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REPORT

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Assessing kinetic and epitopic diversity across orthogonal monoclonal antibody generation platforms

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ABSTRACT

The ability of monoclonal antibodies (mAbs) to target specific antigens with high precision has led to an increasing demand to generate them for therapeutic use in many disease areas. Historically, the discovery of therapeutic mAbs has relied upon the immunization of mammals and various in vitro display technologies. While the routine immunization of rodents yields clones that are stable in serum and have been selected against vast arrays of endogenous, non-target self-antigens, it is often difficult to obtain species cross-reactive mAbs owing to the generally high sequence similarity shared across human antigens and their mammalian orthologs. In vitro display technologies bypass this limitation, but lack an in vivo screening mechanism, and thus may potentially generate mAbs with undesirable binding specificity and stability issues. Chicken immunization is emerging as an attractive mAb discovery method because it combines the benefits of both in vivo and in vitro display methods. Since chickens are phylogenetically separated from mammals, their proteins share less sequence homology with those of humans, so human proteins are often immunogenic and can readily elicit rodent cross-reactive clones, which are necessary for in vivo proof of mechanism studies. Here, we compare the binding characteristics of mAbs isolated from chicken immunization, mouse immunization, and phage display of human antibody libraries. Our results show that chicken-derived mAbs not only recapitulate the kinetic diversity of mAbs sourced from other methods, but appear to offer an expanded repertoire of epitopes. Further, chicken-derived mAbs can bind their native serum antigen with very high affinity, highlighting their therapeutic potential.

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Introduction

Monoclonal antibodies (mAbs) are successful drug moieties showing tremendous biological efficacy and minimal side effects in treating a wide range of diseases. They also provide an engineering platform that has led to new technologies such as antibody-drug conjugates,[1] bispecific antibodies,[2] and the emerging CAR-T cell therapy.[3,4] Antibodies are attractive as therapeutics because they can bind their antigens with high affinities and specificities. An antibody's functional significance is largely dictated by the precise epitope it targets on its antigen, because specific epitopes can convey inhibitory, activating, or no biological activity. While an antibody's affinity can be engineered with a few amino acid changes,[5] epitope specificity is often determined by the ensemble architecture of the complementary-determining regions (CDRs) and the frameworks containing them, rendering it difficult, to almost impossible, to alter an antibody's epitope without drastically perturbing the antibody's paratope. Therefore, given that an epitope defines an antibody's innate property and its functional importance, assessing the epitope diversity within a panel of mAbs is an essential criterion when selecting those with therapeutic potential or as reagents for supporting analytical assays.

Despite the commercial availability of various mAb generation platforms, discovery is still dominated by mouse immunization, as judged by the source of therapeutic mAbs that are currently in the clinic or on the market. The biological similarities shared between human and mouse systems can be leveraged in a positive way, since the in vivo screening that occurs when mAbs are generated via mouse immunization may naturally remove mAbs with undesirable biophysical characteristics.[6] However, because many human antigens of interest are highly homologous with their mouse orthologs, these antigens are often weakly immunogenic, which limits the epitope diversity that can be achieved via the routine immunization of mice or other mammals. Since in vivo proof of mechanism and preclinical safety studies are commonly conducted in mouse or rat models, the use of a human-rodent cross-reactive mAb facilitates such studies. Wherever possible, this is preferred over a surrogate approach, which is often of questionable relevance, or the use of non-human primates, which raises scientific, ethical, and economic issues.[7] In vitro display technology is often employed to generate rodent-human cross reactive mAbs because it bypasses the self-tolerance issues of rodent immunization. However, owing to the lack of an in vivo screen, in vitro-generated antibodies can possess undesirable biophysical and biochemical properties, thereby limiting the utility of these antibodies in therapeutic settings. Additionally, it has been reported that specificity can be negatively altered through

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the in vitro sequence manipulation required for humanization of animal-derived antibodies.[8] These findings reinforce the notion of in vitro antibody discovery or optimization systems being somewhat of a "black box" in which only certain parameters of antibody performance are selected for, whereas in vivo systems have evolved to select for many critical antibody attributes in parallel.

It has been speculated that immunizing an animal that is phylogenetically distant from human may access unique epitopes while still providing an in vivo screening process that removes undesirable clones. The scientific literature contains many examples of antigens that are non-immunogenic in rodents, but generate robust responses in chickens.[9-18] In these cases, achieving a titer is clear evidence of the benefit of using a non-mammalian host. Additionally, chickens may offer an enhanced immune repertoire for many other targets by covering epitopes that are conserved among mammals and hence poorly immunogenic in rodents. Here, we present the first study that critically compares the kinetic and epitopic diversity of mAbs derived from chicken immunization, mouse immunization, and phage display of naïve and synthetic antibody libraries. As model antigens, we chose 2 relatively immunogenic and disparate human proteins, namely proprotein convertase subtilisin/kexin type 9 (PCSK9) and progranulin (PGRN), which are well-conserved across their mouse and rat orthologs (see Fig. S1). The high abundance of these antigens in normal human serum provided a convenient source of their native epitopes, which enabled our KinExA studies. They are also commercially available in purified recombinant form at relatively low cost, which facilitated their use as immunogens, panning reagents, and analytical screening reagents. Due to their monomeric nature they were especially amenable as analytes in our interaction analysis studies. Using label-free biosensors, we compared mAbs from different sources in terms of their binding kinetics toward their specific human antigen, assessed their cross-species reactivity, and performed high-throughput epitope binning assays. Our binning studies provided exquisite resolution of epitope bins and allowed us to identify both overlapping and non-overlapping epitope landscapes of the compared mAb generation platforms.

Results

Chicken immunization produces clones with comparable kinetic diversity as those from traditional mAb generation platforms

Two important biophysical characteristics of a therapeutic mAb are its binding affinity toward its specific antigen and the epitope it targets on that antigen. First, we compared the antigen-binding kinetics of mAbs derived from chicken immunizations with those derived from phage display, comprising both naïve and synthetic antibody libraries (for PCSK9), and mouse immunization (for PGRN). Binding kinetics were obtained using experiments performed on surface plasmon resonance (SPR) biosensors, employing an assay format in which purified recombinant antigens were titrated as monovalent analytes over mAbs that were captured via immobilized anti-human-Fc or anti-mouse-Fc capture reagents, as appropriate. Chicken mAbs were produced in a recombinant scFv-Fc format comprising chicken V regions and human Fc and were captured directly from crude supernatants. Purified mAbs were also analyzed in this way or were amine-coupled to the chip. Representative examples of the range of PCSK9 binding kinetics observed within a panel of chicken mAb supernatants are shown in Fig. 1A. Scatter plots of the apparent association and dissociation kinetic rate constants (k_a and k_d values) obtained toward anti-PCSK9 (Fig. 1B) and anti-PGRN (Fig. 1C) clones from chicken and non-chicken sources showed comparable diversity (see Tables S1 and S2). Thus, for each model antigen studied, the binding kinetics obtained for mAbs derived from chicken immunization or other methods covered a similarly broad range despite minor technical variations across the biosensor experiments, such as the use of different SPR instruments, chip types, mAb immobilization methods, and analyte injection methodologies.

Chicken immunization yields clones that bind native serum antigen with very high affinity

The SPR kinetic analyses described above revealed that all mAb generation platforms tested, including chicken immunization, yielded some clones with apparent K_D values in the low picomolar range. Since kinetic measurements performed on hydrogel-coated surfaces can result in slower apparent k_a values than those observed in solution,[19] it is likely that the SPR measurements overestimated the K_D values of these interactions. Additionally, some clones bound so stably that their k_d values were too slow to resolve within the limits of a capture-based assay.[20] Therefore, to obtain affinity estimates that would more faithfully represent those obtained in solution, a high affinity clone per model antigen/mAb source was chosen for further characterization in the kinetic exclusion assay (KinExA). To provide an even more biologically-relevant measurement, affinities were determined toward the unpurified native forms of these antigens, as available in human serum, using a method described previously.[21]

