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Engineering the Fc region of immunoglobulin G to modulate *in vivo* antibody levels

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We have engineered the Fc region of a human immunoglobulin G (IgG) to generate a mutated antibody that modulates the concentrations of endogenous IgGs *in vivo*. This has been achieved by targeting the activity of the Fc receptor, FcRn, which serves through its IgG salvage function to maintain and regulate IgG concentrations in the body. We show that an IgG whose Fc region was engineered to bind with higher affinity and reduced pH dependence to FcRn potently inhibits FcRn-IgG interactions and induces a rapid decrease of IgG levels in mice. Such FcRn blockers (or 'Abdegs,' for antibodies that enhance IgG degradation) may have uses in reducing IgG levels in antibody-mediated diseases and in inducing the rapid clearance of IgG-toxin or IgG-drug complexes.

The engineering of the variable regions of IgG to generate effective therapeutic antibodies for both targeting and blocking effects is now a widely used approach[1,2]. In contrast, modulation of Fc-receptor function by manipulating the Fc regions of IgGs is relatively unexplored. Here we have investigated such an approach with the aim of developing therapeutics that decrease IgG levels *in vivo*. Such reagents could have potential applications in the treatment of diseases, such as systemic lupus erythematosus, in which autoreactive antibodies play a role[3]. In addition, they may have utility in the clearance of antibodies complexed with toxic molecules such as drugs or toxins.

There is a paucity of methods for modulating IgG levels *in vivo*, primarily owing to the limited understanding of the mechanisms by which antibody concentrations are controlled. However, recent studies have shown that the major histocompatibility complex (MHC) class I–related receptor, FcRn, plays a central role in regulating the transport of IgG within and across cells of diverse origin[4–11]. Thus, in addition to its earlier known activity in the transport of maternal IgG to offspring[12,13], FcRn regulates IgG concentrations both in the serum and throughout the body[14–16]. The current model for FcRn function is as follows: IgGs are taken up into cells, most likely by fluid-phase pinocytosis, as the near-neutral pH of the extracellular milieu is generally not permissive for FcRn-IgG interactions[17,18]. IgGs that bind to FcRn in early, acidic endosomes following uptake are recycled (or transcytosed) and released at the cell surface by exocytosis[11]. In contrast, IgGs that do not bind enter the lysosomal pathway and are degraded[10]. IgGs can fail to be salvaged by FcRn for several reasons. They may have relatively low affinity for FcRn and as a result not compete favorably with other IgGs for interaction, or the IgG concentration may be saturating for FcRn binding. IgG homeostasis in the body is therefore maintained, at least at the level of IgG breakdown, by the saturable nature of FcRn.

The FcRn interaction site on IgG has been mapped using a combination of site-directed mutagenesis, functional analyses and X-ray crystallography[19–22]. It encompasses residues at the CH2-CH3 domain interface of IgG that include Ile253, His310, His435 and, in most mouse isotypes, His436 (ref. 23). In most human IgG isotypes and mouse IgG2b, residue 436 is tyrosine (ref. 23). The IgG-FcRn interaction is also highly pH dependent, with tight binding at pH 6.0, which becomes progressively weaker as near-neutral pH is approached[17,18]. Information concerning the molecular details of the FcRn interaction site offers opportunities to engineer antibodies with altered pharmacokinetics and distribution[24–26].

Rather than using knowledge of IgG-FcRn interactions to generate IgGs with longer (or shorter) serum half-lives[20,24–26], we engineered an antibody that, through its Fc-mediated binding to FcRn, enhances the clearance rates of endogenous IgGs. The rationale for the current study is that modified antibodies with increased affinity for FcRn relative to their wild-type counterparts will inhibit the interaction of endogenous IgGs, thereby increasing degradation of the latter. The inhibitory capacity of the engineered antibody would be further enhanced if the pH dependence of the IgG mutant-FcRn interaction were decreased, because, compared with naturally occurring IgGs, the engineered IgG would bind more stably to FcRn during exocytic events at the cell surface[11]. We have therefore generated a human IgG1 variant that, as a result of mutations in the Fc region, has suitable properties for the blockade of FcRn activity. We show that this engineered antibody inhibits FcRn-mediated recycling of IgG *in vitro* and enhances the clearance rates of endogenous IgGs in mice. Such antibodies, or 'Abdegs' (antibodies that enhance IgG degradation), may have applications in the treatment of antibody-mediated diseases and in other situations where FcRn inhibition is desirable.

