

A Synthetic Mimic of Human Fc Receptors: Defined Chemical Modification of Cell Surfaces Enables Efficient Endocytic Uptake of Human Immunoglobulin-G

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Abstract: Binding of ligands to macromolecular receptors on the surface of mammalian cells often results in ligand uptake through receptor-mediated endocytosis. Certain human leukocytes and epithelial cells express Fc receptors (FcRs) that bind and internalize antibodies through this mechanism. To mimic this process, we synthesized an artificial FcR comprising the membrane anchor *N*-alkyl-3 β -amino-5 α -cholestane linked to a disulfide-constrained cyclic peptide, termed FcIII, known to exhibit high affinity and specificity for the Fc region of human IgG. Treatment of human Jurkat lymphocytes that lack natural FcRs with the synthetic FcR (1 μ M, 1 h) installed an average of $\sim 6.2 \times 10^5$ synthetic receptor molecules per cell surface. These treated cells gained the capacity to internalize human IgG at levels greater than human THP-1 cells that express the natural receptors Fc γ RI and Fc γ RII. By linking binding motifs for circulating ligands to membrane anchors that cycle between the cell surface and intracellular endosomes, minimalistic cell surface receptors can be used to destroy targeted ligands by endocytosis. These small mimics of macromolecular receptors may be useful for controlling the extracellular abundance of ligands involved in disease.

Introduction

Binding of ligands to macromolecular receptors on the surface of mammalian cells typically initiates a complex series of cellular processes. One such process often includes cellular uptake of cell-impermeable ligands by the mechanism of receptor-mediated endocytosis.¹ Antibodies (immunoglobulins) are macromolecular ligands of Fc receptors (FcRs) expressed on the surface of specific cell types. Most of these receptors are transmembrane proteins, but FcRs can also be found anchored to the outer leaflet of the plasma membrane by covalently linked lipids. Cells of the human immune system express FcRs that bind immunoglobulin-G (IgG) with affinities that range from subnanomolar to micromolar K_d values.² This binding results in internalization of IgG and IgG-bound antigens (Figure 1),^{2–4} providing a mechanism for cells to destroy IgG-bound pathogens. FcRs can also protect IgG from catabolism and transport these antibodies across tissue barriers. For example, the neonatal FcR (FcRn, MW = 40 kDa), expressed on certain epithelial cells, enables cellular uptake of IgG at low pH and the delivery of therapeutic maternal IgG to the bloodstream of fetal or newborn animals.⁵ The extracellular domain of this receptor has been shown by X-ray crystallography to bind the hinge region of the IgG Fc fragment (Figure 2, panel A).^{6,7} FcRs play key roles in host defense, inflammation, autoimmune disease, and the efficacy of therapeutic antibodies.⁴

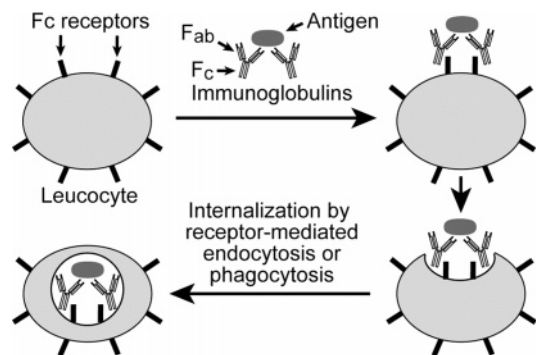


Figure 1. Overview of cellular uptake of IgG by Fc receptors. IgG and IgG-antigen complexes bind Fc receptors on the cell surface. These ligands are internalized by receptor-mediated endocytosis or phagocytosis depending on the FcR and cell type.

