

Improved in vivo anti-tumor effects of IgA-Her2 antibodies through half-life extension and serum exposure enhancement by FcRn targeting

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Abbreviations: Ab, antibody; ABD, albumin-binding domain; ADCC, antibody-dependent cell-mediated cytotoxicity; ASGPR, asialoglycoprotein receptor; CPM, counts per minute; FcR, Fc receptor; FcRn, neonatal Fc receptor; HC, heavy chain; HSA, human serum albumin; LC, light chain; PMN, polymorphonuclear cells

Antibody therapy is a validated treatment approach for several malignancies. All currently clinically applied therapeutic antibodies (Abs) are of the IgG isotype. However, not all patients respond to this therapy and relapses can occur. IgA represents an alternative isotype for antibody therapy that engages Fc α RI expressing myeloid effector cells, such as neutrophils and monocytes. IgA Abs have been shown to effectively kill tumor cells both in vitro and in vivo. However, due to the short half-life of IgA Abs in mice, daily injections are required to reach an effect comparable to IgG Abs. The relatively long half-life of IgG Abs and serum albumin arises from their capability of interacting with the neonatal Fc receptor (FcRn). As IgA Abs lack a binding site for FcRn, we generated IgA Abs with the variable regions of the Her2-specific Ab trastuzumab and attached an albumin-binding domain (ABD) to the heavy or light chain (HC_{ABD}/LC_{ABD}) to extend their serum half-life. These modified Abs were able to bind albumin from different species in vitro. Furthermore, tumor cell lysis of IgA-Her2-LC_{ABD} Abs in vitro was similar to unmodified IgA-Her2 Abs. Pharmacokinetic studies in mice revealed that the serum exposure and half-life of the modified IgA-Her2 Abs was extended. In a xenograft mouse model, the modified IgA1 Abs exhibited a slightly, but significantly, improved anti-tumor response compared to the unmodified Ab. In conclusion, empowering IgA Abs with albumin-binding capacity results in in vitro and in vivo functional Abs with an enhanced exposure and prolonged half-life.

Introduction

Her2 (Her2/neu; ErbB2) is a member of the epidermal growth factor receptor family and its over-expression in certain malignancies such as breast cancer is associated with a worse clinical prognosis.¹ Her2 is the target of the marketed IgG1 monoclonal antibodies (mAbs) trastuzumab (Herceptin[®]) and pertuzumab (Perjeta[®]), and several others under (pre-)clinical development.

Antibodies (Abs) can exhibit direct (Fab-mediated) and indirect (Fc-mediated) anti-tumor effects. Trastuzumab has been shown to induce cytostasis upon binding of the Fab arms to Her2

by inhibiting Her2 downstream signaling.² The dominant Fc-mediated effector mechanism employed by IgG1 Abs, including trastuzumab, is the engagement of Fc γ receptors (Fc γ R) expressed on immune effector cells such as natural killer (NK) cells, macrophages and neutrophils. In vitro studies suggested that NK cells have the highest cytotoxic capacity with human IgG1 Abs.^{3,4}

Despite demonstrated clinical effects, IgG mAb therapy (often in conjunction with other (chemo)therapeutics) rarely results in a complete cure. Partial responses are attributed to several factors: (a) exhaustion of cellular effector mechanisms,⁵ (b) interaction with the non-signaling Fc γ RIIIb,⁶ (c) co-engagement of activating

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Fc γ R and the inhibitory Fc γ RIIb on monocytes resulting in inhibitory signaling⁷ and (d) polymorphisms in Fc γ R such as 131 H/R in Fc γ RIIa and 158 V/F in Fc γ RIIIa, which have been associated with worse clinical outcome upon IgG1 mAb treatment.^{8,9}

Due to the limitations of IgG anti-tumor mAbs, IgA Abs have been investigated as an alternative isotype. IgA in the polymeric form is predominant at the mucosal sites, whereas the monomeric form is mainly found in serum. In humans, monomeric IgA exists as 2 subclasses: IgA1 and IgA2. For IgA2, 3 allotypes have been described: IgA2(m1), IgA2(m2) and IgA2(n). The major structural difference between IgA1 and IgA2 lies within the hinge region, which is 13 amino acids longer in IgA1. The serine/proline/threonine rich hinge region of IgA1 Abs makes them more susceptible to proteolytic cleavage by IgA1 proteases produced by pathogenic bacteria.¹⁰ Furthermore, the glycosylation pattern differs between both subclasses; 5 *O*-linked glycans and 2 *N*-linked glycans are attached to the heavy chain of IgA1 Abs, whereas IgA2 Abs carry 4–5 *N*-linked glycans, but no *O*-glycans.

IgA Abs interact with innate immune effector cells, such as polymorphonuclear cells (PMNs), monocytes, macrophages, granulocytes and Kupffer cells, by binding to the myeloid Fc α RI (CD89) expressed on their surface. For Fc α RI, no polymorphisms affecting IgA binding have been identified yet. Activation of immune effector cells via Fc α RI binding results in destruction of invading pathogens by processes such as oxidative burst, cytokine release and phagocytosis.¹¹ PMNs are the most abundant effector cells in human blood, and they have been demonstrated to readily infiltrate tumor tissue.¹² It has recently been shown that IgA Abs targeting EGFR induce cytotoxicity in vitro with human leukocytes, in particular with isolated PMNs.^{13,14} Additionally, human monocyte-mediated cytotoxicity by IgA Abs is comparable to IgG1 Abs.¹³ An IgG/IgA hybrid Ab molecule, carrying an Fc γ R and Fc α RI recognition site, had superior phagocytic capacity with human macrophages compared to IgG1 Abs.¹⁵ In vivo efficacy of IgA anti-tumor Abs has been demonstrated using human Fc α RI transgenic (Tg) mice.^{14,16} However, to reach an effective Ab concentration in vivo in a long-term tumor model, daily injections of IgA Abs were required to compensate for the short serum half-life of human IgA in mice (~15 hours).¹⁴

The short serum half-life of IgA Abs is partially caused by the rapid clearance via the asialoglycoprotein receptor (ASGPR) recognizing terminal galactose residues.¹⁷ Blockage of the ASGPR with a specific ligand,¹⁴ improved terminal sialylation of IgA¹⁸ and engineered IgA Abs with fewer *N*-glycosylation sites (data not shown) resulted in an extension of the in vivo half-life.

FcRn is an important receptor for placental transport of maternal IgG to the fetus,^{19,20} enhanced phagocytosis,²¹ and recycling or transcytosis of IgG.^{22–25} This pH-dependent recycling pathway mediates the serum half-life of human IgG1 Abs (up to 21 d in humans,²⁶ ~9 d in mice^{27,28}) and albumin (19 d in humans,²⁹ ~2 d in mice).^{30,31} However, it is not engaged by IgA Abs because they lack the FcRn binding site. FcRn can be indirectly targeted, e.g., by fusion of an albumin-binding domain (ABD) to the protein of interest. ABDs are small, 3-helical protein subunits expressed by various gram-positive bacteria. The

most extensively studied ABD is derived from protein G of certain *Streptococcus* strains. Mutational studies resulted in the identification of an ABD with femtomolar affinity for human serum albumin (HSA).³² ABD affinity, rather than valency, contributed to the extension of the in vivo half-life of the investigated protein.³³ To our knowledge, this approach has only been used for small molecules such as single-chain diabodies³⁴ and affibody molecules.^{35,36}

Here, we aimed at targeting the FcRn recycling pathway to extend the serum half-life of full size IgA-Her2 Abs by attaching an ABD. The ABD was attached to either the heavy chain (HC_{ABD}) or light chain (LC_{ABD}) of the IgA Ab to assess whether the attachment site has an effect on Fc α RI binding and subsequent Ab efficacy. We showed that ABD modified IgA Abs exhibit an increased in vivo half-life and significantly enhanced serum exposure. In vitro conjugate formation and cytotoxicity remained unaffected for the IgA-LC_{ABD} Abs upon albumin binding. However, albumin binding by IgA-HC_{ABD} Abs impaired the Fc-mediated engagement of Fc α RI expressing cells. Finally, in a xenograft tumor model we showed that modified IgA1 Abs performed slightly, but significantly, better than unmodified IgA1 Abs.

