Humanization of Lead Chimeric Bispecific Antibody

The lead chimeric bispecific IgG antibody, BS15, was subjected to humanization. CDRs of the anti-FIXa heavy chain, the anti-FX heavy chain and the common light chain were grafted onto homologous human antibody FRs, which were the FRs of V_H3, V_H1 and $V_\kappa1$ subfamilies respectively, by using a conventional CDR grafting approach [28]. A humanized bispecific IgG_4 antibody, termed hBS1, was successfully generated while maintaining FVIII-mimetic activity (supplementary Fig. S3).

Improving FVIII-mimetic Activity of the Bispecific Antibody

Although the humanized bispecific antibody hBS1 enhanced FX activation dose-dependently, demonstrating FVIII-mimetic activity, its therapeutic potential was marginal and its FVIII-mimetic activity needed to be improved for therapeutic application. Therefore, we explored mutations in the CDRs of hBS1 to improve the FVIII-mimetic activity, and we identified several effective mutations in the CDRs of the three chains. Following extensive studies to identify effective combinations of mutations that additively or synergistically improved FVIII-mimetic activity, we successfully generated hBS106. hBS106 demonstrated marked improvement of FVIII-mimetic activity over hBS1, including maximum activity, in the enzymatic assay (Fig. 4A).

During the course of subsequent optimization of hBS106 from the point of other aspects, FVIII-mimetic activity was monitored for each mutation so that the mutation would not compromise the activity. Moreover, further screening for mutations to further improve the activity was performed in parallel with other optimizations. Finally, we successfully generated hBS910, whose activity was even higher than that of hBS106 (Fig. 4A).

Improving Pharmacokinetics of the Bispecific Antibody

To assess the pharmacokinetics of hBS106, the variant with improved FVIII-mimetic activity, the time course of the plasma concentration of this antibody (Fig. 4B) and its pharmacokinetic parameters (supplementary Table S2) were determined in mice. Clearance of hBS106 (67 mL/day/kg) was unexpectedly larger than the clearance of human IgG_4 antibody reported in mice after subcutaneous injection (3–20 mL/day/kg) [29].

A homology model of hBS106 was used to explore the molecular features responsible for the poor pharmacokinetics, and a positive charge cluster was identified on the surface of Fv of the anti-FIXa arm (supplementary Fig. S4). To remove this cluster, we initially attempted subjecting lysine and arginine residues in the cluster to mutagenesis. However, these residues were found to be indispensable for the FVIII-mimetic activity (data not shown). Therefore, we explored the introduction of negatively charged residues near the cluster to neutralize the positive charge, and we identified a Tyr30Glu mutation in the common light chain that achieved this without any reduction in FVIII-mimetic activity. With hBS128, a variant of hBS106 with the single Tyr30Glu mutation, we observed improved plasma concentration and an approximately 4-fold improvement in the clearance compared to hBS106. Furthermore, the isoelectric point (pI) of hBS128 was lowered by introducing multiple mutations in the variable regions. hBS228, a variant of hBS128 with lowered pI, demonstrated further improved plasma concentration and approximately 2-fold improvement in clearance compared to hBS128 (Fig. 4B, supplementary Table S2). During the course of subsequent optimization of hBS228, we constantly made an effort to further improve the pharmacokinetics of the bispecific antibody.

Isoelectric Point Engineering to Facilitate Purification of the Target Bispecific Antibody

Having a common light chain reduces the number of pairs of heavy and light chains to three, and engineering the C_H3 domain enables preferential secretion of heterodimerized heavy chains. However, it is still difficult to completely prevent miss-paired homodimerization in large-scale production. Therefore, a downstream purification process to remove homodimeric byproducts is essential for pharmaceutical development. Ion exchange chromatography (IEC) is the major purification process by which to remove impurities after Protein A purification. The retention of IgG antibodies by IEC is determined by the electrostatic charge of the antibody molecule, which can be measured as its pI. Therefore, the pIs of hBS128 and hBS228, variants with improved pharmacokinetics, and pIs of their homodimeric byproducts were determined by cIEF (Fig. 5A). For both hBS128 and hBS228, the pIs of the bispecific antibody and the homodimeric byproducts

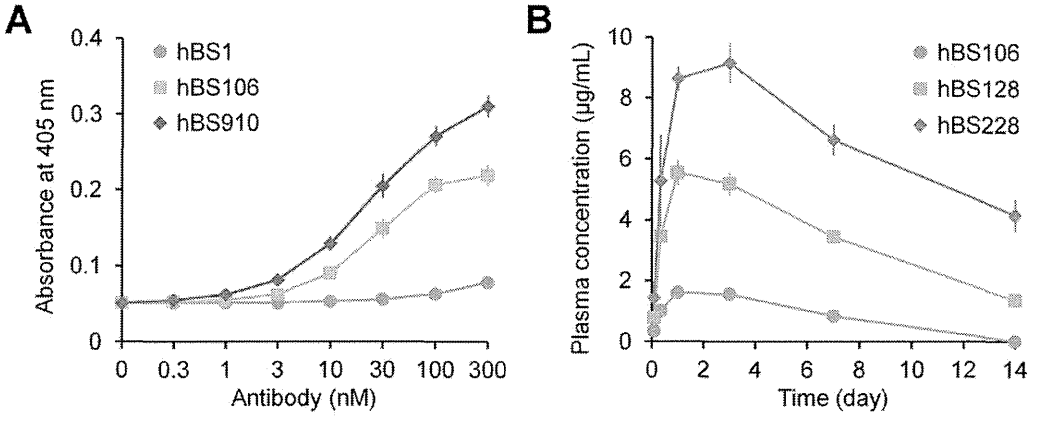


Figure 4. Improvement of therapeutic potential of the bispecific antibody. (A) Improving FVIII-mimetic activity of the bispecific antibody. Effect of hBS1 (circles), hBS106 (squares), and hBS910 (diamonds) on FX activation in the presence of FIXa, FX, and synthetic phospholipid is shown. The Y-axis indicates the absorbance at 405 nm of the chromogenic substrate assay (in many cases, the bars depicting s.d. are shorter than the height of the symbols). (B) Improving pharmacokinetics of the bispecific antibody. Time profiles of plasma concentration of hBS106 (circles), hBS128 (squares), and hBS228 (diamonds) in mice after subcutaneous injection at a dose of 1 mg/kg are shown. All the data were collected in triplicate and are expressed as mean ± s.d.
doi:10.1371/journal.pone.0057479.g004

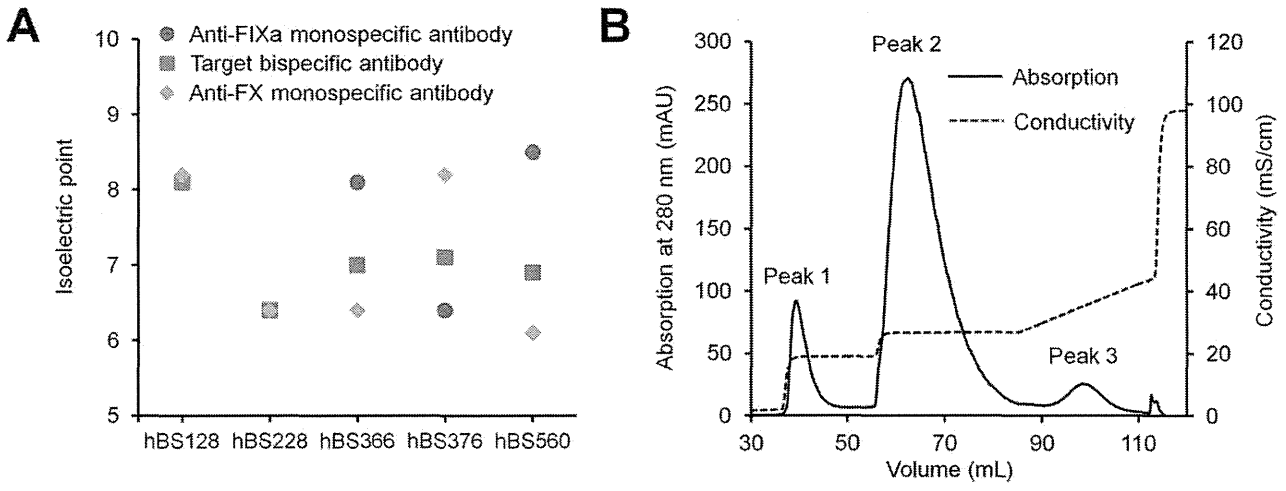


Figure 5. Isoelectric point engineering to facilitate purification of the target bispecific antibody. (A) Isoelectric points of target bispecific (squares) antibodies and homodimeric byproducts (anti-FIXa monospecific antibodies (circles) and anti-FX monospecific antibodies (diamonds)) determined by cIEF. (B) Cation exchange purification chromatogram of the target bispecific antibody of hBS560 from its homodimeric byproducts with step-wise elution with different NaCl concentrations. Peak 1, anti-FX homodimeric antibody; Peak 2, target bispecific antibody; Peak 3, anti-FIXa homodimeric antibody. Each peak area of peak 1, peak 2 and peak 3 was 9.9%, 85.7% and 4.4%, respectively.
doi:10.1371/journal.pone.0057479.g005

were very close to each other, indicating that purification of the bispecific antibody hBS128 or hBS228 from the mixture of homodimeric byproducts is not feasible.

To facilitate the purification of the bispecific antibody, we implemented pI engineering into either one of the heavy chain variable regions to increase the pI difference between the bispecific antibody and the homodimeric byproducts. We successfully generated two variants, hBS366 (pI of the anti-FX heavy chain is lowered) and hBS376 (pI of the anti-FIXa heavy chain is lowered), in which FVIII-mimetic activity was maintained. The difference in the pI between the bispecific antibody and homodimeric byproducts markedly increased (Fig. 5A).

hBS366 was further optimized from the point of solubility (detail in the next paragraph), to generate hBS560. The target molecular form of hBS560 (heterodimeric bispecific antibody) was well separated from the two homodimeric byproducts by using cation exchange chromatography with step-wise elution (Fig. 5B). During the course of subsequent optimization of hBS560, the pI difference between the two heavy chains was carefully maintained. The multidimensionally optimized variant hBS910 was capable of being purified on a large production scale (2500-liter fermentation).

Improving Solubility Properties of the Bispecific Antibody

hBS106, a variant with improved FVIII-mimetic activity, had unexpectedly low solubility, exhibiting either precipitation or liquid–liquid phase separation [30] (supplementary Fig. S5). This lack of solubility was partially due to the positive charge cluster, since the Tyr30Glu mutation described above markedly improved the solubility. However, for hBS376, the variant whose anti-FIXa heavy chain pI was lowered, at concentrations of 4 and 40 mg/mL, precipitation and phase separation still occurred in phosphate buffer of pH 5.5 to 7.0 and NaCl of 100 mM or less (Fig. 6A).

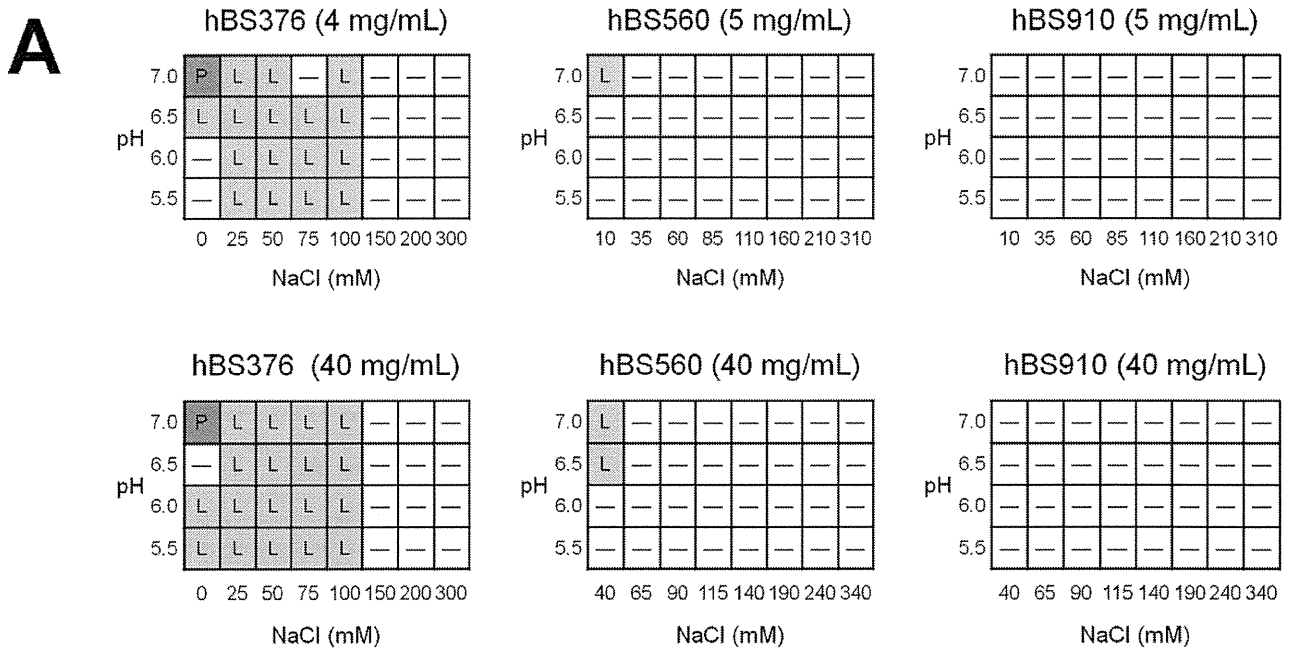
To improve the solubility of hBS376, mutations in the variable regions of hBS376 were explored. Several effective mutations including substitutions of hydrophobic residues into hydrophilic residues were identified, and their combinations successfully generated hBS560. Precipitation and phase separation of hBS560, the variant with improved solubility, was markedly suppressed and occurred only below 40 mM NaCl (Fig. 6A). The solubility of hBS560 was more than 100 mg/mL. During the course of subsequent optimization of hBS560, the effect of mutations on the solubility was constantly monitored and we made further efforts on improving the solubility. We successfully generated hBS910, a multidimensionally optimized variant, which did not exhibit precipitation or phase separation under the conditions tested (Fig. 6A). Furthermore, hBS910 could be concentrated up to at least 200 mg/mL.