The assay format employed for the KinExA experiments is shown in Fig. 2A and was tailored to each studied mAb by the appropriate selection of mAbs for bead-coating and secondary detection (see Methods). A global analysis of the results obtained for the anti-PCSK9 mAb C34 binding to serum PCSK9 is shown in Fig. 2B, returning an apparent K_D of 21 (26 – 18) pM (N = 3) at 23°C. Additionally, the global analysis enabled the quantification of PCSK9 in undiluted serum, which was determined to be 2.0 (2.3 – 1.7) nM or 145 (169 – 122) ng/ml, consistent with values reported in the literature.[22] A similar set of experiments was performed for the anti-PGRN mAb C25, returning an apparent K_D of 1.1 (2.3 – 0.3) pM (N = 2) at 23°C toward serum PGRN (Fig. 2C) The global fit showed that undiluted serum contained an apparent PGRN concentration of 1.3 (1.5 – 1.2) nM, which is consistent with reported values for healthy subjects.[23] Similar serum assays performed on the highest affinity clones derived from non-chicken sources, namely anti-PCSK9 mAb H69 from a phage display library and anti-PGRN mAb M27 from mouse immunization, also

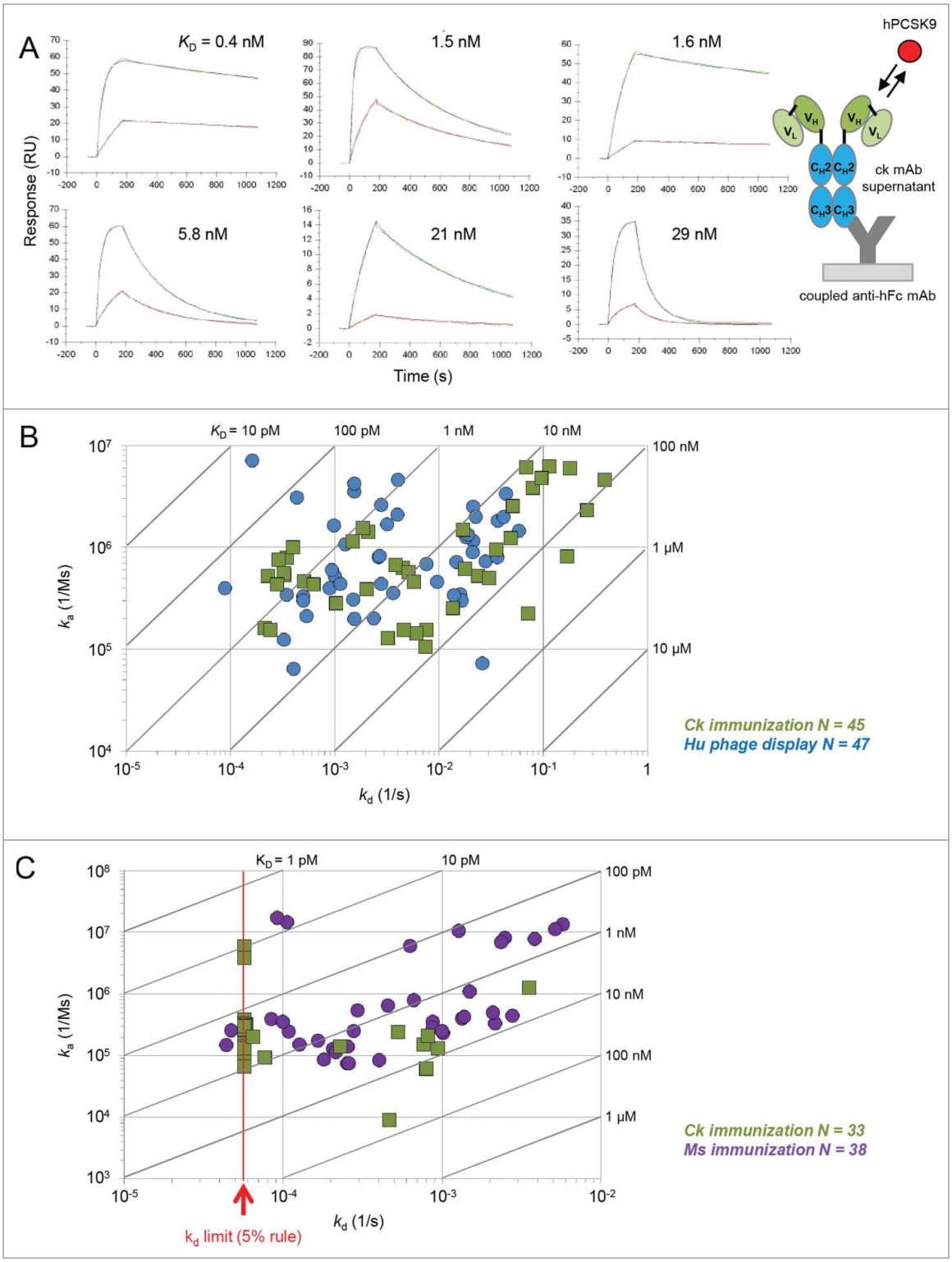

Figure 1. Affinity comparison of mAbs derived from chicken immunizations and other sources for 2 unrelated model antigens, PCSK9 and PGRN. (A) Biacore binding curves and global fits of select anti-PCSK9 mAbs from chicken immunization showing a diverse set of kinetic profiles. The colored curves represent the measured binding responses of hPCSK9 when injected at concentrations of 5 nM (red) and 50 nM (green), with the global fit overlaid in black. (B) Isoaffinity plot comparing anti-PCSK9 mAbs generated from chicken immunization (olive green) with those from human phage display libraries (blue). (C) Isoaffinity plot comparing anti-PGRN mAbs generated from immunizations in chicken (olive green) and mouse (purple). The red dotted line indicates the k_d limit of 5.70×10^{-5} (1/s) that was placed on interactions which showed < 5 % signal decay within the allowed dissociation phase of 15 min, also known as the "5 % rule" (see Methods).[20]

returned apparent affinities in the single digit pM range (data not shown).[23] In addition, these assays confirmed that all mAbs used - on the bead, as titrant, or as secondary - bound native epitopes on their respective serum antigens.