RESULTS

Generation and FcRn binding properties of an Abdeg

An effective Abdeg should have increased affinity for FcRn at both acidic and near-neutral pH relative to its parent wild-type IgG. In earlier studies a mutated variant of human IgG1 was generated in

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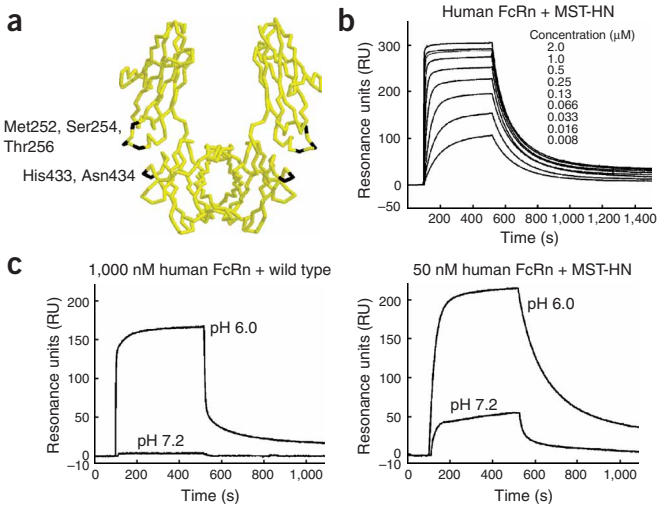

Figure 1 The MST-HN Abdeg binds to human FcRn with increased affinity and reduced pH dependence. (**a**) α-carbon trace of the X-ray crystallographic structure of human IgG1 (Fc region[49]) with location of residues targeted for mutagenesis in the MST-HN mutant (Met252, Ser254, Thr256, His433 and Asn434) indicated in black (drawn using Rasmol, courtesy of Roger Sayle, Bioinformatics Research Institute, University of Edinburgh). (**b**,**c**) SPR analyses of the interaction of human FcRn with wild-type human IgG1 (wild type) or MST-HN Abdeg. Coupling densities for the data shown are 671 RU (MST-HN) or 719 RU (wild-type human IgG1). Recombinant human FcRn was injected over the flow cells at the concentrations indicated in PBS plus 0.01% Tween 20 pH 6.0 (**b**,**c**) or pH 7.2 (**c**) at a flow rate of 10 μl/minute. Sensorgrams showing equilibrium binding analysis of the interaction of human FcRn with MST-HN Abdeg (**b**); interaction of human FcRn with wild-type human IgG1 and MST-HN Abdeg at pH 6.0 and pH 7.2 (**c**). Concentrations of human FcRn used in **c** correspond to about three times the K_{D1} value at pH 6.0 for the respective interactions. Representative sensorgrams of at least duplicate injections are shown (**b**,**c**). All data were zero adjusted and reference-cell subtracted.

which Met252, Ser254, Thr256, His433, Asn434 and Tyr436 were changed to Tyr252, Thr254, Glu256, Lys433, Phe434 and His436 ('MST-HNY') with the goal of increasing serum half-life[26]. The mutations of these residues, which are located at the CH2-CH3 domain interface (**Fig. 1a**), result in increased affinity at pH 6.0 and reduced pH dependence (that is, increased binding at near-neutral pH) for binding to both mouse and human FcRn[26]. With the aim of analyzing the role of residue 436 of human IgG1, which differs between mouse and human IgG1 (histidine in most mouse isotypes; tyrosine in human IgG1[23]) in binding to FcRn, we mutated Tyr436 of human IgG1 to histidine. This mutation resulted in an approximate threefold reduction in affinity for binding to human FcRn (J.Z., F. Mateos and E.S.W., unpublished data), suggesting that the Y436H mutation in the MST-HNY variant might be detrimental to binding. This prompted us to make an engineered variant of human IgG1 containing mutations of Met252, Ser254, Thr256, His433 and Asn434 to Tyr252, Thr254, Glu256, Lys433 and Phe434 (MST-HN; **Fig. 1**). As shown by surface plasmon resonance (SPR) analyses, MST-HN has a substantially increased binding affinity for human FcRn at pH 6.0 (K_{D1} = 15.5 nM for MST-HN; K_{D1} = 370 nM for wild-type human IgG1) and retains significant binding activity at pH 7.2 (**Fig. 1**). We also used SPR to analyze the interaction properties of MST-HN with mouse FcRn. The mutant bound to mouse FcRn with high affinity at pH 6.0 (K_{D1} = 1.2 nM) and retained good affinity at pH 7.2 (K_{D1} = 7.4 nM). The MST-HN variant therefore bound more tightly to mouse FcRn than to human FcRn, consistent with earlier studies indicating that, relative to mouse FcRn, human FcRn generally has lower affinities for IgGs[26,27].