Removing Deamidation Site in the CDR of the Bispecific Antibody

An accelerated stability study revealed that hBS560 exhibited asparagine deamidation in the third complementarity-determining region of the heavy chain (HCDR3) (Asn99) of the anti-FIXa arm after incubation at 40°C for 2 weeks, as shown by the increase in the acidic peak in the cation exchange chromatography analysis (Fig. 6B) [31], and reduction of FVIII-mimetic activity was observed (data not shown). A single mutation of Asn99 to another amino acid to remove this deamidation site was not feasible due to the loss of FVIII-mimetic activity and solubility. Subsequently, a double mutation was explored, and hBS660, in which a His98Arg and Asn99Glu double mutation was introduced to hBS560, was identified to maintain the FVIII-mimetic activity. hBS660 showed no increase in the acidic peak after incubation, demonstrating that the deamidation site was removed (Fig. 6B). During the course of subsequent optimization of hBS660, deamidation was carefully monitored, and successfully generated hBS910, a multidimensionally optimized variant, which did not exhibit deamidation.

Deimmunization of Humanized Bispecific Antibody by Removing T-cell Epitopes

During the course of multidimensional optimization of hBS1, the effects of each mutation on immunogenicity were evaluated by Epibase (Lonza), an *in silico* T-cell epitope prediction system [32]. Any mutation that was predicted to increase the potential



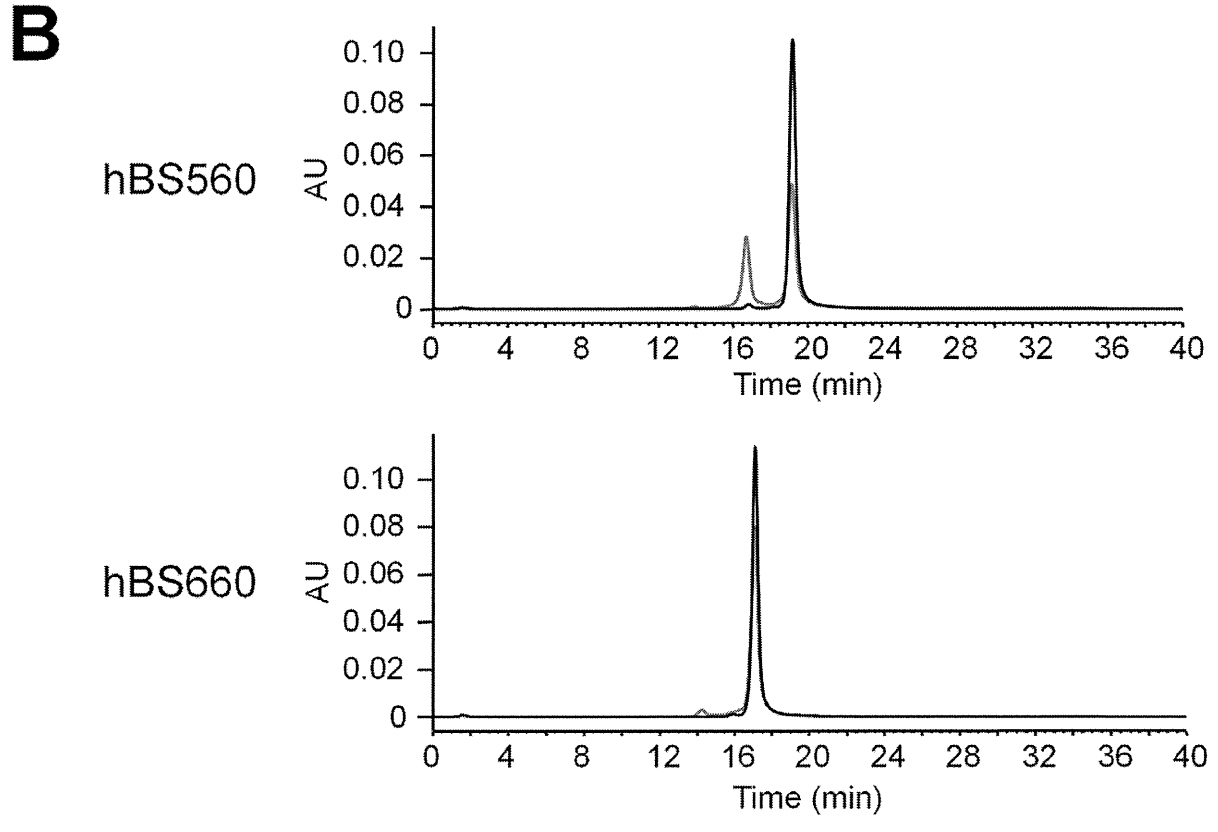


Figure 6. Improvement of pharmaceutical properties of bispecific antibodies. (A) Antibody solution profiles of hBS376 and hBS560 at different antibody concentrations, pH, and NaCl concentrations. The antibody solution under each condition was photographed and the state determined (P, precipitation; L, liquid–liquid phase separation; –, clear liquid). (B) Cation exchange chromatography of hBS560 and hBS660 before (black) and after incubation at 40°C for 2 weeks (red). Acidic peak indicating deamidation at HCDR3 increased after incubation at 40°C for 2 weeks for hBS560. No marked increase of acidic peak was observed for hBS660.
doi:10.1371/journal.pone.0057479.g006

immunogenicity risk was avoided as much as possible. Simultaneously, to generate a bispecific antibody with minimum immunogenicity risk, any mutation that was predicted to decrease the potential immunogenicity risk by reducing the number of T-cell epitopes was screened.

The immunogenicity risk score of hBS910, the multidimensionally optimized variant, was markedly decreased compared to the lead chimeric antibody (BS15) and its humanized version (hBS1), and was comparable to trastuzumab and palivizumab which are non-immunogenic in clinical (supplementary Fig. S6A). Moreover, EpiMatrix (EpiVax), another *in silico* immunogenicity prediction system [33,34], also predicted that the sequence of hBS910 was minimally immunogenic (supplementary Fig. S6B).

Therapeutic Potential of the Multidimensionally Optimized Bispecific Antibody, hBS910

To examine the therapeutic potential of hBS910, its FVIII-mimetic activity was compared with human FVIII by using thrombin generation assay (TGA) [23,35,36] in commercially available human FVIII-deficient plasma which was derived from a single donor with severe hemophilia A without FVIII inhibitors (Fig. 7A). hBS910 dose-dependently increased peak height (defined as the peak concentration of free thrombin) in the same manner as recombinant human FVIII (rhFVIII). The thrombin generation activity of hBS910 was also observed even in plasma of a hemophilia A donor who has FVIII inhibitors, whereas 1 IU/mL of rhFVIII did not exhibit any effects (data not shown). On the other hand, while polyclonal anti-idiotype antibodies to the anti-FIXa Fab or anti-FX Fab of hBS910 completely inhibited the activity of hBS910, they did not interfere with rhFVIII activity at all (data not shown).

To assess the potential for subcutaneous delivery with a long dosing interval, hBS910 was intravenously and subcutaneously administrated to cynomolgus monkeys, and the time course of the antibody plasma concentration and pharmacokinetic parameters were obtained (Fig. 7B, supplementary Table S3). The subcutaneous bioavailability was sufficiently high (86%) and the plasma half-life was approximately 3 weeks. Moreover, hBS910 could be formulated into a 150 mg/mL liquid formulation for subcutaneous delivery in the clinical setting without any significant aggregation or degradation during storage.

Discussion

The lead bispecific antibody was identified from approximately 40,000 different bispecific antibodies. Bispecific antibodies meeting the criteria for FVIII cofactor activity were extremely rare (<0.3%). This seems reasonable since such a bispecific antibody requires simultaneous binding to the appropriate epitope of both FIXa and FX in order to place these two factors into a spatially appropriate position and precisely bring the catalytic site of FIXa close to the cleavage site of FX. Requirement of simultaneous binding to FIXa and FX by a single bispecific antibody was supported by the fact that only a bispecific antibody, and neither monospecific antibodies nor a mixture of them, exhibited FVIII-mimetic activity (supplementary Fig. S7).

Generally, the biological activity of antagonistic antibodies can be improved by increasing the binding affinity to the target antigen [37,38]. The biological activity of agonistic antibodies, on the other hand, is reported to be inversely correlated with the affinity to the antigen, presumably due to the necessity to dissociate from the antigen to repeatedly induce agonistic signals to the target [39]. In the case of our bispecific antibody, the antibody needs to bind to both FIXa and FX with sufficient affinity to promote the

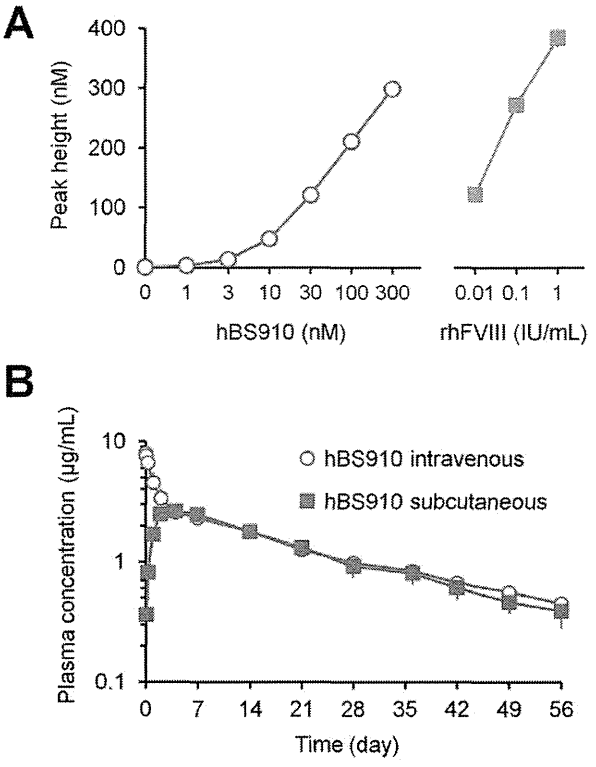


Figure 7. Therapeutic potential of multidimensionally optimized bispecific antibody, hBS910. (A) FVIII-mimetic activity of hBS910 in thrombin generation assay (TGA). Effect of hBS910 (circles) or recombinant human FVIII (squares) on thrombin generation in FVIII-deficient plasma is shown. The reaction was triggered by FXIa, synthetic phospholipid, and Ca^{2+}. The Y-axis indicates the peak height, a thrombin generation parameter (in many cases, the bars depicting s.d. are shorter than the height of the symbols). Data were collected in triplicate for each plasma lot and are expressed as mean ± s.d. (B) Pharmacokinetics of hBS910 in cynomolgus monkeys. Time profiles of plasma concentration of hBS910 after intravenous (circles) or subcutaneous (squares) injection are shown.
doi:10.1371/journal.pone.0057479.g007

interaction between the factors, while after FX activation by FIXa, FXa needs to be rapidly dissociated from the antibody to proceed to the subsequent coagulation reaction and to enable the antibody to turn over. Therefore, we assumed that a simple increase in the binding affinity to both FIXa and FX would not necessarily improve the FVIII-mimetic activity. Consequently, generation of variants that had improved activity required screening of a large number of variants in which mutations had been introduced via structure-based or random mutagenesis. These mutations were mainly introduced to the residues that were predicted to directly or indirectly contact the antigen by the homology modeling of the lead antibody or experimentally identified to affect FVIII-mimetic activity.

To gain insights into the mechanisms underlying the improvement in activity, kinetic analyses of hBS1, hBS106, and hBS910 (bispecific antibodies each with different FVIII-mimetic activity) binding to FIXa and FX were performed by SPR analysis (supplementary Fig. S8A, B). However, because the kinetic parameters of these antibodies were differently affected by the fitting conditions of the sensorgrams, we were not able to obtain meaningful kinetic parameters with which to compare these antibodies. Nevertheless, the binding properties of these antibodies

were obviously different, and these changes might have contributed to the improvement in activity. It appears that the binding affinity of hBS910 to FIXa is weaker than hBS1 and hBS106, and hBS910 has faster association and dissociation rate for FX. Although this tendency might result in rapid turnover of FX activation by the bispecific antibody and explain the highest FVIII-mimetic activity of hBS910, it does not explain the large difference of FVIII-mimetic activity between hBS1 and hBS106. Since bispecific antibody needs to strictly place catalytic site of FIXa to the cleavage site of FX and may require allosteric effect on FIXa in order to mimic the function of FVIII, it can be postulated that the improved FVIII-mimetic activity of hBS106 compared to hBS1 may be derived not only from the changes in the binding kinetics but also from the subtle changes in the binding epitope, binding angle or allosteric effect caused by the mutations introduced.

