Reduction of IgG in nonhuman primates by a peptide antagonist of the neonatal Fc receptor FcRn

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The neonatal Fc receptor FcRn provides IgG molecules with their characteristically long half-lives in vivo by protecting them from intracellular catabolism and then returning them to the extracellular space. Other investigators have demonstrated that mice lacking FcRn are protected from induction of various autoimmune diseases, presumably because of the accelerated catabolism of pathogenic IgGs in the animals. Therefore, targeting FcRn with a specific inhibitor may represent a unique approach for the treatment of autoimmune disease or other diseases where the reduction of pathogenic IgG will have a therapeutic benefit. Using phage display peptide libraries, we screened for ligands that bound to human FcRn (hFcRn) and discovered a consensus peptide sequence that binds to hFcRn and inhibits the binding of human IgG (hiG) in vitro. Chemical optimization of the phage-identified sequences yielded the 26-amino acid peptide dimer SYN1436, which is capable of potently inhibiting the hiG–hFcRn interaction. Administration of SYN1436 to mice transgenic for hFcRn induced an increase in the rate of catabolism of hiG in a dose-dependent manner. Treatment of cynomolgus monkeys with SYN1436 led to a reduction of IgG by up to 80% without reducing serum albumin levels that also binds to FcRn. SYN1436 and related peptides thus represent a previously uncharacterized family of potential therapeutic agents for the treatment of humoraly mediated autoimmune and other diseases.

FcRn antagonist | protein–protein interactions | phage display | autoimmune disease

Over 40 years ago it was recognized that the long serum half-life of IgG decreased with increasing levels of IgG in circulation (1). It was hypothesized that there may exist an IgG receptor responsible for this extended half-life and that this receptor may be saturable, thereby explaining the inverse correlation between IgG concentration and IgG half-life (2). The neonatal Fc receptor (FcRn) was finally cloned and characterized in the 1980s and was eventually determined to be the elusive receptor providing the long half-life to IgG molecules in vivo (3, 4). FcRn is a heterodimer of a MHC-class-I-like heavy chain and β2-microglobulin (β2m) light chain and is broadly expressed in many tissue types but primarily in the vascular endothelium (5, 6). Interestingly, FcRn can bind IgG at pH 6 and not at physiological pH (7.4), and this pH dependence is likely key to the mechanism by which FcRn extends IgG half-lives. It is thought that after uptake of IgG into cells, FcRn can bind to IgG in acidic endosomes, thereby avoiding degradation in the lysosome (4). IgG molecules are then returned to the cell surface by exocytosis (7, 8) and released back into circulation because FcRn has minimal affinity for IgG at extracellular pH 7.4.

Because FcRn can contribute significantly to the longevity of IgG in circulation, it has been suggested that it may also be a therapeutically relevant target for the treatment of autoimmune disease (9, 10). For example, it has been suggested that i.v. IgG therapy (IVIG) exerts its therapeutic effect, at least partially, by saturating FcRn, thus increasing the catabolism of endogenous IgG (11–13). In fact, recent reports have found that mice deficient in FcRn have accelerated catabolism of IgG (14) and are less susceptible to the induction of experimental arthritis (15) and various skin-blasting diseases (16). In addition, IgG-based antagonists for FcRn have been developed to accelerate the catabolism of IgG in rats (17), mice (18), and mice possessing the human FcRn transgene (19). Two of these antagonists have shown efficacy in disease models for rat experimental autoimmune myasthenia gravis (EAMG) (17) and mouse experimental arthritis (19). These studies serve as proof-of-principle that reduction in IgG levels by targeting FcRn can be therapeutically relevant to the treatment of autoimmune diseases.

Herein, we describe a 3.1-kDa peptide, SYN1436, that binds to human FcRn (hFcRn) and inhibits the hFcRn–human IgG (hiG) interaction. The core peptide sequence was discovered by using phage display peptide library screening and possesses no homology to the Fc domain of IgG. The peptide was chemically optimized to enhance its stability in vivo and binding properties for hFcRn. SYN1436 modulates IgG levels in hFcRn transgenic mice and in cynomolgus monkeys and represents a candidate for the treatment of autoimmune or other humorally mediated diseases.