Results

Generation of albumin binding IgA-Her2 antibodies

We generated Her2-targeting IgA1 and IgA2 Abs using the variable region of trastuzumab. IgA1-Her2 and IgA2-Her2 Abs mediated in vitro antibody-dependent cell-mediated cytotoxicity (ADCC) with both human PMNs and mouse effector cells and exerted anti-tumor effects in a xenograft tumor model (Fig. S1). However, in line with previous studies, serum half-life of IgA Abs was shorter than of trastuzumab, thus requiring daily injections to compensate for this. Therefore, we next generated IgA-Her2 Abs with an ABD to extend serum half-life by engaging the FcRn recycling pathway (Fig. 1A). This was done by attaching a 5 kDa ABD derived from streptococcal protein G at the C-terminal end of either the HC or the LC (Fig. 1B). For the attachment to the HC, the tailpiece in the C α 4 domain was substituted with the ABD. All Abs were produced in HEK293F cells and subsequently purified by anti-kappa affinity chromatography and size-exclusion chromatography (SEC; data not shown). Our final panel consisted of 2 unmodified (IgA1 and IgA2) and 4 modified (IgA1-HC_{ABD}, IgA2-HC_{ABD}, IgA1-LC_{ABD} and IgA2-LC_{ABD}) IgA-Her2 Abs. Their purity and integrity was assessed by SDS-PAGE (Fig. S2).

Glycosylation pattern analysis of albumin binding IgA-Her2 antibodies

Matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-mass spectrometry (MS) of enzymatically released and sialic acid-derivatized *N*-glycans was used to determine the main glycosylation properties of all used IgA antibodies³⁷ (Fig. S3 and Table S2). In general, both IgA1 and IgA2 contain *N*-glycan structures ranging from diantennary to tetraantennary. Sialylation is mainly of the α 2,3-linked variant, and ~30% of the

galactoses are occupied by these sialic acids (Table S1). Attachment of the ABD to the LC shifts IgA1 toward an overall higher degree of sialylation, while IgA2 remains relatively the same. Attaching the ABD to the HC, on the contrary, leads in both IgAs to the loss of most of the larger structures (triantennary and tetraantennary species). Fucosylation is found almost exclusively at the core of the *N*-glycans, as determined by MS/MS, and is more abundant in the IgA2 samples than in the IgA1 samples (and in IgA1 mostly on the triantennary and tetraantennary structures).

In vitro characterization of albumin binding IgA-Her2 antibodies

First, we analyzed our panel of unmodified and modified Abs for their capability to bind to immobilized albumin from different species (Fig. 2A). As expected, only modified IgA Abs recognized human, monkey, mouse and rat albumin, whereas bovine serum albumin (BSA) remained unbound (data not shown). The attached ABD had a high binding capacity to albumin derived from mouse, rat and monkey at serum dilutions as high as 1:100,000, corresponding to ~270–330 ng/mL albumin in rodents³⁸ and 400–500 ng/mL albumin in monkeys.^{39,40} However, the best binding capacity was observed for HSA as the detection signal only started to decrease at concentrations lower than 40 ng/mL, which is approximately 1×10^6 times less than found in human serum.

Next, we assessed albumin binding capacity in solution by pre-incubating the panel of IgA Abs in the absence or presence of albumin at various molar ratios and subsequent analysis on HP-SEC (Fig. 2B). Albumin pre-incubation resulted in a shift of the Ab peak (from retention time ~6 min to ~5.5 min) for only the modified IgA Abs. This indicated that both the HC_{ABD} and LC_{ABD} IgA Abs had bound albumin in solution. When antibody and albumin were mixed in a 1:1 molar ratio, a single peak corresponding to a 1:1 complex was observed. By contrast, with a 1:2 ratio of antibody to albumin, a second