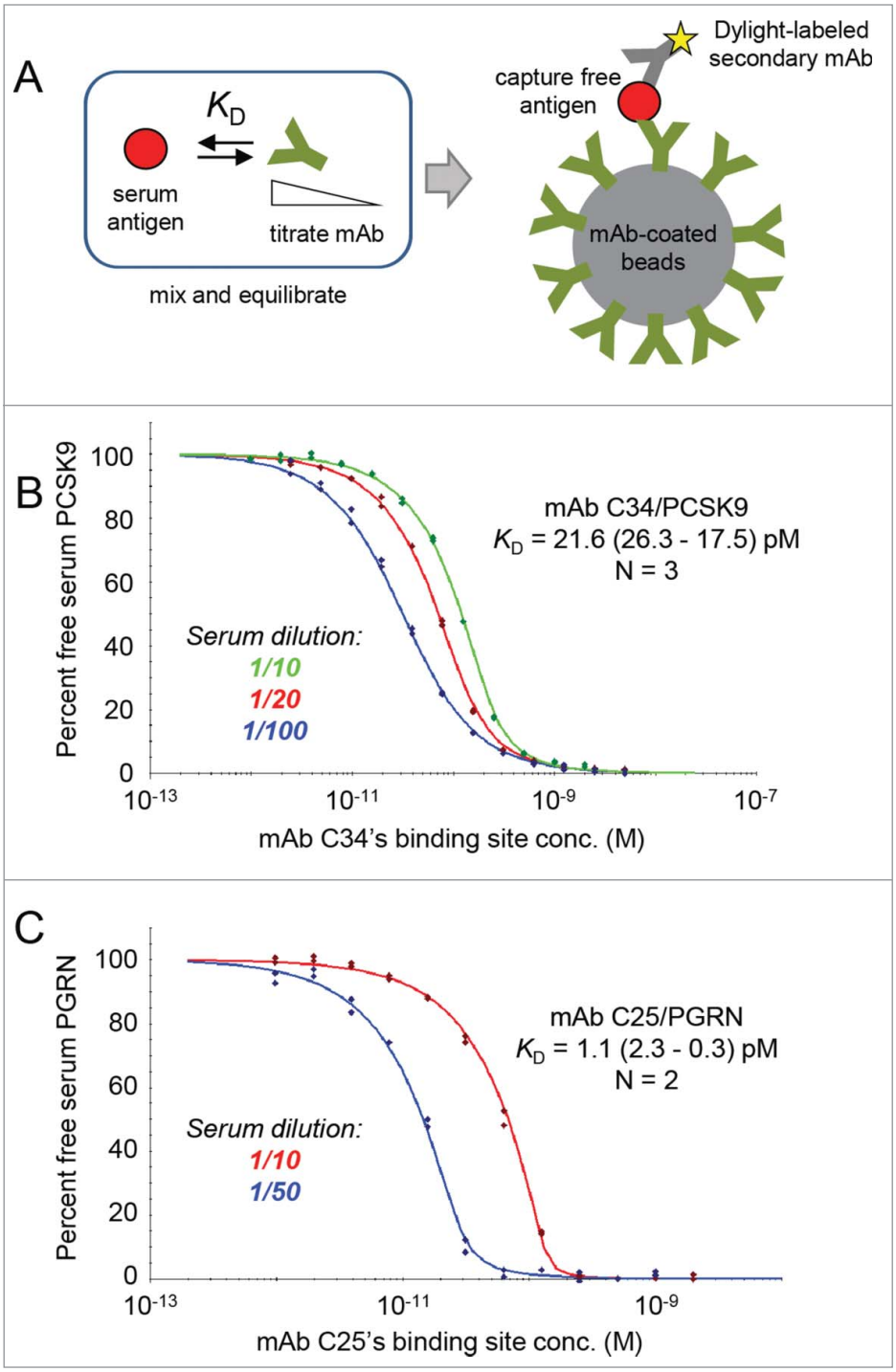

Figure 2. Solution affinity determination of 2 high affinity clones obtained from chicken immunization toward their respective serum antigens. (A) Schematic representation of the KinExA assay set-up. The mAb of interest is titrated into human serum and the equilibrated mixtures are injected over beads absorption-coated with a competing mAb to capture the free antigen. The bead-captured antigen is then detected using a Dylight-labeled sandwiching mAb. (B) Global analysis of mAb C34 binding serum PCSK9. (C) Global analysis of mAb C25 binding serum PGRN. In each case, the reported apparent K_D value is the best fit and 95 % confidence interval of the fit.

Chicken immunization appears to yield clones with broader epitopic coverage than those derived from standard in vivo and in vitro methods

To assess the epitopic diversity produced by chicken immunization, high-throughput epitope binning experiments using array-based SPR imaging were performed as previously described.[24] For each model antigen, a panel of epitopically-diverse mAbs obtained from standard in vivo or in vitro mAb generation methods was assembled, and binned together with the chicken mAbs to provide landmarks for epitope diversity comparison. These binning experiments were performed using a classical sandwich assay format,[25] wherein solution mAbs (analytes) are tested for binding to a monovalent antigen that is

first captured by an array of amine-coupled mAbs (ligands). While purified mAbs were used for the amine-coupling step in these experiments, we found that the chicken mAb supernatants (upon dilution into coupling buffer) could be coupled directly without the need for further purification, because the Expi293™ expression medium used was both serum-free and protein-free.

Anti-PCSK9 mAbs

A sorted heat map of the binning results obtained for a merged panel of 63 anti-PCSK9 mAbs comprising 39 mAbs from chicken immunization and 24 mAbs from human phage display is shown in Fig. 3A. Analytes are named along the header row and this order is transposed to show corresponding ligand names along the far left column. There are more analytes than ligands because not all coupled mAbs performed well in the assay; a few mAbs did not couple at sufficiently high enough capacity or were damaged upon regeneration. The two-dimensional analyte/ligand matrix is sorted using a hierarchical clustering algorithm with self-blocking interactions shown along the diagonal (by the shaded cells with a thick black outline), representing the use of the same mAb in the role of both analyte and ligand. A red cell indicates a blocking analyte/ligand pair, a green cell represents a non-blocking analyte/ligand pair, and a yellow cell indicates an intermediate response (see Methods). Sometimes, 2 mAbs appeared to block one another in an order-dependent fashion, showing a block in one order of addition but not in the other; an "X" is used to flag a pair of mAbs that exhibited this type of asymmetric blocking behavior. The mAbs are colored by library (olive green for chicken immunization or blue for human phage display) to aid their visual discrimination. Alongside each mAb's name is its SPR-derived K_D value toward human PCSK9 (colored with a gradient to highlight the affinity range) and its cross-reactivity toward mouse and rat PCSK9. Overall, we observed that anti-PCSK9 mAbs from both sources were distributed across an intricate network of overlapping epitope bins, with many pairs of mAbs exhibiting asymmetric blocking behavior that complicated the interpretation of the heat map (see Fig. S2). Some chicken mAbs appeared to occupy chicken-only epitope bins, e.g., C14, C25, and C27 together in one bin, and C2 on its own in a second, independent bin. A dendrogram of the sequence lineages for the anti-PCSK9 chicken clones is shown in Fig. 3B alongside their binning heat map (drawn from Fig. 3A, transposed, and resorted). In comparing the binning assignments with the sequence lineages, we observed that multiple clonotypes can populate a single bin. For instance, C25 and C27 are highly related, but C14 is from an independent lineage. On the other hand, overlapping bins are often comprised of independent clones that are part of a sequence-related family, such as the bins represented by the human-specific clusters, C15/C22/C18/C29/C35 and C23/C30/C4/C21/C8/C17/C24, and the human-rat cross-reactive cluster, C43/C47/C44/C46. When a completely unique non-overlapping bin is identified, such as that represented by C9, the antibody sequence is also unique, with no highly similar sequences observed in any other bin.

Anti-PGRN mAbs

A similar binning analysis as described above was performed on PGRN using 32 mAbs from chicken immunization and 20 mAbs from mouse immunization. Fig. 4A shows the sorted heat map for the chicken/mouse merged binning experiment and Fig. 4B is an alternate representation of the same data graphed as a blocking network plot to emphasize the interconnectivities of the bins; mAbs from chicken immunization are shown in olive green and mAbs from mouse immunization are shown in purple. In a blocking network plot, 2 mAbs that block one another are connected by a line, whereas mAbs that do not block one another are not connected by a line. A dotted line indicates a blocking relationship that was observed in only one order of addition, which manifests as an asymmetry in the heat map (see Fig. S2C). MAbs that belong to the same epitope bin, as judged by their sharing the same blocking profile when tested against all other mAbs, are inscribed by an envelope. In contrast to the complex web of bins observed for anti-PCSK9 mAbs (see Fig. S2A), the anti-PGRN mAbs fell neatly into several discrete bins. This likely reflects the different architectures of the 2 model antigens used here, since PCSK9 is relatively globular, whereas PGRN is a multi-subdomain protein composed of 7 small tandem repeats, called the granulins.[26] We corroborated this assumption by epitope mapping the anti-PGRN mAbs and assigning them to specific GEP subdomains via a human-mouse chimeric swap antigen mutagenesis strategy (discussed later). These assignments are also indicated on the network plot shown in Fig. 4B. Taken together, the epitope binning and mapping results showed that chicken mAbs were able to access some of the bins defined by the mouse mAbs (e.g., chicken mAbs C21 and C25 populated the same bin as mouse mAbs M1, M2, M3, M4, M6, M8, M10, and M12). Additionally, some small bins were unique to mouse (e.g., M5 and M13) or chicken (C28, C29, and C32), although it is possible this finding is simply related to a sampling issue rather than one of the species being "unable" to generate a clone that would populate a particular bin. Relative to the PCSK9 clones, we observed fewer cases of asymmetric blocking behavior for the PGRN clones, which simplified the analysis.

We next investigated whether antibody sequence diversity correlated with epitope diversity for the chicken mAbs. A dendrogram of the antibody sequence lineages for these clones is shown in Fig. 4C, alongside the binning heat map (drawn from Fig. 4A, transposed, and resorted) and Fig. 4D shows the corresponding blocking network plot, colored by bin, with GEP assignments indicated. There is a striking correlation between the distribution of chicken clones across the blocking network plot and their sequence lineages.