Results

Phage Display Screen for Inhibitors of hiG–hFcRn Interaction. A unique peptide motif was discovered by using phage display techniques. The phage library was screened with HEK293 cells transfected with hFcRn and hβ2m (293c11) by using competition with hiG to select for phage capable of interfering with the IgG–FcRn interaction at pH 6. The screen yielded a family of related peptides, all of which contained nine residues within the disulfide bond. Each of these sequences, when synthesized as individual peptides, inhibited the binding of hiG to shFcRn immobilized on 96-well plates (SYN722–SYN726, Table 1). A consensus sequence of Gly-His-Phe-Gly-Gly-X-Tyr was observed, where X is preferably a hydrophobic amino acid. The consensus sequence bears no homology to the Fc domain of IgG, and included a cysteine disulfide bond in varying positions relative to the consensus sequence.

Peptide Chemistry. SYN722 was selected for further study as a result of its prevalence in the phage screen. The peptide sequence


Conflict of interest statement: Syntonix has filed a patent application covering the peptides described herein.

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Peptide Sequences Derived from Phage Screening of Cells Expressing Human FcRn

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th># Hits</th>
<th>IC50 (µM)</th>
</tr>
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<tr>
<td>SYN722</td>
<td>QRFCTGHRFGGLYPCNGH</td>
<td>23</td>
<td>36</td>
</tr>
<tr>
<td>SYN723</td>
<td>GGCGVTHFGGIYTCRYQ</td>
<td>4</td>
<td>33</td>
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<td>SYN724</td>
<td>KLPQGCGHFCMCGYQQP</td>
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</tr>
<tr>
<td>SYN725</td>
<td>PSEIYCGHIDGIGYCFNA</td>
<td>3</td>
<td>49</td>
</tr>
<tr>
<td>SYN726</td>
<td>NFSFCGRPGGFGGCYLF</td>
<td>1</td>
<td>33</td>
</tr>
</tbody>
</table>

Consensus

Peptides SYN722–SYN726 were synthesized with flanking AG residues on the N terminus and GTGGGK residues on the C terminus to mimic the nonvariable flanking residues found in the phage library. Critical residues are colored red, much conserved residues are colored green, and cysteine residues are colored yellow. The “# Hits” column represents the number of times that a particular sequence appeared in the sequencing of the selected phage clones. The “IC50” column corresponds to data from the in vitro IgG–FcRn competition ELISA.

Peptide Binding to Soluble Human FcRn by Using SPR. The binding of SYN746, SYN1327, and SYN1436 to shFcRn was studied by using surface plasmon resonance (SPR) by monitoring the binding of the peptides to immobilized shFcRn. SYN746 had a weak affinity for shFcRn with a Kd of \( \approx 5 \) µM at pH 6 and 30 µM at pH 7.4, whereas SYN1327 showed enhanced affinities of 31 nM and 170 nM at pH 6 and 7.4, respectively (Fig. 3A and B and Table 2). In contrast, the interaction of the dimeric SYN1436 with shFcRn was very tight and difficult to measure because of its very high affinity for shFcRn and its very slow off-rate. At pH 6 and 7.4, the equilibrium dissociation constants of SYN1436 for shFcRn were estimated to be \(<1 \) nM (Fig. 3C and Table 2).

Peptide Binding to Soluble Mouse, Rat, and Cynomolgus Monkey FcRn and HLA-A2 by Using SPR. SYN1327 showed equipotent binding to soluble human and cynomolgus monkey FcRn but \( \approx 1,000 \)-fold weaker binding to soluble mouse and rat FcRn at pH 6.0 [see supporting information (SI) Figs. 6 and 7]. This demonstrates a high level of selectivity of this peptide for its target, considering that the extracellular domain of rat and human FcRn are 68% homologous (33). In addition, because FcRn is a MHC-class I-like protein, the possibility of SYN1327 or SYN1436 binding to \( \beta 2 \)m was investigated by using the human MHC Class I protein HLA-A2, which is also associated with \( \beta 2 \)m. We found that there was only a weak nonspecific interaction between the peptides and a surface immobilized HLA-A2 (see SI Figs. 8 and 9).

