clones were expanded and confirmed to carry the correct VHH3 insertion into the heavy-chain locus using PCR for the 5' insertion (B-act-F2, 5'-CTCTGCTAACCATGTTCAT GCCTTC-3' and neo-R1, 5'-AGTGACAACGTCG AGCACAGCT-3') and 3' insertion (huJH4-F2, 5'-TTTGACTACTGGGGCCAAGG-3' and chJC-R45, 5'-GCCCAAAATGGCCCCAAAAC-3') and for the VHH3 gene using primers VHH3-F (5'-GTGAACGTCGGTCCAGGATT -3') and VHH3-R (5-CTCGACCGTCAGCTGGTATC-3') in a quantitative PCR (qPCR) assay. Three independent PGC clones and a pool of 3 other clones were injected into day 3.5 embryos to produce germline chimeras as described.[36] Breeding male chimeras to Cre-expressing hens[40] removed selectable markers and GFP and produced fully transgenic, VHH3-containing birds. Twenty of 25 chimeras transmitted through the germline with 6 chimeras transmitting >50%.

Animal care and handling

All animal experiments were approved by OmniAb, Inc.'s (previously Ligand Pharmaceuticals') Institutional Animal Care and Use Committee. The animal program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Transgenic chicken evaluations

Genotyping

Genotype confirmation was completed on either lysis of comb tissue (collected at hatch) or blood sample (collected at any older timepoint). Tissue lysis was completed in 0.5 mL ATL buffer containing 0.5% Proteinase K with incubation at 56°C overnight with shaking. Ethanol precipitation of DNA was performed on lysed samples and used for downstream genotyping qPCR. For blood samples, lysis was completed with 0.25 µL whole blood in 1 mL of TEN buffer containing 0.005% pronase E with incubation at 37°C overnight with shaking. Lysis reactions were inactivated by incubation at 65°C for 10 minutes and sample used directly for genotyping qPCR. Birds were confirmed twice by genotyping qPCR before proceeding to bird evaluations.

Sample collection and pre-processing

Birds were evaluated for B-cell development at 11 weeks of age, with $n = 1$ chicken serving the wild-type reference control, $n = 3$ from the IgL KO/VHH3 genotype, and $n = 6$ from the tLCi/VHH3 transgenic genotype. These samples were used for analysis on flow cytometry, western blot, RT-PCR, and ELISA evaluations. For each bird, 2 mL of whole blood in ethylenediaminetetraacetic acid anti-coagulant was collected for the isolation of plasma or PBMC. An aliquot of 200 µL of whole blood was centrifuged at 600× g for 10 minutes at 4°C to pellet down red blood cells and collect the plasma sample. Remaining whole blood was layered over 5 mL of Histopaque-1077 (Sigma-Aldrich 10,771) for PBMC isolation following the manufacturer's protocol. The buffy coat containing the PBMCs was collected from the interface and washed once in PBS supplemented with 0.1% bovine serum albumin (BSA) (PBS +0.1% BSA), then aliquoted into 96-well U-bottom plates for immediate downstream flow cytometry or pelleted and stored at −80°C for RT-PCR processing.

Flow cytometry

Plated PBMCs were incubated with primary antibodies diluted in PBS + 0.1% BSA buffer for 1 hour on ice. Anti-chicken primary antibodies were used as follows: monoclonal mouse anti-Bu1 at 5 µg/mL for the chicken B-cell marker Bu1 (Southern Biotech, Birmingham, AL, 8395–01), monoclonal mouse anti-IgM at 5 µg/mL against IgM CH1 (Southern Biotech, 8310–01), monoclonal mouse anti-IgL at 5 µg/mL (Southern Biotech, 8340-01), monoclonal anti-TCR1 (TCRδγ) at 1:800 dilution (Southern Biotech, 8230–01), combined monoclonal anti-TCR2 (TCRαβ/Vβ1) and anti-TCR3 (TCRαβ/Vβ2) at 1:200 dilution (Southern Biotech, 8240-01 and 8250-01), and polyclonal goat anti-IgM at 4 µg/mL (Bethyl Laboratories, Montgomery, TX, A30-102A). Additionally, polyclonal serum against the VHH3 variable region domain included in the transgene design was produced via hyperimmunization of rabbits for transgene-specific antibody detection and used at 1:100 dilution. After primary antibody incubation, cells were washed 3 times in 200 µL/well PBS containing 1% BSA and 0.1% Na-azide (fluorescence-activated cell sorting (FACS) buffer) and then incubated with secondary antibodies conjugated with AlexaFluor647 for 1 hour on ice. The secondary antibodies used were donkey anti-mouse at 5 µg/mL (ThermoFisher, A31571), donkey anti-goat at 4 µg/mL (Abcam, Waltham, MA, AB150131), or goat anti-rabbit at 4 µg/mL (ThermoFisher, A21244). After secondary incubation, cells were washed 3 times with PBS + 0.1% BSA and read on an Attune NxT flow cytometer (Thermo Scientific) for analysis of 10,000 cells per sample. Produced data were processed in FlowJo software (version 10.8.1). Figures were produced in Prism software (version 9).