The FVIII-mimetic activity of 30 nM hBS910 was equivalent to rhFVIII activity at 0.01 IU/mL (1% of normal level), and the activity of 300 nM hBS910 was greater than rhFVIII activity at 0.1 IU/mL (10% of normal level) in the TGA using human FVIII-deficient plasma. The activity was also observed even in the presence of FVIII inhibitors, which is reasonable considering the lack of homology between the sequences of hBS910 and FVIII. Recently, we have demonstrated that FVIII-mimetic bispecific antibody, termed hBS23, exerts hemostatic activity *in vivo* in acquired hemophilia A model using cynomolgus monkey, which was considered sufficient for routine prophylaxis [23]. hBS23 is one of the FVIII-mimetic activity improved variants obtained during the multidimensional optimization to generate hBS910, and has similar FVIII-mimetic activity as hBS106. Kinetic analysis [23] of hBS23 and hBS910 demonstrated that hBS910 showed two times the effect on increasing k_{cat}/K_m compared to hBS23 (unpublished data). In human FVIII-deficient plasma, hBS910 required only two third of plasma concentration to exhibit the equivalent activity to hBS23 in the TGA, suggesting that hBS910 has 1.5 times the activity to promote thrombin burst (unpublished data). When compared with FVIII, this *in vivo* hemostatic effect was consistent with its *in vitro* activity in TGA. Thus, the above results clearly demonstrate that hBS910, which is more potent than hBS23, could exert FVIII-mimetic activity in hemophilia A patients sufficient to achieve routine prophylaxis regardless of the presence of FVIII inhibitors. The amino acid sequence of hBS23 is described in our patent as an antibody name Q153-G4k/J142-G4h/L180-k, and that of hBS910 is described in the patent [Igawa T, Sampei Z, Kojima T, Soeda T, Muto A, et al. Multi-specific antigen-binding molecule having alternative function to function of blood coagulation factor VIII. WO/2012/067176].

It is highly desirable that our bispecific antibody be able to be administered subcutaneously with a long interval between doses. The poor pharmacokinetics of hBS106 in mice was partially attributed to the large positive charge cluster, and the single Tyr30Glu mutation to neutralize this charge cluster markedly improved the pharmacokinetics. Such a charge cluster may increase non-specific binding to the extracellular matrix which would increase the clearance of the molecule. Recently, it was reported that antibodies with lower pI have better pharmacokinetics, and engineering antibodies to lower the pI improved their pharmacokinetics [29]. This approach was successfully applied to our bispecific antibody. hBS910 exhibited a plasma half-life of approximately 3 weeks in cynomolgus monkeys, which is longer than the half-lives of hBS23 (approximately 2 weeks) [23] and other humanized or fully human IgG antibodies [40,41], presumably due to the benefit of pI engineering. Since the half-lives of IgG antibodies are generally longer in humans than in cynomolgus monkeys [42], we expect that hBS910 would have a half-life of at least 3 weeks in humans, which is overwhelmingly longer than that of exogenous FVIII (0.5 days) [4]. Furthermore, hBS910 exhibited 86% subcutaneous bioavailability, consistent with that of other IgG antibodies [24,43,44], providing a huge advantage over exogenous FVIII which requires intravenous administration [5]. This high subcutaneous bioavailability and long half-life of hBS910 strongly supports the feasibility of routine prophylaxis by subcutaneous administration with a long dosing interval.

We utilized an *in silico* T-cell epitope prediction system, Epibase, to minimize the number of T-cell epitopes present in the bispecific antibody. The immunogenicity risk of hBS910 was predicted not only by Epibase but also by EpiMatrix to be comparable with that of non-immunogenic antibodies. Considering that up to 30% of patients develop inhibitors against exogenous FVIII [45], immunogenicity is an important issue in the routine prophylaxis of hemophilia A. Although the true immunogenicity of hBS910 needs to be evaluated clinically, it is noteworthy that two different *in silico* systems predicted hBS910 to be non-immunogenic. However, the possibility that a small number of patients could develop anti-hBS910 antibodies cannot be ruled out. In case hBS910 becomes ineffective owing to development of anti-hBS910 antibodies, it is important that they do not cross-react with FVIII so that exogenous FVIII treatment remains as an alternative. In addition to the fact that there is no homology between the amino acid sequences of hBS910 and FVIII, the risk of such cross-reactivity was found to be negligible because polyclonal anti-idiotype antibodies against hBS910 did not inhibit the thrombin generation activity of FVIII. This demonstrates that the development of anti-hBS910 antibodies would not compromise the use of exogenous human FVIII therapy.

No recombinant bispecific IgG antibody has yet reached the market. One of the reasons is the difficulty in large-scale manufacturing at clinical grade. Identification of a common light chain is an important step for manufacturing asymmetric bispecific IgG antibodies. A previous study utilized a phage display library to identify a common light chain for the two heavy chains against different antigens [26]. However, since high-affinity binding to FIXa or FX would not necessarily result in high FVIII-mimetic activity, selection of a common light chain with potent activity based on the binding affinity by phage display was not feasible. We successfully identified a potent common light chain by a novel FR/CDR shuffling approach. Since we were able to obtain the common light chain (BS15L) that had much better potency than the parental light chain (c1L) from the initial twenty four light chain variants, we did not perform further shuffling of FRs. Because residues in the FR often affects antigen binding of the antibody, we propose that shuffling of both CDRs and FRs would be an efficient and general approach to identify potent common light chains for bispecific antibodies. This novel approach enables incorporation of the beneficial residues from each CDR and FR into a common light chain. Although it might be not suitable for screening common light chain against large panels of heavy chains, this approach can be generally applicable for identification of a common light chain for the selected pair of heavy chains without using a library display system. In our other asymmetric bispecific antibodies with the same molecular format, common light chains for two different heavy chains could be successfully identified by this approach. In some cases, it was possible to directly identify humanized common light chain by performing CDR shuffling on the human FRs. This could be a more efficient approach since it abbreviates the following humanization process. However, since FR residues are often important for antigen binding property, we took more cautious approach in this FVIII-

mimetic bispecific antibody, and performed CDR shuffling on its cognate FRs to identify a potent common light chain, followed by humanization of the identified common light chain.

Although the heavy chain heterodimerization efficiency depended on the expression balance of each heavy chain, our Fc heterodimerization mutations achieved approximately 85% efficiency for hBS560 in the system of unoptimized expression balance (Fig. 5B). Nevertheless, expression of a small amount of monospecific homodimeric antibodies is inevitable even with Fc heterodimerization mutations. In the case of our bispecific antibody, byproducts are anti-FIXa and anti-FX bivalent monospecific antibodies. These byproducts are not simply impurities with no activity, but they have the potential to competitively inhibit the activity of the bispecific antibody by bivalent binding to the factors. Therefore, homodimeric byproducts need to be removed as much as possible by a downstream process. However, the only molecular difference between a homodimeric byproduct and the bispecific antibody is the heavy chain variable region of one arm. Since antibody variable regions generally have similar sequences except for the CDRs, it was assumed that separation of such byproducts from the bispecific antibody by IEC would be difficult. Therefore, we took the advantage of pI engineering, and engineered the heavy chain variable region to increase the pI difference between the bispecific antibody and the byproducts, thereby improving the separation by IEC. pI engineered hBS910 could be actually purified from 2500-liter fermentation by Protein A and IEC using conventional antibody purification processes. Such a novel pI engineering approach would be generally applicable to facilitate the purification of bispecific IgG antibodies.

To realize subcutaneous delivery in a clinical setting, bispecific antibodies need to be formulated into a high concentration (i.e. >100 mg/mL) since the volume that can be subcutaneously injected is generally limited to less than 1.5 mL [46]. However, hBS376 exhibited phase separation even at 4 mg/mL. Phase separation of antibody solutions into an upper phase with low antibody concentration and a lower phase with high antibody concentration not only precludes high concentration formulation but also makes downstream purification processes difficult [30]. It has been recently reported that low solubility of antibodies has been overcome by introducing specific mutations into the molecular surface [47], but mutations to prevent phase separation have not been reported. We demonstrated that phase separation of hBS376 could also be eliminated by introducing multiple mutations into the molecular surface. hBS910 exhibited no phase separation under the conditions tested, and could be concentrated up to at least 200 mg/mL without any issue.

Since FVIII is unstable under liquid formulation, all the marketed FVIII agents are distributed as lyophilized formulation, and therefore require reconstitution before injection. Liquid formulation allows injection without this process, and thus would be much more convenient for the patients. However, monoclonal antibodies stored in aqueous solution often undergo deamidation of the asparagine residues in the CDRs resulting in reduction of the biological activity of the antibody, which was the case for hBS560 [31]. Although the general strategy is to remove the deamidation site by mutating the asparagine residue itself to another amino acid, this strategy was not feasible in the case of hBS560. A simultaneous double mutation approach enabled storage of hBS910 at 40°C for 2 weeks without reduction of activity. Consequently, hBS910 could be stably stored in a patient-friendly 150 mg/mL liquid formulation, which would enable approximately 3 mg/kg subcutaneous delivery in humans (1.5 mL injection for a 75 kg patient). Considering the FVIII-mimetic activity determined by TGA and the pharmacokinetics in cynomolgus monkeys, such a formulation would provide effective prophylaxis by subcutaneous delivery with a long dosing interval.

In conclusion, we have generated a novel humanized anti-FIXa/FX bispecific IgG antibody, hBS910, through a process of identifying the lead candidate from approximately 40,000 bispecific combinations, followed by a multidimensional optimization process to improve both the therapeutic potential and the manufacturability. hBS910 overcomes the two major drawbacks of routine prophylaxis by exogenous FVIII. First, while exogenous FVIII requires frequent intravenous administration, hBS910 can be subcutaneously administered with a long dosing interval. Second, while the development of FVIII inhibitors is a critical issue for exogenous FVIII, our study suggests that hBS910 can be used without fear of developing FVIII inhibitors and can be used in patients who have already developed FVIII inhibitors. We believe that hBS910, with its multidimensionally optimized profile, will provide significant improvement in the quality of life of hemophilia A patients by reducing not only bleeding but also the burden on the patients themselves, their parents, and all medical staff. Potential of hBS910 (investigational drug name; ACE910) in hemophilia A patient is currently being evaluated in clinical study.

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., Ltd. 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). Details of cynomolgus monkey care and maintenance, including shelter and availability of food, water, and environmental enrichment are as follows. Identification of individuals; individual animals were identified by microchip number. Each cage was identified by a cage number indicated on the cage rack, cage type; a stainless steel cage, housing density; 1 animal/cage, temperature; 18°C to 28°C, relative humidity; 35% to 75%, air change frequency; at least 10 times per h, illumination timing; 12 h per day, from 7:00 am to 7:00 pm, feed; cynomolgus monkeys were daily provided approximately 100 g of solid chow (Certified Primate Diet 5048, Japan SLC) and supplementary foods (1/2 peeled banana or 50 g of sweet potato) and drinking water; animal room tap water, provided ad libitum using the automatic water supply system. The studies involved injection of the agents and collection of blood samples which did not require procedures that would cause more than slight or momentary pain or distress to the animals. All injections and blood collection were conducted by trained and qualified primate therapeutic staff in Chugai Pharmaceutical. Provisions were made in the approved protocol for veterinary intervention in the case of any distress or morbidity from the injected agent; however no adverse events occurred during the studies or during the recovery period. Therefore, no scarification of the animals was conducted.

Generation of Anti-FIXa/FX Bispecific Antibodies

Approximately 200 monoclonal antibodies to FIXa or FX were obtained from FIXa or FX immunized mice, rats, and rabbits. The V_H and V_L of those antibodies were then combined with engineered human IgG_2 or IgG_4 that included mutations to facilitate Fc heterodimerization. These engineered human IgG_2 or IgG_4, which included the knobs-into-holes mutations [26], were generated by introducing the same substitutions as those of IgG_1 knobs-into-holes to the corresponding positions of IgG_2 and IgG_4

heavy chains, and were used through the screening for the lead identification. Anti-FIXa/FX bispecific were generated by HEK293 cells co-transfected with mixture of four plasmids encoding anti-FIXa heavy and light chain and anti-FX heavy and light chain (supplementary Fig. S1). At the screening step for lead identification, we used knobs-into-holes mutations which could achieve at least 90% efficiency of the two heavy chain heterodimerization. If the assembly of a heavy chain with two light chains occurs with equal probability, 25% of heavy chain heterodimeric antibodies will have the correct heavy and light chain pair [25]. Theoretically, in the supernatant of transfected cells, this would result in at least approximately 20% (90% ×25% = 22.5%) of antibodies to be the target bispecific antibody, and less than 3% (10% ×25% = 2.5%) of antibodies to be homodimeric antibodies with a correct heavy and light chain pair. Transfected cells were cultured in 96-well culture plates, and either filtrated culture supernatants or Protein A purified antibodies were used for evaluation. We also generated bispecific antibodies with a common light chain, anti-FIXa monospecific IgG consisting of anti-FIXa heavy chain and a common light chain, and anti-FX monospecific IgG consisting of anti-FX heavy chain and a common light chain by the method described above. The engineered human IgG_4 including the knobs-into-holes mutations was used during the early stage of the multidimensional optimization, and a different engineered IgG_4 including electrostatic steering mutations [25] was used during the late stage optimization. By using the engineered IgG_4, approximately 85–95% of the Protein A purified antibody would be the target bispecific antibody and the rest would be homodimeric antibodies. For the detailed characterization of hBS910, homodimeric antibodies were removed by ion exchange chromatography.

Screening Bispecific Antibodies for FVIII-mimetic Activity (Enzymatic Assay)

The ability of each antibody to enhance FIXa-catalized FXa generation was evaluated in an enzymatic assay with purified human coagulation factors (Enzyme Research Laboratories). The FXa generation reaction was performed in the presence of 1 nM human FIXa, 140 nM human FX, 20 μM synthetic phospholipid (10% phosphatidylserine, 60% phosphatidylcholine and 30% phosphatidylethanolamine; Avanti Polar Lipids) prepared as previously described [48], and antibodies at room temperature for 2 min in TBS containing 5 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1% (wt/vol) BSA (pH 7.6). The reaction was stopped by the addition of EDTA at appropriate time points. The activity of the FXa generated was determined by absorbance at 405 nm after the addition of chromogenic substrate S-2222 (Chromogenix). Data were collected in triplicate.

