Inhibitory FcγRIIb-Mediated Soluble Antigen Clearance from Plasma by a pH-Dependent Antigen-Binding Antibody and Its Enhancement by Fc Engineering

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Inhibitory FcγRIIb-Mediated Soluble Antigen Clearance from Plasma by a pH-Dependent Antigen-Binding Antibody and Its Enhancement by Fc Engineering


Fc engineering can modulate the Fc–FcR interaction and thus enhance the potency of Abs that target membrane-bound Ags, but it has not been applied to Abs that target soluble Ags. In this study, we revealed a previously unknown function of inhibitory FcγRII in vivo and, using an Ab that binds to Ag pH dependently, demonstrated that the function can be exploited to target soluble Ag. Because pH-dependent Ab dissociates Ag in acidic endosome, its Ag clearance from circulation reflects the cellular uptake rate of Ag/Ab complexes. In vivo studies showed that FcγR but not neonatal FcR contributes to Ag clearance by the pH-dependent Ab, and when Fc binding to mouse FcγRII and III was increased, Ag clearance was markedly accelerated in wild-type mice and FcR γ-chain knockout mice, but the effect was diminished in FcγRII knockout mice. This demonstrates that mouse FcγRII efficiently promotes Ab uptake into the cell and its subsequent recycling back to the cell surface. Furthermore, when a human IgG1 Fc variant with selectively increased binding to human FcγRIIb was tested in human FcγRIIb transgenic mice, Ag clearance was accelerated without compromising the Ab half-life. Taken together, inhibitory FcγRIIb was found to play a prominent role in the cellular uptake of monomeric Ag/Ab immune complexes in vivo, and when the Fc of a pH-dependent Ab was engineered to selectively enhance human FcγRIIb binding, the Ab could accelerate soluble Ag clearance from circulation. We assume such a function would enhance the therapeutic potency of Abs that target soluble Ags. The Journal of Immunology, 2015, 195: 3198–3205.

Immunoglobulin G has a unique interaction with FcRs through its Fc region. Because FcRs are involved in various functions of IgG, Fc engineering to increase FcR binding has been applied to various Ab therapeutics to enhance their therapeutic potency (1, 2). For example, increasing the binding to human (h)FcγRIIa or hFcγRIIa has enhanced the ability of Abs that target tumor cells to induce cytotoxicity or phagocytosis. Moreover, increasing the binding to hFcγRIIb has enhanced the agonistic activity of Abs targeting the TNFR superfamily (3). Although these are examples of how Ab engineering significantly contributed to improving the therapeutic potency of Abs that target membrane-bound Ag, disease-relevant target Ags for a therapeutic Ab also include soluble Ags, such as cytokines and soluble receptors. Nevertheless, Fc engineering to modulate the interaction of Fc with FcγR has so far only been applied to Abs that target membrane-bound Ags.

Recently, we reported recycling Ab, an Ab with a novel modality that accelerates the clearance of targeted Ag in vivo by binding to the Ag at neutral pH and dissociating the Ag in acidic pH (4). This pH-dependent binding property of recycling Ab enables the Ab to bind to Ag in plasma and, after the Ab/Ag immune complex has been taken up into the cell, dissociate the Ag in the acidic endosome (Supplemental Fig. 3B). Because the dissociated Ag is transferred to the lysosome and degraded, the Ag clearance is accelerated and free Ab without the Ag is recycled back to plasma. This is in sharp contrast to the action of a conventional Ab, which continues to bind the Ag in the acidic endosome and thereby prevents soluble Ag from being degraded (Supplemental Fig. 3A) and causes the Ag to accumulate in circulation (5–10).

Recycling Ab can accelerate Ag clearance by dissociating the Ag in acidic endosome, but first the Ag/Ab immune complex must be taken up into the endosome. It has long been said that a large to midsize multivalent immune complex is internalized and cleared.
by hepatic FcγR via multivalent binding and cross-linking of the Fc to FcγR. In contrast, a monomeric immune complex containing a single Fc, that is, a complex of 1:1 or 1:2 formed by one Ab with one or two Ags, is not internalized by FcγR, because the monovalent interaction between Fc and FcγR is weak (11–15). Moreover, studies have shown that FcγR does not affect the clearance of Ab itself, which suggests that FcγR does not contribute to the internalization and clearance of monomeric immune complex in vivo (16, 17). Thus, we previously assumed that the cellular uptake of monomeric immune complexes by recycling Ab was mediated by nonspecific uptake or pinocytosis, not by FcγR-dependent uptake.