Both chicken immunization campaigns with 2 unrelated human antigens yielded rodent cross-reactive clones

All mAbs were screened for their species cross-reactivity using an Octet biosensor equipped with anti-human-Fc or anti-mouse-Fc sensors, as appropriate. Anti-PCSK9 mAbs generated by chicken immunization and human phage display showed very similar distribution in their cross-reaction toward mouse and rat PCSK9. For example, of the 44 chicken mAbs

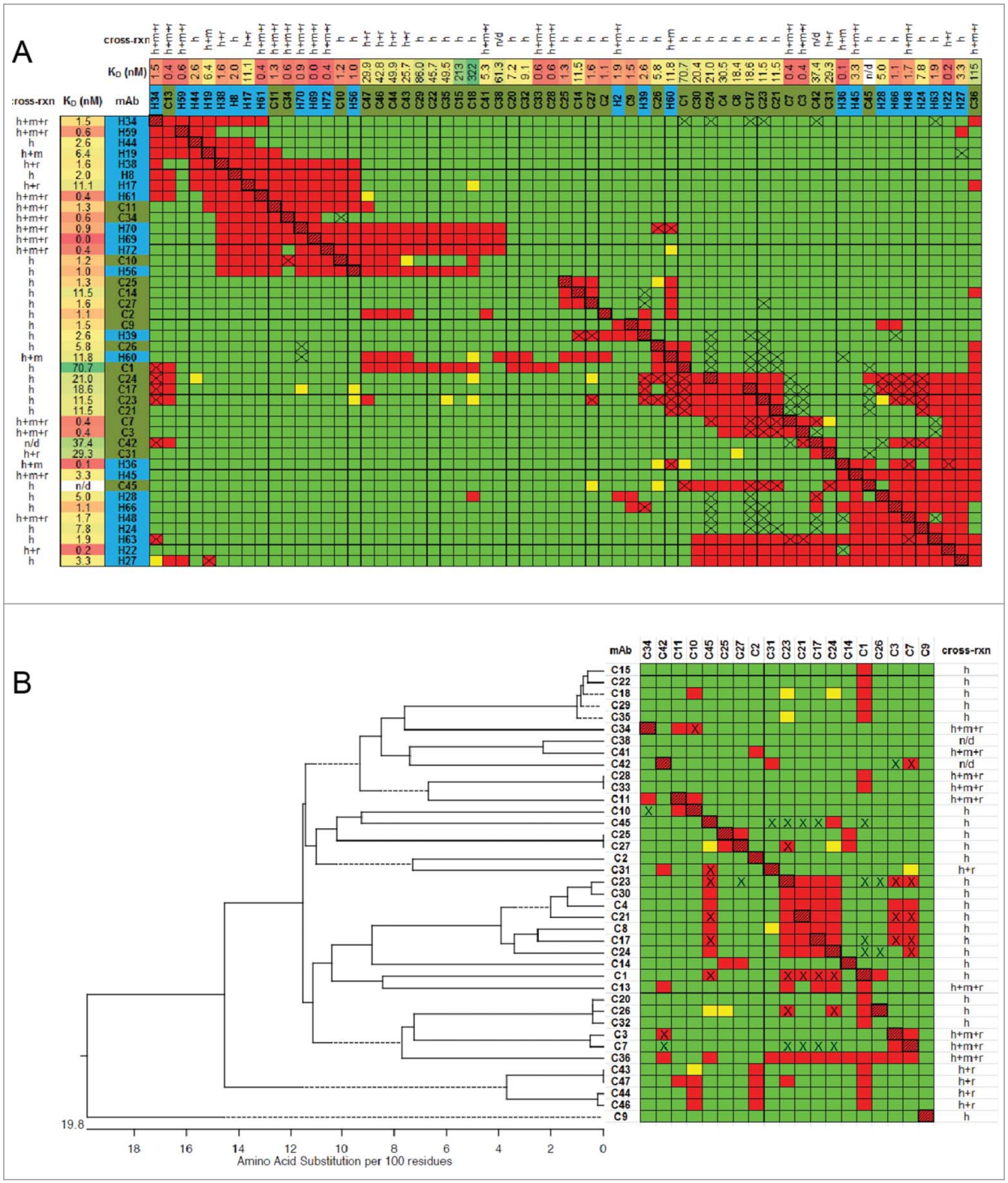

Figure 3. Chicken-human merged binning results for PCSK9. (A) Heat map showing binning assignments for 39 mAbs generated from chicken immunization (olive green) and 24 mAbs generated by human phage display (blue). SPR-derived K_D values toward human PCSK9 are reported (conditionally formatted using a color gradient), along with their Octet-based cross-reaction toward human (h), mouse (m) and rat (r) PCSK9 (n/d = not determined). (B) Dendrogram showing antibody sequence lineages of the chicken mAbs alongside the binning heat map for these clones (drawn from panel A, transposed, and resorted). See Table S1.

that confirmed positive for human PCSK9, 12 crossed to both mouse and rat PCSK9, one crossed only to mouse PCSK9, 5 crossed only to rat PCSK9, and 26 showed no detectable rodent cross-reaction. This distribution was paralleled in the human phage display library (see Table S1). In contrast, in the case of PGRN, only the chicken mAbs showed any cross-reaction toward mouse PGRN, because mAbs generated from mouse immunization were restricted by self-antigen tolerance. Examples of the overlay plots obtained for anti-PGRN chicken mAb supernatants that showed high, medium, or no affinity toward mouse PGRN are provided in Fig. S3A. Consistently, the chicken anti-PGRN clones that showed human-mouse cross-reaction (see Table S2) populated chicken-only bins, e.g., C8, C13, C14, C16, C17, C28, C29, C37, and C40, reinforcing that

these epitopes were uniquely accessed by chickens and not mouse. Interestingly, a given bin may contain sequence-related clones having either cross-reactive or non-cross-reactive binding profiles, suggesting that in some cases only a minor change in an antibody's paratope can allow it to recognize the other species of the antigen (see PGRN bins C8/C30/C38 and C28/C29/C32 in Fig. 4D).

We further epitope mapped the anti-PGRN mAbs and assigned them to a GEP subdomain, using a human-mouse chimeric swap strategy (Fig. S3B). Only mAbs that were specific to human PGRN and did not cross to mouse PGRN were amenable to this analysis because it relied upon using the mouse PGRN as an "inert" framework for swapping out various GEP subdomains and replacing them with their human counterpart (see Fig. S3C for examples of the Octet-based epitope mapping data). The results of these assays are summarized in Table S2 and the assigned GEP subdomain is reported in the heat maps shown in Fig. 4. We observed an excellent correlation between the epitope binning and epitope mapping assignments.

Discussion

Chicken immunization appears to produce clones with diverse kinetic profiles and expanded epitopic repertoires relative to those from standard in vivo and in vitro mAb generation methods

Across independent campaigns that used 2 different purified recombinant human proteins as immunogens, we demonstrated that chicken immunizations can yield clones with very high affinity toward the native serum forms of these antigens. For each model antigen studied, we observed no significant differences in the kinetic diversity of mAbs generated across orthogonal platforms. For PCSK9, SPR assays returned K_D estimates ranging from 0.4 – 322 nM for 45 chicken mAbs and 0.023 – 363 nM for 47 human mAbs (see Table S1). In this case, the use of phage display of human antibody libraries appeared to produce a significantly (17fold) higher affinity clone than any clone generated via chicken immunization. For PGRN, however, SPR returned comparable K_D estimates for clones derived from immunizations in chickens (< 0.01 – 53 nM for 32 chicken mAbs) and mouse (0.01 – 6.5 nM for 38 mouse mAbs), see Table S2. Interestingly, the highest affinity anti-PGRN clones generated in chickens (mAbs C21 and C25) and mouse (mAbs M8 and M27) targeted the same GEP subdomain (E) and populated the same epitope bin. Chicken immunization also produced mAbs that accessed human-rodent cross-reactive epitopes and appeared to show expanded repertoires beyond those offered by standard in vivo and in vitro mAb generation methods. A summary of the epitope coverage observed for PGRN mAbs raised in chickens and mouse is provided in Table 1 (see Fig. 4 for more detail).

When assessing the binding kinetics and affinities of the mAbs used in this study, we performed a relatively high throughput screen via SPR and further characterized the highest affinity clones in a low throughput manner using the KinExA method. The affinities deduced from both methods are referred to as "apparent" because each is valid within the experimental conditions employed. The KinExA method consistently returned tighter apparent affinities (up to 30-fold tighter) than those estimated by SPR and this discrepancy likely reflects inherent differences in the measurements themselves rather than differences between the recombinant and serum forms of the antigens used in the 2 assays.[21] It should be noted that the KinExA measures solution affinities at equilibrium, whereas the SPR affinities were deduced from the binding kinetics of a solution antigen interacting with an immobilized mAb at a hydrogel surface. Furthermore, since the KinExA experiments were aimed at determining a precise affinity of each studied interaction, each measurement was derived from a global fit of a series of optimized experiments, each of which used a 12-membered titration range. In contrast, the purpose of the SPR experiments was to affinity-rank a large panel of mAbs and so they were conducted in a screening mode that used only 2 analyte concentrations. Therefore, while the SPR screen was helpful in identifying the high affinity binders, it lacked the discriminatory power to resolve them precisely.

