Production, characterization, and in vivo half-life extension of polymeric IgA molecules in mice

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ABSTRACT

IgA antibodies have broad potential as a novel therapeutic platform based on their superior receptor-mediated cytotoxic activity, potent neutralization of pathogens, and ability to transcytose across mucosal barriers via polymeric immunoglobulin receptor (pIgR)-mediated transport, compared to traditional IgG-based drugs. However, the transition of IgA into clinical development has been challenged by complex expression and characterization, as well as rapid serum clearance that is thought to be mediated by glycan receptor scavenging of recombinantly produced IgA monomer bearing incompletely sialylated N-linked glycans. Here, we present a comprehensive biochemical, biophysical, and structural characterization of recombinantly produced monomeric, dimeric and polymeric human IgA. We further explore two strategies to overcome the rapid serum clearance of polymeric IgA: removal of all N-linked glycosylation sites creating an aglycosylated polymeric IgA and engineering in FcRn binding with the generation of a polymeric IgG-IgA Fc fusion. While previous reports and the results presented in this study indicate that glycan-mediated clearance plays a major role for monomeric IgA, systemic clearance of polymeric IgA in mice is predominantly controlled by mechanisms other than glycan receptor clearance, such as pIgR-mediated transcytosis. The developed IgA platform now provides the potential to specifically target pIgR expressing tissues, while maintaining low systemic exposure.

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The presence of the JC in polymeric IgA is required for binding pIgR on the basolateral side of the epithelium and for active transport to the apical side of mucosal tissues. Upon transcytosis, the extracellular domain of pIgR is proteolytically cleaved, creating what is known as the secretory component (SC), which remains covalently attached to the polymeric IgA heavy chain through a disulfide bond between Cys467 in pIgR and Cys311 in one HC. This complex is deemed secretory IgA (sIgA), the main determinant of mucosal immunity.

In humans, there are two IgA isotypes, IgA1 and IgA2, distinguished by a 13-residue extension in the hinge region of the IgA1 HC that is absent in IgA2 molecules. Both isotypes are abundant in all organs and tissues, except in the intestines where IgA2 is predominant and in the serum where IgA1 monomer is found almost exclusively. There are three allotypes of IgA2: m1, m2, and mn. The m2 and mn allotypes form canonical light chain (LC)-HC disulfides, whereas the presence of a proline at position 221 of the HC in IgA2m1 results in LC-LC disulfide bond formation instead. Mutation of proline 221 in the IgA2m1 allotype to arginine (P221R), which is found in the m2 and mn allotypes, restores the canonical LC-HC linkage.

IgA has been reported for human IgA1 or IgA2m1 isotypes at 30–70 µg/L. To overcome these low production yields, stable cell lines have increased expression levels of monomeric IgA1, IgA2m1 and IgA2m2 to 110–190 mg/L. Whereas human IgG1 typically has only two N-linked glycosylation sites, one in each CH2 domain, human IgA contains multiple glycosylation sites that can be susceptible to glycan heterogeneity. IgA has multiple O-linked glycosylation sites in the hinge region and two N-linked glycosylation sites (Figure 1A, boxed). The JC also contains one N-linked glycosylation site (Figure 1B, boxed).

Assembly of the three polypeptide chains (LC, HC, and JC) leads to multiple oligomeric states and further contributes to the overall complexity of recombinant polymeric IgA (Figure 1C). With the increasing size of an IgA oligomer comes not only an increased number of glycosylation sites but also the potential for more glycan heterogeneity. To date, most studies have focused on recombinant monomeric IgA, likely due to the added complexities of polymeric IgA production. IgA has previously been shown to have a short circulating half-life (<1 day to ~4 days) in multiple species. This is not surprising given that, unlike IgG, IgA does not bind the neonatal receptor, FcRn, and therefore, cannot undergo endosomal recycling and escape from lysosomal degradation. In addition to the lack of FcRn binding, immature N-linked glycans can also contribute to shorter serum half-lives of recombinant IgA by making them susceptible targets of carbohydrate-specific, endocytic enzymes.
receptors such as the asialoglycoprotein receptor (ASGPR) and mannose receptor. These scavenging receptors, which are highly concentrated in the liver, recognize glycoproteins bearing incompletely sialylated N-linked glycans and remove them from circulation.

To investigate the therapeutic potential of recombinant IgA oligomers, we expressed, purified, and characterized both dimeric and tetrameric IgA molecules. We confirmed their functional properties by measuring binding to antigen, FcαRI and plgR, as well as by demonstrating their transcytosis capability in vitro. However, in vivo we observed that our polymeric IgA antibodies demonstrated fast serum clearance in mice. Other studies have reported rapid clearance of recombinant monomeric IgA molecules, and successful approaches to overcome rapid catabolism include removal of N-linked glycosylation sites through mutagenesis, increasing sialylation of the N-linked glycans by strain engineering, and FcRn-mediated half-life extension strategies. As previous studies have mainly focused on monomeric IgA, we applied two different approaches to overcome rapid serum clearance of polymeric IgA in mice. We engineered an aglycosylated polymeric IgA2m2, as well as a dimeric IgG1-IgA2m1 Fc fusion. Both formats bound antigen and plgR, were able to transcytose in vitro and successfully demonstrated increased serum exposure in mice, with the IgG1-IgA2m1 Fc fusion showing the most improved exposure. To our knowledge, this is the first report of an IgA oligomer that has been engineered to exhibit improved pharmacokinetic (PK) properties in mice.

**Results**

**Analysis of factors affecting IgA oligomer formation**

Recombinant production of monomeric IgA is well understood and can be achieved by coexpression of LC and HC, similar to the production of IgG. In contrast, the assembly of polymeric IgA requires coexpression of LC, HC, and JC, and the resulting IgA oligomeric states are less well characterized. To gain a better understanding of the assembly process of IgA oligomers, we characterized the expression of various human IgA isotypes and allotypes, including IgA1, IgA2m1, IgA2m1.P221R (disulfide stabilized LC-HC pairing) and IgA2m2 (Figure 1A). The murine variable domains of an anti-mouse interleukin-13 (mIL-13) antibody were cloned as chimeras with the human kappa LC and IgA variable domains of an anti-mouse interleukin-13 (mIL-13) anti-

