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REPORT

Elimination of plasma soluble antigen in cynomolgus monkeys by combining pH-dependent antigen binding and novel Fc engineering

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ABSTRACT

A conventional antibody targeting a soluble antigen in circulation typically requires a huge dosage and frequent intravenous administration to neutralize the antigen. This is because antigen degradation is reduced by the formation of antigen–antibody immune complexes, which escape from lysosomal degradation using neonatal Fc receptor (FcRn)-mediated recycling. To address this, we developed an antigen-sweeping antibody that combines pH-dependent antigen binding and Fc engineering to enhance Fc receptor binding. The sweeping antibody actively eliminates the plasma antigens by increasing the cellular uptake of the immune complex and dissociating the antigens in the acidic endosome for degradation. Strong antigen sweeping can reduce the dosage, potentially achieve higher efficacy, and expand the scope of antigen space available for targeting by antibodies. In this study, to further improve the sweeping efficacy, we developed a novel antibody Fc variant by enhancing Fcγ receptor IIb (FcγRIIb) binding and modulating charge characteristics for increased cellular uptake of the immune complex, together with enhancing FcRn binding for efficient salvage of the antigen-free antibodies. Our Fc variant achieved strong antigen sweeping in cynomolgus monkeys with antibody pharmacokinetics comparable to a wild-type human IgG_1 antibody. The positive-charge substitutions enhanced uptake of the immune complex by FcγRIIb-expressing cells in vitro, which was completely inhibited by an anti-FcγRIIb antibody. This suggests that the strong in vivo sweeping efficacy improved by the charge engineering is more likely achieved by FcγRIIb-dependent uptake of the immune complex rather than nonspecific uptake. We expect this novel Fc engineering can maximize the antigen sweeping efficacy even in humans and create novel therapeutic antibodies that meet unmet medical needs for patients.

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Introduction

The administration of a conventional antibody targeting a soluble antigen has been reported to induce antigen accumulation in plasma.[1–5] This often happens because of the reduced clearance of the antigen by the administered antibody. After soluble antigens are taken up by cells, free antigens in the acidic endosome are transported to the lysosome for degradation, while the antibody–antigen immune complex is salvaged back to blood circulation by FcRn, which reduces the antigen clearance.[6,7] Since antigen accumulation in plasma requires huge dosage and frequent intravenous administration to effectively neutralize the antigen, antibody engineering to suppress the antigen accumulation is needed to reduce the treatment burden for patients and their caregivers.

Antibodies that pH-dependently bind to antigens have been eagerly studied in recent years,[7–16] as they could be the key to solving this problem. Such pH-dependent antibodies that show weaker affinity at acidic pH than at neutral pH are expected to dissociate the antigens in the acidic endosome.[7,14] After dissociation, while the antigen-free antibody is recycled back into plasma by FcRn, the dissociated antigens are digested in the lysosome, which thus suppresses the antigen accumulation in plasma that often accompanies conventional antibodies.[12–14]

We previously reported on sweeping antibodies, which combine pH-dependent antigen-binding with enhanced affinity to FcRn or FcγRIIb.[17–19] A sweeping antibody actively eliminates its soluble antigens from plasma by increasing the Fc receptor-mediated cellular uptake of the immune complex, which then increases the number of dissociated antigens in the endosome for degradation. Therefore, the sweeping antibody is expected to achieve lower antibody dosage and higher efficacy by increasing the neutralization capacity per antibody, and thus expand the space of antigens targetable by antibody therapeutics. However, novel antibody engineering to further enhance the sweeping efficiency is still in high demand because sweeping efficiency for some antigens by previously reported technologies could be insufficient.

Several studies show the surface charge of the antibodies had some effect on their pharmacokinetics and functions.[12,20–24] Because surface-charge engineering of the variable and constant regions of a pH-dependent anti-

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C5 antibody suppressed the accumulation of the antigen,[12] the interaction between the cell membrane's negative charge and the antibody's positive charge can be a driving force for increasing the uptake of the immune complex and the number of the antigens degraded in the lysosome. However, positive charges in the antibody have been reported to increase antibody clearance, which must be carefully considered when engineering antibodies for therapeutic applications.

The number of the IgG antibody molecules in the immune complex also affects the sweeping efficiency. When targeting a multimeric antigen, the antibodies can form an immune complex containing multiple Fc regions, and its cellular uptake is accelerated by its binding avidity with multiple Fc receptors.

In this study, we developed a novel Fc variant by combining selectively enhanced FcγRIIb binding, charge engineering, and enhanced FcRn binding to maximize the sweeping efficiency. We used a pH-dependent antigen-binding antibody targeting latent myostatin, a soluble dimer antigen, so that immune complexes each containing multiple antibody Fc regions can form in vivo. This antibody engineering exhibited strong antigen sweeping in cynomolgus monkeys without compromising good antibody pharmacokinetics.

We also conducted an in vitro study to understand the mechanism through which cellular uptake was enhanced by charge engineering. The positive-charge substitutions increased the amount of the antibody binding to human FcγRIIb on the cell surface, which was strongly suppressed by an anti-FcγRIIb antibody. This suggests that the charge engineering further improved in vivo sweeping efficacy mainly through FcγRIIb-mediated cellular uptake, and nonspecific cellular uptake is largely not involved. This novel Fc engineering can realize stronger antigen sweeping efficacy even in humans and provide novel therapeutic antibodies that meet unmet medical needs for patients.