We previously reported that the intracellular uptake of a monomeric immune complex of pH-dependent Ab with human soluble (hs)IL-6R could be accelerated by enhancing the neonatal FcR (FcRn) binding at neutral pH, but the innate mechanism of intracellular uptake of the monomeric immune complex was not studied in detail (4, 18). In this study, to investigate the innate uptake pathway, we took advantage of a specific property of pH-dependent Ab to examine the intracellular uptake of immune complexes; namely, that Ag clearance from circulation by pH-dependent Ab in vivo equates to the cellular uptake rate of a complex. Because our studies in wild-type mice revealed an unexpected contribution of FcγR to the uptake and Ag clearance even in the case of a monomeric immune complex, we extended the study to investigate whether engineering the Fc to increase the binding affinity to FcγR would enhance Ag clearance in wild-type and various FcγR knockout mice and, furthermore, we sought to confirm that when the Fc is engineered to selectively increase the binding to specific human FcγR, the therapeutic potential of pH-dependent binding Abs against soluble Ags can be enhanced.

Materials and Methods

Ethics statement

Animal 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 Accreditation and Accreditation of Laboratory Animal Care International (http://www.aaalac.org).

Generation of anti–IL-6R Abs with increased binding affinity to mFcγRs at neutral pH

A pH-dependent binding Ab against hsIL-6R (PH-IgG1) was generated from a non-pH-dependent hsIL-6R binding Ab (NP-H-IgG1), as previously described (4). To increase the binding affinity to mouse FcγRs at neutral pH, various Fc-engineered variants were generated by site-directed mutagenesis of hIgG1 and mouse (mIgG1). Effective mutations were identified and combined to generate Fc variants with increased binding affinity to FcγRs at neutral pH. The generated variants were assessed for their binding affinity to recombinant mFcγRs (19) at pH 7.4 using Biacore T200 (GE Healthcare). The interaction of each variant with FcγRs was monitored using Biacore instruments (GE Healthcare), as previously described (20). Ab variants were captured on a CM5 sensor chip (GE Healthcare) on which protein A/G (Thermo Scientific) had been immobilized, and FcγRs were then injected. The binding of each variant to each FcγR was normalized by the amount of Ab captured on the sensor chip and was expressed as a percentage of that of IgG1. Kinetic analysis was performed by global fitting of binding data with a 1:1 Langmuir binding model using Biacore evaluation software (GE Healthcare). Fc variants with the desired affinity to mFcγRs were identified. Abs against hsIL-6R with pH-dependent Ag binding and their Fc variants were expressed transiently using HEK293 cells and purified by protein A.

Animals

C57BL/6J mice (wild-type mice) were purchased from Charles River Laboratories and hFcRn transgenic (Tg) mice were licensed from The Jackson Laboratory (supplier’s reference, B6.Cg-Fcgr2tm1Scn/Fccgr2tm1Scn; Tg[FCGRT] 32Dcr/Dcr3). C57BL/6J mice deficient in γ-chain subunits of the FcγRI, FcγRII, and FcεRI receptors (mFcγRIγ-chain knockout mice; supplier’s reference, FcγRIγ-chain−/−; B6.129P2-Fcεr1tm1Lkm/J) and FcγRIII knockout mice (supplier’s reference, FcγRIIa−/−; B6.129SA4-Fcγr2b−/−) were purchased from Taconic, and FcγRIIIB knockout mice (supplier’s reference, Fcγr3−/−; B6.129P2-Fcγr3tm1W/L) were purchased from The Jackson Laboratory.

Generation of hFcγRIIB Tg mice

A hFcγRIIB expression vector was constructed by modifying a bacterial artificial chromosome genomic library clone that contains all the exons of the human FcγRIIB gene with ~30-kbp upstream and downstream regions. The hFcγRIIB vector was microinjected into the pronuclei of fertilized oocytes of C57BL/6N (C57BL/6NCr, Charles River Laboratories) mice. Expression of hFcγRIIB in the transgenic mice was analyzed by RT-PCR and flow cytometry.