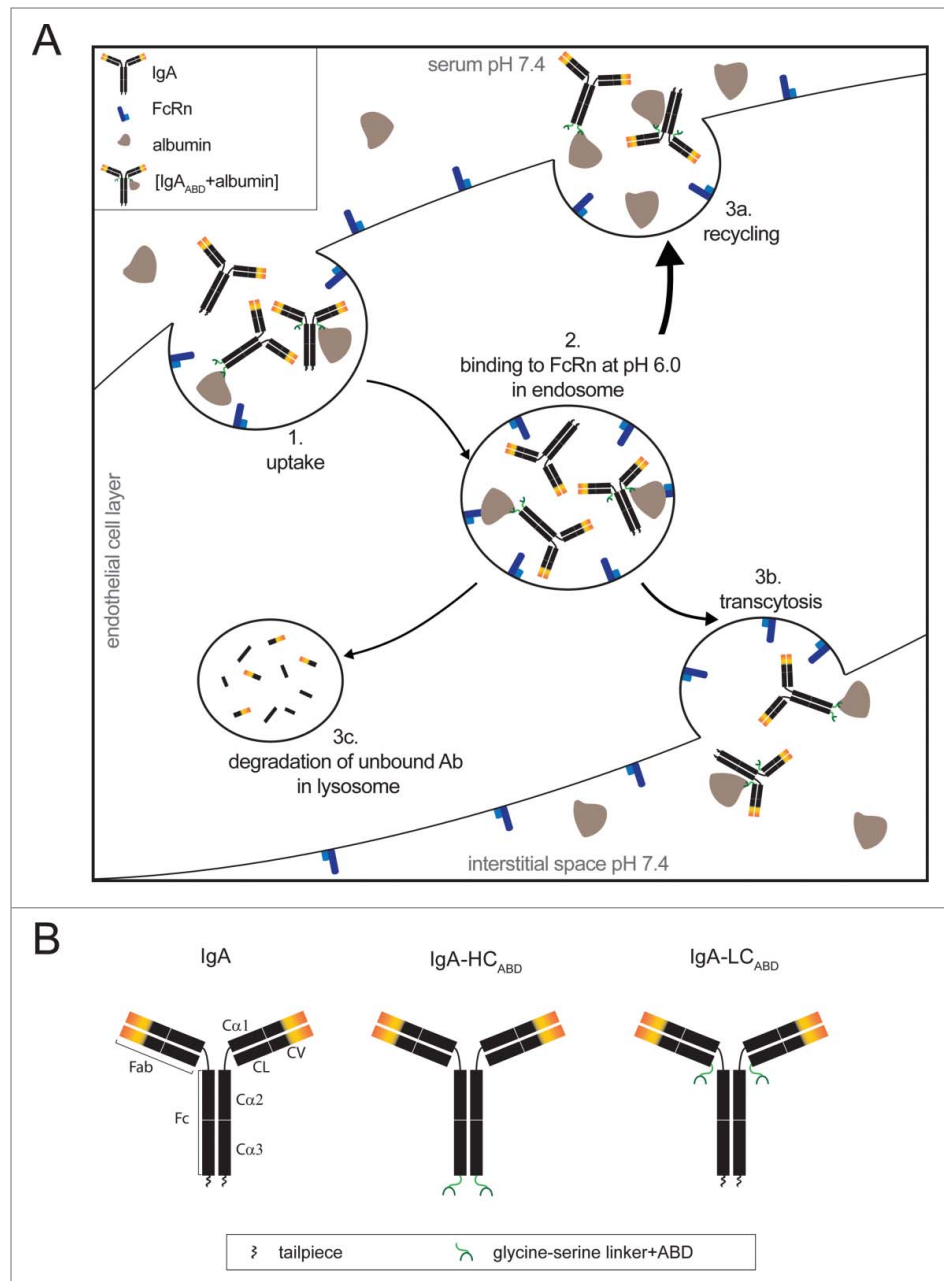


Figure 1. (A) Schematic illustration of neonatal Fc receptor mediated half-life extension. Abs and albumin are internalized by endothelial cells through pinocytosis (1). Upon the decrease of the pH from 7.4 to 6.0 in the endosome, albumin-bound IgA (IgA_{ABD}+albumin) associates with membrane-bound FcRn, whereas unmodified IgA Abs remain unbound (2). IgA_{ABD}+albumin complexes subsequently recycle back into the serum (3a) or undergo transcytosis and are released into the interstitial space (3b). Unmodified IgA Abs are degraded in the lysosome (3c). **(B) Schematic illustration of design of albumin binding IgA-Her2 antibodies.**

peak next to the antibody-albumin complex peak was detected, which corresponds to free albumin. This suggests that only one of the 2 ABDs present in the modified IgA Abs binds albumin.

Additionally, to study whether albumin binding interferes with the simultaneous recognition of Her2 and the FcαRI, we set up a conjugation assay with SKBR3 cells and Ba/F3-FcαRI-eYFP

cells (Fig. 2C). IgA1-based Abs induced higher percentages of conjugates than IgA2-based Abs. For IgA-HC_{ABD} Abs of both subclasses, conjugate formation was impaired in the presence of albumin.

ABD attachment increases the serum exposure and half-life of IgA-Her2 antibodies

Human IgA Abs do not bind to FcRn, and therefore have a roughly 14 times shorter serum half-life compared to human IgG in mice. First, to assess whether the attachment of an ABD to a full-size IgA Ab results in an extended serum half-life, we injected 200 µg Ab intravenously into SCID mice and followed the serum Ab concentrations over time (Fig. 3A and B). The overall serum exposure (Table 1) of IgA1-HC_{ABD} and IgA1-LC_{ABD} was increased to a similar extent (4.7- and 5.2-fold, respectively) compared to unmodified IgA1. IgA2-LC_{ABD} Abs showed a 4.8-fold higher serum exposure compared to unmodified IgA2, which was further pronounced for IgA2-HC_{ABD} Abs (9.6-fold). Whereas the best terminal half-life ($t_{1/2}$) extension was reached with the LC-modified IgA Abs, serum exposure was the least improved for the IgA2-LC_{ABD} Ab (Table 1).

Next, we performed a multi-compartment pharmacokinetics (PK) study to determine distribution and clearance properties of the Abs, which were required for the set-up of our in vivo tumor models. Mice were injected with 200 µg Ab

subcutaneously and the Ab concentrations in both the serum (Fig. 3C and D) and peritoneal cavity (Fig. 3E and F) were monitored. All IgA Abs reached their maximum serum levels within 24 h, with serum levels for the unmodified IgA Abs remaining lower than for the modified IgA Abs. In this experimental setting, both IgA-LC_{ABD} Abs reached a comparable increase in serum exposure (IgA-LC_{ABD} 5.3-fold; IgA2-LC_{ABD} 4.5-fold). The IgA-HC_{ABD} Abs also reached a similar serum exposure, but compared to the matched unmodified subclass, the serum exposure of IgA2-HC_{ABD} Abs was increased 17.9-fold compared to 10.1-fold for IgA1-HC_{ABD} Abs. $t_{1/2}$ was enhanced for all modified IgA Abs to a comparable extend after subcutaneous injection (Table 1). All Abs, except IgA2, were detectable in the peritoneal cavity until at least day 8, although at levels 2 orders of magnitude lower than in serum (Fig. 3E and F). IgA1 Abs were transported to the peritoneal cavity more efficiently compared to IgA2 Abs. Like in the serum, concentrations of the IgA-HC_{ABD} Abs in the peritoneal cavity were the highest, probably reflecting the higher serum exposure of these Abs.

Tumor cell killing ex vivo is comparable between unmodified and IgA-LC_{ABD} antibodies

IgA Abs induce cytotoxicity by the interaction of their Fc tail with Fc α RI expressed on effector cells. To determine if