The MST-HN Abdeg colocalizes with FcRn in endothelial cells

We analyzed the properties of the MST-HN Abdeg using *in vitro* assays with human microvascular endothelial cells (HMEC-1) transfected with mouse FcRn-green fluorescent protein (GFP). HMEC-1 cells were used extensively in our earlier studies to analyze the intracellular trafficking of FcRn and IgGs[10,11]. The uptake of fluorescently labeled MST-HN Abdeg at a concentration of 20 μg/ml (~125 nM) into transfected cells was first compared with that of 20 μg/ml fluorescently labeled human or mouse IgG1 (both wild type). Fluorescence imaging showed that the mutated IgG1 accumulated in the cells to substantially higher levels than either wild-type mouse or human IgG1, which were undetectable under our imaging conditions (**Fig. 2**). Colocalization of the MST-HN Abdeg with mouse FcRn-GFP was extensive, indicating that the Abdeg interacts with FcRn within cells. First, the Abdeg's high affinity and reduced pH dependence for FcRn promotes binding to surface FcRn under the conditions of the assay. This would be expected to facilitate uptake of the Abdeg by receptor-mediated pathways. Second, the Abdeg's binding properties may result in poor release of this IgG during exocytic events involving FcRn at the plasma membrane.

The MST-HN Abdeg can inhibit recycling of IgGs *in vitro*

We next analyzed the ability of the MST-HN Abdeg to inhibit recycling of IgG in human endothelial cells transfected with mouse

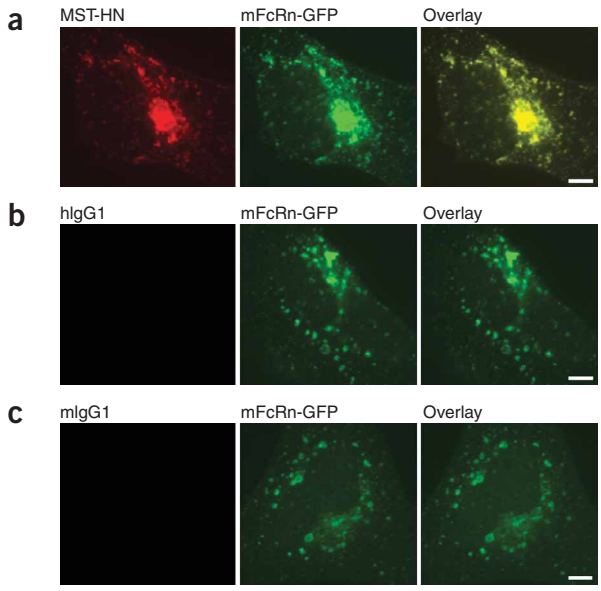

Figure 2 The MST-HN Abdeg accumulates to higher levels in FcRn-GFP–expressing endothelial cells relative to wild-type human or mouse IgG1. Uptake of Alexa 647–labeled MST-HN Abdeg (**a**), wild-type human IgG1 (**b**) or wild-type mouse IgG1 (**c**) by HMEC-1 cells transfected with mouse FcRn (mFcRn)-GFP. Transfected cells were pulsed with 20 μg/ml labeled IgG1 for 60 min, washed, fixed and mounted. The same imaging conditions (exposure times) were used for imaging of Alexa 647–labeled IgGs, and Alexa 647 is pseudocolored in red. Bars, 5 μm.