Results

Design of antibodies for strong antigen sweeping

We prepared two antibody variable region derivatives (non–pH-dependent antibody and pH-dependent antibody) made from the same antibody targeting latent myostatin. The antigen-binding properties of the non–pH-dependent antibody (NpH-IgG$_1$) and pH-dependent antibody (pH-IgG$_1$) were assessed by surface plasmon resonance (SPR) analysis and are shown in Table 1. We confirmed that the antigen binding of these antibodies was sufficiently stable for use in several experiments included in our study (stability data not shown). We also prepared Fc derivatives by introducing amino acid substitutions into IgG$_1$ to selectively enhance FcγRIIb binding, modify the surface charge of the Fc region, and moderately enhance FcRn binding. The antibody design for strong antigen sweeping is shown in Figure 1. The binding activity of these Fc variants to each Fcγ receptor was measured (Table 2, Supplemental Figure S1, S2 and Supplemental Table S1, S2).

Fc engineering to selectively enhance FcγRIIb binding

Previously, we reported that Fc variants with enhanced binding to FcγRIIb showed increased clearance of the soluble antigen in mice.[17] Although a few Fc variants had enhanced human FcγRIIb (huFcγRIIb) binding, none of them is reported to cross-react with cynomolgus FcγRIIb (cyFcγRIIb).[25–27] To predict the sweeping efficacy in humans, we tried to develop a new human Fc variant whose binding levels to huFcγRIIb and cyFcγRIIb were similarly enhanced. This was also needed to reduce binding to activating Fcγ receptors such as FcγRIIIa because it could potentially induce immune activation or cause unwanted adverse effects, depending on the disease or the mode of action of the antibody. Therefore, to make an Fc variant with the desired binding to each FcγR, we selected substitutions G236N, H268D, and A330K, based on our previous work, and introduced them in the Fc region of IgG$_1$.[25] The IgG$_1$ variants V1 (G236N), V2 (G236N/H268D), and V3 (G236N/H268D/A330K) were then produced and their binding activity to each Fcγ receptor was measured (Table 2). V3 showed enhanced binding to both huFcγRIIb and cyFcγIIb, and importantly, the levels of enhancement were comparable (by 3.06-fold and 2.86-fold, respectively). Although the affinity to cyFcγRIIa of V3 was also enhanced because of the high homology between cyFcγRIIa and cyFcγRIIb, this Fc variant could be useful for predicting the sweeping efficacy of the antibodies in humans based on cynomolgus monkey pharmacokinetics studies.

Effect of positive-charge substitutions on cellular uptake of immune complex and antibody pharmacokinetics

We previously reported that charge engineering could reduce antigen accumulation in cynomolgus monkeys.[12] To maximize the antigen sweeping efficacy, we designed Fc variants by introducing positively charged substitutions in the Fc region. We examined and introduced the substitution sets of Q311R/D413K, S400R/D413K, and Q311R/S400R/D413K into the IgG$_1$ Fc region of a pH-dependent antibody to generate the Fc variants pI(A), pI(B), and pI(C), respectively. These substitutions did not greatly affect the binding to human and cynomolgus FcγRs (Table 2).

We examined the antigen uptake efficiency of the pH-dependent antibody (pH-IgG$_1$) and its variants with the positive-charge substitutions in the Fc region, pH-pI(A), pH-pI(B), and pH-pI(C), by an *in vitro* cellular uptake assay, in which we measured the amount of fluorescently labeled antigens taken into the cells expressing human FcγRIIb (Figure 2a) These variants showed improved antigen-uptake activity compared with the wild-type IgG$_1$.

Since the charge characteristics potentially affect the antibody pharmacokinetics, the effect of these substitutions on the antibody clearance *in vivo* was then evaluated using human FcRn transgenic mice.[28] Whereas the antibody clearance was increased in pH-pI(B) and pH-pI(C), that of pH-pI(A) from plasma was mostly unaffected (Figure 2b). Because the antibody plasma half-life was also an important factor for sweeping efficiency, we chose the positive-charge substitutions used in the pH-pI(A) variant for the subsequent combination with the Fc engineering to enhance FcγRIIb binding.

Table 1. The binding profile of NpH-IgG$_1$ and pH-IgG$_1$ to the latent myostatin at pH 7.4 and pH 5.8.

	Human myostatin			Cynomolugus myostatin		
Ab name	KD pH 7.4 (M)	KD pH 5.8 (M)	ratio of KD at pH 5.8/pH 7.4	KD pH 7.4 (M)	KD pH 5.8 (M)	ratio of KD at pH 5.8/pH 7.4
NpH-IgG$_1$	1.78 x 10^{-10}	6.22 x 10^{-11}	0.3	2.24 x 10^{-10}	6.23 x 10^{-11}	0.3
pH-IgG$_1$	2.85 x 10^{-10}	3.48 x 10^{-8}	119.3	3.43 x 10^{-10}	2.59 x 10^{-8}	75.5

The kinetic parameters of NpH-IgG$_1$ and pH-IgG$_1$ against human and cynomolgus latent myostatin were evaluated at 37°C at pH 7.4 or pH 5.8. Kinetic parameters were determined by processing and fitting the data to a 1:1 binding model.

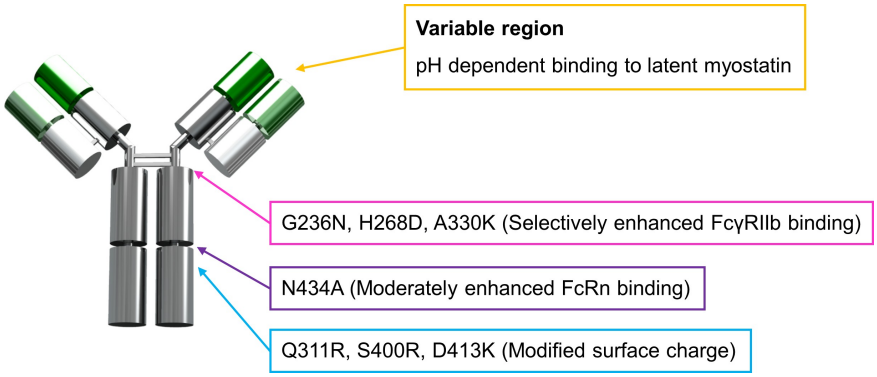

Figure 1. Design of antibodies for strong antigen sweeping.