To construct a small (2.3 kDa) mimic of human FcRs (1), we synthesized a previously reported⁸ cyclic peptide, termed FcIII, linked to an *N*-alkyl derivative of 3 β -amino-5 α -cholestane as a membrane anchor. The FcIII cyclic peptide has been studied by X-ray crystallography (Figure 2, panel B) and binds the hinge region of human IgG with high affinity ($K_i = 25$ nM) and specificity.⁸ The dihydrocholesterol-derived membrane anchor was chosen because structurally similar *N*-alkyl-3 β -amino-5-cholestene derivatives (3 β -cholesteryl amines) can insert into cellular plasma membranes, project linked headgroups from the

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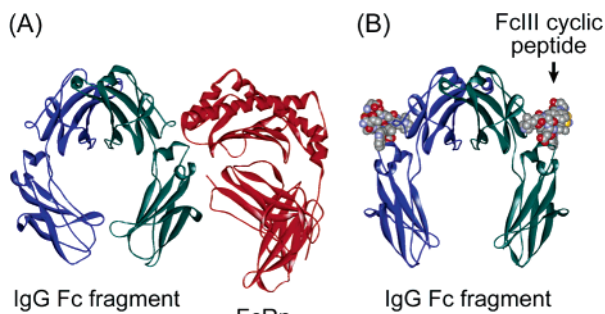
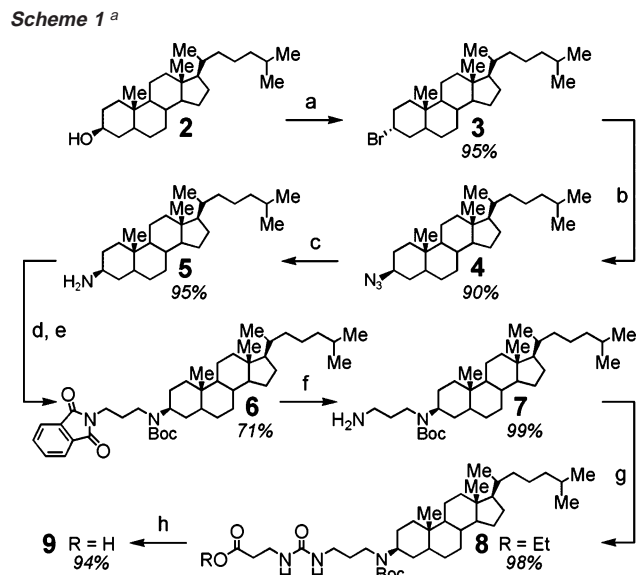


Figure 2. X-ray crystal structures of human IgG Fc regions (blue and green ribbon models) bound to the extracellular fragment of the neonatal Fc receptor (FcRn, panel A, red ribbons, PDB code 111A) and the FcIII cyclic peptide (panel B, CPK models, PDB code 1DN2). Carbohydrates linked to Fc regions were omitted for clarity.

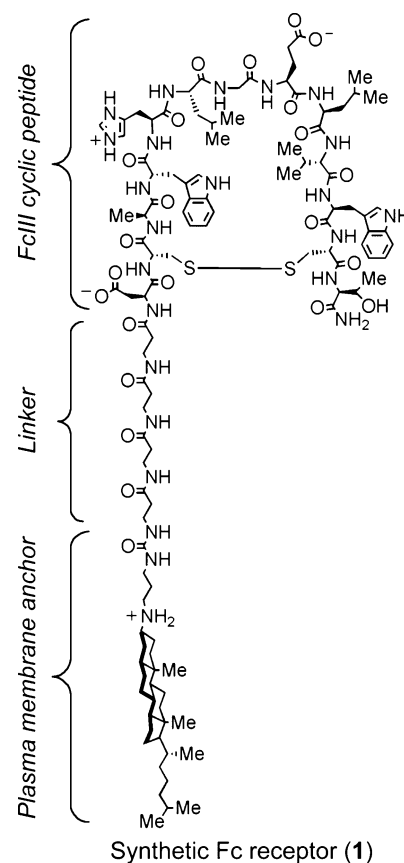
cell surface, and rapidly cycle between the plasma membrane and intracellular endosomes, similar to many natural cell surface receptors.^{9–11} The minimalist synthetic FcR (1) was designed to mimic natural FcRs by insertion into the exofacial leaflet of plasma membranes of living human cells, bind human IgG at the hinge region of the Fc fragment, and promote endocytosis of this macromolecular ligand. Other strategies for minimizing proteins^{12–15} and chemically modifying cell surfaces^{16–18} have also been reported.