It should be noted that for both targets only a small panel of chicken mAbs was generated from a single campaign and was compared to panels of either human phage antibodies or mouse hybridomas that were comprised of clones known to cover the widest possible epitope diversity, as previously defined. Taken together, the results validate chicken immunization as an appealing and robust alternative for generating therapeutic leads as well as analytical reagents. Chicken mAbs may be particularly useful as reagents in anti-drug antibody (ADA) assays to probe clinical samples for possible target interference because a chicken mAb that binds a similar epitope to that of the drug would react only with the target and not the ADA, due to its sequence being very different from that of the drug.

Influence of immunization and screening strategies on recovered antibody profiles

A relatively high frequency (20 % – 30 %) of species cross-reactive clones was observed in the chicken mAb panels for both antigens despite making no special effort to bias toward these specificities. Since only human antigen was used during immunization and screening at the single B cell level, the observed cross-reactivity for these 2 antigens represents the natural frequency produced from chickens. In contrast, for other programs where cross-reactivity has been a specific design goal, chickens have been immunized with antigen from multiple species, and GEMs prepared specifically to screen for this attribute, we have seen species cross-reactivity frequencies as high as 80 % (unpublished data).

We observed that plastic-adsorbed antigen can lead to false positives in preliminary screening, whereas lightly biotinylated antigen more faithfully preserves native epitopes. Many clones that appeared positive on plastic-adsorbed PCSK9 by ELISA did not bind to biotinylated PCSK9 captured onto streptavidin plates, and this correlated with an absence of binding to soluble PCSK9 in our SPR experiments. For example, 70 unique-sequence anti-PCSK9 mAbs that gave strong signal in ELISA to plastic-adsorbed antigen were evaluated by SPR for binding soluble antigen and also by ELISA for binding to biotinylated antigen captured onto streptavidin plates. Of the 47 clones showing

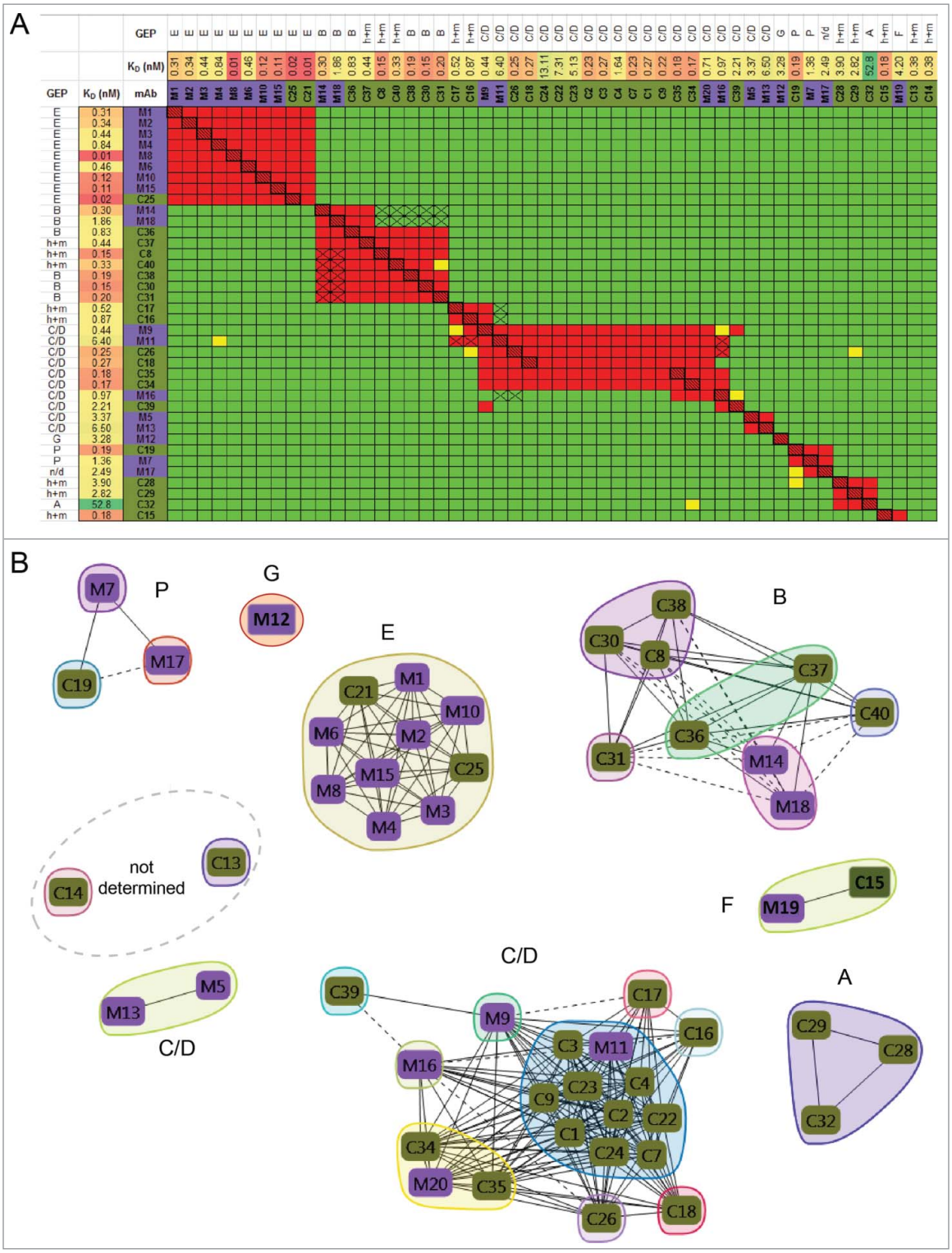

Figure 4. Chicken-mouse merged binning results for PGRN. (A) Heat map showing binning assignments for 32 mAbs generated from chicken immunization (olive green) and 20 mAbs generated from mouse immunization (purple). SPR-derived K_D values toward human PGRN are reported, along with their cross-reaction toward mouse (m) PGRN, and their GEP subdomain assignment (just for the human-specific clones; n/d = not determined). (B) Blocking network plot, colored by mAb library, with GEP assignments indicated (determined empirically, or inferred). (C) Dendrogram of the antibody sequence lineages for the chicken mAbs alongside the binning heat map for these clones (drawn from panel A, transposed, and resorted). (D) Blocking network plot for the chicken clones, colored by bin, with GEP assignments indicated. See Table S2.

detectable binding to soluble antigen, all but one also bound to the biotinylated antigen. Interestingly, the GEM screen was able to detect clones capable of binding soluble PCSK9 as well as those binding only plastic-adsorbed PCSK9, likely due to the aldehyde functional groups present on the latex beads, allowing them to present PCSK9 in both a passively-adsorbed form as well as a covalently-coupled form. In contrast, we did not observe any discordance for PGRN, since almost all of the