**Large-scale purification and biophysical characterization of IgA monomers and oligomers**

Using insights into IgA oligomer formation gained through small-scale expression, we scaled up monomeric, dimeric and tetrameric IgA using CHO transient expression. We isolated the monomeric and dimeric species of IgA1, IgA2m1, IgA2m1.P221R, and IgA2m2, as well as the tetrameric species of IgA2m2 (Figure 3A). Non-reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of these samples shows predominant bands of molecular weights consistent with the expected masses of ~150 kDa, ~310 kDa, and ~610 kDa for an IgA monomer, dimer, and tetramer, respectively (Figure 3B). These expected masses are based on the amino acid sequence without glycosylation and assume incorporation of one JC per oligomer. Molar masses of the purified oligomeric...
species were also measured by size-exclusion chromatography-multi-angle light scattering (SEC-MALS) and found to be consistent with the expected masses of dimeric and tetrameric IgA (Table S1). The reduced SDS-PAGE analysis of the purified IgA samples confirmed the presence of LC and HC bands for monomers at ~25 kDa and ~55 kDa, respectively, whereas in the oligomeric samples a band for JC just below 25 kDa was also detected (Figure 3B). The identity of the LC, HC, and JC were additionally confirmed by mass spectrometry after reduction and enzymatic deglycosylation (data not shown). Negative stain electron microscopy (EM) was also used to further validate the oligomeric state of the isolated species. Negative stain images of the IgA2m2 dimer (Figure 3C) and tetramer (Figure 3D)
confirm the presence of two or predominantly four IgA molecules, respectively. In the dimer, two IgA molecules are linked tail-to-tail by their Fc domains into an elongated particle, whereas in the tetramer, interactions between four Fc domains give rise to a compact complex of four IgA molecules. Raw images of both samples showed the presence of well-behaved, monodisperse particles (Figure S1).

The anti-mIL-13 IgA monomers, dimers, and tetramer bound murine IL-13 with similar affinity as the anti-mIL-13 IgG1 (Table 1), indicating that regions of the antigen-binding fragment (Fab) are properly folded and functional in the recombinant IgAs. As expected, mouse and human plgR binding was only seen for the IgA oligomers, while both monomeric and oligomeric anti-mIL-13 IgA bound with similar affinity to human FcαRI (Table 1). Due to plgR binding capabilities, all IgA oligomers, but not monomers, were capable of transcytosis in vitro using a Madin-Darby canine kidney (MDCK) cell line ectopically expressing human plgR (Figures 4A and 7E). Additionally, the IgA monomers and oligomers all showed increased stability compared to the anti-mIL-13 human IgG1 and similar or increased stability compared to the

Figure 3. Biophysical and structural characterization of recombinant IgA oligomers. (A) Overlay of analytical size-exclusion chromatograms of purified IgA1, IgA2m1, IgA2m1 P221R, and IgA2m2 monomers, dimers, and tetramer. (B) SDS-PAGE analysis of non-reduced (-DTT) and reduced (+DTT) IgA1, IgA2m1, IgA2m1 P221R, and IgA2m2 monomers (M), dimers (D) and tetramer (T). Heavy chain (HC), light chain (LC), and joining chain (*) are indicated in reduced samples, and the LC-LC dimer of IgA2m1 is indicated with an arrowhead. (C-D, upper panels) Reference-free 2D classes from negative stain electron microscopy for (C) IgA2m2 dimer or (D) IgA2m2 tetramer. (C-D, lower panels) A raw image particle compared to its assigned 2D class is presented next to a model of IgA superimposed on the 2D class with the Fc domains (orange) and Fab fragments (green) highlighted.
IgG1 Fab, as measured by differential scanning fluorimetry (DSF) (Figure 4B).

Pharmacokinetic profiles and biodistribution of recombinant IgA

We then studied the serum concentration-time profiles of our recombinant IgA oligomers, and determined those to be comparable to previously reported data using recombinant monomers (Figure 5A and Table 2).\textsuperscript{5,26,38} Very rapid serum clearance (>200 mL/day/kg) was observed after a single administration of recombinant IgA oligomers. Serum purified human IgA monomer exhibited slower overall clearance, and a serum PK profile generally in line with that previously reported for a highly sialylated IgA monomer.\textsuperscript{26} In addition to characterizing the serum concentration-time profiles, we performed a radiolabeled biodistribution study in mice with dual I-125- and In-111-labeled antibodies. The dual tracer approach allowed us to distinguish between intact antibody prior to lysosomal degradation (I-125) and internalized/catabolized antibody (In-111 minus I-125) as previously described (Figure 5B,C).\textsuperscript{39–41} Briefly, iodine rapidly diffuses out of cells and is cleared after iodinated antibodies undergo lysosomal degradation, while In-111-labeled antibodies show intercellular accumulation of In-111 adducts following lysosomal degradation. Since the IgA antibodies cleared so rapidly compared to the IgG1, direct comparisons of tissue distribution data were difficult since raw tissue values represented both interstitial and vascular concentrations. Therefore, intact antibody distribution data were blood corrected as previously described to represent only interstitial concentrations in tissues.\textsuperscript{42} We observed slight enrichment of intact IgA oligomers compared to IgG1 after 1 h in the liver, stomach, small intestine, large intestine, and skin (all pIgR-expressing tissues\textsuperscript{43,44}), albeit at low levels (Figure 5B). We also saw high levels of IgA degradation in the liver and small intestine, across the formats studied after 1 h of dosing (Figure 5C).

To account for the difference in total blood concentrations observed between the formats, the ratio of individual tissue to plasma concentrations was also described (Figure S2). After 1 day, we saw almost no intact IgA antibody left in the tissues (Figure S3) and detected the greatest catabolism in the liver (Figure S4), although catabolism in the small intestine may not have been detected as In-111 does not accumulate very well at later time points in intestinal cells.\textsuperscript{45} We hypothesized that reducing degradation and eventual clearance mechanisms of IgA could further improve uptake of IgA molecules into mucosal tissue compared with IgG.