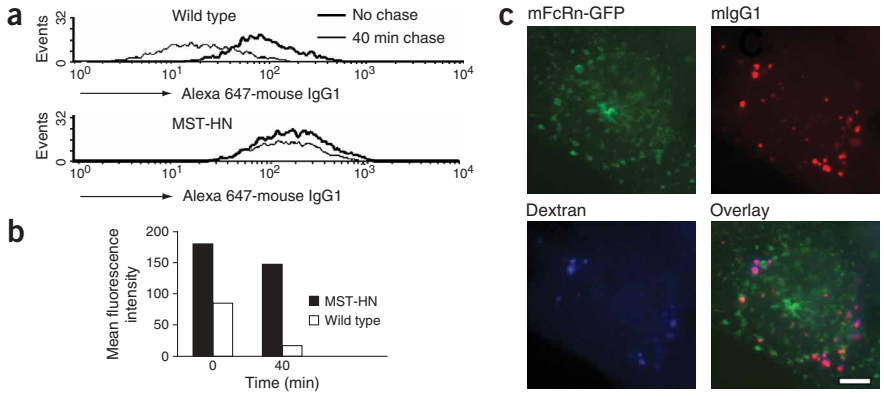

Figure 3 Inhibition of recycling of mouse IgG1 from HMEC-1 cells transfected with mouse FcRn-GFP. (**a**) Histogram plots showing the amounts of Alexa 647–labeled mouse IgG1 at different chase times after pretreatment of cells with either MST-HN Abdeg or wild-type human IgG1. (**b**) Mean fluorescence intensities (background subtracted) of Alexa 647 fluorescence for each time point/treatment for the data shown in **a**. (**c**) Images of mouse FcRn (mFcRn)-GFP–transfected cells to show distribution of Alexa 546–labeled mouse IgG1 and Alexa 647–labeled dextran (lysosomal tracer) after pretreatment with the MST-HN Abdeg. Data are representative of at least two independent experiments. Bar, 5 μm.

FcRn–GFP. Cells were preincubated with the MST-HN Abdeg or wild-type human IgG1 for 60 min, and then fluorescently labeled mouse IgG1 added for a further 60 min. Uptake and recycling of mouse IgG1 at the end of the pulse and after a 40-min chase period, respectively, were quantified by flow cytometry. Immediately after the pulse period, mouse IgG1 had accumulated to higher levels in cells pretreated with the MST-HN Abdeg relative to those pretreated with wild-type human IgG1 (**Fig. 3a**,**b**). In addition, during the chase period substantially more mouse IgG was recycled out of the cells pretreated with wild-type human IgG1 relative to Abdeg-treated cells (**Fig. 3a**,**b**).

We also used fluorescently labeled dextran as a lysosomal tracer to assess the intracellular location of the mouse IgG1 in HMEC-1 cells that had been pretreated with the MST-HN Abdeg. Microscopy analyses of these cells indicated colocalization of dextran and mouse IgG1 (**Fig. 3c**). Taken together, the data show that pretreatment of cells with the MST-HN Abdeg resulted in decreased recycling and accumulation of mouse IgG1 in the lysosomal pathway.

The MST-HN Abdeg can enhance IgG clearance *in vivo*

We also investigated the effect of the MST-HN Abdeg on the clearance rates of IgGs in mice. Mice were injected with radiolabeled (^{125}I) wild-type human IgG1, and 72 h (3 d) later groups of mice were treated with 500 or 200 μg MST-HN Abdeg or 500 μg wild-type human IgG1. **Figure 4** shows the levels of radioactivity remaining in the mice at different times during the experiment. These levels were assessed using whole-body counting and therefore indicate whole-body rather than serum levels of labeled IgG1. After injection of 500 μg MST-HN Abdeg, a rapid decrease in radioactivity levels in the mice was observed (**Fig. 4**). A similar, but less marked effect, was observed for mice treated with 200 μg MST-HN Abdeg (**Fig. 4**). The levels of radioactivity dropped to 12 ± 4.6% (500-μg treatment group) or 20 ± 3.8% (200-μg treatment group) of the injected dose 120 h (5 d) after injection of the MST-HN Abdeg. In contrast, treatment of mice with 500 μg of wild-type human IgG1 had no observable effect on the clearance rate of the radiolabeled IgG1 (**Fig. 4**).