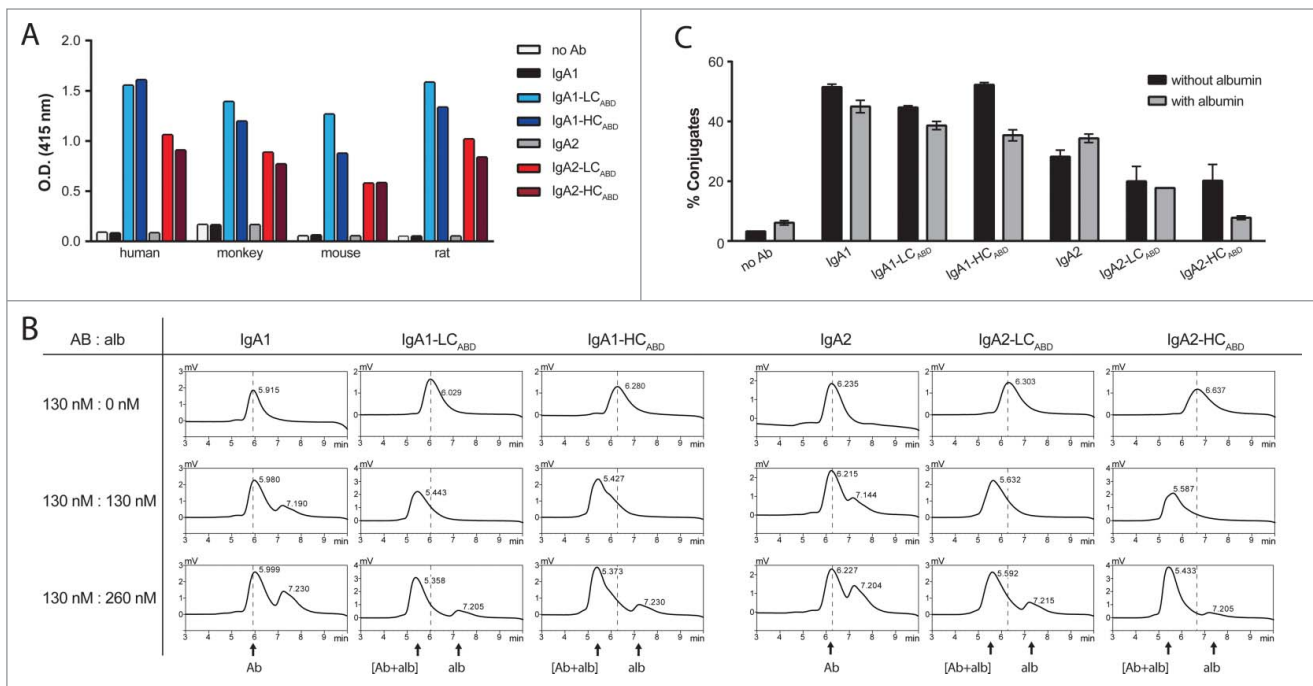


Figure 2. ABD modified IgAHer2 antibodies bind to albumin and simultaneously bind Her2 and Fc α RI. (A) Solid phase binding of unmodified IgA Abs and HC_{ABD} and LC_{ABD} modified IgA Abs (100 ng/mL) to albumin derived from different species (HSA: 40 ng/mL; mouse, monkey and rat serum: 100,000x diluted) measured by ELISA. (B) Antibody-albumin complex formation in solution was determined by pre-incubating unmodified and HC_{ABD} and LC_{ABD} modified IgA Abs (130 nM) in the absence or presence of human albumin (130 nM and 260 nM) and subsequent analyses by HP-SEC. Retention time of Abs in the absence of albumin is indicated by a dotted line. (C) Conjugate formation between Her2 (SKBR3) and Fc α RI (Ba/F3-Fc α RI-eYFP) expressing cells upon simultaneous binding to Abs (20 µg/mL) pre-incubated without (black bar) and with (gray bar) 1 mg/mL HSA (mean \pm SEM).

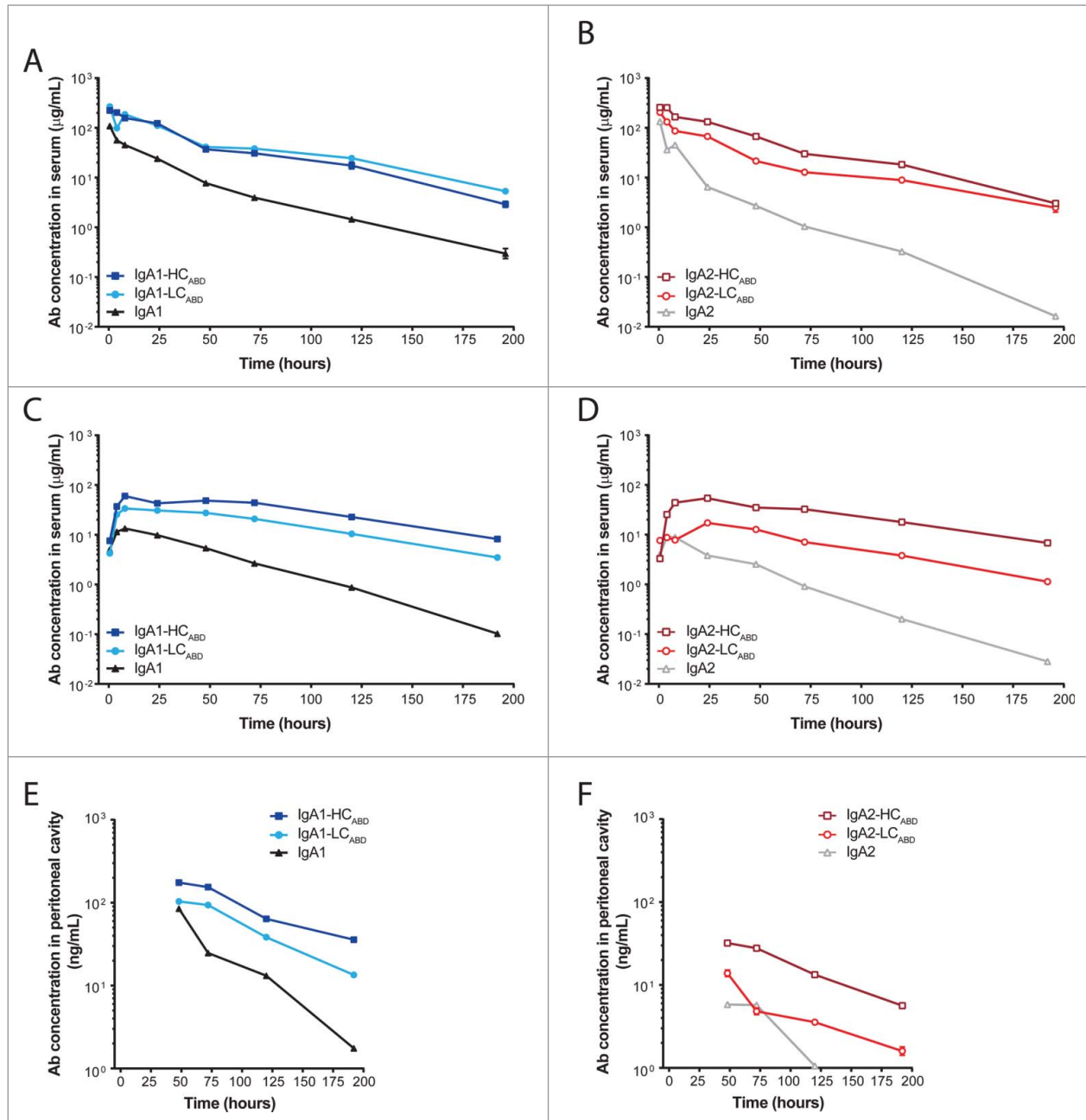


Figure 3. Pharmacokinetic profiles of un-/modified IgA-Her2 antibodies. SCID mice were injected intravenously (A, B) or subcutaneously (C–F) with 200 μg un-/modified IgA1 (A, C, E) and IgA2 (B, D, F) Abs. Ab concentrations in the serum (A–D) and peritoneal cavity (E, F) were determined by ELISA (mean \pm SEM).

Table 1. Exposure (AUC) and terminal half-life ($t_{1/2}$) of un-/modified IgA Abs.

| Antibody | AUC ($\text{h}^* \mu\text{g/mL}$) | | $t_{1/2}$ (h) | |
|------------------------|-------------------------------------|------|---------------|------|
| | i.v. | s.c. | i.v. | s.c. |
| IgA1 | 1741 | 640 | 28.6 | 25.7 |
| IgA1-LC _{ABD} | 9003 | 3371 | 40.1 | 46.5 |
| IgA1-HC _{ABD} | 8201 | 6452 | 32.2 | 49.5 |
| IgA2 | 997 | 299 | 20.4 | 24.0 |
| IgA2-LC _{ABD} | 4786 | 1340 | 49.6 | 45.0 |
| IgA2-HC _{ABD} | 9572 | 5367 | 32.9 | 53.2 |

albumin binding to modified IgA Abs influences tumor cell lysis, we performed a human PMN ADCC assay (Fig. 4). We pre-incubated the panel of IgA Abs either in the absence or presence of albumin before adding them to the freshly isolated PMNs and SKBR3 cells. In the absence of albumin, all IgA1 and IgA2 Abs induced similar tumor cell lysis (Table 2). The unmodified and IgA-LC_{ABD} Abs remained unaffected in their lytic capacity after pre-incubation with albumin; however, IgA-HC_{ABD} Abs induced a lower maximal tumor cell lysis.