Sialylation content on the N-linked glycans of monomeric IgA molecules has been reported to negatively correlate with antibody clearance via specific glycan receptors.\textsuperscript{26} Thus, we analyzed our IgA molecules to determine their overall sialylation content. We

Table 1. Binding affinity of anti-mouse IL-13 IgA and IgG molecules to antigen and receptors.

| K_D (nM)       | Mouse IL-13 | Mouse pIgR | Human pIgR | Human FcαRI |
|----------------|-------------|-------------|------------|-------------|
| IgA1 Monomer   | 0.78 ± 0.05 | NB          | NB         | 425 ± 7     |
| IgA2m1 Monomer | 1.09 ± 0.03 | NB          | NB         | 429 ± 6     |
| IgA2m1 P221R Monomer | 1.16 ± 0.01 | NB | NB | 443 ± 8 |
| IgA2m2 Monomer | 0.70 ± 0.01 | NB          | NB         | 455 ± 5     |
| IgA1 Dimer     | 0.34 ± 0.01 | 2.66 ± 1.45 | 8.80 ± 0.55 | 360 ± 3     |
| IgA2m1 Dimer   | 0.18 ± 0.01 | 2.54 ± 0.15 | 5.05 ± 0.88 | 499 ± 7     |
| IgA2m1 P221R Dimer | 0.84 ± 0.01 | 5.59 ± 0.03 | 15.5 ± 0.10 | 462 ± 4     |
| IgA2m2 Dimer   | 0.81 ± 0.01 | 2.45 ± 0.98 | 13.9 ± 0.10 | 597 ± 5     |
| IgA2m2 Tetramer| 0.97 ± 0.02 | 0.69 ± 0.05 | 1.93 ± 0.02 | 533 ± 5     |
| IgG1           | 0.88 ± 0.05 | NB          | NB         | NB          |

NB: No Binding. All experiments were performed at least n = 3.
Figure 5. Recombinant IgA oligomers demonstrate rapid serum clearance in vivo.
(A) Serum-time concentration profiles of IgA or IgG in mice. The overall serum exposures of Balb/c mice administered with a single 5 mg/kg intravenous (IV) dose of IgA or IgG molecules at 5 min, 15 min, 30 min, 1 h, 1 day, 3 day, 7, and 14 days post dose. All mice were bled retro-orbitally under isoflurane to evaluate serum concentration profile. Human serum IgA monomer was administered at 10 mg/kg and is shown as a dashed line. (B-C) Tissue distribution of IgA or IgG in mice at 1 h post injection. All graphs are means ± SEM for each group with n = 4. %ID/g: Injected Dose/gram. (B) Concentrations of intact antibodies were subtractive blood normalized per tissue, except blood, as $^{125}$I (%ID/g tissue). (C) Concentrations of catabolized antibody values were determined by subtracting the $^{111}$In (%ID/g tissue) from the $^{111}$In (%ID/g tissue).
classified our glycans into categories based on the level of processing, with complex and sialylated being the most desired for our IgA molecules (Figure 6A). Our recombinantly produced dimers of IgA1, IgA2m1, IgA2m1.P221R, IgA2m2 as well as IgA2m2 monomer and tetramer were about 20–50% sialylated (Figure 6B and Table S2). This indicates that the IgA molecules contain incompletely processed glycans that could be recognized by glycan receptors. Additionally, we examined the sialylation content at

Table 2. Pharmacokinetic parameter estimates (Mean) after a 5 mg/kg IV bolus of IgA monomers/oligomers to Balb/C mice.

| Glycans             | C<sub>max</sub> (µg/mL) | AUC<sub>last</sub> (day·µg/mL) | CL (mL/day/kg) | Half Life (Days) |
|---------------------|-------------------------|---------------------------------|----------------|------------------|
| IgA2m2 Monomer      | 76.4                    | 8.70                            | 573            | 0.36             |
| IgA1 Dimer          | 85.2                    | 14.6                            | 341            | 0.31             |
| IgA2m1 Dimer        | 92.5                    | 13.4                            | 373            | 0.26             |
| IgA2m1 P221R Dimer  | 108.0                   | 17.6                            | 285            | 0.27             |
| IgA2m2 Dimer        | 145.0                   | 20.9                            | 239            | 0.31             |
| IgA2m2 Tetramer     | 60.5                    | 6.10                            | 788            | 0.22             |
| Human Serum IgA Monomer<sup>a</sup> | 203.0 | 203.0 | 45.7 | 0.97 |
| IgG1                | 100.0                   | 339.0                           | 14.3           | 2.89             |

AUC<sub>last</sub> = area under the concentration–time curve, last measurable concentration; CL = clearance; C<sub>max</sub> = maximum concentration observed; IV = intravenous;<sup>a</sup>Dosed 10 mg/kg IV bolus to Balb/c mice. Note: As sparse PK analysis was performed for all mouse PK data, data from individual mice per group (n = 3) was pooled and SD was not reported.

Figure 6. Incomplete glycosylation of recombinant IgA molecules. (A) Schematic of N-linked glycan processing. (B) Global N-linked glycan analysis of recombinant IgA and IgA purified from human serum. Glycan analysis was done by mass spectrometric analysis after antibody deglycosylation and subsequent glycan enrichment. While human serum IgA shows greater than 90% sialylation, all recombinantly expressed IgA molecules have less than 60% sialylation. (C) Site-specific N-linked glycan analysis of the IgA2m1 dimer reveals heterogenous glycan composition between the different N-linked glycosylation sites on the IgA2m1 heavy chain (HC) and joining chain (JC).
each site on the IgA2m1 dimer and found that all sites, including the site on the JC, contained incompletely processed glycans, suggesting the incomplete glycan processing does not occur at only one specific site (Figure 6C and Table S3). In stark contrast to our recombinant IgA molecules, IgA purified from human serum has a sialylation content of 95% (Figure 6B and Table S2), and was monomeric as determined by SEC-MALS (data not shown). As serum IgA is known to be predominantly monomeric, it may be enriched for highly sialylated molecules since sialylation content positively correlates with the systemic exposure of antibodies. We hypothesized that this increased sialylation level of the human purified IgA monomer would correlate with decreased serum clearance of the molecule in mice relative to our recombinant IgA monomer. Indeed, we demonstrated this to be true, suggesting that binding to specific glycan receptors in the liver may be an important clearance mechanism for IgA monomer (Figure 5A).

**Improving the pharmacokinetic profile of recombinant IgA through glycosylation site and FcRn engineering**

We took two parallel approaches to reduce the clearance of recombinant polymeric IgA in mice. First, we removed by mutagenesis all five N-linked glycosylation motifs in IgA2m2 and the single site in the JC to produce a molecule without glycans (aglycosylated) (Figure 7A). An aglycosylated IgA polymer will not be recognized by glycan receptors, which allowed us to study PK independent of glycan receptor-mediated clearance mechanisms. For removal of the N-linked glycosylation motifs N-X-S/T in IgA2m2, we mutated the N to A/G/Q or the S/T to A or reverted this to the non-glycosylated IgA1 sequence in the three instances this occurs (Figure 1A). We also mutated the JC residue N49 to A/G/Q or S51 to A (data not shown). We found the individual IgA2m2 mutations N166A, S212P, N263Q, N337T.I338L, T339S, and N459Q, and the N49Q JC mutation to give the highest levels of transient expression (data not shown), but the combination of mutations to remove all five glycosylation sites in IgA2m2 resulted in poor expression and the further addition of the JC chain N49Q mutation completely abolished it (data not shown). In our experience, chimeric immunoglobulins often show lower expression levels in mammalian cells relative to those from a single species. We, therefore, switched the murine anti-mIL13 variable domains to humanized anti-human epidermal growth factor receptor 2 (HER2) variable domains to generate humanized IgAs, but this did not improve transient expression levels (data not shown). Therefore, we produced a CHO targeted integration (TI) stable cell line to increase the expression level of the aglycosylated human anti-HER2 IgA2m2 polymer, which was purified as a mixture of oligomeric species.