FR/CDR Shuffling of the Light Chains

CDRs of three light chains (c1L, c2L and c3L) were shuffled among each other then grafted onto the FRs of either c1L or c3L to generate light chain variants (supplementary Fig. S2A). Since c2L and c3L had identical CDR1 and CDR2 sequences, we generated twenty four light chains (twelve CDR combinations in two FRs) including parental c1L and c3L. Light chain variant genes were generated by assembly PCR, and the twenty four bispecific antibodies with these light chains were prepared as described above. FVIII-mimetic activity of each antibody was evaluated in APTT assay with standard techniques using APTT reagent (Sysmex) and FVIII deficient plasma (Sysmex).

Pharmacokinetic Study of Bispecific Antibodies in Mice

1 mg/kg doses of each bispecific antibody were administered to C57BL/6J normal mice (Charles River) by single subcutaneous injection ($n = 3$ for each group). Blood samples were collected at an appropriate time after each administration. Plasma concentration of bispecific antibodies was determined by human IgG-specific ELISA. Pharmacokinetic parameters were calculated by Win-Nonlin Professional software (Pharsight).

Determination of Isoelectric Point (pI) by Capillary Isoelectric Focusing (cIEF)

cIEF analyses of antibodies were performed with a PA800 plus Pharmaceutical Analysis System and 32 Karat software (Beckman Coulter) as described in the cIEF application guide (PN A78788AA). Briefly, antibody solutions (approximately 1 mg/mL in PBS) were diluted 1:25 with cIEF master mix solution containing cIEF gel, urea, Pharmalyte 3–10, arginine, iminodiacetic acid, and pI markers. A neutral capillary was preconditioned by rinsing with urea solution, and samples were injected and focused. The pI of each of the antibodies was determined from the pI markers.

Separation of Bispecific Antibodies by Cation Exchange Chromatography

Using an AKTAexplorer 10S (GE Healthcare), two HiTrap SP FF 1 mL columns (GE Healthcare) were connected in tandem and equilibrated by 20 mM sodium phosphate, pH 6.0. The elution buffer contained an appropriate concentration of NaCl in this equilibration buffer. The load sample was first prepared from culture supernatant using MabSelect Sure (GE Healthcare), and was then dialyzed with equilibration buffer. The sample antibody solution was applied to the HiTrap SP FF column at 1.5 mg/mL resin. After washing with equilibration buffer, antibodies were eluted with 10 column volumes (CV) of equilibration buffer containing 150 mM NaCl and then with 15 CV of 220 mM NaCl buffer in a stepwise manner, and finally with 15 CV of NaCl buffer in a linear gradient to 450 mM.

Solubility Analysis of Bispecific Antibodies

Stock solutions of bispecific antibodies were prepared by dialysis against water or 50 mM NaCl solutions, followed by ultrafiltration concentration. Samples were prepared by adding formulation stock solution to antibody stock solution using a Hydra II Plus One liquid-handling robot (Matrix). After centrifugation, 1 μL of each sample was stored in an Intelli-Plate 96-2 (Art Robbins) at 20°C for 1 day, and then images of each well were taken by Rock Imager 54 (Formulatrix). The state of each antibody solution was determined as either a clear solution, precipitation, or liquid–liquid phase separation.

Accelerated Stability Study of Bispecific Antibodies

Solutions of bispecific antibodies were dialyzed against PBS, pH 7.4 (Sigma). 1 mg/mL of each antibody solution was stored at 40°C for 2 weeks and then analyzed by cation exchange chromatography (IEC) using BioPro SP columns (YMC) at room temperature. The mobile phase (A) was 20 mM sodium phosphate, pH 5.8, and the mobile phase (B) was 20 mM sodium phosphate and 500 mM NaCl, pH 5.8.

In silico Evaluation of Immunogenicity of the Variable Region of Bispecific Antibodies

T-cell epitope prediction of the bispecific antibody variants and immunogenicity risk scores of antibodies were provided by

Epibase (Lonza). Immunogenicity scales of antibodies were provided by EpiMatrix (EpiVax).

Thrombin Generation Assay (TGA)

Calibrated automated thrombography [35] was employed using a 96-well plate fluorometer (Thermo Fisher Scientific) equipped with a 390/460 filter set, a dispenser, and the analyzing software (Thrombinoscope software version 3.0.0.29; Thrombinoscope) to measure thrombograms. Briefly, each concentration of bispecific antibody or rhFVIII (Bayer Healthcare) was added to FVIII-deficient plasma (<1% FVIII activity) either without inhibitors or with inhibitors against FVIII (George King Bio-Medical). Each concentration of bispecific antibody or rhFVIII was also added to plasma containing polyclonal rabbit anti-idiotype antibodies against anti-FIXa Fab or anti-FX Fab (300 μg/mL each). Into each well was dispensed 80 μL of the plasma, to which was then added 20 μL of the triggering solution containing 0.47 nM human FXIa (Enzyme Research Laboratories) and 20 μM synthetic phospholipid but no Ca^{2+}. For calibration, 20 μL of Thrombin Calibrator (Thrombinoscope) was added instead of the triggering solution. 20 μL of FluCa-reagent prepared from FluCa-kit (Thrombinoscope) was dispensed to initiate the reaction. The thrombograms and peak height were analyzed by the software. Data were collected in triplicate.

Pharmacokinetic Study of hBS910 in Cynomolgus Monkeys

A single dose of 0.3 mg/kg of hBS910 was intravenously or subcutaneously administered to male cynomolgus monkeys ($n = 3$ for each group). Blood samples were collected at an appropriate time after each administration. Plasma concentration of bispecific antibodies was determined by human IgG-specific ELISA. Pharmacokinetic parameters were calculated by WinNonlin Professional software (Pharsight).

Kinetic Analysis of Bispecific Antibodies Binding to FIXa and FX Using Surface Plasmon Resonance

Kinetic analysis of bispecific antibodies was performed by surface plasmon resonance (SPR) using a Biacore T200 system (GE Healthcare). MabSelect SuRe Ligand (Recombinant Protein A; GE Healthcare) was immobilized onto a CM4 sensor chip (GE Healthcare). Then, anti-FIXa or anti-FX monospecific antibodies were injected into flow cell 2 to be captured. Natalizumab (Biogen-Idec Inc.) as a control human IgG_4 antibody was also injected into flow cell 1 to be captured. Then, 0, 80, 160, 320, 640, or 1,280 nM human FIXa or FX dissolved in running buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 0.05% surfactant P20, 2.5 mM $CaCl_2$) was injected at a flow rate of 30 μL/min to monitor the association phase for 120 s and the dissociation phase for 30 s.

Supporting Information

Figure S1 Generation of anti-FIXa/FX bispecific antibodies. Heavy chain variable regions (V_H) of anti-FIXa or anti-FX antibodies were fused with engineered human IgG_2 or IgG_4 constant region having mutations to facilitate Fc heterodimerization. Light chain variable regions (V_L) were fused with human κ or λ constant region. Bispecific antibodies were generated by expression with two pairs of genes, anti-FIXa and anti-FX heavy chain and light chain genes (or a common light chain gene).
(PDF)

Figure S2 FR/CDR shuffling of the light chain. (A) CDRs of three light chains (c1L, c2L and c3L) were shuffled among each other and grafted onto the FRs of c1L and c3L. Each light chain variant was expressed with the selected anti-FIXa and anti-FX heavy chains. (B) Effects of bispecific antibodies (67 nM) with light chain variants on APTT assay in FVIII-deficient plasma are shown. The Y-axis indicates the APTT (s). All the data were collected in duplicate and are expressed as mean.
(PDF)

Figure S3 Effect of humanization of the lead chimeric antibody (BS15) on FVIII-mimetic activity. Effect of chimeric antibody BS15 (circles) or humanized antibody hBS1 (squares) on FX activation in the presence of FIXa, FX, and synthetic phospholipid. The Y-axis indicates the 405 nm absorbance at 120 min of chromogenic development in the chromogenic substrate assay. All the data were collected in triplicate and are expressed as mean ± s.d (in many cases, the bars depicting s.d. are shorter than the height of the symbols).
(PDF)

Figure S4 Positive charge cluster and Tyr30Glu mutation on anti-FIXa Fv of hBS106. The positive charge cluster consists of arginine or lysine residues at Kabat position 60, 61, and 95 in the heavy chain and Kabat positions 24, 27, 31, 53, 54, 61, and 66 in the light chain of hBS106. Tyrosine located at Kabat position 30 in the light chain was mutated to glutamic acid to neutralize the positive charge cluster. Blue, red and gray colored surface indicates positively charged, negatively charged and neutral protein surface, respectively. Red and green line indicates heavy and light chain, respectively.
(PDF)

Figure S5 Precipitation and liquid–liquid phase separation of bispecific antibody solution. Micro CCD camera images of states of bispecific antibody solution showing a clear solution, precipitation, and liquid–liquid phase separation.
(PDF)

Figure S6 *In silico* prediction of immunogenicity of bispecific antibodies. (A) Immunogenicity risk score of BS15, hBS1, hBS910, trastuzumab, and palivizumab predicted by Epibase. (B) Immunogenicity scale of hBS910 and other marketed monoclonal antibodies by EpiMatrix. In both prediction systems, higher score indicates higher risk of immunogenicity in human.
(PDF)

Figure S7 Necessity of bispecific binding to FIXa and FX for FVIII-mimetic activity. Effect of the bispecific antibody (hBS910) (circles), monospecific anti-FIXa antibody (squares), monospecific anti-FX antibody (triangles), or a mixture of the two monospecific antibodies (diamonds) on FX activation in the presence of FIXa, FX, and synthetic phospholipid. The Y-axis indicates the 405 nm absorbance at 30 min of chromogenic development in the chromogenic substrate assay. All the data were collected in triplicate and are expressed as mean ± s.d (in many cases, the bars depicting s.d. are shorter than the height of the symbols). Monospecific antibodies against FIXa or FX antibodies or the mixture of them did not exhibit any detectable activity even at 120 min of chromogenic development.
(PDF)

Figure S8 Surface plasmon resonance analysis of bispecific antibodies binding to FIXa and FX. Sensorgrams of hBS1, hBS106, and hBS910 binding to FIXa (A) and FX (B) at a concentration of 80 nM, 160 nM, 320 nM, 640 nM, and 1280 nM.
(PDF)

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Author Contributions

Provided direction and guidance: YN. Provided the hypothesis of the bispecific antibody, directed and organized the program: K. Hattori. Conceived and designed the experiments: TI T. Kojima T. Kitazawa. Performed the experiments: ZS TI TS YON CM TW ET AM KY AH MF K. Haraya TT SS KE. Wrote the paper: ZS TI.

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REPORT

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Novel asymmetrically engineered antibody Fc variant with superior FcγR binding affinity and specificity compared with afucosylated Fc variant

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Keywords: antibody engineering, ADCC, A/I ratio, Fc engineering, FcγR

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; T_M, melting temperature; mAb, monoclonal antibody; NK, natural killer; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; SPR, surface plasmon resonance; PBMC, peripheral blood mononuclear cells; FcγR, Fc gamma receptor

Fc engineering is a promising approach to enhance the antitumor efficacy of monoclonal antibodies (mAbs) through antibody-dependent cell-mediated cytotoxicity (ADCC). Glyco- and protein-Fc engineering have been employed to enhance FcγR binding and ADCC activity of mAbs; the drawbacks of previous approaches lie in their binding affinity to both FcγRIIIa allotypes, the ratio of activating FcγR binding to inhibitory FcγR binding (A/I ratio) or the melting temperature (T_M) of the C_H2 domain. To date, no engineered Fc variant has been reported that satisfies all these points. Herein, we present a novel Fc engineering approach that introduces different substitutions in each Fc domain asymmetrically, conferring optimal binding affinity to FcγR and specificity to the activating FcγR without impairing the stability. We successfully designed an asymmetric Fc variant with the highest binding affinity for both FcγRIIIa allotypes and the highest A/I ratio compared with previously reported symmetrically engineered Fc variants, and superior or at least comparable in vitro ADCC activity compared with afucosylated Fc variants. In addition, the asymmetric Fc engineering approach offered higher stability by minimizing the use of substitutions that reduce the T_M of the C_H2 domain compared with the symmetric approach. These results demonstrate that the asymmetric Fc engineering platform provides best-in-class effector function for therapeutic antibodies against tumor antigens.

Introduction

Monoclonal antibodies (mAbs) have enormous potential as anticancer therapeutics. MAbs promote elimination of tumor cells by Fab-dependent and Fc-dependent mechanisms, such as interference with signaling pathways, apoptosis induction, complement-dependent cytotoxicity, antibody-dependent cell-mediated phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC).