ELISA

High-binding ELISA plates (Greiner Bio-One 655,061) were coated with 50 µL/well of either 2 µg/mL anti-chicken IgY (Sigma-Aldrich, C2288) or IgM (Sigma-Aldrich, SAB3700236), diluted in PBS. Plates were coated for 1 hour at room temperature or overnight at 4°C. Plates were then blocked with 3% skim milk in PBS blocking buffer at 150 µL/well for 1 hour at room temperature. Plasma samples were diluted to 1:1,000 starting dilution with 5-fold serial dilution in the blocking buffer and then applied to the plate at 50 µL/well. Samples were incubated on the plate for 1 hour at room temperature. Plates were washed 5 times with 300 µL/well PBST wash buffer (PBS containing 0.05% Tween 20), and then secondary was

applied for 1 hour at room temperature. The goat anti-chicken IgM-horse radish peroxidase (HRP) (Bethyl, A30-120P) or rabbit anti-chicken IgY-HRP (Sigma, A90406) was diluted 1:5,000 in blocking buffer and applied to the plate at 50 µL/well. After incubation, the plate was washed 5 times in PBST. The 3,3′,5,5′-tetramethylbenzidine substrate (ThermoFisher 002,023) was applied at 50 µL/well and allowed to incubate for 10 minutes. Color development was stopped with 50 µL/well 1 N HCL and plates read for absorbance at 450 nm in a BioTek Synergy H1 plate reader.

Western blot

Plasma samples were diluted to 1:100 in PBS, then prepared to contain a final concentration of 1X sample loading buffer and 1.5 mm tris-carboxyl-ethyl-phosphene reducing, buffer and heated to 98°C for 10 minutes. Samples were loaded into 4–12% bis-tris NuPage SDS-PAGE gels (ThermoFisher, NP0321) in 2-(N-morpholino)ethanesulfonic acid sodium dodecyl sulfate running buffer at a constant 200 V. Gels were transferred to 0.2 uM nitrocellulose membranes using the iBlot2 7-minute dry transfer system (ThermoFisher). Membranes were immediately blocked in 3% skim milk in PBS blocking buffer for 1 hour at room temperature with shaking. After blocking, membranes were incubated with the primary antibody diluted in blocking buffer for 1 hour. Primary antibodies included polyclonal goat anti-chicken IgM diluted to 1:5,000 (Sigma, SAB3700236) or rabbit anti-VHH polyclonal serum at a dilution of 1:500. After primary incubation, membranes were washed 5 times at 5 minutes with oscillation with PBST buffer, then mouse anti-goat-HRP (Rockland, 18-8814-31) or goat anti-rabbit-HRP (Jackson ImmunoResearch, 111-035-144) secondary antibodies applied at either 1:10,000 or 1:5,000 dilutions, respectively, in blocking buffer for 30 minutes with shaking. After secondary incubation, membranes were washed as previously stated. The West Pico PLUS chemiluminescent substrate (ThermoFisher 34,580) was applied for 15 minutes of development, and then membranes were imaged on a BioRad ChemiDoc XRS+ gel imager under the chemiluminescence filter.

Reverse transcription PCR

Total RNA was extracted from the pelleted PBMC samples using the RNAeasy Plus kit (Qiagen 74,034) using 10^7 cells per sample for extraction and following the manufacturer's protocol. Extracted RNA was resuspended in 30 µL water and quantified using the Nano UV-Vis spectrometer. Quantifications obtained were used for normalization of samples. Then, 2 µL of RNA was used in a reverse transcription/amplification reaction using the Qiagen One-Step RT-PCR kit (Qiagen 210,212). Primers used in these reactions targeted IgM (chVH-F9: 5'-CACCAGTCGGCTCCGCAACCATG-3' and cIgM-CH2-R5 : 5'-GGGATGGGAATCGGGGGACC-3') or IgL (cVL-5'UTR-F: 5'-GACACACAGCTGCTGGGATTC-3' and chIgL-C-R: 5'-CCTGCAGGTGTAGGTCTCGT-3'). RT-PCR parameters consisted of a 30-minute reverse-transcription at 50°C, followed by 40 cycles of 30 seconds of denaturation at 94°C, 1 minute of annealing at 60°C, and 45 seconds of extension at 72°C, with a final extension of 10 minutes. RT-PCR products were loaded and run on 1% agarose-ethidium bromide tris acetate ethylenediaminetetraacetic acid gels and imaged with a BioRad ChemiDoc XRS+ gel imager.

Antibody discovery

Immunizations and sample collection

Omni***dAb*** transgenic chickens were selected for immunization after confirmation of B-cell development in PBMC by flow cytometry and plasma immunoglobulin titer by ELISA (as mentioned above). Birds were immunized with either soluble NKp46 extracellular domain protein (produced in-house) or soluble human progranulin protein (Acro Biosystems PGN-H52H3) at 100 µg of protein per dose per bird via intramuscular administration. Primary immunization contained a 1:1 v/v Complete Freund's Adjuvant (Thermo Fisher 77,140), while boosts contained 1:1 v/v Incomplete Freund's Adjuvant (Thermo Fisher 77,145). Immunization schedule followed an every-other-week pattern of immunization one week with sample blood draw the following week. Upon hyperimmunization and achievement of high titer response (based on ELISA, procedure described below), a final non-adjuvated boost was administered intravenously. Final plasma sample collection and splenocyte harvest was completed 4 days after the final intravenous boost. Spleen outer membranes were removed and parenchyma tissue processed using 40 µM cell strainers into PBS + 0.1 BSA buffer. Single-cell splenocyte samples were then layered over Histopaque-1077 (Sigma-Aldrich 10,771) polysucrose gradients to collect buffy coats containing lymphocytes and mononuclear cells from the total splenocyte samples. Collected cells were cryopreserved in a medium containing 10% FCS and 10% dimethyl sulfoxide, and aliquots were stored in liquid nitrogen until used for screening.