Effect of the combination of enhanced FcγRIIb binding and positive-charge substitutions on soluble antigen clearance in mice

To evaluate the combinatorial effect of the surface-charge engineering with FcγRIIb enhanced binding, pH-V3-pI(A) was constructed by introducing the positive-charge substitutions of pI(A) into pH-V3. We confirm that the binding affinity of pH-V3-pI(A) to huFcγRIIb is comparable to pH-V3 (Table 2). The effect of the Fc engineering on antigen clearance was evaluated in an antigen/antibody co-injection study using all human FcγR transgenic mice.[27] Latent myostatin is reported to be cleaved by proteases and produce mature myostatin.[29–31] To evaluate the effect of Fc engineering on antigen clearance, we need to measure not only latent myostatin concentration in plasma, but also that of mature myostatin. Therefore, we measured total myostatin concentration (mature + latent myostatin) in plasma. Consistent with the previous report using huFcγRIIb transgenic mice,[17] pH-dependent antibody with FcγRIIb-enhanced binding, pH-V3, showed accelerated antigen clearance compared with pH-IgG$_1$ and comparable antibody clearance to pH-IgG$_1$ (Figure 3).

The administration of pH-V3-pI(A) further accelerated antigen clearance compared with pH-V3 or pH-IgG$_1$, although the antibody clearance from plasma was also increased (Figure 3). These data demonstrated that the combination of the positive-charge substitutions and FcγRIIb enhanced binding can effectively increase antigen clearance and suppress antigen accumulation.

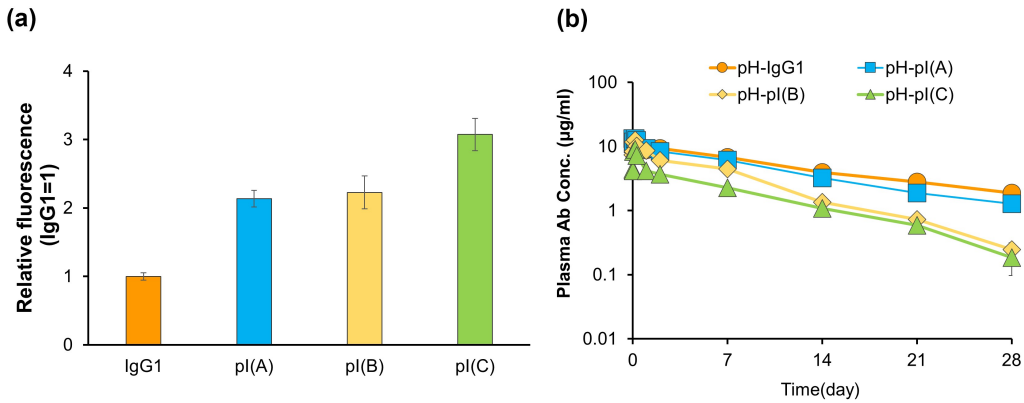

Figure 2. Effect of charge substitutions on cellular uptake into FcγRIIb-expressing MDCK cells and antibody pharmacokinetics in human FcRn transgenic mice (a) PHrodoRed labeled latent myostatin was incubated with each antibody and added to the FcγRIIb-expressing MDCK cell line. The fluorescence intensity of the antigen per cells at 60 min was quantified. Each bar represents the mean ± SD (n = 3 each). (b) The latent myostatin and the antibody were intravenously administered as single doses of 0.5 mg/kg for the latent myostatin and 1 mg/kg for antibody. pH-IgG$_1$, pH-pI(A), pH-pI(B) and pH-pI(c) were each co-injected with the latent myostatin in human FcRn transgenic mice, and the time profiles of plasma antibody concentration were shown. Each point represents the mean ± SD (n = 3 each).

Table 2. The binding profile of Fc variants to human and cynomolgus FcγRs and cynomolgus FcRn.

		Relative binding per units against huFcγR (IgG1 = 1)						Relative binding per units against cyFcγR (IgG1 = 1)							
Fc name	Substitutions	huFcγRIa	huFcγRIIa(H) huFcγRIIb	huFcγRIIa(R)	huFcγRIIIa(V)		huFcγRIIIa(F)	cyFcγRIa	cyFcγRIIa1	cyFcγRIIa2 cyFcγRIIb		cyFcγRIIa3	cyFcγRIIIa(S)	cyFcγRIIIa(R)	KD for cyno FcRn (M) at pH 6.0
$^{IgG}1$	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1.52.E-06
V1	G236N	0.16	0.3	0.47	0.53	0.03	N.D.	0.62	0.9	0.97	0.73	0.87	0.07	0.06	N.E.
V2	G236N/H268D	0.61	0.55	0.69	2.53	0.04	N.D.	0.96	1.45	1.89	2.36	2.15	0.12	0.1	N.E.
V3	G236N/H268D/A330K	0.39	0.91	0.8	3.06	0.06	0.01	0.75	2	2.56	2.77	2.86	0.1	0.09	N.E.
pI(A)	Q311R/D413K	0.91	0.93	0.93	0.92	0.93	0.86	0.91	0.93	0.95	0.95	0.94	0.96	0.95	N.E.
pI(B)	S400R/D413K	0.9	0.96	0.91	0.92	0.91	0.82	0.8	0.94	0.94	0.93	0.93	0.94	0.93	N.E.
pI(C)	Q311R/S400R/D413K	0.78	0.94	0.94	0.87	0.88	0.78	0.94	0.92	0.93	0.96	0.96	0.93	0.91	N.E.
V3-pI(A)	G236N/H268D/Q311R/A330K/D413K	0.36	0.84	0.73	2.85	0.06	0.02	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.
V3-N434A	G236N/H268D/A330K/N434A	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.	0.79	2.1	2.66	2.88	2.97	0.11	0.1	5.26.E-07
V3-pI(A)-N434A	G236N/H268D/Q311R/A330K/D413K/N434A	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.	0.71	1.97	2.52	2.76	2.83	0.1	0.09	3.55.E-07

The binding profile of Fc variants to FcγRs or FcRn was evaluated in SPR. The binding amount of each FcγR was normalized with the amount of the captured antibody and was presented as relative ratio to that of IgG_1 to compare the binding ability of variants. For FcRn, sensorgrams were analyzed by the steady state affinity model to calculate the dissociation constant KD (mol/L). N.D. means not determined. N.E. represents not evaluated.