Second, we engineered two IgG1-IgA Fc fusions in order to exploit FcRn binding as a way to reduce lysosomal degradation (Figure 7B). Previous studies suggested that this approach rescued IgA monomer serum clearance to levels comparable to IgG1. Initially, we made dimeric and tetrameric versions of the previously reported IgG1-IgA2m1 P221R Fc fusion, but observed that these displayed instability in mouse plasma at 4 days (Figure 7C). The primary truncation product eluted in analytical SEC at a similar time to the full-length anti-HER2 IgG1 (trastuzumab), suggesting that this instability was caused by endoproteases cleaving at the IgG1-IgA2m1 P221R Fc junction. We inspected the amino acid sequence of the junction and identified a stretch of positively charged residues that resembled a furin-like cleavage site (Figure S5). To mitigate proteolytic cleavage, we eliminated these positively charged residues at the junction by removal of the C-terminal K447 of the IgG1 heavy chain and started the IgA2m1 Fc with either P221, the native IgA2m1 residue (instead of the P221R mutation), or C242, which deletes the IgA2m1 hinge (Figure S5). The C242 Fc start was also included because it was the first residue of the IgA1 Fc crystal structure construct, so was presumed to be a stable truncated protein. When the reengineered IgG1ΔK-P221 IgA2m1 Fc and IgG1ΔK-C242 IgA2m1 Fc fusions were produced as dimers, both were found to be stable in mouse plasma for up to 4 days (Figure 7C).

The engineered IgA molecules were further characterized by global glycan content, antigen binding, and receptor binding. The aglycosylated anti-HER2 IgA2m2 polymer indeed had no glycosylation, while the anti-mIL-13 IgG1ΔK-P221 IgA2m1 Fc and IgG1ΔK-C242 IgA2m1 Fc fusions contained only ~20% complex, sialylated glycans (Figure S6 and Table S4). The aglycosylated anti-HER2 IgA2m2 polymer was found to have similar binding affinity to human (h)HER2, murine (m)IgR, and hlgR as the glycosylated IgA2m2 tetramer, while it did not bind the IgA-specific hFc receptor, hFcαRI (Table 3). Interestingly, an IgA2m2 tetramer lacking glycosylation on the IgA2m2 HC, but retaining glycosylation on the J-chain was also unable to bind hFcαRI (data not shown), suggesting that glycosylation of the IgA HC is required for receptor binding. Both the anti-mIL-13 IgG1ΔK-P221 IgA2m1 Fc and IgG1ΔK-C242 IgA2m1 Fc dimers had similar binding affinities to mIL-13, mFcRn, and hFcαRI as the anti-mIL-13 IgG1 (Table 3), as well as similar binding affinities to mplIgR, hlpR, and hFcαRI as an IgA2m1 dimer (Tables 1 and 3). Thus, the IgG1-IgA 2m1 Fc fusions retain the desired attributes of both IgG and polymeric IgA.

We then measured the in vitro plgR-mediated transcytosis and serum concentration-time profiles in mice of both of these newly generated formats. The IgG1-IgA2m1 Fc fusions showed the most marked improvements in the overall IgA serum-exposures in mice (Figure 7D and Table 4), yet the lowest levels of in vitro transcytosis compared to the aglycosylated IgA2m2 polymer, which showed the highest level of in vitro transcytosis (Figure 7E).

**Discussion**

IgA has the potential to extend the therapeutic reach of monoclonal antibodies beyond the current functionalities provided by IgG. In part, this is enabled by the versatility of IgA to form both monomeric and oligomeric species. Substantial progress has recently been made on the recombinant production of monomeric IgA, providing a robust path to isolate well-characterized material with increased sialylation content of the N-linked glycans that has resulted in improved serum clearance. While the strong cytotoxic properties of monomeric IgA are an attractive feature for oncology
indications, polymeric IgA is required to reach targets beyond epithelial barriers via pIgR-mediated transcytosis. Prior to our work, only mixtures of recombinantly made monomer and oligomers were used for *in vivo* experiments studying transcytosis. We established a robust expression and purification route allowing the enrichment of dimeric and tetrameric IgA. In particular, modulating the amount of JC DNA used in the transfection or the glycosylation state of the IgA tail region was able to influence the distribution of oligomeric species. Interestingly, the N-linked glycosylation site on a particular IgG1-IgA2m1 Fc fusion and aglycosylated IgA2m2 show improved serum exposures compared to wild-type IgA in vivo and demonstrate the ability to transcytose in vitro.

**Figure 7.** IgG1-IgA2m1 Fc fusions and aglycosylated IgA2m2 show improved serum exposures compared to wild-type IgA in vivo and demonstrate the ability to transcytose in vitro. (A) Schematic of IgA2m2 tetramer with light chain (LC, yellow), heavy chain (HC, blue) and joining chain (JC, green) with 41 N-linked glycosylation sites (gray) (left) or aglycosylated (right). (B) Schematic of IgG1, IgA2m1 dimer, or IgG1-IgA2m1 Fc dimer formats with LC (yellow), IgG1 HC (orange), IgA2m1 HC (blue), JC (green), and N-linked glycosylation (gray). (C) Analytical SEC of iodinated IgG1-IgA2m1 Fc dimers or tetramer after 0 h (black), 24 h (orange) or 96 h (blue) incubation in mouse plasma. The initial IgG1-L-P221R IgA2m1 Fc tetramer and dimer show degradation similar to the peak of anti-HER2 IgG1 (Trastuzumab) control, whereas the reengineered IgG1ΔK-P221 IgA2m1 Fc or IgG1ΔK-C242 IgA2m1 Fc dimers are stable. (D) Serum-time concentration profiles of IgA or IgG in mice. The overall serum exposures of Balb/c mice administered with a single 30 mg/kg IV dose of IgA molecules. The in-house concentration data of a typical human IgG1 (anti-gD) previously dosed as a single intravenous (IV) injection at 30 mg/kg are shown as a dashed line. All mice were bled retro-orbitally or via cardiac puncture under isoflurane to evaluate serum concentration profile. (E) *In vitro* transcytosis of hIgA in MDCK cells transfected with human pIgR.
the IgA tail is the only site that is extremely conserved among species (Figure S7), suggesting this may be a way to control higher order IgA oligomer formation in vivo. For purification of recombinant IgA, we used Protein L affinity chromatography followed by HPLC-SEC and were readily able to separate dimer from tetramer.