Enhanced serum exposure and half-life by modified IgA antibodies improve anti-tumor effect in vivo

To study whether the improved half-life of the modified IgA Abs results in better tumor cell killing in a therapeutic setting in vivo, we tested the Abs in a multi-compartment tumor model. First, we performed a pilot experiment with A431-luc2-Her2 cells injected intraperitoneally and after 7 d treated tumor-bearing SCID mice once with various concentrations of trastuzumab injected subcutaneously. From this we considered 10 µg Ab to be a suboptimal dose for trastuzumab (Fig. S4A and B). The final treatment regimen was determined by modeling IgA2-HC_{ABD}, which had the best PK values (Fig. 3), to find optimal exposure time without accumulation upon repeated treatment (Fig. S4C). Eventually, we injected wild type (WT) and FcαRI Tg tumor-bearing SCID mice with 50 µg IgA Abs subcutaneously once a week, for 5 weeks, starting one week after tumor inoculation. Tumor outgrowth was monitored by serial bioluminescent imaging (BLI). No Fab-mediated anti-tumor effect could be observed, as the tumor outgrowth in Ab treated WT mice was comparable to

the phosphate-buffered saline (PBS)-treated control group (Fig. 5A and B). In Tg mice, IgA1 treatment (Fig. 5C, E and F) resulted in an anti-tumor response, which was similarly improved by both modified IgA1 Abs. The increase in tumor volume over time (from day 6 to day 56) of the unmodified IgA1 Ab was significantly higher than for the modified IgA1 Abs (384 vs. 167 CPM/cm² per day for IgA1-HC_{ABD}, P = 0.0057; 384 vs. 188 CPM/cm² per day for IgA1-LC_{ABD}, P = 0.0106). For IgA2, the effect of the ABD attachment was less pronounced. As shown in Figure 5D-F, only the IgA2-LC_{ABD} Ab appeared effective in therapy until a week after the last treatment (day 41). Until day 41, a significantly lower increase in tumor volume over time was achieved with IgA2-LC_{ABD} Abs compared to the unmodified IgA2 Ab (460 vs. 1124 CPM/cm² per day, P = 0.0003).

To investigate whether the differences between the IgA1 and IgA2 modified Abs could be explained by differences in their transport to the tumor site, we examined Ab distribution into the peritoneal cavity on day 36, one day after the last treatment. As shown in Figure 5G, IgA1 Abs reached the

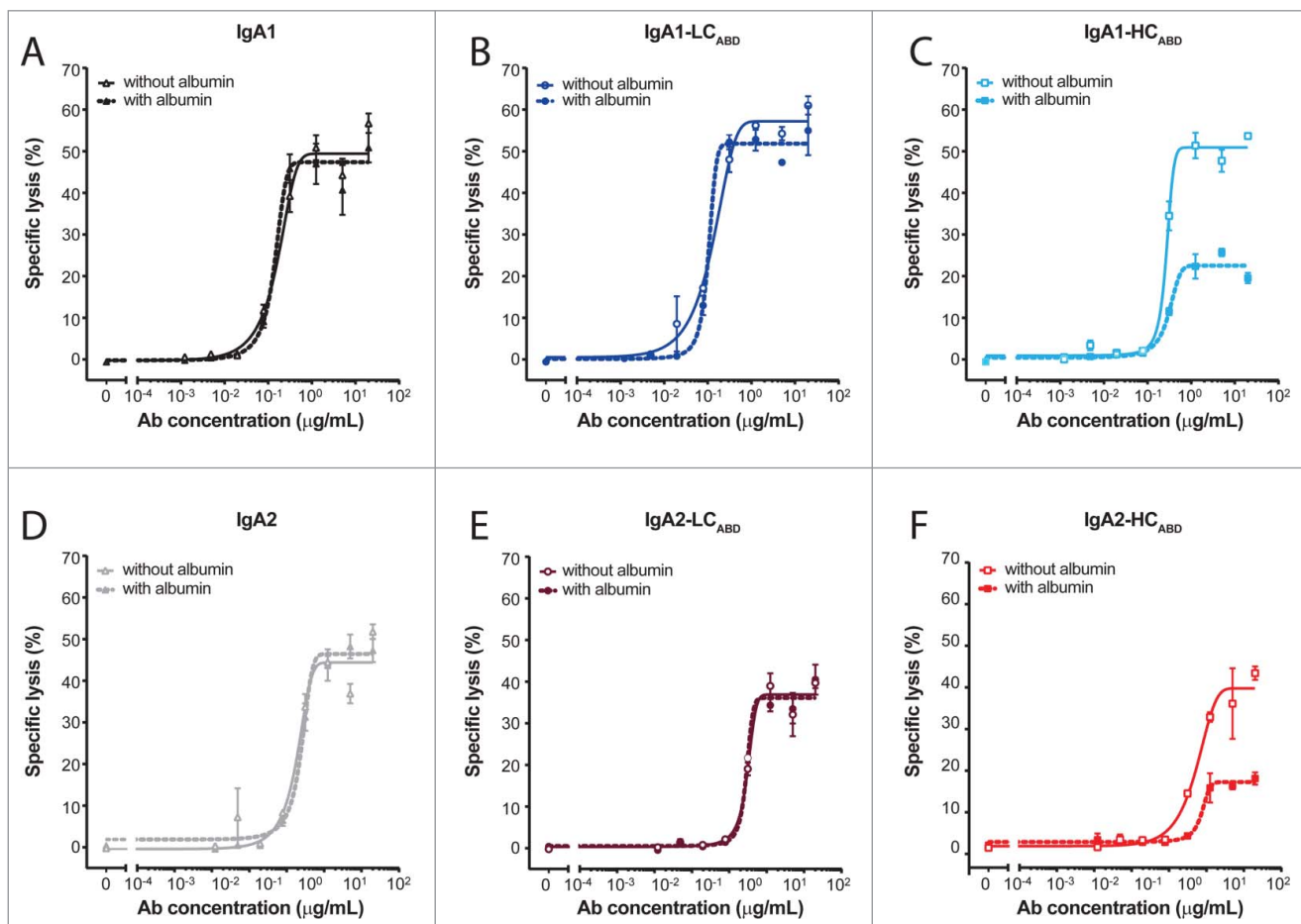


Figure 4. Cytotoxic potential of un-/modified IgA-Her2 antibodies. Specific lysis of SKBR3 cells by IgA (A, D), IgA-LC_{ABD} (B, E) and IgA-HC_{ABD} (C, F) Abs determined in 4 h ADCC assays with human PMNs (E:T = 40:1) (mean ± SEM). Un-/modified IgA1 Abs (A–C) or IgA2 Abs (D–F) were pre-incubated without (solid line) or with (dotted line) human albumin. Results are representative of 4 separate assays.

Table 2. Maximal lytic capacity of un-/modified IgA Abs in human PMN ADCC assay.

| | top plateau; 95% CI [lower-higher] | |
|------------------------|------------------------------------|--------------|
| | without albumin | with albumin |
| IgA1 | 49% [45–54%] | 47% [44–50%] |
| IgA1-LC _{ABD} | 57% [54–60%] | 52% [49–54%] |
| IgA1-HC _{ABD} | 51% [49–53%] | 23% [21–24%] |
| IgA2 | 44% [41–47%] | 46% [43–50%] |
| IgA2-LC _{ABD} | 40% [34–40%] | 36% [34–38%] |
| IgA2-HC _{ABD} | 38% [34–42%] | 16% [14–18%] |

peritoneal cavity more effectively, confirming what we have seen before (Fig. 3E and F). Ab levels found in the serum reflected Ab concentrations in the peritoneal cavity. Furthermore, for each Ab, serum Ab titers one day after the first injection (day 8) and last injection (day 36) were comparable, demonstrating that the dosing regimen indeed did not cause accumulation of the Abs neither in serum nor in the peritoneal cavity (Fig. S5A and B). Both unmodified IgA Abs were mostly cleared or undetectable in serum one day before the last injection (day 34) (Fig. S5C). However, the modified Abs were still detectable in the serum at this time point.