In vivo study of single doses of Abs in a steady-state model of hFcRn Tg mice, wild-type mice, and mFcγR knockout mice

An infusion pump (Alzet) filled with 92.8 g/ml hsIL-6R was implanted under the skin on the back of wild-type mice or hFcRn Tg mice (21) to prepare a mouse model with a constant plasma concentration of hsIL-6R. Monoclonal anti-mouse CD4 Ab, GK1.5 (22) was administered by i.v. injection to inhibit the production of mouse Ab against hsIL-6R by deleting CD4+ T cells. Abs against hsIL-6R were administered at 1 mg/kg to wild-type mice or hFcRn Tg mice with or without a single i.v. injection of 1 g/kg IVIG (CSL Behring) to mimic endogenous hlgG. Plasma anti-hsIL-6R Ab concentration in the presence of hlgG was determined using an anti-idiotypic Ab coated on ELISA 96-well plates, and detected by streptavidin–poly-HRP80 (Stereospecific Detection Technologies) using peroxidase substrate. Plasma total hsIL-6R and Ab concentrations in the absence of hlgG were determined as previously described (4).

In vivo study of single doses of Abs in wild-type mice and an hFcγRIIB Tg mouse coinjection model

In a coinjection model, wild-type mice or hFcγRIIB Tg mice were i.v. given single doses of 50 μg/kg hsIL-6R and 1 mg/kg anti–IL-6R Abs. Plasma total hsIL-6R and Ab concentration in the absence of hlgG were determined as previously described (4).

Results

Uptake mediated by FcγR, not FcεR, contributes to Ag clearance by a pH-dependent IgG1 Ab in mice

To elucidate whether native IgG1 uses a cellular uptake pathway other than nonspecific pinocytosis in vivo, we first evaluated the effect of an excess amount of IVIG on the clearance of Ags by PH-hlgG1 in an hFcRn Tg mouse steady-state model. Characteristics of Abs used in this study are summarized in Fig. 1A. Injection of 1 g/kg IVIG resulted in higher accumulation of Ags after an injection of PH-hlgG1 (Fig. 1B), which indicates that IVIG competes with a monomeric immune complex of PH-hlgG1 for intracellular uptake.

Because IVIG binds to both hFcRn and/or mFcγRs expressed in hFcRn Tg mice, IVIG can compete with either hFcRn- or mFcγR-mediated uptake of an immune complex formed by PH-hlgG1. Therefore, we investigated whether hFcRn and/or mFcγR contributes to the Ag clearance by PH-hlgG1. To test the contribution of hFcRn, we generated a variant of PH-hlgG1 in which hFcRn binding is abrogated [PH-hlgG1-FcRn(−)] (20). Injection of PH-hlgG1-FcRn(−) to hFcRn Tg mice exhibited an Ag accumulation level similar to PH-hlgG1, which demonstrates that hFcRn does not contribute to the uptake of a monomeric immune complex of PH-hlgG1 (Fig. 1B). Next, we generated a variant of PH-hlgG1 in which mFcγR binding is abrogated [PH-hlgG1-FcγR(−)] and injected it into hFcRn Tg mice. Ag accumulation with the PH-hlgG1-FcγR(−) Ab was increased over that of PH-hlgG1 and was similar to that of PH-hlgG1 in the presence of IVIG, but was not itself affected by IVIG (Fig. 1B). These results demonstrate that mFcγR contributes to the intracellular uptake of monomeric
**FIGURE 1.** FcγR but not FcRn contributes to the Ag clearance of a pH-dependent binding Ab. (A) Ab variants used in (B) and (C) are described. (B and C) Effect of Abs on the total hsIL-6R plasma concentration was evaluated in a steady-state model using hFcRn Tg mice or wild-type mice. Steady-state plasma concentration of ∼20 ng/ml hsIL-6R was maintained using an infusion pump filled with hsIL-6R solution. The time profiles of total hsIL-6R plasma concentration are shown. (B) PH-hIgG1 (■), PH-hIgG1-FcRn(−) (●), and PH-hIgG1-FcγR(−) (○ with dashed line) were i.v. administered to hFcRn Tg mice as single doses of 1 mg/kg, and PH-hIgG1 ( ○) and PH-hIgG1-FcγR(−) ( ● with solid line) were i.v. administered to hFcRn Tg mice as single doses of 1 mg/kg together with 1 g/kg IVIG. Plasma hsIL-6R concentration without Ab was set as baseline ( ○). An asterisk indicates a statistically different level of hsIL-6R between PH-hIgG1 and PH-hIgG1-FcγR(−) on day 7. (C) NPH-mIgG1 ( ○ with solid line), NPH-mIgG1-FcγR(−) (○ with dashed line), PH-mIgG1 ( ■ with solid line), PH-mIgG1-FcγR(−) ( ● with solid line), and PH-mIgG1-FcRn(−) ( △ with solid line) were i.v. administered as single doses of 1 mg/kg. An asterisk indicates statistically different levels of hsIL-6R between PH-mIgG1 and NPH-mIgG1, NPH-mIgG1-FcγR(−), or PH-mIgG1-FcγR(−) on day 7. Each datum point represents the mean ± SD (n = 3 each). Statistical significance was determined by a Dunnett test. *p < 0.05.

immune complexes but hFcRn does not, and that an excess amount of IVIG inhibits mFcγR-mediated internalization by competing for mFcγR binding.