ADCC is induced when effector cells are recruited by the Fc domain engaging with a member of the Fcγ receptor family, which is comprised in humans of FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa and FcγRIIIb isoforms. FcγRI, FcγRIIa, FcγRIIc and FcγRIIIa are activating receptors characterized by the immunoreceptor tyrosine-based activation motif (ITAM), and FcγRIIb is the only inhibitory receptor characterized by ITIM. The receptors are expressed on a variety of immune cells, such as NK cells, monocytes, macrophages and dendritic cells.[1]

Increasing affinity for FcγR enhances ADCC, so Fc engineering is considered to be a promising means of increasing the antitumor potency of mAbs.[2] Previous reports described that follicular lymphoma patients treated with rituximab had, on average, significantly prolonged progression-free survival if they possessed two copies of the high-affinity FcγRIIIa allele, FcγRIIIa^{V158}.[3,4] This result suggests that the efficacy of rituximab is mediated by FcγRIIIa-expressing cells, such as NK cells, and that higher affinity to FcγRIIIa improves the efficacy. Several strategies have been employed to enhance the FcγR binding of mAbs. The first strategy was engineering the glycan moiety attached to Asn297 residue in the Fc domain. Afucosylated IgG1 antibody, which is a mAb without fucose in the N-linked glycan at Asn297, binds to FcγRIIIa with higher affinity and mediates superior ADCC compared with wild-type fucosylated IgG1 antibody.[5,6] The second strategy was introducing amino acid substitutions into the Fc domain. mAbs with triple substitutions, S239D/A330L/I332E, bind to FcγRIIIa with higher affinity and have shown superior

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ADCC activity than wild-type IgG.[7] In addition to enhancing the binding to activating FcγRs, minimizing the interaction with inhibitory FcγR, namely FcγRIIb, is another strategy to enhance the potential of the antibody.[8] Enhanced cancer elimination was observed in FcγRIIb knockout mice compared with that observed in mice expressing FcγRIIb when they were treated with anti-Her2/neu mAb or anti-E-cadherin mAb,[9,10] demonstrating that the ratio of activating FcγR binding to inhibitory FcγR binding (A/I ratio) is an important factor determining the therapeutic efficacy of antitumor antibody.[11] mAb with five substitutions, L235V/F243L/R292P/Y300L/P396L, showed enhanced binding to FcγRIIIa, but not to FcγRIIb, which improved the A/I ratio.[8] In terms of improving the selectivity for a specific FcγR, antibody variants with selectively enhanced binding for FcγRI were reported.[12]

Although these glyco- and protein-engineering approaches have successfully enhanced the effector function of mAbs, each technology has issues to overcome. First, afucosylated antibodies bind with lower affinity to FcγRIIIa^{F158} than to FcγRIIIa^{V158}. As a consequence, the resulting ADCC mediated by NK cells bearing the lower-affinity FcγRIIIa allotype is lower than that mediated by NK cells bearing the higher-affinity allotype, suggesting that afucosylated Fc may not achieve maximum ADCC activity for patients having the lower-affinity allotype.[13] Second, because activating and inhibitory FcγRs have high homology, the S239D/A330L/I332E variant also increased binding affinity against inhibitory FcγRIIb, which would be undesirable for achieving maximum antitumor efficacy considering the A/I ratio. Moreover, the T_M in the C_H2 domain of the S239D/A330L/I332E variant was significantly reduced (by more than 20°C), which could be an issue when the variant is developed as a pharmaceutical product.[14] Third, although the L235V/F243L/R292P/Y300L/P396L variant did not increase the binding affinity against inhibitory FcγRIIb, it had only 10-fold increased binding affinity to FcγRIIIa, which is substantially less than the S239D/A330L/I332E variant, thereby achieving only a moderate A/I ratio.

To date, neither glyco- nor protein-engineering has been able to overcome all these issues. Ideal therapeutic use requires an antibody Fc variant that has higher binding affinity to both FcγRIIIa^{F158} and FcγRIIIa^{V158} and better stability of the C_H2 domain, but that does not increase binding affinity to inhibitory FcγRIIb to maintain a higher A/I ratio. To overcome these issues, in this study we focused on the fact that homodimeric and symmetric Fc domain recognizes monomeric FcγR asymmetrically, which was previously revealed by the structural analysis of Fc fragment with FcγR.[15] Considering that Fc and FcγR interact asymmetrically, we hypothesized that asymmetric Fc engineering would make it possible to design a novel Fc variant with improved affinity against both low- and high-affinity FcγRIIIa allotypes, enhancing ADCC activity compared with previously known protein- or glyco-engineering. In addition, asymmetric Fc engineering would result in fewer substitutions or avoidance of the need for stability-reducing substitutions to minimize the reduction of the T_M of the C_H2 domain. Moreover, asymmetric Fc engineering would allow us to optimize the Fc-FcγR interaction more precisely so as not to increase binding affinity to inhibitory FcγRIIb and to have a higher A/I ratio by discriminating activating FcγRs from inhibitory FcγR.

We designed antibody variants with an asymmetrically engineered Fc domain (asym-mAb) by introducing different substitutions in each Fc domain. Comprehensive mutagenesis in the C_H2 domain has identified several substitutions that increase the binding affinity for FcγRs more strongly when they are introduced in one Fc domain than in both chains. We successfully designed an asym-mAb with htthigher affinity for both FcγRIIIa allotypes and superior or at least comparable ADCC than the previously reported symmetrically engineered antibody (sym-mAb), without increasing the affinity for FcγRIIb or substantially reducing the stability of the antibody. Our results demonstrated a novel approach for optimizing the interaction between Fc and FcγR and confirmed the advantage of that approach when applied therapeutically.

Results

Comparing the binding affinity for FcγRIIIa of asym-mAb and sym-mAb. We screened a set of over 1,000 asym- and sym-mAbs, each with a single substitution in the lower hinge and C_H2 domain, for binding to human FcγRIIIa^{F158} to identify substitutions that enhance FcγRIIIa binding only when they were introduced in one Fc domain. The effect of substitutions in both sym- and asym-mAbs was evaluated using surface plasmon resonance (SPR). We identified several unique substitutions to meet our criteria (binding affinity of asym-mAb > that of sym-mAb). Of them, we selected three single substitutions, L234Y, G236W and S298A, and designed a variant with L234Y/G236W/S298A (YWA) substitutions, to investigate whether asymmetric Fc engineering has any advantages over symmetric Fc engineering. As an example of symmetric Fc engineering, we utilized S239D/A330L/I332E (DLE) substitutions, which were previously reported to increase affinity to FcγRIIIa.[7] We prepared five variants: hemi-DLE variant, variant with DLE substitutions in only one Fc domain; homo-DLE variant, variant with DLE in both Fc domains; hemi-YWA variant, variant with YWA substitutions in only one Fc domain; homo-YWA variant, variant with YWA in both Fc domains and DLE/YWA variant with DLE in one Fc domain and YWA in the other Fc domain. We evaluated the affinity for FcγRIIIa^{F158} of each variant (**Table 1**). The representative sensorgrams are depicted in **Figure S1**.

First, we compared homo- and hemi-DLE variants to evaluate the effect of DLE substitutions in symmetric Fc engineering or asymmetric Fc engineering. The homo-DLE variant increased the affinity for FcγRIIIa 255-fold compared with control mAb1, which only has substitutions to facilitate heterodimerization of two heavy chains, while the hemi-DLE variant increased it only 30-fold. Next, we evaluated the other substitutions, YWA. The homo-YWA variant reduced the affinity 0.47-fold, but the hemi-YWA variant increased it 5.0-fold. YWA substitutions showed a distinctly different effect on the Fc-FcγRIIIa interaction when introduced in one Fc domain than when introduced in both Fc domains. The DLE/YWA

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Table 1. Affinity for FcγRIIIa^{F158} and T_M of antibody variants

Fc variants	Substitutions in heavy chain A	Substitutions in heavy chain B	K_D (μmol/L)	Fold	T_M (°C)	ΔT_M (°C)
control mAb1	-	-	1.4 ± 0.3	1	68	-
hemi-YWA	-	L234Y/G236W/S298A	0.28 ± 0.04	5.0	68	0
hemi-DLE	S239D/A330L/I332E	-	0.046 ± 0.009	30	60	-8
homo-YWA	L234Y/G236W/S298A	L234Y/G236W/S298A	3.0 ± 0.6	0.47	68	0
homo-DLE	S239D/A330L/I332E	S239D/A330L/I332E	0.0055 ± 0.0005	255	48	-20
DLE/YWA	S239D/A330L/I332E	L234Y/G236W/S298A	0.0042 ± 0.0003	333	59	-9

$K_D = K_D$ for FcγRIIIa^{F158}. Fold = K_D (control mAb1)/K_D (Fc variants). T_M means T_M of the C_H2 domain. $\Delta T_M = T_M$ (Fc variants) - T_M (control mAb1). K_D was represented as mean ± SD (n = 3).

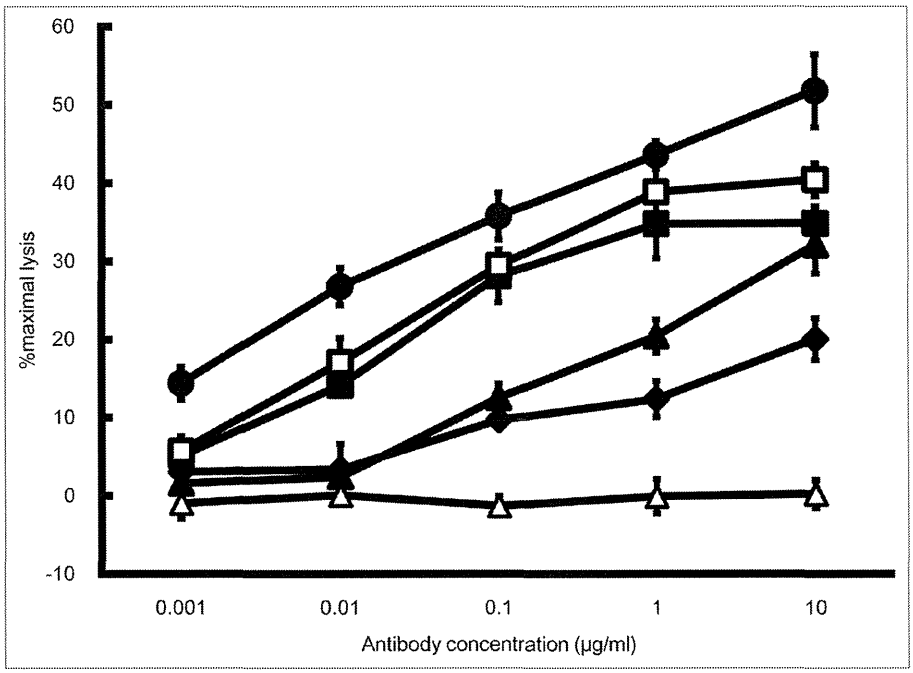

Figure 1. ADCC comparison of asym-mAbs and sym-mAbs. ADCC was determined by percent lysis of SK-Hep-1 cells expressing tumor antigen X at varying concentrations of antibody Fc variants to tumor antigen X using PBMC as effector cells. Mean ± SD of triplicate wells. Black diamond, control mAb1; white square, homo-DLE; black square, hemi-DLE; white triangle, homo-YWA; black triangle, hemi-YWA and black circle, DLE/YWA.

variant showed the highest affinity among evaluated variants, even higher than the homo-DLE variant.

ADCC of antibody variants with asymmetrically engineered Fc. The cellular cytotoxicity of asym-mAb and sym-mAb to tumor antigen X with enhanced FcγRIIIa binding was evaluated using SK-Hep-1 cells expressing tumor antigen X and human PBMC (**Fig. 1**). Homo- and hemi-DLE variants showed higher ADCC than control mAb1, while ADCC of the homo-DLE variant was slightly higher than that of hemi-DLE. On the other hand, the homo-YWA variant showed no detectable ADCC, but the hemi-YWA showed higher ADCC than control mAb1. In ADCC assay, YWA substitutions showed this opposite effect whether they were introduced in both Fc domains or in only one Fc domain. The DLE/YWA variant with the highest FcγRIIIa binding showed the highest ADCC. These results from the ADCC assay were consistent with those obtained in the kinetic analyses of the variants.

Thermostability and accelerated stability study of antibody variants with asymmetrically engineered Fc. T_M of the C_H2 domain of hemi-DLE, homo-DLE, hemi-YWA, homo-YWA and DLE/YWA variants was measured by thermal shift assay (**Table 1**). The T_M of the C_H2 domain of hemi-DLE variant decreased by 8°C from control mAb1 and that of homo-DLE by 20°C. On the other hand, the T_M of hemi-YWA and homo-YWA variants was not significantly reduced, and that of the DLE/YWA variant decreased to the same degree as that of hemi-DLE variant.

After storage for two and four weeks at 40°C at a concentration of 1 mg/ml, the reduction of a monomer peak of each antibody in size-exclusion chromatography was compared

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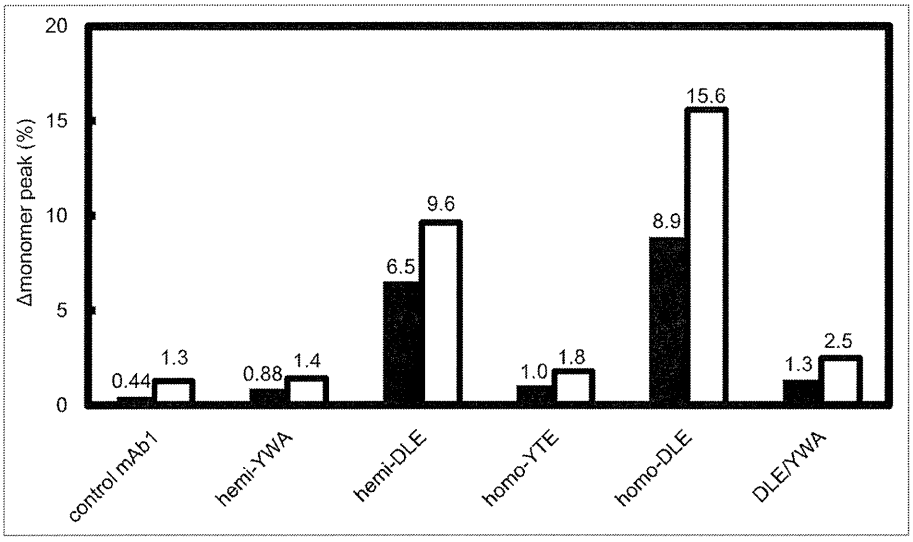

Figure 2. Stability of antibody Fc variants in accelerated stability study. Percentage of the reduction of a monomer peak (Δmonomer peak (%)) of each variant after storage at 40°C in size-exclusion chromatography is shown. Black and white bars represent the average reduction in monomer peaks after 2-week and 4-week storage, respectively. Each experiment was performed twice and individual data were shown in **Table S2**.