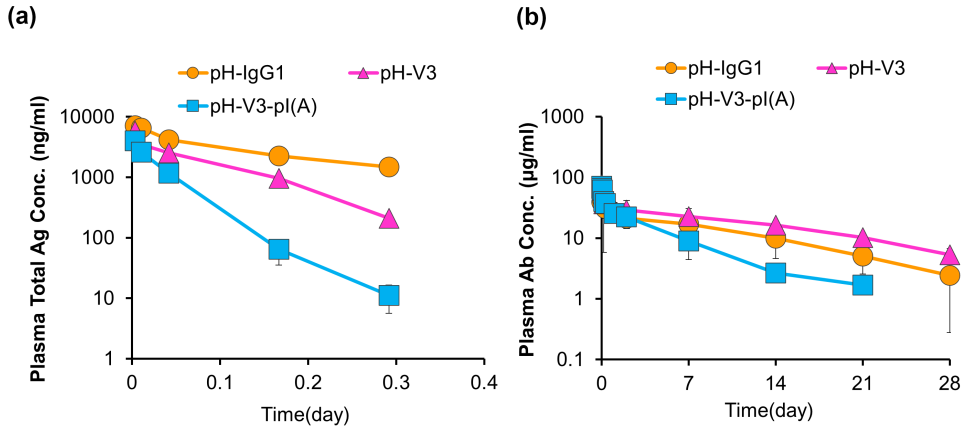

Figure 3. Effect of Fc engineering to enhance binding to human FcγRIIb combined with charge mutations on myostatin clearance and antibody pharmacokinetics in all human FcγR transgenic mice. The latent myostatin and antibodies were intravenously administered as single doses of 0.5 mg/kg for the latent myostatin and 3 mg/kg for antibody. pH-IgG$_1$, pH-V3 and pH-V3-pI(a) were each co-injected with the latent myostatin in all human FcγR transgenic mice, and time profiles of (A) total myostatin plasma concentration and (b) antibody plasma concentration were shown. Each point represents the mean ± SD (n = 6 for pH-IgG$_1$, n = 3 for pH-V3 and pH-V3-pI(A)). Antibody plasma concentration of pH-V3-pI(A) on day 28 was below the level of detection in all animals.

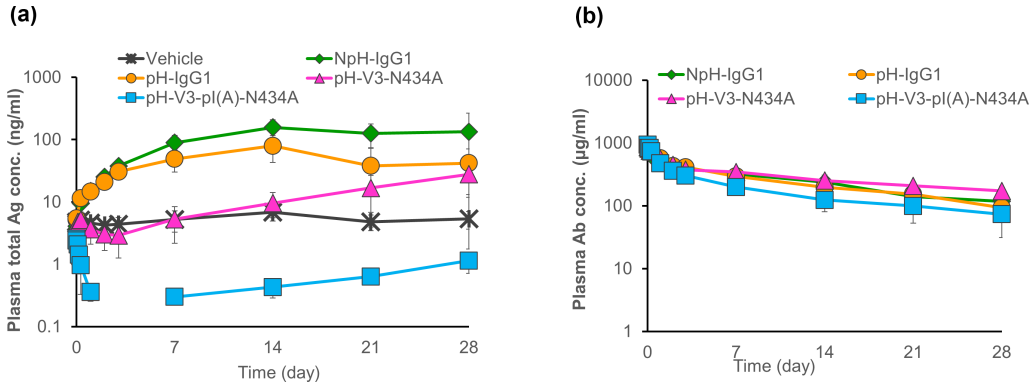

Figure 4. Effect of Fc engineering to enhance binding to human FcγRIIb combined with charge substitutions on myostatin clearance and antibody pharmacokinetics in cynomolgus monkeys (a) The time course of plasma total myostatin concentration after intravenous administration of 30 mg/kg of antibodies in cynomolgus monkey was shown. Each point represents the mean ± SD (n = 4 each). (b) The time course of plasma total antibody concentration after intravenous administration of 30 mg/kg of antibodies in cynomolgus monkey was shown. Each point represents the mean ± SD (n = 4 each). Plasma total myostatin concentration on day 2 to 3 in pH-V3-pI(A)-N434A group was below the level of detection in all animals.

Effect of Fc engineering on endogenous antigen concentration in cynomolgus monkeys

The effect of the Fc engineering on the endogenous antigen clearance was then evaluated in cynomolgus monkeys. We introduced the N434A substitution into pH-V3 and pH-V3-pI(A) to enhance FcRn binding for a longer antibody plasma half-life.[32–37] We confirmed that the N434A substitution did not clearly affect the binding activity to cynomolgus FcγRs, but improved the binding to cynomolgus FcRn at acidic pH (Table 2). As shown in Figure 4, the total myostatin concentration in plasma was