Next, we injected steady-state normal mice with five different Ab variants (Fig. 1C): a pH-dependent anti–hsIL-6R Ab with engineered mIgG1, in which mFcγR binding is abrogated [PH-mIgG1-FcγR(−)]; a non-pH-dependent anti–hsIL-6R Ab with engineered mIgG1 (NPH-mIgG1); a pH-dependent anti–hsIL-6R Ab with engineered mIgG1, in which mFcγR binding is abrogated [NPH-mIgG1-FcγR(−)]; and a pH-dependent anti–hsIL-6R Ab with engineered mIgG1, in which mFcγR binding is abrogated [NPH-mIgG1-FcγR(−)]; and a pH-dependent anti–hsIL-6R Ab with engineered mIgG1, in which mFcγR binding is abrogated [NPH-mIgG1-FcγR(−)]. Consistent with the study in hFcRn Tg mice, PH-mIgG1-FcγR(−) had higher Ag accumulation than did PH-mIgG1, which demonstrates the contribution of mFcγR to the intracellular uptake of a complex formed from an Ag and PH-mIgG1 Ab. In contrast, the plasma Ag concentration of PH-mIgG1-FcRn(−) was the same as that of PH-mIgG1, which means that a monomeric immune complex formed from an Ag with PH-mIgG1 is not internalized by mFcRn. Alternatively, the extent of Ag accumulation induced by NPH-mIgG1 and NPH-mIgG1-FcγR(−) was comparable, which is consistent with the fact that the Ab clearance of NPH-mIgG1 and NPH-mIgG1-FcγR(−) was comparable (data not shown). The different effect of FcγR binding on the levels of Ag accumulated by NPH-mIgG1 and PH-mIgG1 indicates that, although mFcγR contributes to the uptake of a monomeric mIgG1 immune complex, most of the internalized mIgG1 Ab is recycled back to plasma, regardless of whether it still binds hsIL-6R.

Enhancing FcγRII and III binding but not FcγRI and IV binding accelerates Ag clearance by a pH-dependent hIgG1 Ab in hFcRn Tg mice

Having determined FcγR as the receptor responsible for Ag clearance by a pH-dependent Ab, we were motivated to test whether enhancing FcγR binding could accelerate the Ag clearance. Because mice have four different FcγRs, namely mFcγRI, II, III, and IV, Fc engineering enabled us to prepare three Ab Fc variants with different profiles of enhanced mFcγR binding (Table I).

An afucosylated variant of PH-hIgG1 (PH-hIgG1-Fx), which was reported to have selectively higher affinity to mFcγR than to wild-type hIgG1 (23), showed Ag accumulation similar to PH-hIgG1 with 100-fold higher affinity to both mFcγR and hFcRn binding. Because mFcγR-mediated internalization of IVIG inhibits mFcγR-mediated internalization by competing for mFcγR binding.
only marginal reduction of Ag accumulation (Fig. 2). These results demonstrate that Ag clearance by a pH-dependent Ab could be accelerated by enhancing the binding affinity to mFcRyRII and III, and thus suggest that mFcRyRII and/or III are the main contributors to the intracellular uptake of monomeric immune complexes.

To accelerate Ag clearance by enhancing the FcγyR binding, pH-dependent binding is indispensable

To examine whether Ag clearance could be accelerated simply by increasing mFcγRII/III binding without using a pH-dependent Ab, we compared the effect of enhancing the mFcγRII/III binding of a non–pH-dependent binding Ab (i.e., a conventional Ab) with that of a pH-dependent Ab in wild-type mice. In this study, we used wild-type mlgG1 as a control and an engineered mlgG1 with enhanced mFcγRII/III binding (mlgG1-Fx) (Table II). The Ab pharmacokinetics of pH-dependent and non–pH-dependent Abs was comparable when the same C region was used, and enhancing the mFcγRII/III binding accelerated the clearance of the Ab itself ∼5-fold (Fig. 3A). Because the Ag stays bound to a non–pH-dependent Ab and both the Ab and the Ag is cleared from circulation at the same rate, enhanced mFcγRII/III binding also reduced Ag accumulation of a non–pH-dependent Ab ∼4-fold, which is consistent with the 5-fold accelerated Ab clearance. Alternatively, Ag accumulation was reduced by ∼30-fold with the pH-dependent Ab with enhanced mFcγRII/III binding (Fig. 3A). These results demonstrate that just enhancing Fc binding to mFcγRII/III is not enough, and pH-dependent binding is indispensable to effectively accelerate the Ag clearance by enhancing the FcγyR binding.