The MST-HN Abdeg can reduce IgG concentrations *in vivo*

The effect of injection of the MST-HN Abdeg on the levels of endogenous IgGs in the serum of mice was also analyzed. Endogenous (steady-state) serum IgG levels were determined, and mice were subsequently injected with either 500 μg MST-HN Abdeg or 500 μg wild-type human IgG1. Relative to treatment with wild-type human IgG1, injection of the MST-HN Abdeg resulted in significant decreases in endogenous serum IgG levels, which persisted for about 96 h (4 d) (**Fig. 5**).

Taken together, the data show that the MST-HN Abdeg reduced levels of both exogenous and endogenous IgGs in mice when used at doses that were substantially lower than the whole-body load of endogenous IgGs. Further, as we have analyzed the *in vivo* effects of the MST-HN Abdeg on IgG levels in both the serum compartment and whole body, our observations indicate that inhibition of FcRn function by Abdegs acted at diverse sites throughout the body.

DISCUSSION

Manipulating the Fc region rather than the variable regions of IgGs is a relatively unexplored approach whose potential is now beginning to be realized[2]. We have described a route to lower IgG levels *in vivo* by using IgGs engineered in their Fc region to target the IgG-FcRn interaction site. In contrast to earlier studies in which IgG-FcRn interactions were modulated to alter the *in vivo* persistence of a therapeutic antibody[24–26], the Abdeg was designed to alter the levels of endogenous, unmanipulated IgGs by enhancing their clearance rates.

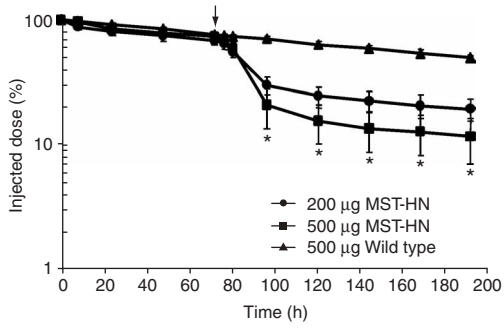

Figure 4 Enhancement of clearance of injected wild-type human IgG1 by MST-HN Abdeg. Swiss Webster mice (six mice/group) were injected with ^{125}I-labeled wild-type human IgG1 and the persistence of the labeled IgG1 monitored by whole-body counting. Mice were injected with 500 μg wild-type human IgG1, or 200 μg or 500 μg MST-HN Abdeg, 72 h later (indicated by arrow). Levels of remaining ^{125}I-labeled human IgG1 were determined at the indicated times. The data shown are means of the remaining radioactivity in the different groups of mice. Error bars indicate s.d. *, data for these time points for mice treated with 500 or 200 μg MST-HN Abdeg are significantly different between the two groups, with $P < 0.03$ (Student's *t*-test). Data are representative of two independent experiments.

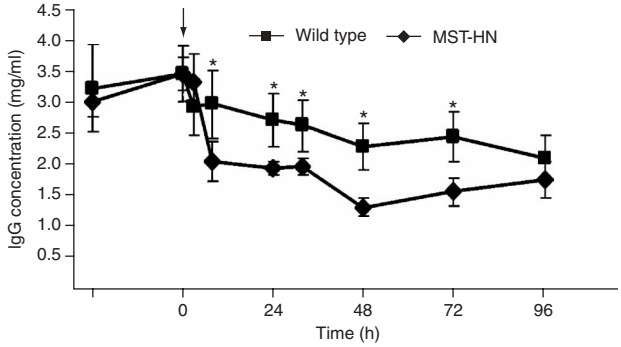

Figure 5 Enhancement of clearance of endogenous IgGs by MST-HN Abdeg. The serum IgG levels in Swiss Webster mice were quantitated by ELISA. Mice (6 mice/group) were subsequently injected with 500 μg wild-type human IgG1 or 500 μg MST-HN Abdeg (indicated by arrow) and serum IgG levels monitored at the indicated times post-injection of these antibodies. Data shown are means of the serum IgG levels in the two different groups of mice, with s.d. indicated by error bars. *, data for these time points are significantly different between the two groups, with a $P < 0.009$ (Student's *t*-test). Data are representative of two independent experiments.