(**Fig. 2**). The monomer peak of hemi-DLE variant decreased ~10% after 4-week storage and that of homo-DLE decreased 16%. The same tendency was observed after 2-week storage. On the other hand, the reduction of monomer peaks of other variants, including DLE/YWA, was comparable with that of control mAb1.

Further optimization of asymmetrically engineered Fc. We further designed more potent asym-mAbs with higher binding affinity to active FcγRs. Further optimization to enhance FcγRIIIa binding was performed based on the result of comprehensive mutagenesis. As a result, we obtained asym-mAb1 containing L234Y/L235Q/G236W/S239M/H268D/D270E/S298A substitutions in one Fc domain and D270E/K326D/A330M/K334E substitutions in the other. We also prepared the reported protein- and glyco-engineered Fc variants to compare their affinity for FcγRs with asym-mAb1. Protein-engineered Fc variants were prepared by control mAb2, an antibody with only substitutions to facilitate heterodimerization of the two heavy chains. Afucosylated IgG1, afucosyl mAb and the homo-DLE variant were prepared as antibodies with enhanced FcγRIIIa binding, and the homo-L235V/F243L/R292P/Y300L/P396L (VLPYLL) variant was also prepared as an antibody with a high A/I ratio, without increased affinity for FcγRIIb.[8] Their increase of affinity for human FcγRs, A/I ratio and the reduction of T_M in C_H2 domain are shown in **Table 2**. K_D and T_M in C_H2 domain of these variants are summarized in **Table S1**.

Compared with the afucosyl mAb, homo-DLE variant and homo-VLPYLL variant, asym-mAb1 demonstrated greatly enhanced affinity for FcγRIIIa. Asym-mAb1 increased affinity for FcγRIIIa^{F158} by 2000-fold and for FcγRIIIa^{V158} by 1000-fold. On the other hand, the afucosyl mAb, homo-DLE variant and homo-VLPYLL variant enhanced affinity for FcγRIIIa^{F158} only by 18-, 286- and 63-fold and affinity for FcγRIIIa^{V158} only by 45-, 126- and 33-fold, respectively. As for the binding to FcγRIIb, the affinity for FcγRIIb of asym-mAb1 and of VLPYLL variant was comparable with that of control mab2.

Asym-mAb1 also demonstrated the highest A/I ratio. Asym-mAb1 increased A/I ratio for FcγRIIIa^{F158} 2000-fold and for FcγRIIIa^{V158} 1000-fold, while the afucosyl mAb, homo-DLE variant and homo-VLPYLL variant enhanced A/I ratio for FcγRIIIa^{F158} only by 7.7-, 43- and 119-fold and that for FcγRIIIa^{V158} only by 20-, 18- and 59-fold, respectively.

Homo-DLE variant reduced the T_M in C_H2 domain by 21°C, while other engineered Fc variants reduced it only by less than 10°C.

ADCC of further optimized asym-mAb. ADCC activity of the optimized asym-mAb was compared with that of afucosyl mAb using human PBMC obtained from four different donors. Asym-mAb1 showed remarkably greater ADCC activity than IgG1, and comparable or slightly higher ADCC activity compared with afucosyl mAb, as shown in **Figure 3 (A, B, C and D)**.

Discussion

Despite the fact that the Fc domain recognizes FcγRs asymmetrically with two distinct interfaces,[15] previous approaches for modifying Fc-FcγR interaction, such as alanine scanning mutagenesis, a protein structure design algorithm and a yeast surface displayed random mutant library screening, focused on modifying the Fc domain in a symmetric manner,[7,8,16] making it difficult to identify substitutions that enhance FcγR binding when they are introduced in only one Fc domain. We investigated such substitutions by comparing single-substituted asymmetric variants and the corresponding symmetric variants in comprehensive mutagenesis and combined the substitutions, L234Y, G236W and S298A, with the desired property that we identified through the investigation.

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Table 2. Relative affinity for FcγRs and T_M in the C_H2 domain of Fc variants

Fc variants	FcγRIa	FcγRIIa^{R131}	FcγRIIa^{H131}	FcγRIIb	FcγRIIIa^{F158}		FcγRIIIa^{V158}		ΔT_M(°C)
	Fold K_D	Fold K_D	Fold K_D	Fold K_D	Fold K_D	Fold A/I	Fold K_D	Fold A/I	
afucosyl mAb	0.53	1.8	0.85	2.3	18	7.7	45	20	-2
homo-DLE	3.4	2.9	1.4	6.7	286	43	126	18	-21
homo-VLPYLL	0.36	0.32	2.2	0.54	63	119	33	59	-1
asym-mAb1	1.0	2.6	4.9	1.0	2167	2188	1054	1032	-6

Fold $K_D = K_D$ (control)/K_D (Fc variants). A/I = (K_D for FcγRIIb)/(K_D for FcγRIIIa^{F158}) or (K_D for FcγRIIb)/(K_D for FcγRIIIa^{V158}). Fold A/I = A/I (Fc variants)/A/I (control). $\Delta T_M = T_M$ in C_H2 domain (control) - T_M in C_H2 domain (Fc variants). In calculating the parameters of afucosyl mAb and protein-engineered Fc variants, those of IgG1 and control mAb2 were used as a control, respectively.

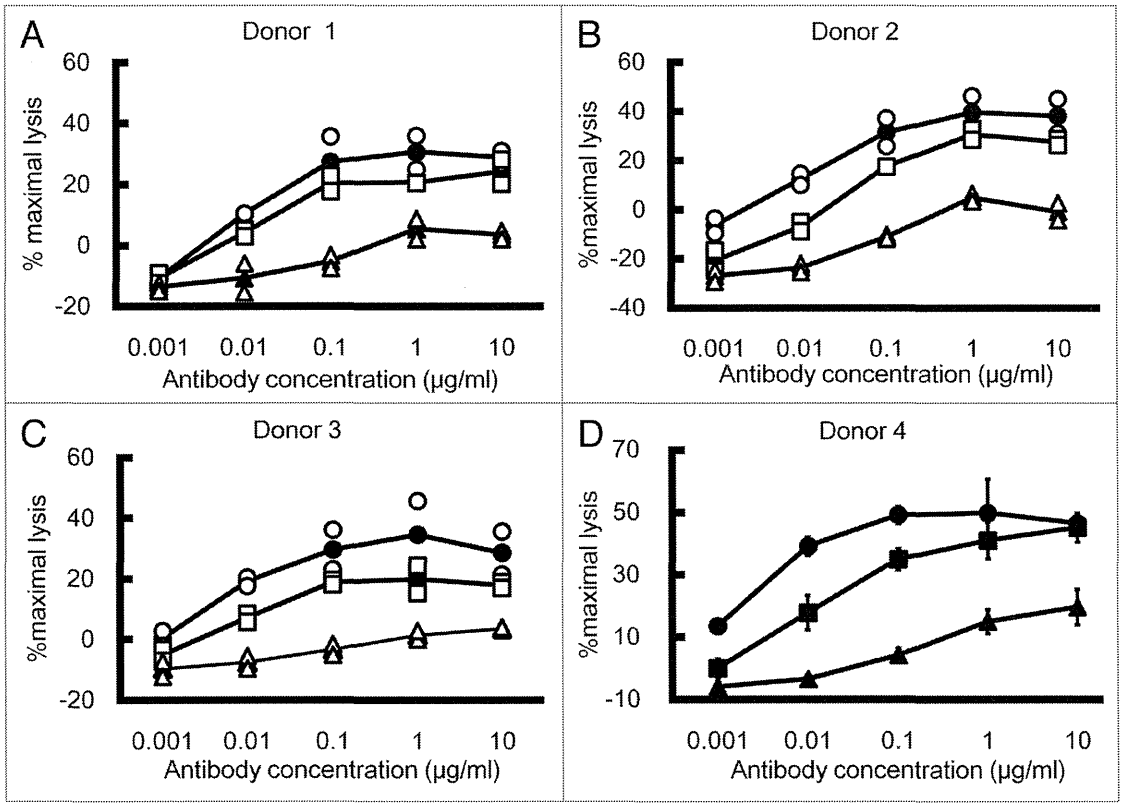

Figure 3. ADCC of antibody Fc variants. ADCC of IgG1, afucosyl mAb and asym-mAb1 was determined by percent lysis of DLD-1 cells expressing tumor antigen Y opsonized at varying concentrations of antibody Fc variants to tumor antigen Y using PBMC obtained from four different donors as effector cells with 10 mg/ml human IgG (A, B and C) or without it (D). Triangle, IgG1; square, afucosyl mAb and circle, asym-mAb1. (**A, B and C**) Open markers indicate individual data and closed makers with lines indicate average (n = 2). (**D**) Mean ± SD of triplicate wells.

We evaluated the FcγR binding and ADCC of homo-YWA, hemi-YWA, homo-DLE, hemi-DLE and DLE/YWA variants. In a previous analysis, DLE substitutions were thought to improve FcγR interaction mainly in one Fc domain.[7] In our results, hemi- and homo-DLE variant enhanced affinity for FcγRIIIa by 30- and 255-fold, respectively, compared with the wild-type antibody. While DLE substitutions in one Fc domain enhanced the binding affinity 30-fold, the gain of affinity by DLE substitution when introduced in the other Fc domain was only 8.5-fold, suggesting that DLE substitutions in each Fc domain contribute positively, but to differing degrees, to enhancing the binding affinity for FcγRIIIa. In contrast to DLE substitutions, YWA substitutions showed a distinct effect. While hemi-YWA variant enhanced affinity for FcγRIIIa 5.0-fold, homo-YWA variant substantially reduced the affinity. This result implies that YWA substitutions stabilize the interaction in one of the two interfaces with FcγRIIIa, but substantially destabilize the interaction in the other interface. The sum of the interactions in each Fc domain was not energetically beneficial, and, as a result, when YWA substitutions were introduced in both Fc domains, they substantially reduced the binding affinity to

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FcγRIIIa. Although the hemi-YWA variant has enhanced binding affinity for FcγRIIIa, the binding affinity was weaker than that of the hemi-DLE variant. These results indicate that YWA substitutions are less potent than DLE substitutions both in hemi-manner and homo-manner; however, the DLE/YWA variant unexpectedly exhibited even higher affinity for FcγRIIIa than homo-DLE variant, suggesting that DLE and YWA enhance FcγRIIIa binding synergistically (not just additively) by stabilizing each of the Fc-FcγRIIIa interfaces simultaneously in a distinct structural environment. Importantly, although the homo-DLE variant was the most potent Fc variant reported to date, the effect of DLE/YWA variant demonstrated that Fc-FcγRIIIa interactions could be further optimized by the novel asymmetric engineering approach. Consistent with the affinity for FcγRIIIa, the DLE/YWA variant exerted stronger ADCC activity than homo-DLE. Among the evaluated variants, higher ADCC showed a general correlation with higher affinity for FcγRIIIa. This correlation demonstrates that asymmetrically engineered Fc could interact with FcγRIIIa expressed on effector cells in a similar manner to symmetrically engineered Fc.

DLE substitutions were reported to decrease T_M of the C_H2 domain by more than 20°C in a homo-DLE variant.[14] In our analysis, the homo-DLE variant did reduce the T_M by 20°C, but the hemi-DLE variant reduced it by only 8°C, less than half of the reduction by homo-DLE. On the other hand, even when YWA substitutions were introduced in both Fc domains, they did not decrease the T_M, and the DLE/YWA variant showed almost the same reduction in T_M as hemi-DLE. These results suggest that substitutions in each Fc domain reduce the T_M of the C_H2 domain independently and that the net reduction is the sum of them. To further clarify the storage stability of asym-mAb, we investigated the storage stability of each variant under accelerated conditions. The monomer peak of the homo-DLE variant was reduced by 16% after 4 weeks of accelerated storage, while that of hemi-DLE was reduced by about 10%, suggesting that DLE substitutions additively reduced the storage stability of the variants. On the other hand, the reduction in monomer peaks of homo-YWA, hemi-YWA and even DLE/YWA variants was comparable with that of wild-type antibody. These studies demonstrate that asymmetric engineering cannot only offer Fc variants with superior ADCC activity compared with the symmetric one, but can also offer Fc variants with higher stability.

By further optimizing the Fc domain in an asymmetric manner, we successfully generated asym-mAb1 variant. During the optimization of the DLE/YWA variant to generate asym-mAb1, we removed DLE substitutions because, despite the fact that DLE substitution significantly contributes to the increased binding affinity to FcγRIIIa, DLE substitutions even in one of the Fc domains significantly reduced the T_M of the C_H2 domain. To the best of our knowledge, asym-mAb1 binds to $Fc\gamma RIIIa^{F158}$ and $Fc\gamma RIIIa^{V158}$ with the highest affinity among any reported Fc engineered mAbs (K_D = 1.2 nM and 0.37 nM, respectively). Consistent with this increased binding affinity to FcγRIIIa, asym-mAb1 demonstrated significantly higher ADCC activity than IgG1, and comparable or slightly superior ADCC activity compared with afucosyl mAb. Asym-mAb1 showed slightly superior ADCC activity than afucosyl mAb in some donors, who might have lower-affinity FcγRIIIa genotype. Notably, asym-mAb1 increased FcγRIIIa binding affinity 1000-fold while maintaining inhibitory FcγRIIb binding comparable with wild-type IgG1. We assumed that this selectivity against FcγRIIb binding was achieved by fine-tuning each Fc-FcγR interaction, resulting in an A/I ratio of 1000 to 2000, which is far superior to the other Fc variants. Moreover, the T_M of the C_H2 domain in asym-mAb1 was 64°C, significantly higher than that of the homo-DLE variant and, though it is slightly lower than wild-type IgG, high enough for pharmaceutical development (**Table S1**). These results demonstrate that our novel asymmetric engineering provides an antibody Fc variant that has the strongest binding affinity for both $Fc\gamma RIIIa^{F158}$ and $Fc\gamma RIIIa^{V158}$ with no increased binding affinity to inhibitory FcγRIIb and with high stability of the C_H2 domain.