SYN1436 Does Not Inhibit the Albumin–shFcRn Interaction by SPR. FcRn is known to bind albumin (20, 21). Therefore, we studied the effect of the peptide on the albumin–FcRn interaction. Neither SYN1436 nor SYN1327 inhibited the binding of shFcRn (1.2 µM) to a sensor chip coated with human serum albumin (HSA, Fig. 3D and SI Fig. 10) by using a peptide concentration of 10 µM. The dimeric peptide SYN1436 appears to have enhanced the binding of shFcRn to albumin, possibly by bridging two shFcRn molecules together, thereby generating a larger response in the SPR instrument. It is also possible that a dimeric form of shFcRn has a higher affinity for HSA. In a separate experiment, it was found that SYN1436 does not bind to a sensor chip coated with HSA at either pH 6 or pH 7.4 up to a peptide concentration of 100 µM (data not shown).

SYN1436 Increases IgG Catabolism in Transgenic Mice. Because SYN1327 did not bind to soluble mouse or rat FcRn by SPR, and SYN1436 had no effect on IgG levels in normal mice or rats (data not shown), the effects of SYN1327 and SYN1436 were tested in transgenic mice (TG32B) that have the murine FcRn 2m genes inactivated and replaced with the hFcRn and h\( \beta 2 \)m genes. We found that there was a significant decrease in IgG levels in the transgenic mice treated with SYN1436 compared to the control mice (Fig. 5A). This decrease was not observed in mice treated with SYN1327 (Fig. 5B). These results suggest that SYN1436 can increase the catabolism of IgG in transgenic mice. In conclusion, SYN1436 appears to be a potential therapeutic candidate for IgG catabolism in transgenic mice.
SYN1436 Lowers Endogenous IgG Levels in Cynomolgus Monkeys. The effect of SYN1436 on endogenous IgG was studied in cynomolgus monkeys by using six different dosing regimens over 4 weeks. Serum concentrations of endogenous IgG were monitored over 77 days (Fig. 5). Once weekly s.c. dosing with 1 mg/kg of SYN1436 lowered serum IgG levels by 60% after 4 weeks of treatment. Serum IgG was reduced in a dose-dependent manner by using the same dosing regimen in separate studies. The concentration of IgG at 120 h was reduced by 90% at 120 h compared with the vehicle control. In addition, 10 mg/kg of SYN1436 reduced the serum half-life of IgG from 114 h (vehicle control) to 34 h.

**SYN1436 Has No Effect on Serum Concentrations of Albumin, IgM, or IgA in Cynomolgus Monkeys.** It was found that SYN1436 did not affect the albumin, IgA, or IgM concentrations in the high-dose group of i.v. dosing SYN1436 at 5 mg/kg three times per week. Each protein was detected at levels approximately ±20% of the predose levels, and deviations from predose levels did not correlate with the observed reduction in IgG levels (see SI Figs. 12 and 13).

**Discussion**

In this report, we describe a peptide that can bind to hFcRn, block the IgG–hFcRn interaction *in vitro*, and modulate IgG levels in hFcRn transgenic mice and in nonhuman primates. By using peptide phage display and multiple rounds of competitive screening, a peptide motif was discovered that inhibits the binding of IgG to hFcRn. The peptide motif contains several conserved residues, but also consists of a cysteine disulfide bond in three different positions with respect to the consensus motif (Gly-His-Phe-Gly-Gly-X-Tyr), where all of the corresponding peptides are nearly equipotent in competing for hFcRn binding. Chemical optimization of one peptide from the consensus sequence resulted in monomeric peptide SYN1327 and dimeric peptide SYN1436.

We first tested the optimized peptides for their ability to inhibit FeRn in transgenic mice and thereby increase the catabolism of IgG. The dimeric peptide SYN1436 accelerated the catabolism of IgG at doses as low as 0.5 mg/kg/day, whereas the monomeric peptide SYN1327 was ineffective at 10 mg/kg/day. Dimerization of the optimized monomeric peptide proved critical to the *in vivo* efficacy of the peptide. This is likely because of the increased avidity effects of the dimer, but may also be a requirement for inhibition of the IgG–FeRn complex *in vivo*. IgG contains two FeRn-binding sites and binds to FeRn in a 2:1 stoichiometry as observed for rat FeRn in solution (23) and by x-ray crystallography (37). More recently, hFcRn dimers have been reported on cell membranes (24). It has been suggested that this 2:1 stoichiometry may be a critical aspect of FeRn function (23), and therefore, a dimeric ligand for FeRn may best inhibit FeRn function *in vivo*. Thus, the lack of activity of monomeric SYN1327 *in vivo* may be due to its weaker affinity for FeRn or its monovalency.