FcRn is known to be involved in transporting IgGs within and across cells of diverse origin, and in so doing, to regulate IgG concentrations and transport throughout the body[4–11,28]. We show that a mutated, human IgG1-derived antibody (MST-HN) with higher affinity for binding through its Fc region to FcRn at both pH 6.0 and 7.2 relative to its wild-type counterpart competes effectively with wild-type IgGs for FcRn-mediated transport. The binding properties of the Abdeg confer the ability to enhance the clearance of endogenous IgGs in mice.

The intracellular routing of FcRn and its IgG ligand has recently been analyzed using live-cell fluorescence imaging[10,11]. Although the steady-state levels of FcRn on the plasma membrane are generally low relative to cytosolic levels[4–6,10,14,29], FcRn is directly involved in exocytic events involving transported IgG at the plasma membrane[11]. Surface expression of FcRn, even if only transient, would allow Abdegs such as MST-HN to bind, which in turn would facilitate more efficient uptake relative to fluid-phase processes. The MST-HN Abdeg therefore has several advantages over naturally occurring, pH-dependent IgGs in competing for FcRn binding. First, it can bind with high affinity at the cell surface. Second, it can outcompete IgGs that have lower affinity for FcRn within the sorting endosome[10]. Third, the mutated IgG1 would not be efficiently released during exocytic events involving FcRn[11]. Thus, as shown here, the MST-HN Abdeg accumulates at substantially higher levels in FcRn-expressing endothelial cells relative to wild-type mouse and human IgG1.

We have shown that the MST-HN Abdeg reduces mouse IgG levels both in serum and in the whole body. FcRn is expressed not only in endothelium but also in epithelia of the gut, lung and kidney[4,7,8]. We therefore expect that Abdegs such as MST-HN will have access to FcRn at a diverse array of body sites, including epithelial barriers.

Enhanced IgG degradation in humans may be desirable in multiple therapeutic situations. For example, Abdegs could be tested for the treatment of diseases caused by high levels of autoreactive antibodies, such as systemic lupus erythematosus and immune thrombocytopenic purpura (ITP)[3,30]. Further, FcRn is directly involved in the maternofetal transport of IgGs[31,32], indicating that Abdegs might also be used to inhibit the passage of deleterious (auto)antibodies to the fetus. Alternatively, it may be possible to couple antibody-mediated clearance of toxins or drugs from the body with the subsequent delivery of an Abdeg to enhance degradation of IgG-antigen complexes. A possible disadvantage of using Abdegs in therapy is that they systemically induce degradation of all IgGs and are relatively nonspecific compared with therapies that target a particular protein. As a consequence, Abdegs might be more attractive as reagents in situations where short-term effects are desired, rather than as a long-term treatment modality. However, it might be possible to develop ways of targeting Abdegs to specific tissues so that local effects predominate.

Intravenous gamma globulin (IVIG) has been used with some efficacy to treat autoimmune diseases such as ITP, myasthenia gravis and multiple sclerosis[33]. However, the mechanisms of action underlying these effects are a matter of debate. Proposed mechanisms include the blockade of Fcγ receptors on phagocytes, anti-idiotypic effects, activation of inhibitory Fcγ receptors and prevention of complement activation[34–36]. It has also been proposed that IVIG enhances clearance of endogenous, disease-associated IgGs[34,37], and recent data in the murine K/BxN arthritis model support this[38]. However, the doses of IVIG that are needed to lower endogenous IgGs are in the range of the whole-body load of IgG. In contrast, owing to its competitive advantage in binding, the effective dose of MST-HN Abdeg (or any other Abdeg with equivalent properties) is much lower. Indeed, in the current study we show that wild-type human IgG1, when used at the same dose as the MST-HN Abdeg, has no effect on the clearance of endogenous IgGs in mice. This lack of effect is observed despite the approximately five- to tenfold higher affinity of (wild-type) human IgG1 relative to mouse IgGs for binding to mouse FcRn[27].