Although asym-mAb1 has a human FcγR binding property superior to other Fc variants, it is challenging to precisely evaluate and compare the therapeutic effects of these Fc-engineered antibody in human using an in vivo pre-clinical murine model system. This is because the structural diversity and expression patterns of murine FcγRs do not correspond to those of human FcγRs.[1] As expected, asym-mAb1 and other Fc variants bound to murine FcγRs with a different specificity and affinity (data not shown), making it impossible to predict their efficacy in human based on results from the murine model. It would be possible to predict the effect mediated by human FcγRIIIa by using human FcγRIIIa-transgenic mice as previously described, but it is still difficult to evaluate the effect mediated through other FcγRs, including the therapeutic advantage of superior A/I ratio.[17] A mouse whose FcγRs were replaced with human FcγRs was recently developed. This mouse recapitulated the unique expression pattern and the functions of human FcγRs that are mediated by human IgG[18] and might enable us to evaluate the effect of our asymmetrically engineered antibody in human more accurately.

Asymmetric bispecific IgG antibody, which binds to different antigens with each arm, was recently reported to be valuable in the field of hemophilia A[19] and approaches to overcome the problems involved in manufacturing this type of IgG antibody has been recently reviewed.[20] Cancer-targeting bispecific antibodies, although not in IgG form, have been investigated to enhance efficacy by targeting two different tumor antigens.[21] Since asymmetric bispecific IgG antibodies targeting two different tumor antigens inevitably require hetero-dimerization of the two heavy chains, our asymmetrically engineered Fc could be applied to such bispecific antibodies without any difficulty. Bispecific IgG antibody is a promising antibody format for next-generation antibody therapeutics, and our novel asymmetrically engineered Fc can be easily applied to the format to achieve maximum anti-tumor efficacy.

In conclusion, we demonstrated that asymmetric Fc engineering provides more effective optimization of the Fc-FcγR interaction and identified an asymmetric Fc variant with the highest binding affinity for both FcγRIIIa allotypes, the highest A/I ratio, and slightly higher ADCC activity than previously reported symmetric Fc variants. In addition, asymmetric Fc engineering minimized the use of substitutions that reduce the T_M of the C_H2 domain. Therefore, our asymmetric Fc engineering

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platform provides best-in-class effector function for maximizing the therapeutic potential of either monospecific or bispecific IgG antibodies against tumor antigens.

Materials and Methods

Preparation of antibodies. The antibody variants used in the experiments were expressed transiently in FreeStyle™ 293 cells (Invitrogen) transfected with plasmids encoding heavy and light chains and purified from culture supernatants using rProtein A Sepharose 4 Fast Flow or rProtein G Sepharose 4 Fast Flow (GE Healthcare). Site-directed mutagenesis of the constant regions of mAbs was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) or In-Fusion HD Cloning Kit (Clontech) and the sequence was confirmed by DNA sequencing. The substitutions to facilitate Fc heterodimerization were introduced to obtain asym-mAbs consisting of different heavy chains.[20] Asym-mAbs were generated by Freestyle™ 293 cells transfected with three plasmids encoding a light chain and two different heavy chains. Sym-mAbs were generated by Freestyle™ 293 cells transfected with plasmids encoding a light chain and a heavy chain. Afucosylated antibody was prepared as previously described.[22]

Construction, expression and purification of FcγRs. The sequence information of genes encoding the extracellular region of human FcγRs was obtained from the National Center for Biotechnology Information (NCBI) and the genes were synthesized. FcγRs were fused with 6x His-tag at the C terminus. Vectors containing FcγRs were transfected into FreeStyle™ 293 cells (Invitrogen). Media were harvested and receptors were purified using cation exchange chromatography, nickel affinity chromatography and size exclusion chromatography. Instead of cation exchange chromatography, anion exchange chromatography was used to purify FcγRIa.

Comprehensive mutagenesis of the Fc domain of IgG1 and evaluation of its binding. Sym-mAb and corresponding asymmab against tumor antigen X, each with a single substitution in only one heavy chain, were prepared for comprehensive mutagenesis assay. They were designed by substituting each of the residues 234–239, 265–271, 285, 296, 298, 300 and 324–337 (EU numbering) in the lower hinge and C_H2 domain with other 18 amino acids excluding cysteine. The Fc variants were expressed in 6-well cell culture plate (Becton, Dickinson and Company) transiently in FreeStyle™ 293 cells (Invitrogen) and purified from the culture supernatants using rProtein A Sepharose 4 Fast Flow or rProtein G Sepharose 4 Fast Flow (GE Healthcare) in a 96-well format. The concentrations of purified the Fc variants were determined by NanoDrop 8000 (Thermo Scientific). The binding activity of those variants to FcγRIIIa^{158F} was quantified by a Biacore instrument. The variants were captured on the CM5 sensor chip (GE Healthcare) on which antigen peptide was immobilized, followed by injection of FcγRs. The binding of each antibody to each FcγR was normalized by the captured amount of each variant on the sensor chip and was expressed as a percentage of that of the antibody without the substitutions.

Kinetic analysis by surface plasmon resonance. The kinetic analysis of antibody variants for human FcγRs was monitored by SPR using a Biacore instrument (GE Healthcare), as previously described.[23] A recombinant protein L (ACTIGEN) was immobilized on CM5 sensor chip (GE Healthcare) using a standard primary amine-coupling protocol. Antibody variants were captured on the chip, followed by injection of FcγRs.

Thermal shift assay. T_M of the C_H2 domain of an antibody was measured as previously described.[24] The SYPRO orange dye (Invitrogen) was diluted into phosphate buffered saline (PBS; Sigma-Aldrich), before being added to the 0.3 mg/ml protein solutions. Fluorescence measurements were employed using a real-time polymerase chain reaction (RT-PCR) instrument, Rotor-Gene Q (QIAGEN). Rotor-Disc 72 was used with 20 μL of solution per well. The fluorescence emission was collected at 555 nm with a fixed excitation wavelength at 470 nm. During the measurement, the temperature was increased from 30°C to 99°C at a heating rate of 4°C/min.

Stability study under accelerated conditions. Antibodies were dialyzed against PBS and diluted to 1 mg/ml. The antibody solutions were stored at 40°C and analyzed before the treatment and after 2 weeks and 4 weeks. A monomer peak area of each antibody was analyzed with size-exclusion chromatography TSK-GEL G3000SWXL column (TOSOH) by SEC-HPLC with UV detection (Waters). The percentage of reduction from initial monomer peak area was calculated and reported using Empower Waters software.

ADCC assay. Cytotoxicity of antibody against antigen X and antigen Y was measured using a standard 4-h ^{51}Cr release assay and calcein-AM release assay, respectively.[6,25,26] Peripheral blood mononuclear cells (PBMC) were purified from whole human blood of healthy donors and used as effector cells. For ^{51}Cr-release assay, we used SK-Hep-1 cells transfected with tumor antigen X as target cells. Target cells were labeled with 1.85 MBq of ^{51}Cr at 37°C for 1 h in a CO_2 incubator. For calcein-AM release assay, DLD-1 cells expressing tumor antigen Y were labeled with calcein solution at 37°C for 2 h in a CO_2 incubator. 10 mg/ml human IgG (Sanglopor, CSL Behring K.K.) was added to mimic endogenous IgG in human. The number of tumor antigen X expressed on the cell surface was 9.8×10^4 per cell and that of tumor antigen Y was 3.7×10^5 per cell.

Antibody solution was mixed with target cells (1×10^4 cells) and then effector cells were added to the solution at 50:1 PBMC/target cell ratio. The solution was incubated in a CO_2 incubator at 37°C for 4 h. Supernatant was harvested and its radioactivity (in ^{51}Cr release assay) or the fluorescence emitted from its released calcein (in calcein-AM release assay) was quantified. Calculating the percentage of specific cell lysis from experiments was done using the following equation: % specific lysis = 100 × (mean experimental release - mean spontaneous release) ÷ (mean maximal release - mean spontaneous release). "Mean experimental release" is radioactivity in ^{51}Cr release assay or fluorescent emission in calcein-AM release assay of the supernatant from the reaction solution with antibody variants. "Mean spontaneous release" is radioactivity in ^{51}Cr release assay or fluorescent emission in calcein-AM release assay of the supernatant from the reaction solution without anvtibody. "Mean maximal release" is measured from the prepared supernatant by lysing the target cells with 2% NP-40.

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Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interests to disclose.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/mabs/article23452

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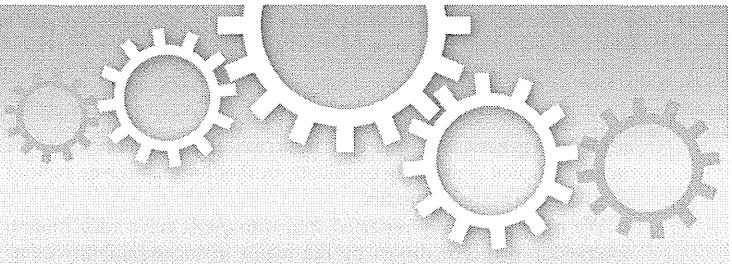

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Novel genetically-humanized mouse model established to evaluate efficacy of therapeutic agents to human interleukin-6 receptor

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For clinical trials of therapeutic monoclonal antibodies (mAbs) to be successful, their efficacy needs to be adequately evaluated in preclinical experiments. However, in many cases it is difficult to evaluate the candidate mAbs using animal disease models because of lower cross-reactivity to the orthologous target molecules. In this study we have established a novel humanized Castleman's disease mouse model, in which the endogenous interleukin-6 receptor gene is successfully replaced by human *IL6R*, and human IL6 is overexpressed. We have also demonstrated the therapeutic effects of an antibody that neutralizes human IL6R, tocilizumab, on the symptoms in this mouse model. Plasma levels of human soluble IL6R and human IL6 were elevated after 4-week treatment of tocilizumab in this mouse model similarly to the result previously reported in patients treated with tocilizumab. Our mouse model provides us with a novel means of evaluating the in vivo efficacy of human IL6R-specific therapeutic agents.

Worldwide trends in the development of therapeutic agents are towards the use of molecularly targeted drugs such as monoclonal antibodies (mAbs), which have revolutionized therapy for many intractable diseases. However, novel issues have emerged when evaluating their preclinical efficacy and safety[1–3]. It is usually difficult to evaluate the efficacy of therapeutic mAbs in animal experiments because, in most cases, they have no or low cross-reactivity to orthologous molecules of animals other than primates (phylogenetically the closest species to human). This means that systems using smaller experimental animals, such as mice and rats, are not applicable despite their obvious advantages. These advantages are that they are well-characterized after a long history of contributing to thousands of studies in various research fields of medical science, and their smaller body sizes require relatively small amounts of candidate agents. This latter advantage is especially useful at the early stage of drug development when a wider variety of drug candidates needs to be screened to select the best agent. As for the use of primates, this has been limited by disease outbreak risks, legislative changes and logistical problems with supply[2]. Moreover, it has also been pointed out that even an examination using primates would not be sufficient to perfectly predict clinical outcomes[1].

Some reviews propose the use of genetically engineered rodents and/or surrogate antibodies in order to predict the efficacy and safety of drug candidate antibodies in preclinical studies[1–3], but various attributes of the two tools need to be taken into account. For example, it will be costly and time-consuming to develop surrogate antibodies only for preclinical animal experiments and, even then, the surrogate antibodies would not necessarily work in the same manner as fully developed therapeutic antibodies. Genetic engineering in mice is a powerful technique to make loss-of-function or gain-of-function mutants for analyzing in vivo gene function and to develop animal models for human diseases, but the type of transgenic mouse established needs to correspond to its end purpose. To evaluate the pharmacokinetics, pharmacodynamics, in vivo efficacy, etc. of a drug, we must produce a genetically humanized mouse by the gene knock-in technique, in which a human target gene would be substituted and controlled to express in a similar spatial and temporal pattern to that of the endogenous orthologous gene. By

using such genetically humanized mice, we can expect to evaluate the in vivo efficacy of the drug candidate antibodies themselves, instead of using surrogate antibodies.