In conclusion, ABD addition to full-size IgA Abs significantly increased their serum exposure. This resulted in an increased Ab concentration in the peritoneal cavity, and provided modified IgA1 Abs and IgA2-LC_{ABD} Abs with significantly better therapeutic potential than unmodified parental Abs of the same subclass.

Discussion

Monomeric IgA Abs represent promising therapeutic agents for the treatment of cancer.^{13,14,16,41} However, in vivo, daily injections are required to compensate for their short half-life in mice.¹⁴ In this study, we generated and evaluated albumin binding IgA-Her2 Abs regarding in vitro and in vivo functional activity and half-life extension in mice.

ABD modified IgA Abs bound to albumin derived from different species immobilized on a surface and to HSA in solution. The attachment of the ABD had no effect on the lytic capacity of human PMNs in the absence of albumin. However, ABD attachment to the HC resulted in an impaired ADCC efficacy in vitro after pre-incubation of the Abs with albumin. This is possibly due to interference of the bound albumin with Fc α RI binding, which occurs at the interface of C α 2 and C α 3. As both IgA subclasses were equally affected, neither their structural differences nor the additional *N*-linked glycosylation sites in IgA2 are likely to play a role. This is in line with previously published literature stating that Fc α RI binding by IgA remains unaffected by the antibodies' glycosylation state.⁴² The partial reduction of the ADCC capacity of IgA-HC_{ABD} Abs compared to the unmodified IgA Abs suggests that the binding of one albumin, as

determined by HP-SEC, does not interfere with both Fc α RI recognition sites on the IgA Fc tail.

In our PK studies, all IgA Abs carrying an ABD exhibited an augmented serum exposure compared to the unmodified IgA Abs, suggesting that albumin binding and subsequent recycling by FcRn occurred in vivo. The attachment of an ABD to small molecules was demonstrated previously to result in the extension of their half-lives in mice. The involvement of FcRn mediated recycling was confirmed by performing PK studies in FcRn knockout mice.⁴³ However, the reduced renal clearance due to their increased molecular weight (>60 kDa) contributed further to the extension of the half-life, a mechanism that is irrelevant for our full-size IgA Abs.

So far, few PK studies directly comparing IgA1 and IgA2 Abs have been performed in mice. We detected less IgA2 than IgA1 in the serum of mice after intravenous and subcutaneous injections. However, glycosylation analysis of the antibodies has shown that sialylation is higher for IgA2 than for IgA1. In addition, antennarity is higher for IgA1 than for IgA2, leading to more exposed galactoses. This is counter-intuitive to literature, as it has been shown that clearance of plasma IgA1 Abs is less affected by the clearance via the ASGPR than of IgA2 Abs,⁴⁴ possibly due to the higher degree of terminal sialylation of *N*-linked glycans of monomeric serum IgA1.^{45–49} Indeed, when the glycosylation pattern of IgA2 Abs was modified, either by increasing the degree of terminal sialylation,¹⁸ or by reducing the number of glycosylation sites (data not shown), an extended in vivo half-life in mice was achieved. However, comparing these half-life data obtained in different studies is complicated since distinct mechanisms were altered and different recombinant protein production systems were used. It is known that expression cell lines differ in their expression of sugar-modifying enzymes such as sialyltransferases.⁵⁰ Furthermore, we found that IgA2-HC_{ABD} Abs had a higher serum exposure compared to IgA2 and IgA2-LC_{ABD} Abs, possibly due to the replacement of the tailpiece with the ABD, resulting in the removal of an *N*-linked glycosylation site at Asn 459 and subsequent loss of most tri- and tetraantennary glycan species. Addition of the ABD causes albumin binding but also glycosylation changes, and the contribution of either property to the different kinetics remains to be elucidated.

Our modified IgA Abs did not reach the same half-life and exposure as IgG1 Abs. This, however, was not unexpected as the half-life of human IgG1 in mice is suggested to be overestimated due to a 10^{–51} to 85^{–52} fold higher affinity of murine FcRn for human IgG1 compared to murine IgG1. Using values from literature, we estimated a roughly 14-fold difference in half-life of human IgG1 compared to human IgA in mice. However, albumin in mice has a half-life of ~2 days. With our LC-modified IgA Abs, we reached a similar terminal half-life. Thus, to enhance the in vivo half-life of human IgA Abs in mice to facilitate pre-clinical testing, a combination of both strategies is likely needed: (a) glyco-engineering toward full sialylation to avoid quick hepatic clearance, and (b) FcRn engagement to guarantee successful recycling.