It has been previously demonstrated that increasing the level of sialylation on recombinantly expressed IgA monomer reduces serum clearance by overcoming glycan receptor-mediated catabolism.26 As an alternative strategy, we eliminated the contribution of glycan to the serum clearance of oligomeric IgA and produced fully aglycosylated IgA2m2 polymer. The lack of N-linked glycans did not affect binding to pIgR, as assessed by surface plasmon resonance. Surprisingly, in our in vitro MDCK transcytosis assay, we observed significantly improved transcytosis compared to glycosylated tetramer or dimer. This improvement was not observed in a previous study with human IgA1 dimer that had N-linked glycans removed from the antibody Fc region, but still had the carbohydrate present on the murine J-chain.27 One explanation might be that the lack of glycan completely eliminates the binding to glycan receptors, thus providing unperturbed and more efficient transcytosis via pIgR binding. Interestingly, binding of tetrameric IgA2m2 to FcαRI was dependent on glycosylation, which is in sharp contrast to the lack of effect on binding observed for monomeric IgA2m1 that was engineered to contain a reduced number of glycosylation sites.26 Although we did not evaluate the cytotoxic effects of polymeric IgA in this study, an IgA therapeutic that can transcytose without activating FcαRI is very desirable for inflammatory diseases, as this would prevent pro-inflammatory responses from neutrophil migration.50

Glycosylated IgA oligomers and monomers produced recombinantly in our study cleared rapidly from serum similar to what was previously observed.26,37 Therefore, to better understand the contribution of having glycan, we generated an IgA polymer without glycans (aglycosylated) and directly compared its in vivo PK profile to the glycosylated version. The aglycosylated IgA2m2 polymer showed no appreciable difference in overall mouse serum exposure compared to the glycosylated polymer (<twofold). This suggests that having N-linked glycan plays a minimal role in contributing to the clearance of IgA polymers in mice. While further studies are necessary to understand why IgA oligomer without glycans does not improve serum clearance, we speculate that pIgR-mediated transcytosis or clearance may play a significant role in determining the overall serum concentrations and the fate of polymeric IgA in mice. Indeed, the equilibrium binding affinity of tetrameric IgA to pIgR is at least in the picomolar range allowing for efficient binding to the abundant pIgR receptor, followed by transcytosis. However, a detailed biodistribution study looking at the tissue distribution profile will be needed to better interpret the serum concentration-time profiles of the molecules and the disposition of glycosylated polymeric IgA. One important caveat to studying PK properties and biodistribution of a polymeric IgA molecule in rodents is that expression patterns of pIgR differ between rodents and humans, potentially confounding eventual clinical translation. In particular, high expression of pIgR in the hepatocytes of rodents and rabbits have been associated with biliary clearance mechanisms of polymeric IgA,34 not thought to occur in humans, where plgR expression is found instead in cells of the bile duct.33 Therefore, the exact role that plgR plays in the biodistribution and clearance of a polymeric IgA molecule in mice needs to be separately evaluated.

A separate strategy that we took to avoid accelerated serum clearance and improve exposure was via engineering IgG1-IgA2m1 Fc fusions. The ability to bind FcRn may enable recycling in the endosome, avoiding sorting for lysosomal degradation. Previous reports with monomeric IgG-IgA Fc fusions reported serum clearance comparable to IgG.7,35 In addition, an improvement in PK properties was observed for monomeric IgA that was fused to albumin binding peptides.25 Since we did not observe a large contribution by removing glycans from the IgA polymers, we produced glycosylated fusion proteins. While our dimeric IgG1-IgA2 Fc fusions showed improved overall serum exposures, it was not comparable to IgG as observed for the monomeric IgG-IgA Fc fusions.7,35 We demonstrated by surface plasmon

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### Table 3. Binding affinity of IgG1-IgA2m1 Fc fusion dimers and aglycosylated IgA2m2 polymer to antigens and receptors.

|            | Mouse IL-13 | Human HER2 | Mouse plgR | Human FcRn* pH 6.0 | Mouse FcαRI | Human FcαRI |
|------------|-------------|------------|------------|-------------------|-------------|-------------|
| Anti-HER2 IgA2m2 Tetramer | NB | 0.21 ± 0.08 | 0.35 ± 0.01 | 0.50 ± 0.06 | NB | 1,590 ± 200 |
| Anti-HER2 IgA2m2 Polymer Aglycosylated | NB | 0.27 ± 0.07 | 0.55 ± 0.10 | 0.72 ± 0.06 | NB | NB |
| Anti-IL-13 IgG1K-P221 IgA2m1 Fc Dimer | 1.46 ± 0.33 | NB | 3.32 ± 1.07 | 7.67 ± 0.42 | 6,800 ± 556 | 8,400 ± 294 |
| Anti-IL-13 IgG1K-C242 IgA2m1 Fc Dimer | 1.38 ± 0.15 | NB | 3.32 ± 1.62 | 4.41 ± 1.72 | 7,400 ± 830 | 9,900 ± 838 |
| Anti-IL-13 IgG1 | 1.15 ± 0.17 | NB | NB | 7,800 ± 499 | 9,800 ± 1,081 | NB |
| Human Serum IgA Monomer | NB | NB | NB | NB | 7,800 ± 499 | 9,800 ± 1,081 |

NB: No Binding. *Kd was calculated using steady-state kinetics. All experiments were performed at least n = 3.