Inhibitory receptor mFcγRII is the main contributor to Ag clearance by a pH-dependent Ab in mice

The studies using PH-mlgG1-Fy and PH-mlgG1-Fx (Figs. 2, 3A) suggested that mFcγRII and/or III contribute mainly to the Ag clearance achieved by a pH-dependent Ab. However, in hFcRn Tg mice or wild-type mice it was difficult to examine the effect of mFcγRII and III separately. Because mFcγRII and III have high sequence homology, it was not feasible to selectively enhance the binding affinity to one or the other. To distinguish between the contribution of mFcγRII and III, we used wild-type mice and three types of knockout mice that lacked either a common γ-chain (FcγRII knockout mice), FcγRII (FcγRII knockout mice), or FcγRIII (FcγRIII knockout mice). We engineered mlgG1 to prepare two variants: one with diminished binding to all mFcγRs [mlgG1-FcγR(−)] and one with 100-fold enhanced binding to both mFcγRII and III (mlgG1-Fy) (Table II). The pH-dependent Abs with mlgG1, mlgG1-FcγR(−), and mlgG1-Fy [PH-mlgG1, PH-mlgG1-FcγR(−), and PH-mlgG1-Fy] were injected to wild-type mice, FcR γ-chain knockout mice, FcγRIII knockout mice, and FcγRIII knockout mice, respectively (Fig. 4).

PH-mlgG1-Fy markedly accelerated the Ag clearance and reduced Ag plasma concentration to a level below the baseline in wild-type mice. The increased Ag clearance shown by PH-mlgG1-Fy in wild-type mice was mostly diminished in FcγRII knockout mice, but it was largely maintained in FcR γ-chain knockout mice and FcγRIII knockout mice. The difference in Ag clearance among the different mice was not significant when using PH-mlgG1-FcγR(−), which lacked mFcγR binding. These results demonstrate that mFcγRII, which is an inhibitory FcγR, contributes strongly to the Ag clearance by a pH-dependent Ab in mice, which indicates that mFcγRII contributes to the intracellular uptake of monomeric immune complexes.
Fc engineering to selectively enhance the hFcγRIIb binding accelerates the Ag clearance by a pH-dependent hIgG1 Ab in hFcγRIIb Tg mice

To evaluate in vivo efficacy, we generated eight lines of hFcγRIIb Tg mice and confirmed their expression of hFcγRIIb mRNA and protein by RT-PCR and flow cytometry (data not shown), respectively. Two of these lines, nos. 90 and 23-1, were used for further study. To translate the enhanced Ag clearance of a pH-dependent Ab when binding affinity to mFcγRII is increased into the clinical situation, we generated a pH-dependent Ab with an hlgG1 variant that had selectively increased binding affinity to hFcγRIIb, and not to any other hFcγRs or mFcγRs (PH-v12) (24). PH-v12 shows ∼100-fold enhanced binding to hFcγRIIb compared with PH-hlgG1 (Table I). The effect of the increased binding to hFcγRIIb on Ag clearance by this pH-dependent hlgG1 Ab was evaluated in hFcγRIIb Tg mice.

Because a technical issue of hFcγRIIb Tg mice made it impossible to establish an hsIL-6R steady-state model using an infusion pump, we assessed the effect of the PH-v12 Ab on Ag clearance with an Ag/Ab coinjection study, as previously described (4, 18). To compare mFcγRII and hFcγRIIb, we first evaluated PH-mlgG1 and PH-mlgG1-Fy in the Ag/Ab coinjection study using FcγRIII knockout mice, because PH-mlgG1-Fy in FcγRIII knockout mice results in selective increased binding to mFcγRII. As observed in the hsIL-6R steady-state model, PH-mlgG1-Fy enhanced the Ag clearance over that of PH-mlgG1 (Fig. 5A) without significantly compromising the Ab half-life (Fig. 5B). Next, we evaluated PH-hlgG1 and PH-v12 in the Ag/Ab coinjection study using hFcγRIIb Tg mice. Similar to PH-mlgG1-Fy in wild-type mice, PH-v12 also accelerated the Ag clearance over that of PH-hlgG1 in both hFcγRIIb Tg mouse lines nos. 90 and 23-1 (Fig. 5C, data for no. 23-1 not shown), but not in wild-type mice (Supplemental Fig. 1). Furthermore, increasing the binding affinity to either mFcγRII or hFcγRIIb did not alter the Ab pharmacokinetics (Fig. 5D).