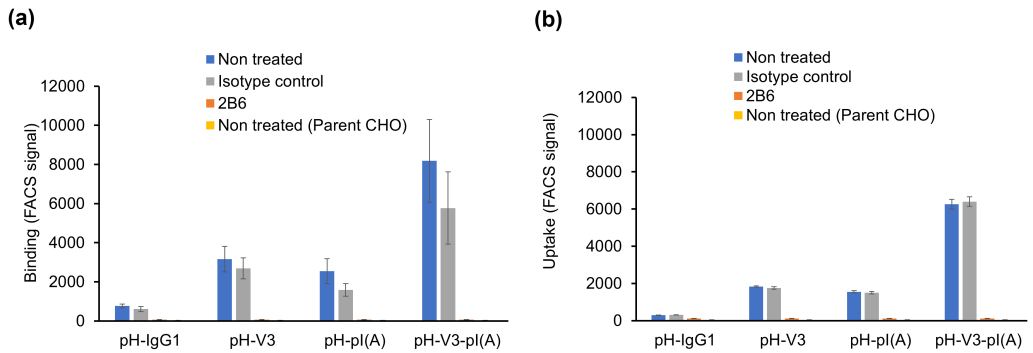

Figure 5. Binding and uptake of antigen-antibody complex on huFcγRIIb-expressing CHO cells. Alexa Fluor 488-labeled antigen-antibody (pH-IgG$_1$, pH-V3, pH-pI(a) and pH-V3-pI(A)) complex was incubated with huFcγRIIb-expressing CHO cells or parent CHO cells for 20 min at 4°C for binding (A) or 37°C for uptake (b), and fluorescence intensity was measured by flow cytometer after washing cells with phosphate-buffered saline or acidic medium (pH 3.5), respectively. Binding and uptake in huFcγRIIb-expressing CHO cells or parent CHO cells are shown in blue and yellow, respectively. Binding and uptake in huFcγRIIb-expressing CHO cells in the presence of anti-huFcγRIIb antibody (2B6) or its isotype control antibody are shown in Orange and gray, respectively. Each bar represents mean ± SEM (n = 3).

greatly elevated by administration of NpH-IgG$_1$, while pH-IgG$_1$ showed less total myostatin accumulation, demonstrating that the pH-dependent antigen-binding property (Table 1) was effective in suppressing antigen accumulation. pH-V3-N434A showed significantly lower total myostatin concentration than pH-IgG$_1$ and reduced it to the baseline level, indicating that the antigen clearance can be accelerated in cynomolgus monkeys by enhancing the binding to Fcγ receptors. Moreover, administration of pH-V3-pI(A)-N434A accelerated the antigen clearance even more strongly and reduced the total myostatin concentration below the baseline, whereas the antibody clearance was not obviously affected. The clearance of pH-V3-pI(A)-N434A was 4.7 mL/d/kg, whereas that of NpH-IgG$_1$ and pH-IgG$_1$ was 3.2 and 3.5 mL/d/kg, respectively (Figure 4). These data showed that combining pH-dependent antigen binding and Fc engineering technologies can strongly reduce the plasma antigen concentration in cynomolgus monkeys.

Enhancement of FcγRIIb-mediated cellular uptake by Fc engineering

It is known that the clearance of antigen–antibody immune complexes from plasma is mainly mediated by liver sinusoidal endothelial cells and/or Kupffer cells that express FcγRIIb.[38,39] To elucidate the underlying mechanism of the effect of the charge substitutions on the in vivo antigen sweeping, we evaluated the binding and the cellular uptake of the immune complex by Chinese hamster ovary (CHO) cells that did or did not stably express huFcγRIIb. Alexa Fluor 488-labeled antigen and the tested antibodies, pH-IgG$_1$, pH-V3, pH-pI(A), and pH-V3-pI(A), were incubated with the cells, and the cellular fluorescence intensity was measured by flow cytometer. Compared with pH-IgG$_1$, pH-V3 and pH-pI(A) showed significantly higher binding and uptake, and that pH-V3-pI(A) was especially high. In contrast, they show little binding and uptake in the parent CHO cells (Figure 5). Furthermore, the enhanced binding and uptake by the Fc-engineered variants, not only in pH-V3, but also in pH-pI(A) and pH-V3-pI(A), were completely inhibited by an anti-huFcγRIIb antibody (2B6) that recognizes the Fc binding site of huFcγRIIb,[40] although they were not inhibited by the isotype control antibody (Figure 5). These results indicate that the positive-charge substitutions further enhance FcγRIIb binding and increase FcγRIIb-mediated cellular uptake, rather than nonspecific uptake.

Discussion

In this study, we developed a novel Fc variant, V3-pI(A)-N434A, by combining enhanced binding to both human and cynomolgus FcγRIIb (V3), positive-charge substitutions (pI(A)), and improved FcRn binding (N434A). Combined with the pH-dependent antigen-binding property, enhanced binding to FcγRIIb (V3) significantly improved antigen sweeping from plasma in cynomolgus monkeys, which was further enhanced by the charge engineering (pI(A)).

To identify effective positive charge substitutions and their combinations, we selected and changed the amino acids found on the surface of the antibody Fc region based on the antibody structure. We then selected the desired combinations of substitutions by evaluating the efficiency of antigen uptake into cells expressing human FcγRIIb, as well as the antibody pharmacokinetics profiles and physicochemical properties. Basic amino acids are positively charged in circulating blood (neutral pH), which is where the uptake of immune complex occurs. Since the plasma membrane is negatively charged, the interaction between the cell membrane's negative charge and the antibody's positive charge can be a driving force for increasing the uptake of the immune complex.

We carefully monitored the physicochemical properties of the Fc variants during the engineering procedure, and we confirmed that the Fc variants were sufficiently stable in vivo to sweep antigens for weeks. This Fc engineering did not influence the thermal stability and aggregation (data not shown). We also confirmed that our positive charge substitutions did not strongly influence nonspecific binding (data not shown). We used similar Fc engineering in GYM329 and confirmed that the developability and sufficient stability of that antibody.[29] In addition, we examined the C1q binding of pH-V3 and pH-V3-pI(A)-N434A and determined that these engineered Fc variants have greatly reduced binding to C1q (data not shown). It was theorized that some of the substitutions to enhance FcγRIIb binding may influence C1q binding because they exist in the C1q interaction region of IgG antibodies.