Mouse and human FcRn have significant differences in binding specificities[39]. The molecular basis for this has recently been determined by site-directed mutagenesis and binding studies[40]. However, despite these differences, relative to wild-type human IgG1 the MST-HN mutant has increased affinity and reduced pH dependence for binding to both mouse and human FcRn. These binding properties for human FcRn suggest that the MST-HN mutant might have uses as an Abdeg in humans. In addition, other engineered IgG variants that share binding properties with the MST-HN mutant[26] may have activities as Abdegs.

In summary, we show that it is possible to generate an IgG with a mutated Fc region that efficiently inhibits FcRn function. As a consequence, this Abdeg reduces the levels of IgGs *in vivo*. The similarities between human and mouse FcRn function suggest that analogous approaches may be effective in humans. The use of engineered antibodies that, through their Fc region, are effective inhibitors of FcRn function therefore offers a potential therapeutic modality for the treatment of antibody-mediated diseases.

METHODS

Mice. Swiss Webster mice (females, 6–8 weeks old) were purchased from Harlan Sprague and housed in the Animal Resources Center at UT Southwestern Medical Center. All procedures with mice were approved by the Institutional Review Board at UT Southwestern.

Expression vectors. The expression vector for a humanized anti-lysozyme heavy chain (human IgG1[41]) was generously provided by Jefferson Foote. The human IgG1 constant region was mutated by the PCR with mutagenic oligonucleotides and splicing by overlap extension[42]. The following mutations were made: Met252 to Tyr, Ser254 to Thr, Thr256 to Glu, His433 to Lys and Asn434 to Phe ('MST-HN'). Sequences of mutagenic oligonucleotides are provided in **Supplementary Data** online. After mutagenesis, altered regions were sequenced to ensure insertion of the desired mutation without second site

mutations. The mutated Fc gene was recloned into the final expression construct using standard methods.

A plasmid-encoding mouse FcRn linked to enhanced GFP was generated using an analogous strategy to that described for human FcRn and the vector pEGFP-N1[10] (Clontech). The mouse FcRn α-chain gene was isolated from murine SV40-transformed endothelial cells[43]. A mouse β2-microglobulin expression plasmid was generated using the PCR to reclone the β2-microglobulin gene from an insect cell vector[18] into pCB7 (ref. 44) (generously provided by Michael Roth, UT Southwestern Medical Center).

Generation of transfectants for antibody expression. NSO cells expressing a human anti-lysozyme specific light chain (HuLys5 (ref. 41): generously provided by Jefferson Foote) were transfected by electroporation, and transfectants selected as described[41]. Culture supernatants were screened for expression of antibody by enzyme-linked immunosorbent assay (ELISA) using hen egg lysozyme (Sigma)–coated plates and horse radish peroxidase (HRP)–conjugated anti-human Fc (Sigma). Positive clones were expanded further for protein production.

Expression, purification and labeling of recombinant proteins. Recombinant human IgG1 proteins (wild type and MST-HN variant: both lysozyme specific) were purified from culture supernatants using lysozyme-sepharose as described[41]. Human IgG1 was radiolabeled with ^{125}I using iodogen according to methods previously described[45].

The mouse (IgG1) anti-lysozyme antibody, D1.3, was purified from culture supernatants of the D1.3 hybridoma[46] (generously provided by Roy Mariuzza, Center for Advanced Research in Biotechnology, Rockville, MD, USA) using lysozyme-sepharose. Mouse or human IgG1 (wild type or mutants) were labeled using Alexa 546 or Alexa 647 carboxylic acid, succimidyl ester and methods recommended by the manufacturer (Molecular Probes).

Recombinant, soluble human and mouse FcRn were expressed in High-Five cells (Invitrogen) infected with recombinant baculoviruses and protein purified as described[27].

Surface plasmon resonance analyses. Binding analyses of human or mouse FcRn with immobilized IgGs were carried out as described previously[27,40]. However, the loss of pH dependence of the MST-HN mutant-mouse FcRn interactions necessitated the use of 100 mM glycine, 100 mM NaCl, pH 2.8 buffer to 'strip' the flow cells after each injection at pH 6.0 (rather than the pH 7.2 buffer used in earlier analyses for FcRn-IgG interactions[27]).