Single B-cell screening and cloning

Single B-cell screening was completed on cryopreserved cells using either the GEM assay method[41] (US Patents: 8,030,095 and 8,415,173) or the xPloration method[42] (US Patents: 10227583; 11085039; 11473081; and 12024705). For the GEM method, 5 µM latex-aldehyde beads (Thermo Fisher, A37306) were coated with 37.6 µg soluble protein per 100 µL of bead solution in PBS buffer overnight at 4°C on a rotator. After overnight coating, beads were washed and blocked with 3% skim milk in PBS for 1 hour, followed by 5 washes with PBS, and then finally brought back to original bead volume. Coated beads were stored at 4°C until use in gel encapsulated micro-environment (GEM) screening. The GEM method facilitates single B-cell screening by encapsulating single B cells with antigen-coated beads into agarose gel micro-droplets. B cells secreting antibodies specific to the antigen are detected by a fluorophore-labeled secondary antibody and manually screened/extracted under the microscope. GEMs containing the B cells and antigen-coated beads were prepared, incubated for 3 hours at 37°C in RPMI media supplemented with 10% FCS and Glutamax, and pre-mixed with the 2 µL/mL goat anti-chicken IgM-DyLight 594 secondary antibody (Novus

Biologicals, NBP2-60690DL594). GEMs were washed 3 times in CO_2-Independent Media containing 10% FCS and Glutamax media before viewing under the microscope. GEMs containing single B cells and positive staining on beads were extracted for cloning. For the xPloration screening method, the antigen target protein is coated onto M450 Dynabeads (Thermo Scientific 14044) following manufacturer's coating procedures. Cells were washed in PBS supplemented with 0.1% BSA and mixed with coated beads at a ratio of 4800 cells per 1 µL of coated beads in RPMI base media-containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and glutamine supplemented with 10% FCS and pre-mixed with goat anti-chicken IgM-DyLight 650 secondary antibody (Novus Biologicals, NBP2-60690DL650) at 1:1000 dilution. The cell-bead-secondary mix was spread onto a microcapillary chip containing 1.5 million capillaries, a 1% agarose overlay was applied, and the entire chip was incubated for 2 hours at 37°C. After incubation, the chip was loaded into the xPloration instrument and screened for binding to the antigen-coated beads for each capillary well. Contents of individual positive wells were laser-extracted from the chip for cloning. After extraction of antigen-specific single B cells (either by GEM or xPloration), cells were lysed using TurboCapture mRNA kits (Qiagen 72,251) following manufacturer's instructions for single-cell mRNA preparations. Reverse transcription PCR was performed on the samples to amplify the entire heavy variable region using primers in the 5' UTR: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTCACC-AGTCGGCTCCGCAACCATG and IgM CH2 exon: 5'-GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGT-GCATGGTGACGAAAAG-3' regions. This was followed by a nested PCR using primers with overhangs in the VH-leader: 5-TCGAACCCTTGCTAGCCGCCATGGAGTTTGGGC TTAGCTGGATC and JH-region: 5'-TGAGGAGACGGTGA CCTGGGT. These amplified VH regions were then inserted into a mammalian expression vector containing an IgG1 CH2-CH3 human Fc domain using the In-Fusion method (Takada Bio 638,911), creating a sdAb-Fc format, also referred to as HcAb. Clones were transfected into Expi293 cells for protein expression and used for downstream analysis. Control antibodies to PGRN representing in-house clones from wild-type chicken[21] and transgenic OmniChicken® and OmniClic® animals[27–29] and to NKp46 (NKp46–1, NKp46–2, NKp46–3, and NKp46–4; US patent application US20240034816 and PDB ID 6IAP) from OmniClic and OmniFlic® were generated as scFv-Fc (PGRN) or full IgG (NKp46) constructs with a human IgG1-Fc and purified by standard protein A chromatography, as described for the Omni***dAb*** antibodies.

Antigen-specific ELISAs

ELISA for antigen-specific binding followed the same standard procedures as previously mentioned, but with these stated modifications. Plates were coated with the target antigen at 2 µg/mL. Detection of antigen-specific binding antibodies utilized either the goat anti-chicken IgM-HRP (Bethyl, A30-120P) for screening during the immunization stage or rabbit anti-human-Fc-HRP (Rockland, 609–4303) during the monoclonal screening phase. During screening for immunoresponse to antigen during the immunization stage, plasma samples were applied at a starting dilution of 1/100 in blocking buffer (for PGRN immunizations) or 1/500 (for NKp46 immunizations), followed by 7-rounds of a 5-fold serial dilution. During the monoclonal screening phase, the transfected Expi293 supernatant was initially screened for antigen-binding using a sample dilution of 1/50. After initial qualitative screening, these clones were sequenced to sort out clones with unique sequences. Representative clones for each unique sequence group were then selected for normalized ELISA analysis and downstream flow cytometry, kinetics, epitope binning, and functional assays. Normalized ELISA for unique sequence clone representatives were applied at a starting dilution of 1 µg/mL, followed by 3-rounds of 5-fold serial dilutions to observe dose–response binding curves. Selected unique sequence clones which maintained strong binding were selected to move forward for further evaluation.