The dramatic effects of SYN1436 in cynomolgus monkeys demonstrate that, by inhibiting the natural IgG recycling mecha-

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**Table 2. In vitro data for anti-FcRn peptides and Fc as a control**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>$K_d$ (SPR)* pH 6, nM</th>
<th>$K_d$ (SPR)* pH 7.4, nM</th>
<th>IC$_{50}$ (IgG competition ELISA: binding to shFcRn) pH 6, nM</th>
<th>IC$_{50}$ (IgG competition FACS: binding to 293c11 cells) pH 6, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc</td>
<td>31 ± 17 (n = 3)*</td>
<td>No binding</td>
<td>69 ± 29 (n = 4)</td>
<td>149 ± 28 (n = 3)</td>
</tr>
<tr>
<td>SYN746</td>
<td>5,100</td>
<td>30,000</td>
<td>40,000 ± 15,000 (n = 4)</td>
<td>18,000 (n = 2)</td>
</tr>
<tr>
<td>SYN1327</td>
<td>31 ± 4 (n = 3)</td>
<td>170 ± 20 (n = 3)</td>
<td>570 ± 200 (n = 11)</td>
<td>510 (n = 2)</td>
</tr>
<tr>
<td>SYN1436</td>
<td>&lt;0.5</td>
<td>&lt;0.8</td>
<td>2.4 ± 1.4 (n = 13)</td>
<td>2.8 ± 2.0 (n = 3)</td>
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</table>

*shFcRn immobilized on the SPR chip surface.

*Apparent $K_d$ data for Fc fitted to a 1:1 steady-state affinity model for comparison to peptides.
anism with a targeted peptide antagonist, IgG catabolism is accelerated, and overall IgG levels can be rapidly reduced. This reduction occurred despite ongoing IgG biosynthesis, which is regenerated at a constant rate regardless of IgG concentrations (25). In addition, it was shown that, once treatment was stopped, IgG levels eventually returned to concentrations that approached predose levels.

Interestingly, FcRn knockout mice retain only ∼10–25% of the level of endogenous IgG compared with the background strain (14) (see SI Fig. 14). This is consistent with two human patients who have recently been identified as having a mutation in their β2m gene, which would lead to poor or nonfunctioning FcRn. These patients possessed IgG serum concentrations of ∼10–25% of their other family members (26, 27). These data suggest that IgG levels of 10–25% of normal may be the minimum concentration of circulating IgG that can be achieved by targeting FcRn with an antagonist; this level of reduction in IgG is approached with SYN1436 treatment in cynomolgus monkeys.

FcRn has also been implicated as the receptor responsible for the long half-lives of serum albumin (20, 21). We studied the possibility of the peptides interfering with the albumin–FcRn interaction and found that the peptides described herein did not disrupt this interaction either in vitro by using SPR methods or when administered to cynomolgus monkeys. Although SYN1436 inhibited the IgG–FcRn interaction in vivo, FcRn remained functional in regulating albumin levels. Indeed, it has been recently reported that the IgG and albumin binding sites on FcRn are distinct (28).

Human IgG binds to FcRn at pH 6 but exhibits minimal binding at pH 7.4. This pH-dependent property of IgG enables the molecule to be taken up nonspecifically into acidic endosomes of a cell, bind to FcRn for rescue from the degradative lysosomal pathway, and be released at the cell surface at physiological pH (7). In contrast, SYN1436 binds to FcRn at both pH 6 and pH 7.4 and is thus ideally suited to compete for FcRn at both the cell surface (pH 7.4) and within the cell (pH 6). Such pH-independent binding may be critical for successful inhibition of the IgG-FcRn interaction in vivo.