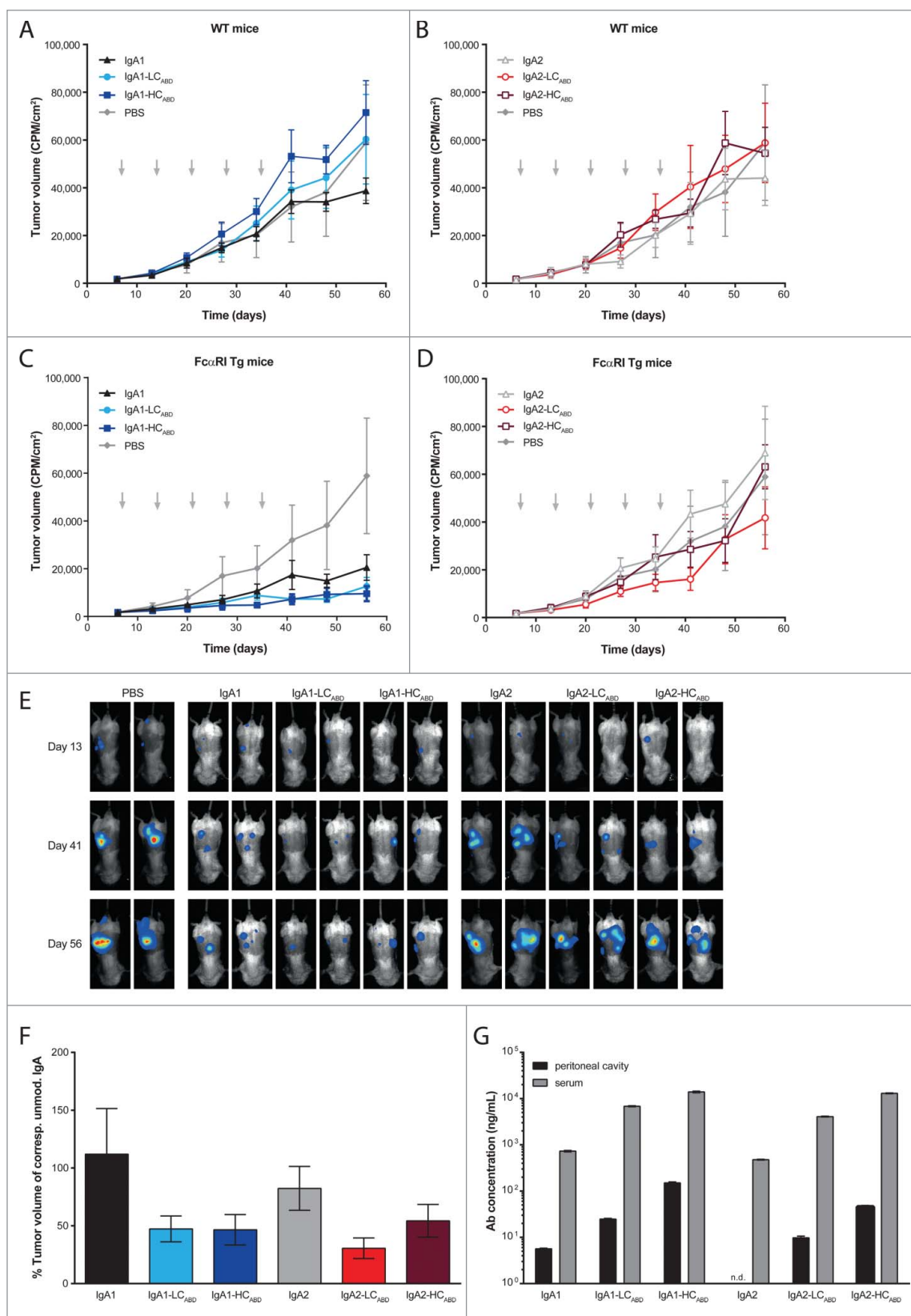


Figure 5. (For figure legend, see next page 95)

Mechanisms contributing to the clearance of IgA Abs are similar between humans and mice. Based on studies in non-human primates, the catabolism of IgA Abs in humans has been suggested to be predominantly mediated by hepatic uptake via the ASGPR. In humans, a 3.5-fold difference in serum half-life of human monomeric IgA1 and IgA2 Abs (5–9 days and 4–5 d⁵³) compared to IgG1 Abs (21 days²⁶) was found. To extrapolate our findings to the human context, we anticipate that mainly glyco-engineering of IgA Abs is required to achieve an effective half-life. However, next to recycling and phagocytosis, the FcRn plays a role in transcytosis of IgG over epithelial cell layers. Thus, for improved transcytosis of IgA Abs to reach tumor tissues, targeting FcRn might be helpful. However, the binding of an albumin to a full-size Ab significantly increases molecular weight, thereby restricting tissue penetration. As an alternative to the indirect targeting of FcRn, small peptides directly interacting with FcRn have been developed.^{54–56}

So far, no studies with ABD fusion molecules have been performed in humans. Possible immunogenicity of the ABD because of its bacterial origin is conceivable, but might be minimal due to its small size. Additionally, ABDs are part of the normal bacterial flora and thus have evolved to escape immune response. Furthermore, a successful deimmunization of an ABD has been reported.⁵⁷

Due to the extension of the in vivo half-life and enhancement of Ab exposure upon ABD attachment, we were able to design an in vivo therapeutic study with less frequent Ab injections compared to a previous study with IgA Abs.¹⁴ We detected more IgA1 than IgA2 in the peritoneal cavity of tumor-bearing mice, possibly explaining the better anti-tumor response with IgA1 Abs. Ab concentrations in serum for each modification were lower for IgA2 Abs than for IgA1 Abs, but not as pronounced as in the peritoneal cavity. These findings suggest that albumin binding to IgA might trigger FcRn-mediated transcytosis, and that this process is somehow more effective for IgA1 Abs than IgA2 Abs. Furthermore, although IgA1-HC_{ABD} had ~2-fold higher Ab concentrations in the peritoneal cavity compared to IgA1-LC_{ABD}, both Abs performed similarly with respect to the therapeutic activity. Thus, the higher exposure reached with IgA1-HC_{ABD} Abs in the peritoneal cavity may result in the best possible anti-tumor response, whereas with the IgA1-LC_{ABD} Ab probably no full tumor opsonization was achieved due to suboptimal dosing. Among the IgA2 Abs, tumor growth was significantly impaired until day 41 with the LC modified variant. The response and Ab titers with IgA2-LC_{ABD} in the peritoneal cavity

were comparable to the unmodified IgA1 Ab, suggesting that the multi-compartment xenograft model used is likely to be suboptimal for the study of the in vivo efficacy of modified IgA2 Abs.

In conclusion, ABD-modified IgA Abs showed in vitro and in vivo anti-tumor effects, further strengthening the concept of therapeutic IgA Abs. As demonstrated here, the major in vivo limitation of their short half-life can be successfully addressed by Ab engineering strategies. For future strategies it would be advised to use albumin-deficient mice for ABD-modified IgA therapeutics, as injected HSA has a half-life of even 20 days,⁵⁸ therefore fewer injections of IgA will be needed.

Material and Methods

Cell lines

A431 cells (ATCC) were lentivirally transduced with a luciferase-GFP construct and then retrovirally transduced with Her2 to generate A431-luc2-Her2 cells. A subclone with good in vivo outgrowth and stable Her2 expression was further used. A431-luc2-Her2 cells and SKBR3 cells (ATCC) were cultured in RPMI 1640+HEPES+glutamine (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies) at 37°C/5% CO₂. Ba/F3-FcαRI-eYFP cells were generated by co-electroporation of pEGFP-N1 containing the FcαRI(wt)-YFP sequence (lacking the internal eGFP) and pSG5-CMV-Hygro (0.28V; capacitance 960 µFD) into Ba/F3 cells. Stable cell lines were cultured in RPMI 1640 Glutamax + HEPES (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 µg/ml G418 (Invitrogen) and murine IL-3 (kindly provided by Paul Coffey, UMC Utrecht). HEK FreeStyleTM293F cells (Invitrogen) were cultured in GIBCO FreeStyleTM 293 expression medium (Invitrogen) at 37°C/8% CO₂ on an orbital shaker (125 rpm).

Mice

Human FcαRI Tg mice were generated at the UMC Utrecht⁵⁹ and were backcrossed to an immunodeficient SCID background (Charles River) and maintained as hemizygous. Transgene-negative littermates were used as controls. All experiments were approved by the Animal Ethical Committee of the UMC Utrecht.

Antibody production and purification

The variable region of the HC and LC for the generation of IgA-Her2 Abs is derived from trastuzumab (Accession number DB00072). Sequences coding for the albumin binding domain

Figure 5. (See previous page) Therapeutic efficacy of un-/modified IgA-Her2 antibodies in a multi-compartment xenograft model. FcαRI Tg or WT SCID mice were injected intraperitoneally with 10⁵ A431-luc2-Her2 cells. On day 6 tumor growth was measured by BLI and mice were randomized. Starting on day 7, mice were treated once weekly (arrow) with 50 µg un-/modified IgA Abs or PBS. Tumor outgrowth was measured one day before treatment by BLI. Tumor volume (CPM/cm²) over time for each measurement (5–10 mice/group) for WT SCID mice (A,B) and FcαRI Tg SCID mice (C,D) for (A,C) IgA1, IgA1-HC_{ABD}, IgA1-LC_{ABD} and PBS or (B,D) IgA2, IgA2-HC_{ABD}, IgA2-LC_{ABD} and PBS (mean ± SEM). (E) Representative BLI images of FcαRI Tg SCID mice treated with IgA Abs and WT SCID mice treated with PBS 6 d after first treatment (day 13), 6 d after last treatment (day 41) and 15 d after last treatment (day 56). (F) Tumor volume (CPM/cm²) of FcαRI Tg SCID mice on day 41 (mean ± SEM). (G) Ab concentrations in peritoneal cavity and serum of mice on day 36 (mean ± SEM; n.d. not detectable).