### Table 4. Pharmacokinetic parameter estimates (Mean) after a 30 mg/kg IV bolus of IgA oligomers to Balb/C mice.

| Drug | Cmax (µg/mL) | AUClast (day*µg/mL) | CL (mL/day/kg) | Half Life (Days) |
|------|--------------|---------------------|---------------|-----------------|
| Anti-HER2 IgA2m2 Tetramer | 618 | 48.9 | 613 | 0.87 |
| Anti-HER2 IgA2m2 Polymer Aglycosylated | 1064 | 159 | 321 | 0.68 |
| Anti-IL-13 IgG1K-P221 IgA2m1 Fc Dimer | 963 | 176 | 237 | 3.42 |
| Anti-IL-13 IgG1K-C242 IgA2m1 Fc Dimer | 740 | 177 | 192 | 1.94 |
| Anti-IL-13 IgA2m1 Dimer | 1372 | 115 | 431 | 0.41 |

AUClast = area under the concentration–time curve, last measurable concentration; CL = clearance; Cmax = maximum concentration observed; IV = intravenous. Note: As sparse PK analysis was performed for all mouse PK data, data from individual mice per group were pooled and SD was not reported.
resonance that the dimeric IgG1-IgA2m1 Fc fusions can bind FcRn and plgR, albeit having reduced in vitro transcytosis in our MDCK model. While the binding to FcRn extended the terminal half-life (Table 4), IgG1-IgA2m1 Fc dimer interaction with plgR may have provided a clearance mechanism, particularly in the early phase, which resulted in plgR-mediated transcytosis or clearance, contributing to the reduced serum concentrations compared to IgG. More follow up in vivo studies are needed to understand whether the in vitro assays we developed to measure plgR-mediated transcytosis can be used as an accurate predictor of tissue partitioning in vivo and how changes in serum exposures observed with the novel IgA formats correlate with tissue distribution and partitioning. Of note, healthy mice, rhesus macaques, and humans all have a high plgR expression in the intestine, so it will be interesting to see if this correlates to the best tissue delivery.43,44,51

IgA in serum is constituted predominantly of IgA1 monomer secreted from bone marrow cells, while polymeric IgA2 is secreted from plasma cells in the lamina propria at the location of transcytosis.5 The high affinity between polymeric IgA and plgR may naturally lead to fast scavenging of polymeric IgA from circulation, providing effective clearance of harmful antigens from the circulation as IgA-antigen complexes.52 In a therapeutic setting, this may be exploited to restrict drug activity to a defined tissue, something that may be of particular benefit when agonizing cytokine receptors. The fast clearance of polymeric IgA via plgR from serum in mice is further supported by the approximately 20-fold increased IgA concentrations in the serum of plgR-deficient non-obese diabetic (NOD) mice.53 As only polymeric IgA, but not IgA1 monomer, binds plgR for transcytosis, this increased concentration of IgA in serum must be reflective of increased polymeric IgA concentrations. Future biodistribution studies will show if removing clearance through glycan receptors contributes to more selective binding to plgR. Regardless, characterization of an aglycosylated IgA therapeutic molecule is easier compared to a highly glycosylated counterpart. In addition, finding a balance between serum exposure and transcytosis is essential to be able to efficiently deliver a therapeutic molecule to the mucosa. The combination of an aglycosylated IgA molecule that binds FcRn may provide a promising path forward for an IgA therapeutic.

Materials and methods

Plasmid cloning and sequence alignments

Antibody variable domain sequences used include a humanized anti-human HER2 antibody54 and a murine anti-murine IL-13 antibody (Genentech). Protein sequences of human IgA constant heavy chains IgA1, IgA2m1, and IgA2m2, other IgA species, and human J chain were obtained from UniProt (www.uniprot.org) or NCBI (www.ncbi.nlm.nih.gov/protein). P221R is a mutation in IgA2m1 that stabilizes the light chain-heavy chain disulfide as previously reported.54 Genes encoding a fusion of the antibody variable domains to the human light chain and human IgA1, IgA2m1, and IgA2m2 heavy chain constant domains were synthesized and cloned into the mammalian pRK vector.55 Site-directed mutagenesis was used to introduce point mutations. All plasmids were sequence-verified. Sequence alignments were done using GSeqWeb (Genentech) and Excel (Microsoft).

Small-scale antibody expression and purification

Expr293T™ cells were transiently transfected at the 30 mL scale with 15 µg of DNA of both LC and HC for IgA monomers or a total of 30 µg of DNA of varying ratios of LC, HC, and JC for IgA oligomers.56,57 IgAs were affinity-purified in batch with Protein L (GE Healthcare) as all antibodies contained kappa light chains. Proteins were washed with phosphate-buffered saline (PBS), eluted with 50 mM phosphoric acid pH 3.0, and neutralized with 20x PBS pH 11. Protein L eluate was characterized by analytical SEC-HPLC ( Tosoh Bioscience LLC TSKgel SuperSW3000 column, Thermo Scientific Dionex UltiMate 3000 HPLC). A constant volume was loaded on the column, and the area under each curve was quantitated using Chromelonde Chromatography Data System software (Thermo Scientific).

Large-scale antibody expression and purification

IgA, IgG, and IgG-IgA Fc fusions were transiently expressed in CHO DP12 cells as previously described.58 For low expressing clones, TI stable cell lines were generated. IgG and IgG-IgA Fc fusions were affinity-purified using MabSelect Sure (GE Healthcare), followed by SEC with a HiLoad Superdex 200 pg column (GE Healthcare). IgAs were affinity-purified using Capto L (GE Healthcare) followed by SEC. For IgA samples where DNA ratios successfully biased expression to mainly one oligomeric state, a HiLoad Superdex 200 pg column (GE Healthcare) was used for SEC. For IgA samples containing complex mixtures of oligomers, a 3.5 µm, 7.8 mm x 300 mm Xbridge Protein BEH 450 Å SEC column (Waters) was used for better separation of dimer and tetramer peaks.

SEC-MALS

Polymeric IgAs were run over a 3.5 µm, 7.8 mm x 300 mm Xbridge Protein BEH 200 Å SEC column (Waters) and directly injected onto a DAWN HELOS/Optilab T-rEX II (Wyatt) MALS detector for molar mass determination and polydispersity measurement.

Differential scanning fluorimetry

DSF was performed as described previously.59

In vitro transcytosis assay

MDCKII cells (European Collection of Authenticated Cell Cultures, Salisbury, U.K.) cells were transfected with retrovirus containing cDNA coding for the human plgR gene (Retro-X, Takara Bio; OriGene, Rockville, MD). Expression of the plgR gene was confirmed by qRT-PCR and Western Blotting. MDCKII cells expressing plgR were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher, Carlsbad, CA), and 2 µg/ml Puromycin (Takara Bio, Mountain View, CA). For the
transcytosis assay, cells were seeded on 0.4 μm Millicell 24-well cell culture insert (Millipore, Burlington, MA) and cultured for 4 days. On the day of the experiment, the cells were washed twice with FluoroBrite DMEM (Thermo Fisher), and 6 μg of IgA molecules was added to the basolateral compartments. After 24 h of incubation, media from both apical and basolateral compartments were collected for analysis by ELISA.