The strong antigen sweeping of pH-V3-pI(A)-N434A motivated us to determine the underlying cellular mechanism. Consistent with the enhanced antigen clearance in the pharmacokinetic study, the binding and uptake of Alexa Fluor 488-labeled antigen by huFcγRIIb expressing CHO cells were increased in pH-V3 and pH-pI(A) compared with pH-IgG$_1$, and even more strongly increased in pH-V3-pI(A). Interestingly, the anti-huFcγRIIb antibody completely suppressed the binding and uptake of the immune complexes that had been enhanced by the Fc engineering technologies, not only the enhancement of FcγRIIb binding, but also the positive-charge substitutions, although they were not inhibited by the isotype control antibody. Furthermore, the lack of binding and uptake in the parent CHO cells suggested that the charge substitutions of these variants have little effect on nonspecific binding and uptake. These results indicated that the effect of these charge substitutions is to increase the binding of the antibodies to FcγRIIb on the cell surface rather than to promote nonspecific binding. This greatly increases antigen clearance from plasma in cynomolgus monkeys.

The composition of the antigen–antibody immune complex is likely to contribute to the strong antigen sweeping. Since the latent myostatin forms a dimer in plasma, the administration of the antibody can produce an immune complex that is composed of a few antigens and a few antibodies. Therefore, when the immune complex of pH-V3-pI(A)-N434A is formed, it can exhibit avidity binding to FcγRIIb and contain a few-fold more positive-charge residues than the antigen-free antibody. The effect of positive-charge substitutions on the immune complex is much stronger than that of the antigen-free antibody, leading to the strong antigen sweeping with a minimal effect on antibody pharmacokinetics. Based on this evidence, our novel Fc engineering seems to be advantageous for

sweeping antibodies that can form immune complexes containing multiple antibodies and sweeping efficiency may be reduced in the case of monomer antigen.

The pharmacokinetics of antibodies are also an important factor in sweeping efficiency. Although the effect of the charge substitutions is weaker for the antigen-free antibody than for the immune complex, its influence on antibody clearance must be carefully considered, as suggested by several reports which showed that the antibody charge may influence its pharmacokinetics profile.[20–23] To minimize the negative effect on antibody pharmacokinetics, we conducted pharmacokinetics screening using human FcRn transgenic mice and selected pI(A), which showed a slightly accelerated but acceptable antibody clearance. We also applied the N434A substitution, which was previously reported for FcRn-mediated elongation of the antibody half-life.[36,37] As the difference in antibody clearance of pH-V3-pI(A)-N434A compared to NpH-IgG$_1$ and pH-IgG$_1$ was within 1.5-fold in cynomolgus monkey, the N434A substitution is able to keep antibody clearance by enhancing the FcRn binding of the antigen-free antibody in the acidic endosome in spite of the introduced positive charge substitutions. Therefore, due to the enhanced FcRn binding, we expect that these charge substitutions can be applied clinically without critically affecting antibody pharmacokinetics. We previously reported that enhancing FcRn binding was effective to sweep soluble antigens.[18] However, we believe that the N434A substitution used in this study is not strong enough to contribute to antigen sweeping. Although FcRn-enhancing mutation M252Y/S254T/T256E (YTE) showed stronger binding ability to FcRn than N434A (YTE > AAA > N434A),[36,41] it did not enhance the antigen clearance.[18]

To the best of our knowledge, this is the first report showing Fc variants that exhibit enhanced binding to both human and cynomolgus FcγRIIb similarly. The Fc region of the antibody is desired to have cross-reactivity to non-human animals, especially to cynomolgus monkeys, which have restricted gene diversity and similar FcγR expression patterns,[42,43] so that the results obtained in non-human animals can be extrapolated into humans more accurately. In addition, the amount of endogenous IgG in cynomolgus monkeys, which competes with FcγRIIb binding, is much higher than in mice and comparable in humans.[44–46] Cynomolgus monkeys are better suited to accurately predict sweeping efficiency and antibody pharmacokinetics in humans, which means the good cross-reactivity of our novel Fc to cyFcγRs is very useful for predicting these profiles in humans based on the data obtained from cynomolgus monkey studies. However, extrapolation to humans is still not ideal, for it is difficult to obtain an Fc variant that can distinguish cyFcγRIIb from cyFcγRIIa due to their high homology. As shown in Table 2, substitutions that increase cyFcγRIIb binding also enhance cyFcγRIIa binding, whereas the variant V3 successfully distinguishes huFcγRIIb from huFcγRIIa. This difference can make it harder to extrapolate monkey data to humans, especially when FcγRIIa is involved in the main mechanism of action.

In terms of antigen sweeping, pH-V3-N434A did not strongly sweep the antigen in cynomolgus monkeys. We can further engineer the Fc variant for even stronger binding to cyFcγRIIb, which is likely to achieve even stronger antigen sweeping. As discussed in the previous paragraph, however, increasing the cyFcγRIIb binding most likely enhances the cyFcγRIIa binding as well, even though these variants distinguish huFcγRIIb from huFcγRIIa. As a result, there is a large difference between huFcγRIIa binding and cyFcγRIIa binding, making it difficult to extrapolate the monkey data to humans. Given these limitations, strong antigen sweeping achieved through a combination of positive-charge substitutions and FcγRIIb-enhanced binding can greatly broaden the clinical applicability of sweeping technology.

In conclusion, we developed novel Fc variants combining positive-charge substitutions, selectively enhanced binding to both human and cynomolgus FcγRIIb, and the N434A substitution. Combined with pH-dependent antigen-binding, our Fc variant achieved strong antigen sweeping in cynomolgus monkeys with good antibody pharmacokinetics, comparable to wild-type IgG$_1$. We expect this novel Fc engineering to achieve stronger antigen sweeping efficacy even in humans, enabling the development of novel therapeutic antibodies that can meet the unmet medical needs of patients.