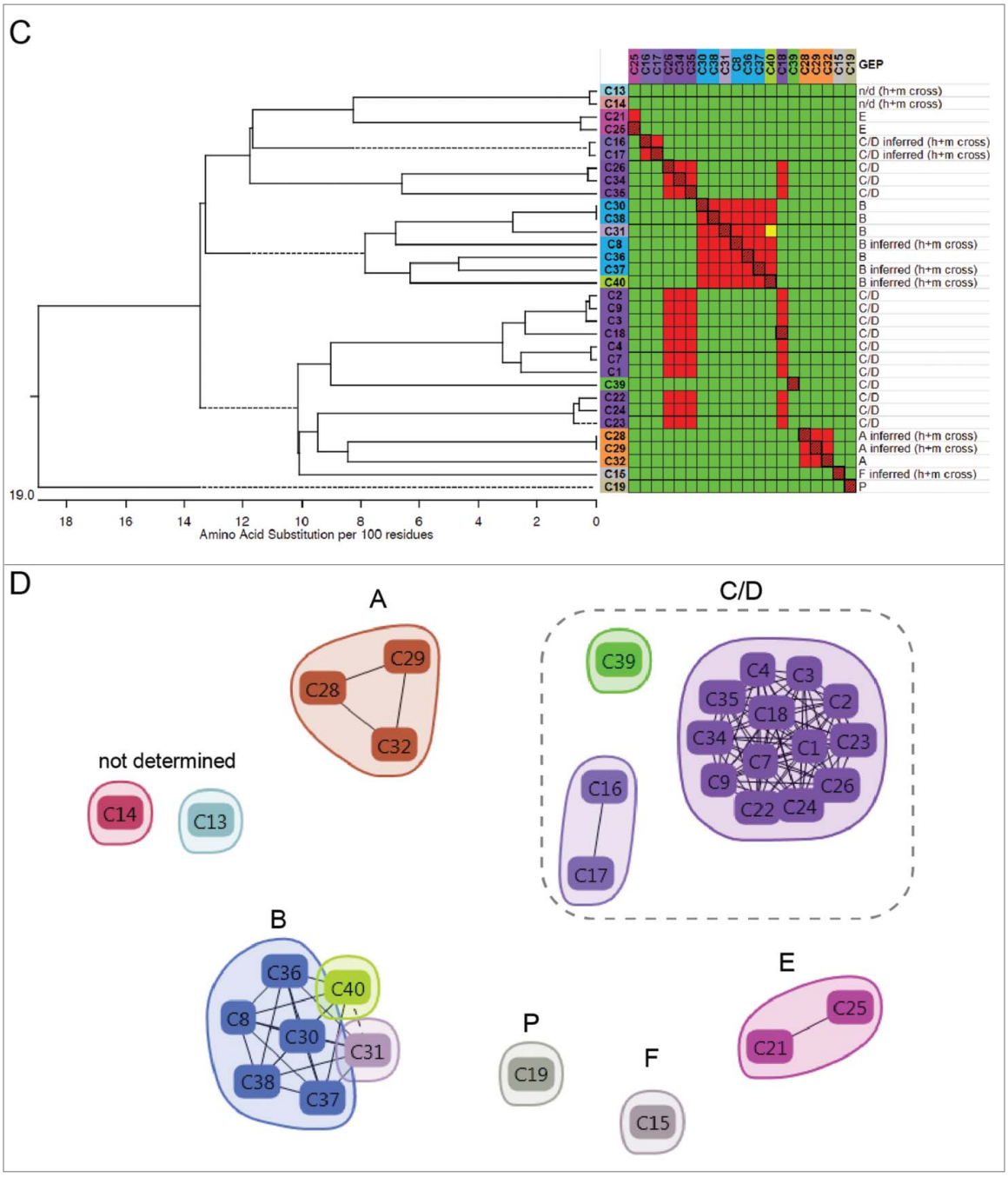

Figure 4. (Continued)

clones we generated were able to bind both streptavidin-captured biotinylated antigen and plastic-adsorbed antigen in ELISA.

Sourcing therapeutic leads from chicken immunization

MAbs are claiming an increasing share of the new drug landscape, and most pharmaceutical companies and many biotech companies have invested in mAb generation from both traditional and novel technologies. With this ubiquity, there is an inclination to consider mAbs as commodities. Here, we challenge that view and demonstrate that a large panel of mAbs to a single target can have impressive kinetic and epitopic diversity, and that both the mAb source and the screening/selection methods have an important effect on the quality of the clones obtained. We performed a high resolution functional characterization of a panel of mAbs from a novel source, namely immunized chickens, directed against 2 independent antigens, and have found a significant enhancement of epitopic coverage relative to what is offered by traditional approaches, including both mouse hybridoma and phage display. It is likely that our general findings will extend to other targets, and that the mining of chicken immune repertoires will be complementary, and not redundant, with existing discovery campaigns. For any given therapeutic target, it is not clear from what source the "best" drug candidates will come, but it is reasonable

Table 1. Comparison of the epitope coverage observed for anti-PGRN mAbs raised via the immunization of chickens or mouse. *Of the 14 chicken mAbs that showed human/mouse crossreactivity, 8 were mapped to a GEP subdomain (by inferring from the binning data shown in Fig. 4B) and are included in the A – P tally, and 6 were not assigned to a GEP subdomain.

Immunized species #mAbs that map to GEP subdomain:	Chicken	Mouse
A	3	4
B	9	2
C/D	17	6
E	2	9
F	1	2
G	1	2
P	1	2
not assigned	6	0
#human/mouse crossreactive mAbs	14*	0
total # mAbs tested in epitope mapping	40	27
#non-overlapping bin clusters	7	7
#species-only bin clusters	2	2
#chicken/mouse shared bin clusters	5	
total # mAbs tested in epitope binning	32	20

to expect that probing diverse repertoires from multiple sources with powerful analytical tools will produce favorable results.

The chicken mAbs described in this study are derived from wild-type chickens and thus contain V regions composed of chicken sequences that would likely be immunogenic in therapeutic applications if not humanized. Humanization of chicken mAbs has been achieved through a classical CDR grafting approach,[27,28] and more recently through framework shuffling.[29] Concurrently, significant progress has been made toward the development of genetically-engineered chickens that generate human sequence mAbs upon immunization.[30-32] There is preliminary evidence that mAbs from such chickens can readily produce species cross-reactive antibodies and populate some of the same "chicken only" bins for the model antigens described here (unpublished data), all of which indicates that there will be exciting opportunities for sourcing therapeutic leads directly from chicken immunization. While it is common practice to optimize lead candidates in vitro to remove potential sequence liabilities, mature affinity, and enhance solubility, in vivo methods offer the advantage of allowing the sampling of sequence space to occur within a live animal, thereby reducing the need for engineering, and associated risk, that occurs ex vivo. From this perspective, any experimental animal generating human sequence antibodies is highly desirable, and an engineered chicken perhaps particularly so, if it can recapitulate the immune recognition attributes that we have reported here with wild-type chickens.

Methods

Immunization of chickens

A total of 4 female white leghorn chickens were used for this study, 2 per model antigen, all starting immunization at 8 – 9 weeks of age. For the PCSK9 program, 2 birds were each immunized with 160 μg recombinant hPCSK9 (Avi-His tagged or His-tagged, prepared in-house) bi-weekly for 4 boosts followed by a final boost, which was increased to 320 μg and administered intravenously. For the PGRN program, 2 birds were immunized with 75 μg recombinant hPGRN (R&D systems 2420-PG or Sino Biologicals 10826-H08H) bi-weekly for 5 boosts intramuscularly followed by a final boost, which was increased to 100 μg and administered intravenously. For PCSK9, all intravascular boosts were mixed with CpG and LPS. For both programs, all intramuscular boosts were mixed with an equal volume of Imject Freund's complete adjuvant (VWR PI77140) for the initial boost and mixed with an equal volume of Imject Freund's incomplete adjuvant (VWR PI77145) for all subsequent boosts.

Serum ELISA

Sera were collected bi-weekly during the immunization to determine plasma titer. ELISA plates were adsorption-coated with antigen, as described below. Diluted sera was incubated for 2 h at room temperature then washed off with phosphate-buffered saline (PBS) + 0.05 % Tween-20 (PBST+). One hundred microliters of rabbit anti-chicken IgY HRP (Sigma A9046) diluted 1:5,000 with 3 % dry milk in PBST (PBSM) was added and incubated for 1 h at room temperature. Plates were washed 3 times with PBST+ and developed with 50 μl of TMB and stopped with 50 μl 1 N HCl. ELISA plates were read at 450 nm using the BioTek plate reader. Final titers achieved were 1:312,000 for PCSK9 (both birds) and 1:12,500 for PGRN (both birds).

ELISA with adsorption-coated antigens

High binding ELISA plates were incubated with 2 μg/ml of PCSK9 or PGRN in PBS overnight at 4°C. Plates were washed 3 times with PBST+ and blocked with PBSM for 1 h at room temperature. Dilutions of HEK293 transfected cell supernatant was added to the plate and incubated for 2 h at room temperature then washed 3 times with PBST+. One hundred microliters of rabbit anti-human Fc HRP (VWR RL609-4303) diluted 1:5000 with PBSM was added and incubated for 1 h at room temperature. Plates were washed with PBST+ and developed with 50 μl of TMB and stopped with 50 μl 1 N HCl. ELISA plates were read at 450 nm using the BioTek Synergy H1 Hybrid Reader.