FcRn binds to two sites on IgG that are not equivalent[47,48]. Data were therefore fitted to a two-site binding model involving negative cooperativity[40]. This generated estimates for two dissociation constants (K_{D1} and K_{D2}) that are taken to represent occupancy of the higher affinity site (K_{D1}) followed by occupancy of the lower affinity site (K_{D2})[40].

Analyses of *in vitro* properties of IgGs. HMEC-1 cells (from the Centers for Disease Control; generously provided by F. Candal) were cotransfected with mouse FcRn-GFP and mouse β2-microglobulin expression plasmids using Amaxa nucleofector technology (Amaxa) and incubated in phenol red–free Hams 12K medium depleted of bovine IgGs as described[10]. To assess IgG uptake, about 20 h after transfection cells were incubated with 20 μg/ml of Alexa 647–labeled, wild-type human or mouse IgG1, or mutated human IgG1 (MST-HN) at 37 °C in a 5% CO_2 incubator for 60 min. Cells were then washed, fixed and mounted for fluorescence microscopy as described[10].

To assess the recycling of mouse IgG1, transfected cells were incubated with 20 μg/ml wild-type or mutated (MST-HN) human IgG1 for 60 min as above, and Alexa 647–labeled mouse IgG1 was added at a concentration of 250 μg/ml for a further 60 min. Cells were then washed and processed immediately (no chase) or incubated in phenol red–free Hams 12K medium for a further 40 min. After each treatment, cells were trypsinized, washed and stored on ice. Amounts of cell-associated Alexa 647–labeled mouse IgG1 at each time point were quantified by flow cytometry using a FACScaliber. Data were processed using WinMDI2.8 (J. Trotter, Scripps Research Institute, La Jolla, CA, USA). To assess the intracellular location of mouse IgG1 in MST-HN pretreated transfectants, cells were pretreated as above and then incubated with 250 μg/ml Alexa 546–labeled mouse IgG1 and Alexa 647–labeled dextran (Molecular Probes) for 60 min. Cells were washed, chased in medium for 40 min, and then washed, fixed and mounted for fluorescence microscopy analyses.

Fluorescence microscopy. Images were acquired using a Zeiss Axiovert 200M inverted fluorescence microscope with a 100× PlanApo objective as described previously[10]. Data were processed using custom written software in the programming language MatLab (MIAtool/LABSoft p1.0.0; http://www4.utsouthwestern.edu/wardlab/miatool).

ELISA. ELISA was used to determine total serum IgG levels in mice. We coated 96-well plates with rabbit anti-mouse IgG (heavy-chain specific; Zymed) and then nonspecific sites were blocked with 1% bovine serum albumin in PBS. Dilutions (1:40,000–1:120,000) of serum samples were made in PBS and then added to the wells. Bound mouse IgGs were detected using HRP-conjugated rabbit anti-mouse IgG (heavy- and light-chain specific; Zymed). A standard curve was generated using purified mouse IgGs (The Binding Site).

Analyses of the *in vivo* effects of mutated human IgG1 molecules. To assess the effect of the mutated (MST-HN) human IgG1 on the clearance of ^{125}I-labeled wild-type human IgG1, mice were injected intraperitoneally with ^{125}I-labeled human IgG1 (wild type) and levels of radioactivity assessed by whole-body counting (using an AtomLab 100 Dose Calibrator). 72 h later, mice were injected intravenously with 500 μg of either wild-type human IgG1, or 500 or 200 μg mutated (MST-HN) human IgG1. Six mice per treatment group were used. Levels of radiolabeled human IgG1 were determined at the indicated times after injection of wild-type or mutated IgG1 by whole-body counting.

To analyze the effects of the mutated (MST-HN) human IgG1 on the levels of serum IgGs in mice, IgG levels in serum samples taken at two different time points (24 h apart) were determined by ELISA (above). Mice were then injected intravenously with 500 μg wild-type human IgG1 or 500 μg mutated (MST-HN) human IgG1. Six mice per treatment group were used. Serum levels of endogenous (mouse) IgGs were determined by ELISA at the indicated times after injection.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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