Materials and methods

Ethics statement

Mice studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical Co. under the approval of the company's Institutional Animal Care and Use Committee. The company is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (http://www.aaalac.org). Cynomolgus monkey studies were also performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Shin Nippon Biomedical Laboratories Ltd. under the approval of the company's Institutional Animal Care and Use Committee.

Protein preparation

Recombinant latent myostatin and anti-latent myostatin antibodies were prepared by the method described previously.[29] For the preparation of FcγRs, the genes encoding the extracellular region of human and cynomolgus FcγRs were synthesized based on the sequence information obtained from the National Center for Biotechnology Information or from the results of cDNA cloning conducted in house, respectively. FcγRs were fused with His-tag at their C-terminus. FreeStyle293-F cells (Invitrogen) were transfected with the vectors encoding FcγRs. The receptors were purified from the harvested culture supernatants by using ion exchange chromatography, nickel affinity chromatography, and size exclusion chromatography.

Affinity measurement to latent myostatin

The NpH-IgG$_1$ and pH-IgG$_1$ antibodies were derived from anti-latent myostatin antibodies.[29] The kinetic parameters of these antibodies against human and cynomolgus monkey latent myostatin were evaluated at 37°C at pH 7.4 or pH 5.8 using a BIACORE® T200 instrument. ProA/G (Pierce) was

immobilized onto all flow cells on a CM4 chip using the amine coupling kit (GE Healthcare). Anti-latent myostatin antibody and analytes were prepared in ACES pH 7.4 (20 mM ACES, 150 mM NaCl, 1.2 mM $CaCl_2$, 0.05% Tween 20, 0.005% NaN_3). Antibody was captured onto the sensor surface by ProA/G, and human and cynomolgus latent myostatin was used as analyte. Kinetic parameters were determined by processing and fitting the data to a 1:1 binding model using BIACORE® T200 Evaluation software, version 2.0 (GE Healthcare).

Generation of Fc variants and analysis of FcγRs and FcRn binding

The Fc variants were generated by introducing substitutions in the human IgG_1 constant region of an anti-latent myostatin antibody. The binding profile of these antibodies to FcγRs or FcRn was evaluated using BIACORE® T200 (GE Healthcare). Phosphate buffer pH 7.4 was used as the running buffer for the evaluation of FcγRs, whereas phosphate buffer pH 6.0 was used for that of FcRn, and the measurements were conducted at 25°C. Mouse anti-human IgG kappa light chain (BD Biosciences, 555789) was immobilized onto a Series S Sensor Chip CM5 (GE Healthcare) by the amine-coupling method. Antibodies were then captured on the chip to interact with each FcγR or FcRn (prepared in house) that had been diluted with the running buffer, and the binding amount of antigen was measured. The binding amount of each FcγR was normalized with the amount of the captured antibody and was presented as relative values to IgG_1 to compare the binding ability of variants. For FcRn, the sensorgrams obtained as measurement results were analyzed by the steady state affinity model using the BIACORE® Evaluation Software to calculate the dissociation constant KD (mol/L).

Screening of charge substitutions using MDCK stable cell line

To evaluate the rate of internalization into a human FcγRIIb-expressing cell line, we established an MDCK (Madin-Darby canine kidney) cell line constitutively expressing human FcγRIIb. Antigen–antibody complexes were formed in a culture solution with the antibody concentration being 10 mg/mL and the pHrodoRed (Life Technologies) labeled latent myostatin concentration being 2.5 mg/mL. Then these solutions were added to culture plates containing MDCK stable cell line and incubated for one hour. The fluorescence intensity of the antigen inside the cells was quantified using InCell Analyzer 6000 (GE healthcare). PHrodoRed was used to specifically detect the internalized antigen present in the acidic environment. The amount of antigen internalized per cells was presented as relative values to human IgG_1.

Animals

For the cynomolgus monkey study, 2–4 years old Macaca fascicularis (cynomolgus monkey) from Cambodia (Shin Nippon Biomedical Laboratories Ltd., Japan) were used. Human FcRn transgenic mice were licensed from The Jackson Laboratory (supplier's reference, B6.Cg-*Fcgrt*^{tm1Dcr}Tg(FCGRT)32Dcr/DcrJ).[28] Mice with entirely humanized FcγRs kindly provided by Prof. Jeffrey V. Ravetch were bred and used in this study under the license agreement with The Rockefeller University (B6.Cg-Del(1Fcgr2b-Fcgr3)1Rav-*Fcgr1*^{tm1Hoga}-Tg(FCGR1A)#Jgjw-Tg(FCGR2A)11Mkz-Tg(FCGR2B)#Rav-Tg(FCGR3A)1156Rav-Tg(FCGR3B)1373Rav).[27]

In vivo study

For the cynomolgus monkey study, 30 mg/kg of antibody was administered as a single dose. For human FcRn transgenic mice, 1 mg/kg of pH-IgG_1, pH-pI(A), pH-pI(B) and pH-pI(C) were co-administered with 0.5 mg/kg of the mouse latent myostatin at a single dose of 10 ml/kg into the caudal vein. For all human FcγR transgenic mice, 3 mg/kg of pH-IgG_1, pH-V3 and pH-V3-pI(A) were co-administered with 0.5 mg/kg of the human latent myostatin at a single dose of 10 ml/kg into the caudal vein. In-house prepared anti-CD4 antibody (10 mg/kg) was also administered three times (every 10 days) at a dose of 10 ml/kg into the caudal vein to suppress anti-drug antibody in all human FcγR transgenic mice. The collected blood was centrifuged immediately to separate the plasma. Since the anti-CD4 antibody had human IgG Fc, which can affect the enzyme-linked immunosorbent assay (ELISA) results, we specifically used liquid chromatography electrospray ionization tandem mass spectrometric (LC/ESI-MS/MS) to detect test antibodies in all human FcγR transgenic mice. The dose was elevated to 3 mg/kg for each test antibody in all human FcgR transgenic mice because LC/ESI-MS/MS has a higher detection limit than ELISA.