Antigen-specific flow cytometry for NKp46 clones

A randomly selected subset of NKp46-binding HcAbs was screened for the ability to bind to native NKp46 protein on transfected CHO-K1 cells (KYinno Bio, KC-1789). The CHO-K1 cells (parental and NKp46-expressing) were cultured in Ham's F12K media (Gibco 21,127,022) supplemented with 10% FBS. The NKp46-expressing CHO-K1 cells were grown with 10 µg/mL puromycin selection. On the day of flow cytometry, cells were removed from the adherent surface with cell dissociation buffer (Corning, 25-066-CI) for 10 minutes at 37°C, washed with FACS buffer (400× g for 10 minutes at 4°C), and counted using a hemocytometer. For each cell type, washed cells were resuspended in 250 µL human Fc block (BD 564,220) and incubated at room temperature for 10 minutes, protected from light. Cells were plated at 1.5×10^5 cells/well at 200 µL/well in FACS buffer and pelleted before applying primary antibodies. NKp46-binding antibodies were applied to the well at a starting dilution of 10 µg/mL, with three 5-fold serial dilutions, at 50 µL/well and incubated for 45 minutes on ice. A NK-binding mouse anti-CD335 monoclonal positive control antibody was also included in the assay (Invitrogen, 16-3359-82) at the same working concentrations. After primary incubation, the cell plate was washed twice with FACS buffer and the secondary applied at 50 µL/well for 45 minutes on ice, protected from light. The secondary antibodies used were goat anti-human IgG-AlexaFluor 647 (Thermo, A21445) or goat anti-mouse IgG-AlexaFluor 647 (Thermo, A21235) at 1:500 dilutions. After secondary incubation, cell plates were washed twice and resuspended in 200 µL of FACS buffer before processing for analysis on the Attune NxT flow cytometer (Thermo Scientific).

IFNγ release assay

CD56/CD16-positive human primary NK cells (Lonza, 2W-502) were cultured in ImmunoCult NK Cell Expansion media (Stemcell Technologies, 100-0711) for 10–14 days according to manufacturer's recommended protocols at 37°C with 5% CO_2. On the day of testing, cells were harvested and seeded into sterile 96-well microplates at a density of 150,000 cells/well in 50 µL of culture media. A randomly selected subset of NKp46-binding clones (same clones as above) was tested at 30 nM and controls applied at 100 nM, 30 nM, 10 nM, 3 nM, 1 nM, 0.3 nM, and 0.1 nM. Reference antibodies from the patent literature were

included (NKp46-1, NKp46-2, and NKp46-3; US20240034816). Ionomycin (Sigma-Aldrich, I0634) was used as an assay positive control and applied at a final concentration of 1 µM. A human IgG antibody isotype control (ThermoFisher, 02-7102) was also included and applied at a final concentration of 30 nM (matching the concentration used for experimental samples). Samples were run in 4 replicates over 2 separate assay runs, totaling 8 replicates per sample. Treatments were prepared at 2X the final testing concentration in media, with 50 µL of each 2X concentration sample applied to the wells containing the 50 µL of cells (1:1 v/v) for 24 -hour treatment incubation. After incubation, plates were briefly centrifuged at 500× g for 10 minutes and 50 µL of supernatant collected to a fresh 96-well microplate for ELISA processing. IFNγ concentrations of samples were determined using a commercially available human IFNγ ELISA kit (Abcam, AB300323), following the manufacturer's protocol, and included standard curve controls. Sample supernatants were diluted to 1:20 for ELISA. Results were measured on a BMG Clariostar plate reader, and absorbance values were converted to concentration using the IFNγ standard curve calculated from the linear fit regression equation. Statistical significance was calculated in comparison to the media-only control.

Evaluation and developability of antigen-specific antibody clones

Sequence analysis

AA sequence diversity of antigen-specific HcAbs was completed by multiple sequence alignment of the heavy-chain variable regions and germline sequences using the IMGT numbering system to identify the CDR regions (CDR1: 27–38, CDR2: 56–65, and CDR3: 105–117). At each position, the occurrence of all non-germline animo acids was calculated and plotted using Logomaker,[43] dmslogo, funcgroup,[44] and ANARCI.[45] Pseudogene usage identification of antigen-specific clones was completed by first locating regions of gene conversion on the DNA sequence. Queried CDR sequences with 4 bp or more identity match to the germline pseudogene sequence were considered a result of gene conversion. Sequence fragments were required to be 100% match to the germline pseudogene sequence to be considered. Sequences 3 bp or less were considered too short to definitively confirm gene conversion and may be a result of somatic hypermutation. Some germline pseudogenes had matching stretches of sequences, at which point the specific pseudogene origin for that gene conversion event could not be determined and was considered an undefined gene conversion with multiple potential pseudogene sources. Analysis of gene conversion usage and pseudogene origin was completed with the SequenceMatcher tool from Biopython.[46] The phylogenic tree was generated by first aligning sequences using Clustal Omega (ClustalO), calculating pairwise distances based on the sequence identity of the entire variable heavy chain region and constructing the tree with the UPGMA algorithm from the Biopython Phylo TreeConstruction package. The tree was visualized in a circular style, with the clones on the leaves of the tree colored to match the bin color used in the binning experiments. Sequence lineages were defined as a group of antibody sequences with up to the 2 AA edit distance in the concatenated CDR1 and CDR2 regions and up to 2 AA edit distance in the CDR3 region. The lineage identifications (ID) were ranked, with the lineage containing the largest number of unique sequences ranked at the top as Lineage ID 1.