ELISA with biotinylated antigens

Antigens were biotinylated using a 1:1 or 3:1 molar ratio of linker:protein, using EZ-Link™ NHS-LC-LC-biotin (Pierce 21343). High binding ELISA plates were incubated with 2 μg/ml streptavidin in PBS overnight at 4°C. The streptavidin-coated plates were then washed with PBST+ and incubated for 1 h with 1 μg/ml biotin-PGRN or 1 μg/ml biotin-PCSK9. Plates were washed 3 times with PBST+ and blocked with PBSM for 1 h at room temperature. Supernatants from 2-ml transfections of HEK293 cells were diluted 1:50 followed by 3 5-fold dilutions. Fifty microliters of diluted supernatant were added to the wells and incubated for 2 h at room temperature

and then washed 3 times with PBST+. One hundred microliters of rabbit anti-human Fc HRP (VWR RL609-4303) diluted 1:5,000 with PBSM was added and incubated for 1 h at room temperature. Plates were washed with PBST+ and developed with 50 μl of TMB and stopped with 50 μl 1 N HCl. ELISA plates were read at 450 nm using the BioTek Synergy H1 Hybrid Reader.

Quantification ELISA

The specific mAb in crude supernatants (Expi293^{TM} Expression Medium, ThermoFisher Scientific A1435101) was quantified via ELISA, as follows. High binding ELISA plates were incubated with 2 μg/ml of rabbit anti-human Fc (VWR RL609-4103) in PBS overnight at 4°C. Plates were washed 3 times with PBST+ and blocked with PBSM for 1 h at room temperature. Supernatants from 2-ml transfections of HEK293 cells were diluted 1:500 followed by 3 5-fold dilutions. Fifty microliters of diluted supernatant were added to the wells. Fifty microliters of control scFv-Fc starting at 500 ng/ml followed by 3-fold serial dilutions was added for the standard curve. Primary mAbs were incubated for 2 h at room temperature and then washed off 3 times with PBST+. One hundred microliters of rabbit anti-human Fc HRP (VWR RL609-4303) diluted 1:5,000 with PBSM was added and incubated for 1 h at room temperature. Plates were washed with PBST+ and developed with 50 μl of TMB and stopped with 50 μl 1 N HCl. ELISA plates were read at 450 nm using the BioTek Synergy H1 Hybrid Reader. Concentrations were back-calculated using the 5-parameter logistic non-linear regression curve fitting model.

Screening single B cells using the GEM assay

A single lymphocyte screening and recovery method, the Gel-Encapsulated Microenvironment (GEM) assay (US Patents 8030095 and 841517382), was used to isolate antigen-specific mAbs from immunized chickens. The GEM assay involves placing single mAb-secreting lymphocytes in proximity with reporters, which can be cells or beads. The secreted mAb diffuses locally within the GEM and has the opportunity to bind to the reporters. Bound mAb can be detected either directly through the use of a secondary mAb, or by eliciting a response in the reporter that generates a visual signal. Each GEM may contain multiple types of reporters that can be differentiated from each other based on color. In this study, GEMs were prepared with aldehyde-latex beads coated directly with antigen or streptavidin. Streptavidin beads were subsequently complexed with biotinylated antigen. In addition, for a specificity control, streptavidin beads without biotinylated antigen were also prepared (on alternatively colored beads) to screen out non-specific clones, but this was generally unnecessary because streptavidin-binding mAbs were extremely rare. We did not use non-human forms of either antigen on beads, although that can be an effective strategy for enriching for species cross-reactive mAbs.

Cloning, expression, and initial characterization of recombinant mAbs

Antigen-positive GEMs were isolated and chicken antibody V genes amplified through RT PCR and cloned into the mammalian expression vector pF5a (Promega) in scFv-Fc format (with Fc derived from human IgG1 sequence). Plasmids containing recombinant scFv-Fc from the GEM harvests were transiently transfected into HEK293 cells and clonal supernatants were harvested. Supernatants were tested for antigen binding activity on both plastic-adsorbed antigen as well as streptavidin-captured biotinylated antigen, as described above. All clones that were confirmed as binding their respective targets were fully sequenced to avoid redundancies. The initial cloning and expression yielded 124 and 106 unique sequence hits for PCSK9 and PGRN, respectively.

Small scale mAb purifications

On the basis of initial SPR results, some clones were chosen to be purified so that they could be studied further in other assays. We captured scFv-Fc from 1 ml of crude supernatant on Protein G SpinTrap Columns (GE Healthcare) and eluted per the manufacturer's protocol. Eluate was concentrated and buffer exchanged using Vivaspin concentrators (10KD MWCO) and purified mAb was quantified by light absorbance measurement.

Generation of non-chicken mAbs

Human anti-PCSK9 mAbs were generated from both naïve and synthetic human antibody libraries using previously published phage panning protocols.[33,34] Four rounds of panning were performed with biotinylated hPCSK9 and each library contained a theoretical diversity of approximately 10^{11} clones. Outputs were combined and a total of 96 unique clones were reformatted from scFv fragments into full-length human IgG1 and purified by protein A chromatography. Mouse anti-PGRN mAbs were generated via standard hybridoma technology using a single Balb/c mouse that was immunized with 50 μg recombinant hPGRN (R&D systems, 2420-PG) mixed with Gerbu adjuvant with weekly intraperitoneal (i.p.) boosts for 5 boosts total. The final boost was administered i.p. without adjuvant in PBS. The fusion titer was 1:3,000 after Day 52 and yielded over 100 mAbs that confirmed positive by Biacore. All mAbs were purified by protein A chromatography.

Kinetic experiments

Kinetic experiments were performed on SPR biosensors at 25°C in a running buffer of PBS + 0.01 % Tween-20 (PBST) for the ProteOn, or PBST+ for the Biacore. Anti-PCSK9 mAbs derived from human phage display libraries were analyzed on a ProteOn XPR36 equipped with NLC sensor chip (BioRad, Hercules, CA) using a one-shot kinetic method.[35] The capture surfaces for these experiments were prepared by coating the analyte channels with ~2,000 RU biotinylated goat anti-human IgG Fc-specific mAb. Purified anti-PCSK9 mAbs were captured at 2 μg/ml along 6 parallel ligand channels to levels of ~200 RU (with <3 % variation along a channel). Purified recombinant

hPCSK9 (100, 20, 4, 0.8, 0.16, and 0 nM) was injected along the analyte channels for 3 minutes allowing 15-min dissociation time. Capture surfaces were regenerated with 75 mM phosphoric acid. Similarly, the binding kinetics of purified recombinant hPGRN to purified mouse anti-PGRN mAbs were determined in a one-shot kinetic mode on the ProteOn, but using low capacities of amine-coupled mAbs on GLC or GLM sensor chips. Binding data were processed and analyzed in ProteOn Manager software; the one-shot kinetic data were interspot-referenced and double-referenced and fit globally to a simple Langmuir model to determine their apparent association and dissociation kinetic rate constants (k_a and k_d values) and their ratio was used to derive the K_D value of each antigen/mAb interaction, where $K_D = k_d/k_a$. The analyte's "nominal" concentration representing its total protein content, as determined by light absorbance and appropriate extinction coefficient, was used as input value in the kinetic fitting software.

A Biacore T200 equipped with CM4 sensor chips (GE LifeSciences) was used to perform a 2-concentration kinetic screen of each model antigen binding to crude supernatants containing chicken mAbs. The capture surfaces for these experiments were prepared by amine-coupling the anti-human IgG Fc-specific antibody mentioned above to saturating levels (of ~8,000 RU) on all 4 flow cells. Crude supernatants were diluted in running buffer up to 20-fold (to final 1 – 5 μg/ml) and captured onto individual flow cells (2, 3, and 4) leaving flow cell 1 unmodified to serve as a reference channel. Purified recombinant human antigens (PCSK9 or PGRN) were screened as analytes at 0, 5, and 50 nM for 3 min, allowing 15 min dissociation time. Capture surfaces were regenerated with glycine pH 1.7. Binding data were analyzed globally in the T200 software using a Langmuir model with mass transport. The 5 % rule was applied when reporting limits for k_d values that were too slow to resolve precisely within the allowed dissociation time.[20] Accordingly, 5 % signal decay must be observed within the allowed dissociation phase to place a limit on its k_d value. For example, using a dissociation phase time (t) of 15 min, the slowest k_d value that can be resolved precisely is $k_d = \ln(1/0.95)/(60 \times t) = 5.70 \times 10^{-5}$ (1/s).