Reduction of serum IgG concentrations through blockade of binding to FcRn may be useful in treating humorally mediated autoimmune diseases (17, 19), and therefore, SYN1436 or related peptides may represent a previously uncharacterized therapeutic modality. As a comparison, immunoadsorption and plasmapheresis are available treatment options for those suffering from autoimmune diseases such as myasthenia gravis (MG) to reduce a patient’s immunoglobulins (29). Treatment with a FcRn-targeted therapy such as SYN1436 may provide an alternative to these therapies. In addition, a recent report identified serum IgG as an inhibitory component of antibody-dependent cellular cytotoxicity (ADCC) by binding to CD16; a hypothesized mechanism by which antitumor monoclonal antibodies exert their cytotoxic effect (30). Pretreatment of these patients with a FcRn antagonist such as SYN1436 may reduce these inhibitory antibodies to a level that renders subsequent antitumor antibody treatment more effective.

The inhibition of protein–protein interactions is a major challenge for the pharmaceutical industry (31). Because such interactions cover large protein surfaces, it is difficult to design successful inhibitors that are not large proteins themselves. Peptides (<50 aa) represent a prime source for the discovery of inhibitors of protein–protein interactions as a result of their moderate size and structural diversity. Furthermore, recent progress in the development of peptide drugs has addressed many of the shortcomings typically associated with peptides, including peptide stability, half-life, and noninvasive delivery (32). The application of peptide phage display technology coupled with chemical optimization represents a powerful approach to target such large protein surfaces.

In summary, we have identified a series of peptides that target hFcRn and inhibit the hIgG–hFcRn interaction. Disruption of this molecular complex in cynomolgus monkeys resulted in increased catabolic rates for IgG and, hence, temporarily reduced serum IgG levels. The reduction of IgG may be helpful in the treatment of patients with a variety of IgG-mediated autoimmune diseases such as MG, immune thrombocytopenic purpura, and systemic lupus erythematosus.

Materials and Methods
Transfection of HEK293 Cells with hFcRn for Screening and Binding Studies.
Full-length human FcRn (33) was cloned into the mammalian expression vector pcDNA6 (Invitrogen). Human β2m (34) (hβ2m) was cloned into the mammalian expression vector pcDNA3 (Invitrogen). HEK 293 cells (cat. no. CRL 1573; American Type Culture Collection) were transfected with hFcRn and hβ2m by using SuperFect Transfection Reagent (Qiagen) according to the manufacturer’s recommended protocol. Clonal populations of stably transfected cells were established, and the clone with the highest hFcRn/hβ2m

Fig. 3. SPR peptide binding experiments. (A–C) SPR data for binding of serial twofold dilutions of SYN746 (A, 50 × 10⁻³ nM to 400 nM), SYN1327 (B, 1,000 nM to 2 nM), and SYN1436 (C, 20 nM to 0.04 nM) to immobilized shFcRn at pH 6.0. (D) Relative equilibrium response for binding of shFcRn to immobilized HSA at pH 6.0 in the presence of 5 mM HSA or 10 mM SYN746, SYN1327, or SYN1436.

Fig. 4. Catabolism of hIgG in TG328 mice. Human IgG was injected i.v. at t = 0, followed by i.v. injections of test article at t = 24, 28, 72, and 96 h. Test article vehicle (diamonds), 10 mg/kg SYN1327 (filled circles), and SYN1436 at 0.5 mg/kg (open circles), 1 mg/kg (filled triangles), 2.5 mg/kg (open triangles), 5 mg/kg (filled squares), and 10 mg/kg (open squares). Note that the vehicle and SYN1327 groups are nearly superimposed.
expression level that exhibited the most robust binding to hIgG was designated as ‘293c11.’

**Phage Display Screening.** Filamentous phage libraries were obtained from Dyax. The libraries TN-9-FV (3.2 × 10^5 independent transformants), TN10-X (2 × 10^6 independent transformants), TN-11-I (2.7 × 10^6 independent transformants), and TN-12-I (4.1 × 10^6 independent transformants) were used in the screen. These libraries code for cyclic disulfide peptides with 9–12 aa enclosed within the disulfide loop. The libraries were screened by using standard protocols (35). Briefly, 293c11 cells were incubated with the phage libraries, washed with buffer A [PBS (pH 6) and 10 mM EDTA] to remove nonbinding phage and treated with 66 μM hIgG to elute the phage bound to FcRn. The eluted phage were titrated, amplified, and rescreened by using the same process, with an additional subtraction step using untransformed cells. After three rounds of selection, the phage were evaluated by using an IgG–shFcRn competition ELISA (below). DNA from positive phage hits was sequenced, and the corresponding amino acid sequences are shown in Table 1 (and see SI Text).