Immunogenicity analysis

To examine the potential for immunogenicity, an *in silico* evaluation was performed on a subset of antigen-specific Omni***dAb*** antibodies selected to encompass a diversity of sequences to four different targets. The antibody sequences were analyzed as overlapping 9-mer segments and screened with EpiVax software for the presence of Class II (HLA-DR)-restricted human leukocyte antigen (HLA) ligands and putative T-cell epitopes.[47,48] The software calculates the binding potential to the most common HLA molecules within HLA "supertypes". The total number of putative T-cell epitopes is used to generate an EpiMatrix score, which is further adjusted for T-regulatory (Tregitope) binding sites. Binding to T-regulatory cells may reduce immunogenicity and thus offset the raw EpiMatrix score. Tregitope-adjusted EpiMatrix scores of <−15 are considered to have low immunogenicity and scores <−30 are considered to have minimal immunogenicity. Putative T-cell epitopes identified in the EpiVax screen were further characterized with the JanusMatrix software.[49] Activation of T-cells requires peptides to bind HLA binding clefts on antigen presenting cells and present the MHC:peptide complex to T-cell receptors (TCR). The simultaneous binding results in two 'faces': TCR-facing AA residues and major histocompatibility complex (MHC)-facing AA residues. TCR-facing residues that are highly conserved within the human proteome are less likely to generate an immune response, whereas novel sequences may be recognized as foreign and activate T-cell responses. The JanusMatrix algorithm matches the predicted T-cell epitopes to human proteins. A high JanusMatrix Human Homology Score (>5) suggests a reduced immunogenic potential.[50]

Antibody purification

HcAbs were high-throughput-purified from the Expi293 supernatant using a Hamilton Nimbus equipped with PhyTip Protein A Columns (Biotage, PTH-91-40-07). Columns were equilibrated in PBS pH7.4 before capturing sdAb-Fc antibodies. The columns were subsequently washed twice in PBS pH7.4. Samples were eluted off the column in 50 mm sodium citrate pH 3.0 and 150 mm sodium chloride and immediately neutralized by the addition of 0.5 M sodium phosphate pH 9. Neutralized solutions were buffer exchanged into PBS pH 7.4 in high throughput 96-well desalting plates (ThermoFisher, #89807).

Thermal stability and aggregation

Differential scanning fluorimetry (UNCLE-0330, Unchained Labs, Pleasanton, CA) was used for the assessment of the melting temperature (T_m) and aggregation temperature (T_{agg}) on selected NKp46 and PGRN antibody clones in sdAb-Fc format. The samples, which are in PBS pH 7.4, were loaded onto the microcuvette arrays at a low injection volume (9 µL)

and in a broad concentration range from 0.05 to 5 mg/ml. A temperature ramp from 20°C to 95°C was applied with a 1.0°C/min increment, incubation time of 180 seconds, and a plate hold of 60 seconds. Change in the 350/330-nm absorbance ratio was used to analyze T_m and T_{agg}. Intrinsic protein fluorescence (excitation: 266 nm, emission: 280–450 nm) was monitored for measurement of T_m. The aggregation curve was generated through measurement of temperature-dependent intensity of static light scattering (excitation: 266 nm). Static light scattering at 473 nm was monitored for measurement of T_{agg}. All data were analyzed directly with UNcle Analysis Software (V.6.0), and T_m and T_{agg} were determined using 350/330 nm absorbance ratio or SLS473 (counts.nm) vs. temperature (°C) plots, respectively.

AC-SINS

Both polyclonal goat anti-human IgG Fc (Jackson ImmunoResearch, #109-005-098) (capture) and whole-goat IgG (Jackson ImmunoResearch, #005-000-003) (non-capture) antibodies were buffer exchanged into 20 mm potassium acetate (pH 4.3) buffer and normalized to 0.4 mg/mL. The two antibodies were mixed at a ratio of 4:1 of capture:non-capture. Antibodies were incubated with gold nanoparticles (Ted Pella Inc. #15705) at a ratio of 9:1 of gold nanoparticle to antibody for 2 hours at room temperature. Empty sites were then blocked by the addition of 0.1 µM (final conc.) poly-ethylene glycol methyl ether thiol (2000 MW, Sigma-Aldrich #729140). The coated and blocked particles were passed through a 0.22 µm polyvinylidene fluoride membrane (Millipore Sigma #SLGVV255F) and eluted with 10% starting volume of PBS. To assess self-association, 10 µL nanoparticles were mixed with 100 µL purified antibody at 50 µg/mL in PBS, pH7.4 in a 96-well polypropylene plate. As a control, buffer was mixed with coated and blocked gold nanoparticles. Absorbance was measured on a BioTek Synergy H1 plate reader from 510 to 570 nm in increments of 1 nm. The peak absorbance wavelength was calculated manually using Excel. Control buffer peak absorbance wavelength was subtracted from the sample peak absorbance wavelength, and these data were plotted.

Analytical SEC

Analytical SEC-HPLC was performed for Omni***dAb*** samples using a Superdex 200 Increase 5/150 GL column (Cytiva, GE28-9909-45) on an UltiMate 3000 hPLC (Thermo Scientific). Concentrations of antibodies ranged from 0.2 to 1 mg/mL with a couple outliers below 0.1 mg/mL and above 1.5 mg/mL. The buffer formulation for both the sample and running buffer was PBS pH 7.4 with a flow rate of 0.25 mL/min at room temperature over 15 minutes.