We have previously reported that transgenic mice with human interleukin-6 (hIL6) driven by the major histocompatibility complex class I *H-2L*^{d} gene promoter develop symptoms similar to Castleman's disease in human[4–6] such as lymphadenopathy, massive immunoglobulin G1 plasmacytosis, splenomegaly, mesangial proliferative glomerulonephritis, thrombocytopenia, leukocytosis, anemia and muscle atrophy[7,8]. We also demonstrated that a mAb to mouse IL-6 receptor, the surrogate antibody MR16-1, completely blocked their symptoms[8]. These findings indicate that neutralization of IL-6 signaling by a mAb to IL-6 receptor would be an effective therapeutic strategy for IL-6-related diseases. However, it is not possible to use these transgenic mice to evaluate the in vivo efficacy of drug candidate antibodies directly because they express murine IL-6 receptor (*Il6ra*) instead of human IL-6 receptor (h*IL6R*). A possible solution is to use a double transgenic mouse established by crossing an *H-2L*^{d}-h*IL6* transgenic mouse with an h*IL6R* transgenic mouse. As far as we know, two lines of h*IL6R* transgenic mice were previously reported[9,10]. However, these h*IL6R* transgenic mice cannot be used to evaluate therapeutic mAbs because they express not only hIL6R but also endogenous mouse Il6ra, which is well known as responding to human IL6. Therefore, it is necessary to neutralize or disrupt the endogenous mouse *Il6ra* before evaluating drug efficacy. Moreover, these h*IL6R* transgenic mice express extremely higher levels of hIL6R, driven by relatively stronger promoters. Therefore we predict that using these h*IL6R* transgenic mice to evaluate the therapeutic efficacy of neutralizing antibody to hIL6R would be difficult because the antibody, mediated by antigen, would disappear extremely rapidly from blood.

In this study we have generated a novel Castleman's disease mouse model, in which, in addition to the *H-2L*^{d}-h*IL6* transgene described above, mouse endogenous *Il6ra* gene is successfully replaced by h*IL6R* with the gene knock-in technique to establish a humanized ligand-receptor system for IL6 in mice. We have also demonstrated that symptoms of this model were almost completely blocked by administering tocilizumab, a humanized antibody against hIL6R[11]. These results demonstrate that genetically humanized mice will be powerful tools for directly evaluating in vivo efficacy of not only mAbs but also a wide variety of future therapeutic agents that are highly specific to human target molecules.

Results

Establishing a human IL6R knock-in mouse. The scheme for generating an h*IL6R* gene knock-in mouse is presented in Fig. 1a. Correctly targeted ES cell clones with the targeting vector were microinjected into the blastocysts of C57BL/6J (B6) mouse to make chimera mice. Male chimera mice were crossed with B6 females to obtain offspring with the hIL6R knock-in locus. Genomic PCR analysis of the offspring revealed that the full length of h*IL6R* cDNA with a floxed neomycin resistant gene (*neo*) cassette was correctly inserted in the target region by homologous recombination, and the knock-in allele was transmitted through the germline. To establish the h*IL6R* knock-in allele without the *neo* cassette, the Cre expression plasmid vector was microinjected into the pronuclei of fertilized eggs[12] that were obtained by crossing male heterozygous knock-in mice with C57BL/6J females. PCR product, amplified with the primer set depicted in Fig. 1a, reduced the size from 4.2 kb to 2.7 kb; this difference of 1.5 kb indicates the length of the *neo* cassette excised from the knock-in allele (Fig. 1b). Heterozygous mice without the *neo* cassette were intercrossed to obtain homozygous knock-in mice. This strain of the h*IL6R* knock-in mouse has been named B6;129S6-*Il6ra*$^{tm1(hIL6R)Csk}$. No apparent abnormalities were observed in h*IL6R* knock-in mice.

The results of RT-PCR for h*IL6R* or mouse *Il6ra* cDNA show that each reaction amplified the specific target correctly; that is, in the cDNA samples of homozygous *Il6ra*$^{hIL6R/hIL6R}$ mice, the human-specific *IL6R* target sequence was exclusively amplified and the mouse *Il6ra* sequence was not and, in the cDNA samples of wild-type (*Il6ra*$^{+/+}$) littermates, the mouse-specific *Il6ra* sequence was amplified and the h*IL6R* sequence was not. Signal intensities detected in the same organs were almost similar between h*IL6R* in *Il6ra*$^{hIL6R/hIL6R}$ mice and mouse *Il6ra* in *Il6ra*$^{+/+}$ mice (Fig. 1c).

Plasma soluble hIL6R in homozygous *Il6ra*$^{hIL6R/hIL6R}$ mice was detected at a range of 15 ng/mL–30 ng/mL (Fig. 1d), which is substantially similar to that reported in human[13–15]. Soluble hIL6R levels in heterozygous *Il6ra*$^{hIL6R/+}$ mice were at a range of 8 ng/mL–24 ng/mL, about half of those in homozygous *Il6ra*$^{hIL6R/hIL6R}$ mice, which indicates that soluble hIL6R levels in plasma would be dependent on the gene-dosage of knocked-in h*IL6R*. As determined by the plasma levels of serum amyloid A (SAA), which is produced by the hepatocytes in response to IL6[16], the h*IL6R* knock-in mice can respond to human IL6 but not mouse Il6, whereas wild type mice can respond to both human IL6 and mouse Il6 (Fig. 1e).

Establishing a humanized Castleman's disease model mouse. We have crossed the h*IL6R* knock-in mouse and the *H-2L*^{d}-*hIL6* transgenic mouse to establish a humanized Castleman's disease mouse model, which is named B6(Cg);129-*Il6ra*$^{tm1(hIL6R)Csk}$-Tg(IL6)40Csk. Enlargement of systemic lymph nodes and splenomegaly, typical symptoms of Castleman's disease[4–6], were observed in h*IL6* transgenic mice whether their *Il6ra* gene alleles were wild-type (*Il6ra*$^{+/+}$) (Fig. 2b) or humanized (*Il6ra*$^{hIL6R/hIL6R}$) (Fig. 2c). Histological observation revealed that the number of plasma cells and white pulps were increased in the spleen of both *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice (Table 1, Fig. 3b), as compared to h*IL6* non-transgenic control mice (*Il6ra*$^{hIL6R/hIL6R}$ mice), shown in Fig. 3a.

Treatment with an hIL6R-neutralizing antibody in a humanized Castleman's disease mouse model. We then examined whether this novel humanized Castleman's disease mouse model can be used to evaluate the efficacy of hIL6R-specific therapeutic agents. We treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice and *Il6ra*$^{+/+}$-h*IL6* transgenic mice with tocilizumab and MR16-1 (Fig. 2a). As we previously reported, tocilizumab has a neutralizing activity specifically against hIL6R but not against mouse Il6ra, whereas MR16-1 has a specific neutralizing activity to mouse Il6ra but not to hIL6R[17].

The spleen weights (mean ± SD) markedly increased to 0.26 ± 0.03 g in the vehicle-treated *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice. These increased spleen weights were significantly different from those of the hIL6 non-transgenic *Il6ra*$^{hIL6R/hIL6R}$ mice group (0.08 ± 0.01 g). Treatment with tocilizumab markedly prevented the development of splenomegaly in male *Il6ra*$^{hIL6R/hIL6R}$-h*IL6* transgenic mice (Fig. 2c; Supplementary Fig. S1): the spleen weights at the end of 4-week treatment were decreased to 0.14 ± 0.03 g, 0.14 ± 0.02 g and 0.13 ± 0.03 g in groups treated with 0.1, 0.25 and 0.5 mg/body of tocilizumab, respectively. These values were not significantly different to those of the h*IL6* non-transgenic *Il6ra*$^{hIL6R/hIL6R}$ mice group. Spleen weights of the group treated with MR16-1, an antibody to mouse Il6ra (0.34 ± 0.11 g) increased to the same level as the vehicle-treated group.

In contrast, in *Il6ra*$^{+/+}$-h*IL6* transgenic mice tocilizumab treatment does not show preventive effects on the splenomegaly (0.45 ± 0.26 g) observed in the vehicle-treatment group (0.34 ± 0.09 g), whereas MR16-1 markedly prevented splenomegaly at a dose of 0.1 mg/body, with spleen weights decreasing to 0.12 ± 0.01 g. These values were not significantly different to those of h*IL6* non-transgenic *Il6ra*$^{+/+}$ mice (0.08 ± 0.01 g) (Fig. 2b; Supplementary Fig. S1). The spleen weights also displayed great interindividual variability in *Il6ra*$^{+/+}$-h*IL6* transgenic mice treated with tocilizumab (Fig. 2b).

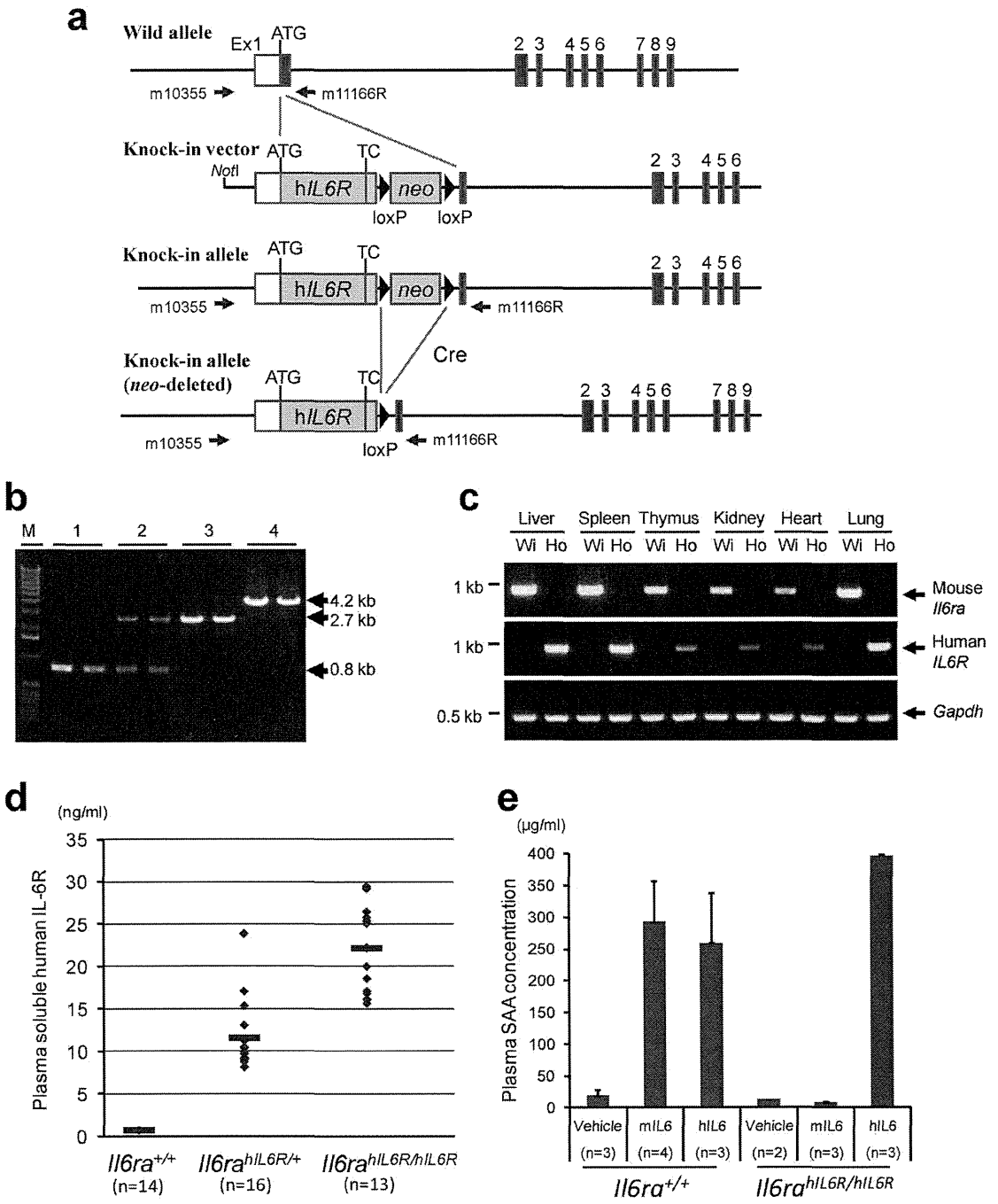

Figure 1 | Generation of human IL6 receptor (*IL6R*) gene knock-in mouse. (a) Schematic representation of the knock-in strategy for the h*IL6R* gene. A knock-in vector was constructed by inserting h*IL6R* cDNA with *neo* cassette flanked by two loxP sites into the mouse *Il6ra* genomic locus in the frame of a BAC genomic clone. A knock-in allele and a *neo*-deleted knock-in allele are also shown. Arrows indicate PCR primers (m10355 and m11166R) for genotyping. TC, terminal codon. (b) A representative result of genotyping to confirm the *neo*-depleted h*IL6R* knock-in allele and homozygosity of the h*IL6R* knock-in allele. Wild-type allele and knock-in allele were detected as signals of 0.8 kb and 4.2 kb, respectively, whereas knock-in allele after removing *neo* cassette was detected as a signal of 2.7 kb. M, DNA molecular marker. Numbers above the gel denote the mouse genotypes, (1) $Il6ra^{+/+}$, (2) $Il6ra^{hIL6R/+}$, (3) $Il6ra^{hIL6R/hIL6R}$ and (4) $Il6ra^{hIL6R(neo)/hILR6(neo)}$. (c) Representative results of RT-PCR analysis for tissue distribution of $Il6ra^{+/+}$ (Wi) and $Il6ra^{hIL6R/hIL6R}$ (Ho) mice. (d) Plasma levels of soluble hIL6R in $Il6ra^{+/+}$ (n = 14), $Il6ra^{hIL6R/+}$ (n = 16) and $Il6ra^{hIL6R/hIL6R}$ mice (n = 13). (e) Species-specific ligand response was confirmed after intraperitoneal injection of mouse Il6 (mIL6) or human IL6 (hIL6) in $Il6ra^{+/+}$ and in $Il6ra^{hIL6R/hIL6R}$ mice. Ligand responses were evaluated by the elevation of plasma SAA levels after injection of vehicle (n = 3), mIL6 (n = 4) and hIL6 (n = 3) in $Il6ra^{+/+}$ and those of vehicle (n = 2), mIL6 (n = 3) and hIL6 (n = 3) in $Il6ra^{hIL6R/hIL6R}$.