Measurement of total antibody concentration in the pharmacokinetic study

To measure the concentration of the administered antibodies in monkeys, anti-human IgG antibody (Antibody Solutions, AS75-P) was immobilized onto maxisorp 96-well plates overnight before incubating in blocking buffer. Antibody calibration curve samples, quality control samples, and plasma samples were incubated on plates for 1 hour at room temperature before washing. Next, anti-human IgG-horseradish peroxidase (HRP) (Southern Biotech, 9040–05) was added and incubated for 30 min at room temperature before washing and ABTS substrate was incubated for 10, 20, and 30 min before detection with microplate reader at 405 nm.

To measure the concentration of the administered antibodies in human FcRn transgenic mice, Goat Anti-Human IgG (γ-chain specific) Biotin conjugate (Southern Biotech, 2040–08) was added to react for 1 hour at room temperature. Then, Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) was added to react for 1 hour at room temperature, and chromogenic reaction was carried out using TMB One Component HRP Microwell Substrate (BioFX Laboratories) as a substrate. After stopping the reaction with 1 N sulfuric acid (Showa Chemical), the absorbance at 450 nm was measured by a microplate reader. The antibody concentrations were calculated based on the response of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices).

The concentration of the administered antibodies in all human FcγR transgenic mice was measured by LC/ESI-MS /MS. Antibody calibration curve samples, quality control samples, and plasma samples were added to Ab-Capture Mag (ProteNova) and allowed to incubate for 2 hours at room temperature. Next, the magnetic beads were recovered from samples and washed. After washing, the magnetic beads were suspended in 7.5 mol/L urea, 8 mmol/L dithiothreitol, and 1 μg/mL lysozyme (chicken egg white) in 50 mmol/L ammonium bicarbonate and the suspended samples were incubated for 45 min at 56°C. Then, 500 mmol/L iodoacetamide was added and the samples were incubated for 30 min at 37°C in the dark. Next, Lysyl Endopeptidase digestion was carried out by adding Lysyl Endopeptidase for Biochemistry (Wako) and the samples were incubated for 3 hours at 37°C. Subsequently, tryptic digestion was carried out by adding sequencing grade modified trypsin (Promega). Samples were allowed to digest while mixing overnight at 37°C, and quenched by adding 10% trifluoroacetic acid. LC/ESI-MS/MS was performed using a Xevo TQ-S triple quadrupole instrument (Waters) equipped with 2D I-class UPLC (Waters). The concentration in mice plasma was calculated from the calibration curve using the analytical software Masslynx Ver.4.1 (Waters).

Measurement of total myostatin concentration in the pharmacokinetic study

The concentration of the total (mature + latent) myostatin in mouse and monkey plasma was measured by electrochemiluminescence. BIOTIN TAG labeled anti-mature myostatin antibody was coated onto a MULTI-ARRAY 96-well streptavidin plate (Meso Scale Discovery) before incubating in blocking buffer for 2 hours at room temperature in the cynomolgus monkey study. In-house prepared anti-mature myostatin antibody was immobilized to a MULTI-ARRAY 96-well plate by incubating overnight at 4°C in mouse studies. Myostatin calibration curve samples, quality control samples, and plasma samples were incubated with Glycine-HCl (pH 2.5) for 10 min, then, the samples were applied to the antibody plate and incubated for 1 hour on the plate at room temperature before washing. Next, SULFO TAG labeled anti-mature myostatin antibody (in-house prepared) was added and the plate was incubated for 1 hour at room temperature before washing in the cynomolgus monkey study. BIOTIN TAG labeled anti-mature myostatin antibody (in-house prepared) was added and the plate was incubated for 1 hour at room temperature before washing, and then the SULFO TAG labeled streptavidin (Meso Scale Discovery) was added and the plate was incubated for 1 hour at room temperature before washing in mice plasma. Read Buffer T (x2) (Meso Scale Discovery) was immediately added to the plate and the signal was detected by SECTOR Imager 2400 (Meso Scale Discovery). The total myostatin concentration was calculated based on the response of the calibration curve using the analytical software SOFTmax PRO (Molecular Devices).

Uptake and binding assay of immune complex

The human latent myostatin was labeled with Alexa Fluor 488 (Invitrogen). The Alexa Fluor 488-labeled human latent myostatin and anti-latent myostatin antibodies, pH-IgG_1, pH-V3, pH-pI(A) and pH-V3-pI(A) were mixed by equal ratio so that the final concentrations of them were 67 nM. Then the antigen–antibody mixture solution was incubated with 2×10^5 huFcγRIIb-expressing CHO cells or parent CHO cells for 20 min at 4°C for binding and 37°C for uptake. To inhibit FcγRIIb-mediated binding and uptake, anti-huFcγRIIb antibody (Clone 2B6; Creative Biolabs, TAB-036WM), which was reported to recognize the Fc binding site of huFcγRIIb,[40] and its isotype control antibody (R&D systems, MAB002) were used. After incubation, cells were washed with phosphate-buffered saline for binding or acidic medium (pH 3.5) for uptake to remove cell surface-bound antigen and antibody. Cellular fluorescence was measured by FACS Canto II (Becton, Dickinson and Company). Binding and uptake amount are represented by the geometric mean of the fluorescence intensity.

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Disclosure statement

All the authors are employees and/or shareholders of Chugai. YH, HK, TK and TI are listed as inventors of the patent, "Anti-myostatin antibodies, polypeptides containing variant Fc regions, and methods of use" (WO/2017/104783). These do not alter the authors' adherence to all the mAbs policies on sharing data and materials.

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