Biosensor analysis

Binding kinetics, epitope mapping, and epitope binning experiments were performed by high-throughput surface plasmon resonance on Carterra's LSA platform equipped with HC-30 M sensor chips. The method has been previously described in detail elsewhere.[27] To calculate binding affinity, a capture kinetic method was used on a chip coupled with goat anti-human IgG Fc-specific polyclonal (Southern Biotech, 2047–01) capture reagent. Purified recombinant His-tagged PGRN (R&D systems) and NKp46 (prepared in-house) were used as monovalent analytes and prepared as a 3-fold dilution series with top concentrations of 300 nM or 1 µM. Epitope mapping of anti-PGRN HcAbs to specific granulin subdomains was accomplished using a chimeric swap strategy as described previously.[21] A detailed map describing the chimera constructs, linker regions, and chimera binding patterns used for mapping assignments is shown in Figure S4. Epitope binning experiments were performed using a classical sandwich method as described previously merged with a curated panel of antibody standards to guide bin assignments. For PGRN binning, the standards represented a panel of previously characterized clones of known specificities, whereas for NKp46 binning, the standards represented so-called 'anchor' clones from in-house unpublished OmniFlic and OmniClic repertoires generated by standard protein-based immunizations and literature benchmarks (NKp46-1, NKp46-2, NKp46-3, and NKp46-4; see "single B-cell screening and cloning section" above). Anti-His mAb was used as a universal sandwiching control for all epitope binning experiments. For NKp46 binning, a 'many-on-few' approach was used where a large panel of HcAb analytes (in solution) was tested for blocking against a small, curated panel of ligands (on chip) comprising anchor clones and a limited set of HcAbs that tolerated multiple cycles of acid-regeneration. Since a comprehensive pairwise analysis was not performed for NKp46 hcAbs, they were not tested for mutual competition against one another, so they were assigned to 'blocking profiles' (herein referred to as 'bins'). In this definition, HcAbs belonging to the same blocking profile do not necessarily block one another but show the same blocking pattern when tested against the ligand set.

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Abbreviations

AA	amino acid
AC-SINS	affinity-capture self-interaction nanoparticle spectroscopy
aSEC	analytical size exclusion chromatography
BiP	immunoglobulin binding protein
bp	base pair
BRL	buffalo rat liver
BSA	bovine serum albumin

CDR	complementarity determining region
CH	heavy chain constant domain
CHO	Chinese hamster ovary
cJH	chicken JH gene
CL	light chain constant domain
cVH	chicken VH gene
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
Fc	fragment crystallizable
FCS	fetal calf serum
FD	final draw
FDA	Food and Drug Administration
FR	fragment region
GC	gene conversion
GEM	gel encapsulated microenvironment
GFP	green fluorescent protein
GRAVY	grand average of hydropathy
GRN	granulin domain
HcAb	heavy chain only antibodies
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
ID	identification
IFNγ	interferon gamma
IgG	immunoglobulin G
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IgM	immunoglobulin M
Ig_{NAR}	immunoglobulin new antigen receptor
IgY	immunoglobulin Y
IMGT	international ImMunoGeneTics information system
K_D	equilibrium dissociation constant
KO	knock out
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
n/d	not determined
NK	natural killer
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PGC	primordial germ cell
PGRN	progranulin
PI	pre-immune
P-VHH	pseudogene of variable heavy domain of heavy chain Omni***dAb*** transgene
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
scFv	single chain variable fragment
sdAb	single domain antibodies
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHM	somatic hypermutation
T_{agg}	aggregation temperature
TCR	T-cell receptor
tLCi	truncated light chain transgene
T_m	melting temperature
VH	variable domain of heavy chain
VHH	variable heavy domain of heavy chain-only antibody
VHH3	variable heavy domain of heavy chain Omni***dAb*** transgene
VL	variable domain of light chain
WT	wild-type

References

1. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R. Naturally occurring antibodies devoid of light chains. Nature. 1993;363(6428):446–448. doi:10.1038/363446a0.
2. Greenberg AS, Avila D, Hughes M, Hughes A, McKinney EC, Flajnik MF. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. Nature. 1995;374(6518):168–173. doi:10.1038/374168a0.
3. Vu KB, Ghahroudi MA, Wyns L, Muyldermans S. Comparison of llama VH sequences from conventional and heavy chain antibodies. Mol Immunol. 1997;34(16–17):1121–1131. doi:10.1016/S0161-5890(97)00146-6.
4. Riechmann L, Muyldermans S. Single domain antibodies: comparison of camel VH and camelised human VH domains. J Immunological Methods. 1999;231(1–2):25–38. doi:10.1016/S0022-1759(99)00138-6.
5. Nguyen VK, Muyldermans S, Hamers R. The specific variable domain of camel heavy-chain antibodies is encoded in the germline. J Mol Biol. 1998;275(3):413–418. doi:10.1006/jmbi.1997.1477.
6. Nguyen VK, Hamers R, Wyns L, Muyldermans S. Loss of splice consensus signal is responsible for the removal of the entire CH1 domain of the functional camel IGG2A heavy-chain antibodies11This work was supported by the VLIR, VIB and FGWO. Mol Immunol. 1999;36(8):515–524. doi:10.1016/S0161-5890(99)00067-X.
7. Arbabi-Ghahroudi M. Camelid single-domain antibodies: promises and challenges as lifesaving treatments. Int J Mol Sci. 2022;23(9):5009. doi:10.3390/ijms23095009.
8. Sang Z, Xiang Y, Bahar I, Shi Y. Llamanade: an open-source computational pipeline for robust nanobody humanization. Structure. 2022;30(3):418–429.e3. doi:10.1016/j.str.2021.11.006.
9. Arras P, Yoo HB, Pekar L, Schröter C, Clarke T, Krah S, Klewinghaus D, Siegmund V, Evers A, Zielonka S. A library approach for the de novo high-throughput isolation of humanized VHH domains with favorable developability properties following camelid immunization. mAbs. 2023;15(1). doi:10.1080/19420862.2023.2261149.
10. Drabek D, Janssens R, de Boer E, Rademaker R, Kloess J, Skehel J, Grosveld F. Expression cloning and production of human heavy-chain-only antibodies from murine transgenic plasma cells. Front Immunol. 2016;7. doi:10.3389/fimmu.2016.00619.
11. Teng Y, Young JL, Edwards B, Hayes P, Thompson L, Johnston C, Edwards C, Sanders Y, Writer M, Pinto D, et al. Diverse human VH antibody fragments with bio-therapeutic properties from the crescendo mouse. N Biotechnol. 2020;55:65–76. doi:10.1016/j.nbt.2019.10.003.
12. Clarke SC, Ma B, Trinklein ND, Schellenberger U, Osborn MJ, Ouisse L-H, Boudreau A, Davison LM, Harris KE, Ugamraj HS, et al. Multispecific antibody development platform based on human heavy chain antibodies. Front Immunol. 2018;9:3037. doi:10.3389/fimmu.2018.03037.
13. Bélanger K, Tanha J. High-efficacy, high-manufacturability human VH domain antibody therapeutics from transgenic sources. Protein Eng Des Sel. 2021;34. doi:10.1093/protein/gzab012.
14. Rossotti MA, Bélanger K, Henry KA, Tanha J. Immunogenicity and humanization of single-domain antibodies. FEBS J. 2022;289(14):4304–4327. doi:10.1111/febs.15809.
15. Barakat S, Berksöz M, Zahedimaram P, Piepoli S, Erman B. Nanobodies as molecular imaging probes. Free Radic Biol Med. 2022;182:260–275. doi:10.1016/j.freeradbiomed.2022.02.031.
16. Ma J, Xu X, Fu C, Xia P, Tian M, Zheng L, Chen K, Liu X, Li Y, Yu L, et al. CDH17 nanobodies facilitate rapid imaging of gastric cancer and efficient delivery of immunotoxin. Biomater Res. 2022;26(1). doi:10.1186/s40824-022-00312-3.
17. Takeuchi T. Structural, nonclinical, and clinical features of ozoralizumab: a novel tumour necrosis factor inhibitor. Mod Rheumatol. 2023;33(6):1059–1067. doi:10.1093/mr/road038.
18. Deiteren A, Bontinck L, Conickx G, Vigan M, Dervaux N, Gassiot M, Bas S, Suratt B, Staudinger H, Krupka E. A first-in-human, single and multiple dose study of lunsekimig, a novel anti-TSLP /anti- IL -13 NANOBODY® compound, in healthy volunteers. Clin Transl Sci. 2024;17(6):17. doi:10.1111/cts.13864.