**Electron microscopy**

Purified anti-mIL-13 IgA2m2 dimer and tetramer samples were first crosslinked by incubating in 0.015% glutaraldehyde (Polysciences, Inc.) for 10 min at room temperature. Once fixed, the samples were diluted using Tris-buffered saline to achieve a concentration of 10 ng/μL. Then, 4 μl of each sample was incubated for 40 s on freshly glow discharged 400 mesh copper grids covered with a thin layer of continuous carbon before being treated with 2% (w/v) uranyl acetate negative stain (Electron Microscopy Sciences). IgA dimers and tetramers were then imaged using a Tecnai Spirit T12 (Thermo Fisher) operating at 120 keV, at a magnification of 25,000x (2.2 Å/pixel). Images were recorded using a Gatan 4096 × 4096 pixel CCD camera under low dose conditions. About 5000 particles for both IgA dimer and tetramers were then selected and extracted using the ezboxer.py software within the EMAN2 package using a 128-pixel particle box size. Reference-free 2D classification, within the RELION image software package was used to generate averaged images of both samples.

**Antibody kinetics**

A 96 × 96 array-based Surface Plasmon Resonance (SPR) imaging system (Carterra USA) was used to analyze the kinetics of purified IgA, IgG-IgA Fc fusions, or IgG. Antibodies were diluted at 10 μg/ml in 10 mM sodium acetate buffer pH 4.5 and using amine coupling, were directly immobilized onto a SPR sensorprism CMD 200M chip (XanTec Bioanalytics, Germany) using a Continuous Flow Microspotter. Antigens diluted in running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% Tween 20, 1 mM EDTA) were injected at various concentrations for 3 minutes and allowed to dissociate for 10 minutes, with regeneration between cycles using 10 mM glycine pH 2.5. Antigens were from R&D Systems (mIL-13, 413-ML-025/CF; mpIgR, 2800-PG-050; hFcaRI, 3939-FA-050), Sino Biologicals (hHER2, 10004-H08H), or Genentech, co-expressed with species specific beta-2 microglobulin (m/hFcRn). The data was processed with the Wasatch kinetic software tool.

**Global N-linked glycan composition analysis (LC-MS analysis)**

Ten μg of each IgA sample were denatured with 8 M guanidine HCl at 1:1 volume ratio and reduced with 100 mM dithiothreitol (DTT) for 10 min at 95°C. Samples were diluted with 100 mM Tris HCl, pH 7.5, to a final concentration of 2 M guanidine HCl, followed by overnight N-linked deglycosylation at 37°C with 2 μl of P0705S PNGase F (New England BioLabs). After deglycosylation, 150 ng of each sample were injected onto an Agilent 1260 Infinity LC system and eluted by an isocratic gradient of 2% to 32% solvent B (solvent A: 99.88% water containing 0.1% formic acid and 0.02% trifluoroacetic acid (TFA); solvent B: 90% acetonitrile containing 9.88% water plus 0.1% formic acid and 0.02% TFA). The HPLC system was coupled via an Agilent G4240A Chip Cube MS system to a G6520B Q-TOF mass spectrometer. The samples were glycan enriched and separated using porous-graphitized carbon columns built within a G4240-64025 mAb-Glyco chip in the Chip Cube MS system. Data acquisition: 1.9 kV spray voltage; 325°C gas temperature; 5 l/min drying gas flow; 160 V fragmentor voltage; 65 V skimmer voltage; 750 V oct 1 RF Vpp voltage; 400 to 3000 m/z scan range; positive polarity; MSI centroid data acquisition using extended dynamic range (2 GHz) instrument mode; 333.3 ms/spectrum and 3243 transients/spectrum. Acquired mass spectral data were searched against a glycan library in the Agilent MassHunter Qualitative Analysis software utilizing a combination of accurate mass with a mass tolerance of 10 ppm and expected retention time for glycan identification. N-linked glycans were label-free quantified relative to all identified N-linked glycans within each sample based on the AUC in the extracted compound chromatogram of each glycan.

**N-linked glycopeptide site mapping analysis (LC-MS/MS analysis)**

Ten μg of IgA was reduced with 10 mM DTT at 37°C for 1 h, alkylated with 10 mM iodoacetamide at room temperature for 20 min, digested with 0.2 μg of trypsin (Promega) and 0.2 μg of chymotrypsin (Thermo Fisher Scientific) separately at 37°C overnight, quenched with 0.1% TFA and subjected clean up with C18 (3M Empore C18 extraction disks) stage-tip (50% acetonitrile, 49.9% water, 0.1% TFA). A quantity of 200 fmol of the sample was injected onto a Waters NanoAcquity UPLC system via an autosampler and separated at 45°C on a Waters Acquity M-Class BEH C18 column (0.1 mm × 100 mm, 1.7 μm resin). A gradient of 2% to 40% solvent B was used for elution (solvent A: 99.9% water, 0.1% formic acid; solvent B 99.9% acetonitrile, 0.1% formic acid).

Separated peptides were analyzed on-line via nanospray ionization into an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) using the following parameters for data acquisition: 60000 resolution; 375–1600 m/z scan range; positive polarity; centroid mode; 1 m/z isolation width with 0.25 activation Q and 10 ms activation time and a collisional voltage of 35. Data were collected in data-dependent mode in which precursor ions being analyzed in the Orbitrap and the top 15 most abundant ions were sent to the ion trap for fragmentation. Acquired mass spectral data were searched against the candidate protein sequence using Protein Metrics Byonic software and analyzed in Protein Metrics Byologic software. Peptide identification for each glycosylation site was manually validated based on a combination of MS2 fragmentation spectra, extracted ion chromatograms (XIC), and retention time. N-linked glycopeptides were label-free quantified relative to the unmodified peptide by the integration of peak areas in the XICs.
Mouse studies

Female Balb/C mice (6–8 weeks old) were obtained by Charles River laboratories. Upon arrival, all mice were maintained in a pathogen-free animal facility under a standard 12 h light/12 h dark cycle at 21°C room temperature with access to food and water ad libitum. All mice received a single intravenous (IV) injection of either IgG or IgA antibody. Blood samples (150–200 µL) were collected via either via retro-orbital sinus or cardiac puncture under isoflurane anesthesia at various times post injection. Samples were collected into serum separator tubes. The blood was allowed to clot at ambient temperature for at least 20 min. Clotted samples were maintained at room temperature until centrifuged, commencing within 1 h of the collection time. Each sample was centrifuged at a relative centrifugal force of 1500–2000 x g for 5 min at 2–8°C. The serum was separated from the blood sample within 20 min after centrifugation and transferred into labeled 2.0-mL polypropylene, conical-bottom microcentrifuge tubes.