Results

Chemical Synthesis. Fmoc solid-phase synthesis using Cys residues protected as acid-stable *t*-Bu disulfides was employed to prepare a protected precursor of the FcIII cyclic peptide with three additional β -alanine linker subunits. This peptide was capped on solid phase with a protected derivative of 3β -amino- 5α -cholestane (9) synthesized as shown in Scheme 1. 3β -cholestanol (dihydrocholesterol, 2) as a building block for the construction of the membrane anchor of 1 differs from cholesterol by lacking the C5 double bond. We chose to study derivatives of 2 as a membrane anchor because the synthesis of 3β -amino- 5α -cholestane (5) shown in Scheme 1 proved to be more efficient than our previously reported synthesis of 3β -amino- 5α -cholestene (3β -cholesterylamine).^{9,10} Additionally, this cholesterol analogue (2) is considered a partial functional substitute for cholesterol and can support the proliferation of cholesterol-deficient human cells in culture.¹⁹ A related synthesis of 5 from 2 has also been reported by Cushman.²⁰ Addition of TFA to the resin-bound peptide capped with 9 resulted in cleavage from the solid support with concomitant deprotection



^a Reagents and conditions: (a) CBr_4 , PPh_3 , THF; (b) NaN_3 , DMSO, 80 °C; (c) PPh_3 , THF/ H_2O (10:1); (d) *N*-(3-bromopropyl)phthalimide, K_2CO_3 , DMF, 60 °C; (e) $(\text{Boc})_2\text{O}$, Et_3N , CH_2Cl_2 ; (f) NH_2NH_2 , EtOH, 50 °C; (g) ethyl 3-isocyanatopropionate, DIEA, CH_2Cl_2 ; (h) LiOH, $\text{CH}_3\text{OH}/\text{THF}/\text{H}_2\text{O}$ (3:2:1).



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of acid-labile protecting groups. The resulting uncyclized peptide bearing protected Cys residues was purified by reverse-phase (RP)-HPLC. The Cys protecting groups were cleaved with dithiothreitol, the peptide was cyclized in the same pot by addition of DMSO,²¹ and the synthetic FcR (1) was again purified by RP-HPLC.

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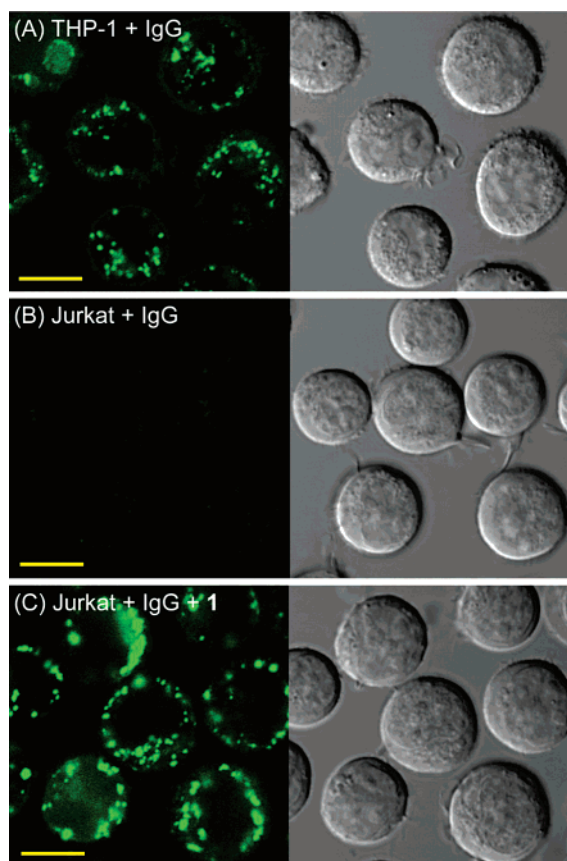


Figure 3. Confocal laser scanning (left panels) and differential interference contrast (right panels) microscopy of living human cells. Cells in panels A–C were treated with fluorescent human IgG ($0.5 \mu\text{M}$) for 4 h prior to microscopy. (Panel A) THP-1 cells that express natural Fc receptors. (Panel B) Jurkat lymphocytes that lack expression of Fc receptors. (Panel C) Jurkat lymphocytes treated with synthetic Fc receptor **1** ($1 \mu\text{M}$) for 1 h, washed to remove unincorporated receptor, followed by addition of fluorescent IgG for 4 h. Scale bar = $10 \mu\text{m}$.