**Synthesis of Monomeric Peptides.** Peptides SYN722-SYN726, SYN746, and SYN1327 were synthesized by standard Fmoc/tBu protocols on Rink amide AM resin (Novabiochem) and commercially available amino acids. Peptides were cleaved from the resin as C-terminal amides by using 95% TFA in 5% water with 0.1% TFA as the eluent. Peptide identity and purity were confirmed with analytical RP-HPLC coupled with electrospray MS. Peptide identity and purity were confirmed with analytical RP-HPLC coupled with electrospray MS.

**Synthesis of SYN1436.** SYN1436 was synthesized by reacting the peptide resin containing the sequence NH2-Arg(Phe)-Phe-Pen(Thr)-Thr(Phe)-Gly-His(Thr)-Phe-Gly-Sar-NMeLeu-Tyr(tBu)-Pro-Cys(Thr)-resin with 0.5 eq of succinic acid, 1 eq of PyBOP, and 2 eq of diisopropylpropylamine for 18 h. This results in the covalent attachment of adjacent peptides on the resin via amide bonds via the N-terminals (36). The resulting peptide dimer was cleaved from the resin and the disulfide bonds were formed at a peptide concentration of 0.1 mg/ml in 10 mM sodium phosphate (pH 7.5) with 20% DMSO for 18 h at room temperature (rt). The reaction mixture was purified by HPLC, residual TFA was removed by using a PL-HCO3 ion-exchange column (Polymer Labs), and the material was lyophilized from 5% acetic acid to afford the acetate salt as a white powder. Analysis of the product was performed by LCMS. The proper disulfide connectivity was confirmed by treating SYN1436 with trypsin (5% w/v) for 30 min at pH 8. Cleavage of the two Arg–Phe bonds in SYN1436 was observed, and the corresponding molecular mass of the truncated monomers of 1,355 Da confirmed that the proper disulfide bonds had formed (see SI Text and Figs. 15 and 16).

**Protein Expression.** All soluble FcRn proteins were expressed by using the glutamine synthetase expression system in CHOK1SV cells (Lonza Biologics), and purified as described (37, 38). Briefly, DNA containing the hFcRn sequence truncated at amino acid 274 and the hIgM sequence were cloned into a pEE12.4/6.4 as a double-gene vector (DGV). The shFcRn-hIgM-pEE12.4/6.4-DGV was transfected into CHOK1SV cells in adherent culture by electroporation, and selection was initiated 24 h later with a range of concentrations of methionine sulfoximine (MSX) from 25 to 100 μM. The cells were adapted to serum-free suspension, and the shFcRn–hIgM protein was purified as described (37, 38). This procedure was applied to the cloning, expression, and purification of soluble mouse, rat, and cynomolgus monkey sFcRn–βm. The function of the sFcRn proteins was confirmed by surface plasmon resonance (SPR) analysis of the binding of soluble IgG to immobilized sFcRn. In all cases, the proteins bound IgG at pH 6 with affinities comparable to those reported in the literature and showed very weak or no binding at pH 7.4 (data not shown). The fc fragment of IgG1 used here was a control expressed recombinantly in CHO cells as described above and purified by protein A affinity chromatography.

**In Vitro hIgG–sFcRn Competition ELISA.** shFcRn was biotinylated with two eq of sulfosuccinyl-LC Biotin (Pierce), by incubating the reagent with shFcRn for 2 h on ice, followed by dialysis into PBS. ReactiBind Neutravidin-coated 96-well plates (Pierce) blocked with BSA were washed twice with 200 μl per well of Buffer B [PBS (pH 7.4), 0.5% BSA IgG-free, and 0.05% Tween-20]. Biotinylated shFcRn (1 μg/ml in Buffer B) was added to each well, sealed, and incubated at 37°C for 2 h. The plate was washed with 200 μl per well of Buffer C (100 mM Mes (pH 6), 150 mM NaCl, 0.5% BSA IgG-free, and 0.05% Tween-20) and then 60 μl per well of 3 mM hIgG pooled IgG subtypes, Cabicochem) in Buffer C, and 40 μl per well of the peptide competitors in 3 mM hIgG was added to the plate. After incubation at 37°C for 2 h, liquid was aspirated from the plate, 100 μl per well of a 110,000 dilution of HRP-conjugated goat F(ab’)2 anti-human IgG F(ab’)2 fragment-specific (Jackson ImmunoResearch) in Buffer C was added and the plate incubated for 30 min at rt before washing four times with 200 μl per well of ice-cold Buffer C. SureBlue TMB substrate solution (KPL) was added (100 μl per well), and the plate was incubated at rt until color developed (5–10 min). The reaction was terminated by the addition of TMB stop solution (100 μl per well), and the absorbance was measured at 450 nm.