Discussion

In this study, we report the involvement of inhibitory FcγRII in the intracelluar uptake of monomeric immune complex in vivo and show that this function can be exploited in a therapeutic Ab that targets soluble Ag by engineering enhanced FcγRII binding into the Fc region. These findings were only revealed by using a pH-dependent Ab that dissociates the Ag in acidic endosome, a property that enabled us to separately evaluate 1) the intracellular uptake rate by measuring Ag clearance, and 2) the recycling property of the Ab after internalization by comparing Ag clearance and Ab clearance.

First, by using various pH-dependent Abs, we revealed that inhibitory FcγRII is capable of intracellular uptake of monomeric immune complexes without cross-linking the receptor (which is reflected by the accelerated Ag elimination mediated by FcγRII), and that after internalization of immune complexes followed by dissociation of the Ag, it does not transfer the Ab into the lysosome but rather the Ab is efficiently recycled back to the cell surface (which is reflected by the rather long half-life of Ab compared with Ag) (Supplemental Fig. 3C). This recycling property of the monomeric immune complex after FcγRII-mediated internalization makes it difficult to examine FcγRII-mediated internalization in vivo using a non pH-dependent Ab (i.e., a conventional Ab), and we assume that this is the reason for this observed paradox of a significant Ag clearance with a poor Ab clearance.

Table II. Mutations and FcγR binding affinity of mlgG1 Fc variants

<table>
<thead>
<tr>
<th>Fc Variant</th>
<th>Mouse FcγRI</th>
<th>Mouse FcγRII</th>
<th>Mouse FcγRIV</th>
<th>Human FcγRIIb</th>
<th>Mutations</th>
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<tbody>
<tr>
<td>mlgG1</td>
<td>ND</td>
<td>1.1 × 10⁻⁷</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>mlgG1-FcγR(−)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>mlgG1-Fx</td>
<td>ND</td>
<td>4.6 × 10⁻¹⁰</td>
<td>6.5 × 10⁻¹⁰</td>
<td>8.7 × 10⁻⁷</td>
<td>NT</td>
</tr>
<tr>
<td>mlgG1-Fy</td>
<td>ND</td>
<td>1.2 × 10⁻⁹</td>
<td>3.6 × 10⁻⁹</td>
<td>ND</td>
<td>NT</td>
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<tr>
<td></td>
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<td></td>
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<td>S239D/K268D/A327D</td>
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</table>

The $K_D$ of mlgG1 and Fc variants and the mutations introduced in the Fc region are shown. Mutation sites in the Fc region are described in EU numbering.

ND, not detected; NT, not tested.

FIGURE 3. pH-dependent Ag binding is required for FcγR-mediated Ag clearance. The effect of pH-dependent Ag binding on FcγRII- or III-mediated Ag clearance and Ab pharmacokinetics in a normal mouse steady-state model with hsIL-6R concentration of ∼20 ng/ml is shown. NPH-mlgG1 (●), NPH-mlgG1-Fx (∆), PH-mlgG1 (■), and PH-mlgG1-Fx (□) were i.v. administered as single doses of 1 mg/kg. Time profiles of (A) Ab plasma concentration and (B) total hsIL-6R plasma concentration are shown. Each datum point represents the mean ± SD (n = 3 each). An asterisk indicates statistically different levels of hsIL-6R between PH-mlgG1-Fx and NPH-mlgG1-Fx or PH-mlgG1 on day 7. Statistical significance was determined by a Dunnett test. $^*p < 0.05$. 

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why FcγRII was not thought to be involved in the process of internalizing a monomeric immune complex.