Only animals that appeared to be healthy and that were free of obvious abnormalities were used for the study. All animal work performed was reviewed and approved by Genentech’s Institutional Animal Care and Use Committee.

IgA ELISA for transcytosis and pharmacokinetic studies

IgA antibody levels were measured by sandwich ELISA. Wells of 384-microtiter plates were coated overnight at 4°C with 2 µg/ml of goat anti-human Kappa antibody (SouthernBiotech, Cat# 2060-01) in 25 µl of coating buffer (0.05 M sodium carbonate, pH 9.6), followed by blocking with 50 µl of 0.5% bovine serum albumin (BSA) in PBS for 2 h at 37°C. Samples (25 µl) diluted in sample buffer (1x PBS, pH 7.4, 0.5% BSA, 0.35 M NaCl, 0.05% Tween20, 0.25% CHAPS, 5 mM EDTA) were then added to the blocked plates and incubated for 2 h at room temperature. Following incubation, 25 µl of horseradish peroxidase-conjugated goat anti-human IgA (SouthernBiotech, Cat# 2053-05) were added and incubated for 1 h at room temperature. The plates were then incubated with 25 µl of 3,3',5,5'-tetramethylbenzidine (Moss, Cat# TMBE-1000) for 15 min, and the reaction was stopped with 25 µl 1M H₂PO₄. Absorbance was measured at 450 nm with a reduction at 630 nm using a plate reader. Between steps, plates were washed six times with 200 µl of washing buffer (0.05% Tween-20 in PBS). As a reference for quantification, a standard curve was established using serially diluted stock material (20 ng/ml) diluted in 0.3 M ammonium acetate pH 7 at 37°C for 1 h. Purification of all radioimmunoconjugates was achieved using NAP5 columns equilibrated in PBS and confirmed by SEC.

Antibodies were radioiodinated using an indirect iodogen addition method. The radiolabeled proteins were purified using NAP5™ columns (GE Healthcare Life Sciences, cat. 17–0853-01) pre-equilibrated in PBS. Following radioiodination, the labeled antibodies were characterized by SEC-HPLC to compare to the unlabeled antibodies. Samples were injected onto an Agilent 1100 series HPLC (Agilent Technology, Santa Clara, CA) and a Yarra SEC-3000, 3 µm 300 mm x 7.8 mm (Phenomenex, Torrance, CA, cat. 00H-4513-K0) size exclusion columns connected in series and eluted with PBS pH 7.0 at a flow rate of 0.8 mL/min for 20 min. Elution was monitored by absorption at 280 nm and by measuring the radioactivity of the eluted fractions in an in-line Gabi gamma counter (Elysia-Raytest, Germany).

Tissue distribution study design and analysis

The protocol, housing, and anesthesia were approved by the Institutional Animal Care and Use Committees of Genentech Laboratory Animal Resources, in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care regulations.

Female BALB/c mice in a 20–30 g body weight range and 6–7 weeks age range were obtained from Jackson/West (CA). Six groups of 12 mice each were used for this study. To prevent thyroid sequestration of ¹²⁵I 100 µL of 30 mg/mL of sodium iodide was intraperitoneally administered 1 and 24 h prior to dosing. All mice received a single IV injection consisting of a mixture of ¹²⁵I- and ¹¹¹In-labeled antibodies (5 µCi of each) plus the respective unmodified antibodies for a total dose of 5 mg/kg. Cohorts of four mice were bled retro-orbitally under Isoflurane (inhalation to effect) at 5 min, 15 min, 30 min, 1 h, 4 h, 12 h, 1 day, 2 days, and 3 days after injection. At 1 h, 1 day, and 3 days; four animals were euthanized under anesthesia of ketamine (75–8 mg/kg)/xylene (7.5–15 mg/kg) by thoracotomy. The following tissues collected, rinsed in cold PBS, blotted dry, weighed and frozen: brain, liver, lung, kidney, spleen, heart, stomach, small intestine, muscle, skin, fat, large intestine. Sample radioactivity was counted for radioactivity using a 1480 WIZARD™ Gamma Counter in the energy windows for 11,n (245 keV; decay t/2 = 2.8 days) and 11 (35 keV; decay t/2 = 59.4 days) with automatic background and decay correction. Data were analyzed and graphed using GraphPad Prism (version 7.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Mouse plasma stability

Mouse plasma (with anti-coagulant Lithium Heparin) was obtained from BioIVT (Westbury, NY), and a buffer control was made by mixing BSA (Sigma-Aldrich; St. Louis, MO, cat. A2058) with PBS (PBS + 0.5% BSA). Radiolabeled antibodies were mixed into mouse plasma or buffer control at 5 µCi of radiolabeled tracer, and then incubated in an incubator set at 37°C with 5% CO₂. At the set time point of 0, 24, and 96 h of incubation, the
samples were removed from the incubator and stored at −80°C freezer until analysis.

The samples were analyzed by SEC-HPLC method described above with a 1:1 sample dilution in PBS. The result chromatograms were compared between the time points to monitor the changes from the parent peak at time zero.

**Abbreviations**

- ASGPR: asialoglycoprotein receptor
- CHO: Chinese hamster ovary
- dIgA: dimeric IgA
- FcR: Fc receptor
- FcγR: Fc gamma receptor
- FcRn: neonatal Fc receptor
- LC: light chain
- HC: heavy chain
- JC: joining chain
- slgA: secretory IgA
- PK: pharmacokinetic
- plgR: polymeric immunoglobulin receptor

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**Author Contributions**

TNL, SR, JAZ, MLM, and CS devised the conceptual framework of the study. TNL performed in vitro antibody characterization. FF, SV, SL, and AG carried out small-scale protein purification and analysis. JAZ and MLM performed large-scale protein purification and analysis. AE and CC performed EM experiments. DBP performed and analyzed ELISA data from in vitro transcytosis assays and non-radioactive PK experiments. SR coordinated and analyzed all non-radioactive PK studies. DM and AB performed and analyzed radioactivity PK studies. WP and WS analyzed glycan occupancy by mass spectrometry. VY conducted the mouse plasma stability experiments. TNL, MLM, and CC made the figures. TNL, SR, MLM, and CS wrote the manuscript. All authors read and approved the final manuscript.

**Conflict of interest**

All authors are current or former employees of Genentech, Inc., a member of the Roche Group, and may hold stock and options. This work was funded and conducted by Genentech, Inc.

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