**IgG-Peptide FACS Competition Assay Using Transfected HEK293 (293c11) Cells.** A humanized IgG1 mAb, Synagis (MedImmune) was labeled with Alexa Fluor488 (A488 Protein Labeling kit, Invitrogen) according to the manufacturer’s suggested protocol. The 293c11 cells grown in complete DMEM (Invitrogen) containing 5 μg/ml blasticidin and 250 μg/ml G418 (Biogen) were sedimented by centrifugation and resuspended in Buffer D (Dulbecco’s PBS plus 10 mM EDTA) at a concentration of 3 × 10^6 cells per milliliter. Cells (0.1 ml) were transferred to each well of a 96-well plate and then centrifuged for 5 min, and the supernatants were decanted. Peptide competitors dissolved in 90 μl of Buffer D were added to the plate. A488-Synagis (10 μl) was added to each well such that the final concentration of A488-Synagis was 100 nM. The plate was incubated on ice for 1 h. The plate was centrifuged for 5 min, and the cell pellet was washed once with 100 μl of Buffer D and centrifuged again. The cells were resuspended in 200 μl of Buffer D, and bound fluorescence was quantified by using a flow cytometer.
with ethanolamine for reference subtraction. Experiments were performed in running buffer (50 mM phosphate, 100 mM sodium chloride, and 0.01% surfactant P20 (pH 6.0 or 7.4)). For analysis of peptide monomer binding, sFcRn or HLA-A2 was coated to a final density of 3,500–5,000 response units (RU). Dilutions of peptide monomer in running buffer were injected over the FcRn-CMS (or HLA-A2-CMS) chip at 20 μl/min for 2 min. The peptides were then dissociated from the chip for 2.5 min with running buffer. Remaining peptide was removed from the chip with a 30-second injection of HBS-P buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, and 0.005% vol/vol P-20) at 30 μl/min. For analysis of SYN1436, shFcRn or HLA-A2 was coated to a density of 2,000–2,500 RU. Dilutions of the peptide were injected over the FcRn-CMS chip at 30 μl/min for 10 min. Peptide was dissociated from the chip for 10 min with running buffer. Any remaining peptide was removed from the chip with two 60-second injections of 50 mM Tris-HCl, 100 mM NaCl, and 0.01% surfactant P20 (pH 9.0) at 100 μl/min. Sensorgrams were generated and analyzed by using BiaEval software version 3.1 (Biacore AB). The equilibrium RU observed for each injection was plotted against the concentration of peptide. The equilibrium Kd values were derived by analysis of the plots by using the steady-state affinity model included in the BiaEval software.

HSA was coated to a final density of 2,000–3,000 RU. For blocking FcRn binding to HSA-CMS chip, sample buffer contained 1.2 μM hFcRn. Samples containing either no additive, 5 μM HSA, or 10 μM peptide were prepared and injected over the HSA-CMS chip.

Transgenic Mice. Transgenic mice were obtained from The Jackson Laboratory. The endogenous murine FcRn and β2m genes were inactivated and replaced with the human FcRn and the human β2m genes (murFcRn −/−/−, murβ2m −/−/−, hucFcRn +/+/+), hucβ2m +/+/+). These mice are referred to herein by the strain name TG32B.