19. Jin B-K, Odongo S, Radwanska M, Magez S. NANOBODIES®: a review of diagnostic and therapeutic applications. Int J Mol Sci. 2023;24(6):5994. doi:10.3390/ijms24065994.
20. Ratcliffe MJH. Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. Dev Comp Immunol. 2006;30(1–2):101–118. doi:10.1016/j.dci.2005.06.018.
21. Abdiche YN, Harriman R, Deng X, Yeung YA, Miles A, Morishige W, Boustany L, Zhu L, Izquierdo SM, Harriman W. Assessing kinetic and epitopic diversity across orthogonal monoclonal antibody generation platforms. mAbs. 2016;8(2):264–277. doi:10.1080/19420862.2015.1118596.
22. Banik SSR, Kushnir N, Doranz BJ, Chambers R. Breaking barriers in antibody discovery: harnessing divergent species for accessing difficult and conserved drug targets. mAbs. 2023;15(1). doi:10.1080/19420862.2023.2273018.
23. Schusser B, Collarini EJ, Pedersen D, Yi H, Ching K, Izquierdo S, Thoma T, Lettmann S, Kaspers B, Etches RJ, et al. Expression of heavy chain-only antibodies can support B-cell development in light chain knockout chickens. Eur J Immunol. 2016;46(9):2137–2148. doi:10.1002/eji.201546171.
24. Leighton PA, Ching K, Reynolds K, Vuong CN, Zeng B, Zhang Y, Gupta A, Morales J, Rivera GS, Srivastava DB, et al. Chickens with a truncated light chain transgene express single-domain H chain-only antibodies. J Immunol. 2024;212(11):1744–1753. doi:10.4049/jimmunol.2300617.
25. Muyldermans S, Atarhouch T, Saldanha J, Barbosa JARG, Hamers R. Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. Protein Eng Des Sel. 1994;7(9):1129–1135. doi:10.1093/protein/7.9.1129.
26. Reynaud CA, Dahan A, Anquez V, Weill JC. Somatic hyperconversion diversifies the single Vh gene of the chicken with a high incidence in the D region. Cell. 1989;59(1):171–183. doi:10.1016/0092-8674(89)90879-9.
27. Ching KH, Berg K, Morales J, Pedersen D, Harriman WD, Abdiche YN, Leighton PA. Expression of human lambda expands the repertoire of OmniChickens. PLOS ONE. 2020;15(1):e0228164. doi:10.1371/journal.pone.0228164.
28. Ching KH, Berg K, Reynolds K, Pedersen D, Macias A, Abdiche YN, Harriman WD, Leighton PA. Common light chain chickens produce human antibodies of high affinity and broad epitope coverage for the engineering of bispecifics. mAbs. 2021;13(1). doi:10.1080/19420862.2020.1862451.
29. Ching KH, Collarini EJ, Abdiche YN, Bedinger D, Pedersen D, Izquierdo S, Harriman R, Zhu L, Etches RJ, van de Lavoir M-C, et al. Chickens with humanized immunoglobulin genes generate antibodies with high affinity and broad epitope coverage to conserved targets. mAbs. 2018;10(1):71–80. doi:10.1080/19420862.2017.1386825.
30. Lefranc M-P. IMGT, the international ImMunoGeneTics information system. Cold Spring Harb Protoc. 2011;2011:pdb.
31. Desmyter A, Decanniere K, Muyldermans S, Wyns L. Antigen specificity and high affinity binding provided by one single loop of a camel single-domain antibody. J Biol Chem. 2001;276(28):26285–26290. doi:10.1074/jbc.M102107200.
32. Liu Y, Caffry I, Wu J, Geng SB, Jain T, Sun T, Reid F, Cao Y, Estep P, Yu Y, et al. High-throughput screening for developability during early-stage antibody discovery using self-interaction nanoparticle spectroscopy. mAbs. 2014;6(2):483–492. doi:10.4161/mabs.27431.
33. Jain T, Sun T, Durand S, Hall A, Houston NR, Nett JH, Sharkey B, Bobrowicz B, Caffry I, Yu Y, et al. Biophysical properties of the clinical-stage antibody landscape. Proceedings of the National Academy of Sciences of the United States of America; Washington, DC,USA. Vol. 114. 2017. p. 944–949.
34. Dimitrov L, Pedersen D, Ching KH, Yi H, Collarini EJ, Izquierdo S, van de Lavoir M-C, Leighton PA, Kormann MSD. Germline gene editing in chickens by efficient CRISPR-Mediated homologous recombination in primordial germ cells. PLOS ONE. 2016;11(4):e0154303. doi:10.1371/journal.pone.0154303.