Previous studies using conventional Abs have shown that FcγR binding does not affect Ab pharmacokinetics (16, 17), and they suggested that FcγR does not contribute to the uptake of a monomeric immune complex. Thus, it seemed plausible that FcγR binding is not involved in the Ag clearance of a conventional Ab because Ag bound to the Ab exhibits almost the same clearance as the Ab itself. Indeed, our study also showed that FcγR binding does not affect the Ag clearance of a conventional mIgG1 Ab in vivo (Fig. 1C). However, because these findings are based on the use of a conventional Ab that recycles most of the complexes back into circulation after FcγR-mediated internalization (Supplemental Fig. 3D), the actual ability of FcγR to take up monomeric immune complexes into the cell in vivo could not be evaluated. Alternatively, when we used a pH-dependent Ab, which dissociates the Ag within acidic endosome from where it is transferred to lysosome and degraded, the Ag clearance directly reflected the uptake of immune complexes into the cell (because all the internalized Ags are transferred to lysosome and degraded) and, consequently, our study could evaluate the intracellular uptake of immune complexes by FcγR.

This study showed that both abrogating Fc binding to FcγR and administering a large amount of endogenous hlgG i.v. reduced the amount of hsIL-6R eliminated by a pH-dependent Ab, whereas abrogating Fc binding of non–pH-dependent Ab to FcγR did not affect the level of hsIL-6R (Fig. 1). This indicates that, in the case of wild-type IgG1, FcγR contributes to the uptake of monomeric immune complexes into the cell in vivo could not be evaluated.

FIGURE 4. mFcγRII is the main contributor to the Ag clearance by a pH-dependent binding Ab. The effect of mFcγRII and mFcγRIII on Ag sweeping is shown in a mouse steady-state model with hsIL-6R concentration of ∼20 ng/ml. PH-mIgG1 (●), PH-mIgG1-FcγR(−) (□), and PH-mIgG1-Fy (△) were i.v. administered as single doses of 1 mg/kg in (A) wild-type mice, (B) common γ-chain knockout mice, (C) FcγRII knockout mice, and (D) FcγRIII knockout mice. Time profiles of total hsIL-6R plasma concentration in each type of mouse are shown. Each datum point represents the mean ± SD (n = 3 each). In (A), (B), and (D), an asterisk indicates statistically different levels of hsIL-6R between PH-mIgG1-Fy and PH-mIgG1 or PH-mIgG1-FcγR(−) on day 7. Statistical significance was determined by a Dunnett test. *p < 0.05.

FIGURE 5. A pH-dependent binding Ab with Fc engineering to selectively increase the hFcγRIIb binding enhanced Ag clearance while maintaining Ab pharmacokinetics in hFcγRIIb Tg mice. The effect of Abs on the total hsIL-6R plasma concentration was evaluated in a coinjection model. hsIL-6R and Ab were i.v. administered as single doses of 50 μg/kg for hsIL-6R and 1 mg/kg for Ab. PH-mIgG1 (●) and PH-mIgG1-Fy (□) were each coinjected with hsIL-6R in FcγRIII knockout mice, and time profiles of (A) total hsIL-6R plasma concentration and (B) Ab plasma concentration are shown. PH-hIgG1 (●) and PH-v12 (□) were each coinjected with hsIL-6R in hFcγRIIb Tg mice, and time profiles of (C) total hsIL-6R plasma concentration and (D) Ab plasma concentration are shown. Each datum point represents the mean ± SD (n = 3 each).
We applied the findings on FcγRII gained by our study, that is, that a pH-dependent Ab could accelerate Ag clearance in an FcγRII-dependent manner, to enhance the therapeutic potential of an mAb. We have recently shown that when Fc is engineered to confer FcRn binding at neutral pH, monomeric immune complexes can be taken up into the cell in an FcRn-dependent manner, and this will accelerate the Ag clearance of a pH-dependent Ab (18). However, this study showed that FcRn does not contribute to the uptake of monomeric immune complexes formed by wild-type hlgG1 (Fig. 1B), which is not surprising given that wild-type hlgG1 has negligible binding affinity to hFcRn at neutral pH (18). Alternatively, wild-type IgG1 does bind to FcγR at neutral pH (29–31), which is consistent with our finding that monomeric immune complexes can be taken up into the cell in an FcRγ-dependent manner. Therefore, enhancing this natural IgG1 uptake pathway by increasing the Fc binding affinity to FcγR also enables us to increase the Ag clearance of a pH-dependent Ab (Fig. 2). As our study using FcγR knockout mice revealed (Fig. 4), immune complexes were mainly taken up by mFcγRII, so the Ag clearance of a pH-dependent Ab could be successfully accelerated by increasing the binding affinity to mFcγRII to enhance Ag clearance was not observed when a non–pH-dependent, or conventional, Ab was used (Fig. 3B)—because Ag stays bound to the Ab within acidic endosome and is efficiently recycled back to the cell surface as an immune complex after mFcRγ-mediated internalization—and note that this novel application of Fc engineering to increase the binding affinity to mFcγRII and thus enhance the clearance of soluble Ag could only be revealed using a pH-dependent Ab.