IgG Catabolism in TG22B Mice. TG22B mice (four animals per group) were injected i.v. with 500 mg/kg hIgG (pooled IgG subtypes; MP Biomedical) at t = 0 h. The mice were then injected i.v. at t = 24, 48, 72, and 96 h with vehicle, 10 mg/kg of SYN1327, or SYN1436 at doses of 0.5, 1, 2.5, 5, or 10 mg/kg. The vehicle was 20 mM sodium acetate (pH 5.0). Blood samples were taken just before injections at 24, 48, 72, and 96 h and at 30, 120, and 168 h. An IgG ELISA was used to detect the levels of hIgG in the serum at each time point. Goat anti-hIgG [Fab] (Pierce) was used as a capturing agent, the conjugate goat anti-hIgG[Fab]-HRP (Pierce) was used for detection, and TMB One-Component substrate (BioFX) was used for ELISA development. Sulfuric acid was used to stop the development reaction, the UV absorbance was measured at 450 nm, and the concentration of IgG at each time point was determined from a standard curve. IgG half-lives were estimated by using the software WinNonLin (Pharsight, v.4.1 or 5.1).

IgG Catabolism in Cynomolgus Monkeys. Cynomolgous monkeys (six groups, three animals per group, average body weight 4.2 kg) were treated with SYN1436 (injections of 1 ml in water; pH 5) and endogenous IgG levels were monitored until day 77. The following dosing regimens were used: (i) four s.c. injections of 1 mg/kg, once a week; (ii) 12 s.c. injections of 1 mg/kg, three times a week; (iii) 12 i.v. injections of 1 mg/kg, three times a week; (iv) four s.c. injections of 5 mg/kg, once a week; (v) 12 s.c. injections of 5 mg/kg, three times a week; or (vi) seven i.v. injections of 5 mg/kg, three times a week. Blood samples were collected (before any dosing) on days 1–5 during the first week, three times a week in weeks 2–5, once a week in weeks 6–8, and once at week 11 (day 77). Serum was prepared and stored at −80°C until the ELISA analyses were performed to determine the levels of endogenous cynomolgus monkey IgG, IgM, IgA, and albumin (see SI Text). Endogenous monkey IgG was detected by using an FC ELISA that used rabbit anti-monkey IgG (fc-specific Sigma–Aldrich) as a capture antibody and rabbit anti-monkey IgG-HRP conjugate (Sigma–Aldrich) as the detection antibody. Serum albumin was detected by using an ELISA that used rabbit anti-monkey serum albumin (Nordic Immunology) as a capture step and goat anti-human albumin-HRP conjugate (Academy Bio-Medical) for detection. Serum IgA concentrations were determined by using an ELISA that used a goat anti-monkey IgA antibody (KPL) as a capture step and goat anti-mouse IgM-HRP (BD) for detection. Serum IgA concentrations were determined by using an ELISA that used goat anti-monkey IgA antibody (KPL) as a capture step and goat anti-mouse IgA-HRP for detection.

All studies in animals were conducted by using protocols approved by Syntonyx’ Institutional Animal Care and Use Committee (IACUC) or New Iberia Research Center’s IACUC, following all National Institutes of Health guidelines for the care and use of research animals.

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Figure 13

Figure 14

SYN1327 - FcRn interactions across 3 different species

Figure 15
SYN1327 binding across Human FcRn or HLA

Figure 16

SYN1327-FcRn Biaevaluation

SYN1327 - Human FcRn, 3780 RU
SYN1327 - Cynomolgus FcRn, 4249 RU
**Figure 16**

**SYN1327 binding across Human FcRn or HLA**

**SYN1327-FcRn Biaevaluation**

**SYN1327-FcRn Biaevaluation**

**SYN1327-FcRn Biaevaluation**
Figure 17.

**SYN1436 binding across Human FcRn or HLA**

![Graph showing SYN1436 binding across Human FcRn or HLA](image-url)
Figure 9

![Graph showing serum IgG levels for C57BL/6, FcRn KO, and TG32B](image)

Figure 10

![Graph showing serum IgG levels for SYN 1436, 5 mg/kg](image)

Figure 11.
Figure 12.
Figure 12.
Figure 9

![Graph showing Serum IgG levels for different strains.](image)

Figure 10

![Graph showing the effect of SYN 1436, 5 mg/kg on Serum IgG levels.](image)

Figure 11.
Figure 7

Molecular Weight = 430.47
Exact Mass = 430
Molecular Formula = C_{16}H_{30}N_{8}O_{6}

Molecular Weight = 1354.63
Exact Mass = 1353
Molecular Formula = C_{64}H_{87}N_{15}O_{14}S_{2}