Solution affinity determination of chicken mAbs toward serum antigen

The affinity of select chicken mAbs toward their native unpurified antigen, as available in human serum, was determined using the KinExA method as described previously,[21] with the following modifications. A KinExA 3200 equipped with autosampler was used (Sapidyne, Boise, IH) at 23°C in a running buffer of PBST + 0.01 % sodium azide. Pooled human serum from healthy donors was purchased from Biological Specialty Corp, Colma, PA (lot X1467). To study the anti-PCSK9 mAb C34, PMMA beads were adsorption-coated with mAb H103 (from human naïve phage display library), chosen because it competes with mAb C34 for binding to PCSK9. MAb H36 (from human synthetic DNA phage display library) was Dylight-labeled and used as secondary detection because it does not compete with mAb C34 (or mAb H103) for binding to PCSK9. Serum was diluted 10-, 20-, or 100-fold in sample buffer (running buffer + 1 g/l BSA) and titrated with mAb C34 as a 12-membered 2-fold serial dilution with top at 5 nM or 2nM binding sites. Samples were allowed to equilibrate for up to 48 h and sample volumes were optimized to yield signal 100 % amplitudes between 0.69 V and 0.96 V.

A similar rationale was used to select appropriate reagents to study the anti-PGRN mAb C25. Beads were absorption-coated with mAb C21 because it competes with mAb C25 for binding to PGRN and mAbs C17 or C18 were Dylight-labeled for use as secondary detection reagents because they do not compete with mAbs C25 or C21 for binding to PGRN. Serum was diluted 10-fold or 50-fold in sample buffer and titrated with mAb C25. Due to the negligible non-specific binding obtained with these capture and detection reagents, we could work at very low signal amplitudes, corresponding to signal 100 % values of only 0.02 V.

KinExA data were fit using the N-curve tool in the Sapidyne software with drift correction, inputting the titrant's concentration as reference standard (representing the titrated mAb's nominal binding site concentration as determined by light absorbance). The global analysis computed the best fit values and 95 % confidence intervals for both the apparent K_D of the mAb's interaction with its specific serum antigen, and the concentration of serum antigen.

Epitope binning experiments

Epitope binning experiments were performed in a running buffer for PBST at 25°C on an array-based SPR imager (IBIS MX96, Netherlands), as described previously.[24] Briefly, a 48-channel continuous flow microspotter (CFM) from Wasatch Microfluidics, Inc. was used to amine-couple 96 purified mAbs onto a sensor chip surface in 2 consecutive prints. Different chip types were used, depending upon the experiment; SensEYE COOH or Xantec CMD 50L, 200M, or 500M. The CFM was primed with PBST. The top of the chip was then activated with a freshly mixed aqueous solution of 12 mM EDC and 3 mM sulfo-NHS and then coupled with 48 mAbs at a final concentration of ~2 μg/ml in 10 mM sodium acetate pH 4.5 supplemented with 0.01 % Tween-20, allowing 5 min per step. The bottom of the chip was functionalized similarly and then the chip was docked in the SPR imager for in-line quenching with 1 M ethanolamine pH 8.5. When less than 96 unique mAbs were available, mAbs were printed on more than one spot coordinate to provide intra-assay replicates. A classical sandwich epitope binning script was used to inject antigen (16 nM hPCSK9 or hPGRN) followed immediately by a mAb analyte (either 5 μg/ml purified mAb or crude chicken supernatant diluted up to 20-fold in running buffer). Surfaces were regenerated with a "Pierce/salt blend" comprising a 2:1 v/v mixture of Pierce's IgG elution buffer pH 2.8 and 4 M NaCl for the PCSK9 experiments, or 75 mM phosphoric acid for the PGRN experiments. In a single experiment, 96 mAb analytes arrayed in a microplate were injected in consecutive binding cycles, interspersing a buffer analyte (instead of mAb analyte) every 12 cycles, to facilitate the data processing.

Epitope binning data were processed in SPRint software and then analyzed in the Wasatch binning tool, where 2 data transformations were applied; first, the binding responses obtained on each spot were normalized to 1 at the end of the antigen

binding step, and then a report timepoint was set at the end of the mAb analyte step to read out the "sandwiching response," relative to the response of the buffer blank analytes at this timepoint, which was nominally set to zero. A threshold was set above this value, such that normalized responses <0.2 were considered "blocked" (red) and normalized responses >0.3 were considered "not blocked" (green). Normalized responses falling within these limits (0.2 – 0.3) were considered "intermediate" (yellow). Threshold settings were adjusted, where appropriate. The data were groomed to remove non-ideal interactions from the matrix, such as inactive or barely active ligands, and ligands or analytes that appear universally blocked or universally not blocked. A heat map of the analyte/ligand matrix was then generated based upon the threshold settings. It was auto-sorted to provide the best visual clustering of bins and ambiguous interactions or apparent blocking asymmetries were then examined by hand and a proper assignment made. Self-blocking interactions are indicated by a shaded cell inscribed by a thick black box and asymmetric blocking interactions are marked with an "X." The heat map was also exported into Excel for further curation by hand, as needed. The software used the heat map to create a blocking network plot, which is an alternate graphical representation of blocking relationships within the antibody panel, independent of the heat map's sort order. In this type of plot, a line between 2 mAbs indicates a blocking relationship confirmed in both orders of addition, and a dotted line represents a blocking relationship observed in only one direction, either because the other direction was not tested (due to inactive ligand) or was ambiguous or gave an asymmetric/conflicting result. By grouping antibodies with the same blocking profile to all others in the panel, epitope bins and their inter-connectivities can be displayed.

Octet screen to assess cross-species reactivity

An Octet Red384 equipped with anti-hFc or anti-mFc sensors (Pall-Fortebio, Menlo Park, CA) was used to capture mAbs directly from crude chicken supernatants, typically diluted up to 20-fold in running buffer (HBS pH 7.4 + 0.01 % Tween-20 + 1 g/l BSA), or at 10 μg/ml purified mAbs for the non-chicken comparator panels. Human, mouse, and rat PCSK9 (prepared in-house) and human and mouse PGRN (R&D systems or Sino Biologicals) were screened as analytes at 100 nM, allowing 5 min association and dissociation time. The sensors were regenerated with 75 mM phosphoric acid and re-used several times within an assay. Binding responses were Y-aligned to zero immediately prior to the binding step of interest (mAb capture or antigen capture) and exported into Biaevaluation v4.1.1 for further analysis. Report points taken at the end of the antigen-binding step were used to assess rodent-human cross-reactivity.

Epitope mapping using a chimeric swap strategy

Anti-PGRN mAbs that were specific for human PGRN and showed no cross-reaction toward mouse PGRN were assigned to a GEP subdomain using a human-mouse chimeric swap strategy. Thus, 5 chimeric swap mutants were designed (see Fig. S3B) using the mouse PGRN (accession NP_032201) as an "inert" framework and swapping out various GEP domains for their human counterparts (accession NP-002078). The five chimeras comprised the following amino acid sequences (h, human; m, mouse): chimera 1: hT18-A123 and mV136-L602; chimera 2: mT31-A135, hI124-Q358 and mI369-L602; chimera 3: mT31-Q368 and hA359-L593; chimera 4: mT31-L69, hG58-V200 and mS213-L602; chimera 5: mT31-P286, hA276-P504 and mP516-L602. The chimeric DNAs were cloned into the HindIII-NotI sites of pSecTag2/hygroA vector (ThermoFisher), in frame with the N-terminal mouse IgG kappa secretory signal sequence and the C-terminal myc-6xHis epitope tag. The constructs were expressed by transient transfection of HEK293 cells using standard protocols, and the conditioned media (Expi293 expression media, Life Technologies) was harvested 5 days later, and filtered through a 0.2 μM filter. The C-terminal 6xHis-tag was used for on-line purification of the chimeric proteins using an Octet HTX biosensor equipped with streptavidin sensors (Pall-Fortebio, Menlo Park, CA). Epitope mapping experiments were performed in a running buffer of PBST + 1 g/l BSA. The sensors were coated with 5 μg/ml biotinylated anti-His mAb (R&D systems BAM050) and used to capture the chimeric mutants directly from the crude supernatant, diluted up to 10-fold in running buffer. As controls, 3 μg/ml purified human PGRN (Sino Biological) or mouse PGRN (R&D systems) were captured onto parallel sensors. Anti-PGRN mAbs were screened as analytes over anti-His-captured chimeras or anti-His-captured PGRN controls using purified mAbs at 10 μg/ml or crude chicken mAb supernatants diluted up to 20-fold in running buffer. The anti-His-coated sensors were regenerated with 75 mM phosphoric acid after each binding cycle and re-used several times within an assay. The data were processed by aligning the sensorgrams to zero at the start of each capture step and report point values were computed at the end of each binding step. The sensorgrams were grouped by mAb analyte and visualized as color-coded plots.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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