As mentioned in the Introduction, the use of Fc engineering to modulate Fc–FcγR interaction has been limited to membrane-bound Ags and, to the best of our knowledge, this is the first report regarding soluble Ags. With the clinical application of this approach in mind, we confirmed that Fc engineering to enhance hFcγRIIb binding could also accelerate the Ag clearance of a pH-dependent Ab in hFcγRIIb Tg mice (Fig. 5). Importantly, the half-life of the Fc-engineered Ab was comparable to that of wild-type hlgG1, which indicated that the Ab was efficiently recycled back to plasma after hFcγRIIb-mediated internalization. As previously reported, our Fc engineering could selectively enhance the binding to inhibitory hFcγRIIb over that to other activating FcγRs, including the highly homologous Arg131 allele of hFcγRIIa (24, 32). Because activating hFcγRIIa is highly expressed on platelets and contributes to platelet activation (33, 34), we think that selective enhancement of FcγRIIb binding would be important for clinical application from the point of safety and pharmacokinetics.

In conclusion, the present study revealed a hitherto unknown function of inhibitory FcγRIIb to take up monomeric immune complexes into the cell and subsequently recycle them back to the cell surface with high efficiency. We demonstrated that this newly discovered function of inhibitory FcγRIIb could be exploited to accelerate the clearance of soluble Ag when the Fc of a pH-dependent Ab, not a conventional Ab, is engineered to increase the binding affinity to inhibitory FcγRIIb.

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Disclosures
All authors are full-time employees, part-time employees, or contractors of Chugai Pharmaceutical Co. Ltd.

References
Figure S1: A pH-dependent binding antibody with Fc engineering to selectively increase hFcγRIIb binding did not enhance antigen clearance in wild-type mice.

The effect of antibodies on the total hsIL-6R plasma concentration was evaluated in a co-injection model. hsIL-6R and antibody were intravenously administered as single doses of 50 µg/kg for hsIL-6R and 1 mg/kg for antibody. PH-hIgG1 and PH-v12 were each co-injected with hsIL-6R in wild-type mice and time profiles of (A) total hsIL-6R plasma concentration and (B) antibody plasma concentration are shown. Each data point represents the mean ± s.d. (n=2 or 3 each).
Figure S2: Sensorgrams of Fc variants binding to FcγRs

SPR sensorgrams for representative set of Fc variants as described in Table I, II are shown. X-axis represents time (sec) and y-axis represents binding response (RU). Each analysis were conducted as described in Materials and Method under several different concentrations of FcγR.
Supplemental Figure 3

Figure S3: Proposed mode of action of pH-dependent antibody in comparison with conventional antibody. (A) Conventional antibody bound to soluble antigen is slowly taken up by nonspecific pinocytosis. Since wild-type IgG1 antibody has weak FcγRII binding affinity, this interaction is diminished under high endogenous IgG competition. Antigen-antibody complex binds to FcRn in acidic endosome, and is recycled back to the cell surface and released from FcRn back to circulation. (B) pH-dependent binding antibody (recycling antibody) bound to soluble antigen is also nonspecifically taken up by pinocytosis as conventional antibody, and binds to FcRn in acidic endosome, while antigen is dissociated from the antibody, transferred into lysosome and degraded. Antibody is recycled back to the cell surface by FcRn, released from FcRn back to circulation and binds to another antigen, allowing single antibody to bind to antigen multiple times. (C) pH-dependent antibody with FcγRII binding capability bound to soluble antigen is taken up by FcγRII-mediated endocytosis. In acidic endosome, antigen is dissociated from the antibody, transferred into lysosome and degraded. Antibody is recycled back to the cell surface and released from FcγRII back to circulation to bind to another antigen. Enhanced FcγRII binding allows rapid FcγRII-mediated uptake and enhances lysosomal antigen degradation rate. (D) Conventional antibody with FcγRII binding capability bound to soluble antigen is taken up by FcγRII-mediated endocytosis. Antigen stay bound to the antibody in acidic endosome, and antigen-antibody complex is recycled back to the cell surface and released from FcγRII back to circulation. Although enhanced FcγRII binding allows rapid FcγRII-mediated uptake, but it does not significantly enhances lysosomal antigen degradation rate.