(ABD-H³³) attached to the HC or the kappa LC by a glycine-serine linker (9GS) were synthesized by Shinegene (Shanghai, China). Antibody sequences were cloned into Lonza expression vectors with the following backbones: pEE14.4-kappaLC, pEE14.4-IgA1 and pEE14.4-IgA2(m1) (vector sequences available upon request). All IgA-Her2 Abs were produced by transient transfection of HEK293F cells with the HC coding plasmid, the LC coding plasmid and pAdvantage (Accession Number U47294; Promega) using 293fectin transfection reagent (Invitrogen) following the manufacturer's instructions. Ab containing supernatants were harvested 4 d after transfection. Abs were purified as described before.¹³ Briefly, Abs secreted into the supernatant were isolated by anti-kappa affinity chromatography (HiTrap KappaSelect, GE-Healthcare) and bound protein was eluted using 0.1 M glycine pH 2.5 (VWR International) and directly neutralized with 1 M Tris-HCl pH 8.8 (Roche Diagnostics). Elution fractions were subsequently subjected to SEC (HiPrep 26/60 Sephacryl S-300 High Resolution, GE-Healthcare). Ab concentrations were determined using the following formula: OD values at 280 nm/correction factor 1.4. The production yield of the unmodified and modified Abs varied between 30 to 70 µg antibody/mL; the ABD-modified Abs tended to be in the lower part of the range (30 to 50 µg antibody/mL).

Antibody concentration determination in serum samples

Maxisorp NUNC plates (Sanbio) were coated overnight at 4°C with 0.5 µg/mL goat IgG anti-human kappa (Southern Biotech) in PBS followed by a blocking step with 1% BSA (Roche Diagnostics) in 0.05% Tween 20 (Immunologic) in PBS. Samples and standards were diluted in 1% BSA/0.05% Tween 20 in PBS and incubated on the microtiter plate for 1.5 h at room temperature. Horseradish peroxidase (HRP)-labeled goat IgG anti-human IgA (1:2,000; Southern Biotech) was used for 1 h at room temperature for detection. Plates were developed using ABTS substrate (Roche Diagnostics) and read on a Multiscan RC (Thermolab systems) at 415 nm.

Antibody binding to albumin

HSA (Albuman, Sanquin) or serum derived from different species (SCID mouse, Wistar rat, cynomolgus monkey, kindly provided by the Animal Facility Utrecht and Synthron Biopharmaceuticals BV) was coated on Maxisorp NUNC plates (Sanbio) overnight at 4°C followed by a blocking step with 1% BSA in 0.05% Tween 20 in PBS. 100 ng/mL IgA Abs diluted in 1% BSA in 0.05% Tween 20 in PBS were added to the wells and incubated for 1.5 h at room temperature. HRP-labeled anti-human IgA (1:2,000; Southern Biotech) was used for 1 h at room temperature for detection. Plates were developed for 10 min using ABTS substrate (Roche Diagnostics) and read on a Multiscan RC (Thermolab systems) at 415 nm.

Conjugate assay

SKBR3 cells labeled with CellTrace violet (Life Technologies) were incubated for 45 min on ice with 20 µg/mL Abs pre-incubated without or with 1 mg/mL HSA for 1 h at room

temperature. After extensive washing with cold fluorescence-activated cell sorting (FACS) buffer, Ba/F3-FcαRI-eYFP cells were added in a 1:1 ratio and the cell mix was incubated for another hour on ice. Cells were fixed with 1% paraformaldehyde (Klinipath) in PBS and subsequently analyzed on a FACS CantoII (BD Biosciences). % conjugates was calculated as (events double positive cells / events (double positive cells + CellTrace violet⁺ tumor cells))*100%.

HP-SEC

130 nM IgA Abs were pre-incubated at various molar ratios with HSA diluted in PBS for at least 1 h at room temperature. Formation of albumin-antibody complexes was analyzed by HPLC size-exclusion chromatography (HP-SEC) (YarraTM 3u SEC-2000 column; Phenomenex) with 100 mM sodium phosphate/150 mM NaCl pH 6.8 as running buffer and a flow rate of 0.35 mL/min.

Human PMN ADCC

ADCC was measured using a chromium-release assay as previously described.⁴¹ Briefly, SKBR3 cells labeled with 100 µCi ⁵¹chromium (per 10⁶ cells) were added to Abs pre-incubated for 1 h in PBS or HSA (final concentration in assay 1 mg/mL) in round-bottom microtiter plates (Corning Inc.). PMNs were isolated from blood from healthy donors (MinidonorDienst UMC Utrecht) by ficoll-histopaque density gradient and added with an effector-to-target (E:T) ratio of 40:1 in a final assay volume of 200 µL/well. The cells were incubated for 4 h at 37°C/5% CO₂. Subsequently, the supernatant was transferred to a Deepwell LumaPlate (Perkin Elmer) and counted in a liquid scintillation counter (MicroBeta; Perkin Elmer). Lysis was calculated using the following formula: % lysis = ((counts of sample - minimal release)/(maximum release - minimum release))*100. Culture medium was used to determine minimal release and 3% TritonX-100 (Roche Diagnostics) to determine maximum release.

PK study

FcαRI Tg or WT SCID mice were injected intravenously or subcutaneously with 200 µg IgA Abs (3 mice/group). Blood was collected by tail vein cut or cheek pouch from alternating mice at indicated time points. Blood samples were processed and serum stored at -20°C. Peritoneal lavages were performed with 1 mL PBS at indicated time points. The human IgA Ab concentrations in the sera and peritoneal lavage samples were determined by ELISA. Serum exposure and t_{1/2} was calculated with WinNonlin 6.3 (Certara, Princeton, USA).

Multi-compartment xenograft model

FcαRI Tg/WT male SCID mice were injected intraperitoneally with 1 × 10⁵ A431-luc2-Her2 cells in 100 µL PBS on day 0. On day 6, tumor size was assessed by BLI (PhotonImager, Biospace Lab) and the mice were randomized into different treatment groups (5–10 mice/group). 50 µg Ab/mouse was injected subcutaneously weekly (day 7, 14, 21, 28, 35) and tumor outgrowth was followed by serial BLI. Image analysis was performed

using M3Vision software (Biospace Lab). To assess the PK of the Abs, 2 additional tumor-bearing mice per Ab type were used. Blood samples were obtained a day after the first treatment (day 8) and a day before (day 34) and after the last (day 36) treatment (1 mouse/time point). Peritoneal lavage samples in 2 mL PBS were obtained 24 h after the first injection and 1 day after the last injection (1 mouse/time point). Ab concentrations in peritoneal lavages and serum samples were determined as described above.

Statistical analysis

Data were graphed and analyzed using GraphPad Prism 6.0. (Graph Pad Software, CA, USA). In the xenograft model, linear regression analysis was performed to estimate tumor outgrowth over time in each treatment group and the slopes were compared using an F-test. A P value <0.05 was considered significant.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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