35. Schusser B, Collarini EJ, Yi H, Izquierdo SM, Fesler J, Pedersen D, Klasing KC, Kaspers B, Harriman WD, van de Lavoir M-C, et al. Immunoglobulin knockout chickens via efficient homologous recombination in primordial germ cells. Proc Natl Acad Sci USA. 2013;110(50):20170–20175. doi:10.1073/pnas.1317106110.
36. Collarini EJ, Leighton PA, M-CVd L. Production of transgenic chickens using cultured primordial germ cells and gonocytes. Microinjection. 2019;1874:403–430.
37. Song Y, Duraisamy S, Ali J, Kizhakkayil J, Jacob VD, Mohammed MA, Eltigani MA, Amisetty S, Shukla MK, Etches RJ, et al. Characteristics of long-term cultures of avian primordial germ cells and gonocytes. Biol Reprod. 2014;90(1):1–8. doi:10.1095/biolreprod.113.113381.
38. van de Lavoir M-C, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessaro TM, Swanberg SE, et al. Germline transmission of genetically modified primordial germ cells. Nature. 2006;441(7094):766–769. doi:10.1038/nature04831.
39. Thyagarajan B, Olivares EC, Hollis RP, Ginsburg DS, Calos MP. Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. Mol Cell Biol. 2001;21(12):3926–3934. doi:10.1128/MCB.21.12.3926-3934.2001.
40. Leighton PA, Pedersen D, Ching K, Collarini EJ, Izquierdo S, Jacob R, van de Lavoir M-C. Generation of chickens expressing cre recombinase. Transgenic Res. 2016;25(5):609–616. doi:10.1007/s11248-016-9952-6.
41. Izquierdo SM, Varela S, Park M, Collarini EJ, Lu D, Pramanick S, Rucker J, Lopalco L, Etches R, Harriman W. High-efficiency antibody discovery achieved with multiplexed microscopy. Microscopy (Oxford, Engl). 2016;65(4):341–352. doi:10.1093/jmicro/dfw014.
42. Chen B, Lim S, Kannan A, Alford SC, Sunden F, Herschlag D, Dimov IK, Baer TM, Cochran JR. High-throughput analysis and protein engineering using microcapillary arrays. Nat Chem Biol. 2016;12(2):76–81. doi:10.1038/nchembio.1978.
43. Tareen A, Kinney JB, Valencia A. Logomaker: beautiful sequence logos in Python. Bioinformatics. 2020;36(7):2272–2274. doi:10.1093/bioinformatics/btz921.
44. Crooks GE, Hon G, Chandonia J-M, Brenner SE. WebLogo: a sequence logo generator. Genome Res. 2004;14(6):1188–1190. doi:10.1101/gr.849004.
45. Dunbar J, Krawczyk K, Leem J, Baker T, Fuchs A, Georges G, Shi J, Deane CM. SAbDab: the structural antibody database. Nucleic Acids Res Suppl. 2014;42(D1):D1140–6. doi:10.1093/nar/gkt1043.
46. Cock PJA, Antao T, Chang JT, Chapman BA, Cox CJ, Dalke A, Friedberg I, Hamelryck T, Kauff F, Wilczynski B, et al. Biopython: freely available python tools for computational molecular biology and bioinformatics. Bioinformatics. 2009;25:1422–1423.
47. Jawa V, Cousens LP, Awwad M, Wakshull E, Kropshofer H, Groot ASD. T-cell dependent immunogenicity of protein therapeutics: preclinical assessment and mitigation. Clin Immunol. 2013;149(3):534–555. doi:10.1016/j.clim.2013.09.006.
48. Jawa V, Terry F, Gokemeijer J, Mitra-Kaushik S, Roberts BJ, Tourdot S, Groot ASD. T-Cell dependent immunogenicity of protein therapeutics pre-clinical assessment and mitigation–updated consensus and review 2020. Front Immunol. 2020;11:11. doi:10.3389/fimmu.2020.01301.
49. Moise L, Gutierrez AH, Bailey-Kellogg C, Terry F, Leng Q, Hady KMA, VerBerkmoes NC, Sztein MB, Losikoff PT, Martin WD, et al. The two-faced T cell epitope. Hum Vaccin Immunother. 2013;9(7):1577–1586. doi:10.4161/hv.24615.
50. Mattei AE, Gutierrez AH, Seshadri S, Tivin J, Ardito M, Rosenberg AS, Martin WD, Groot ASD. In silico methods for immunogenicity risk assessment and human homology screening for therapeutic antibodies. mAbs. 2024;16(1). doi:10.1080/19420862.2024.2333729.