Biological Evaluation. Human IgG was conjugated to the bright-green fluorophore Alexa Fluor-488 to provide a labeled ligand for cellular uptake experiments. To provide a positive control, human THP-1 monocytes, cells that express the natural FcRs Fc γ RI and Fc γ RII,²² were treated with this fluorescent ligand. As shown in Figure 3, confocal laser scanning microscopy revealed that THP-1 cells substantially internalize fluorescent human IgG within 4 h (panel A). In contrast, human Jurkat lymphocytes, a T-cell line that does not express FcRs, did not show appreciable uptake of this fluorescent protein (panel B). However, treatment of Jurkat lymphocytes with the synthetic FcR (**1**) for 1 h, washing cells to remove unincorporated receptor, and addition of the fluorescent IgG resulted in substantial dose-dependent uptake (panel C). In both THP-1 cells and Jurkat lymphocytes, the internalized fluorescent protein was delivered into defined intracellular compartments. As shown in Figure 4, these compartments were identified as late endosomes and lysosomes by confocal laser-scanning microscopy. Treatment of cells with green fluorescent IgG and red fluorescent DiI-loaded low-density lipoprotein (LDL) revealed substantial intracellular colocalization of the red and green fluorophores. LDL was chosen for these experiments because this lipoprotein complex represents the primary carrier of cholesterol in the

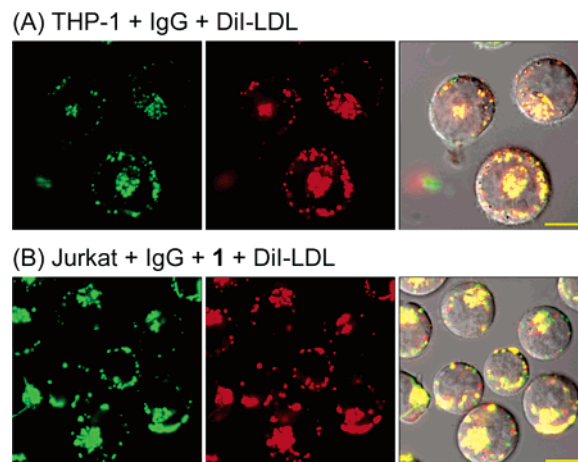


Figure 4. Colocalization of human IgG with DiI-LDL in endosomes and lysosomes visualized by confocal laser scanning microscopy. Cells in panels A and B were treated with the green fluorescent IgG ($0.5 \mu\text{M}$) and red fluorescent DiI-LDL ($0.04 \mu\text{M}$) for 4 h prior to microscopy. (Panel A) THP-1 cells. (Panel B) Jurkat lymphocytes pretreated with **1** ($1 \mu\text{M}$, 1 h). Green fluorescence is shown in the left panels. Red fluorescence is shown in the middle panels. Colocalization of green and red fluorescence is shown in yellow overlaid on the DIC image in the right panels. Scale bar = $10 \mu\text{m}$.

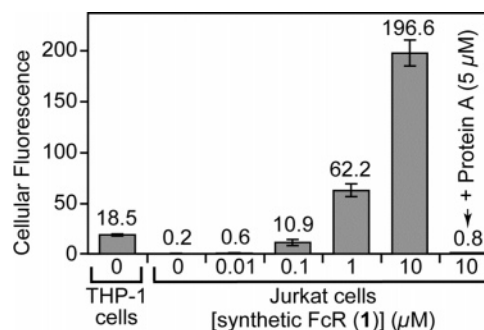


Figure 5. Flow cytometric analysis of uptake of fluorescent human IgG by human cell lines. Each bar represents the median fluorescence of 10,000 living cells. Cells were treated with DMSO alone (1%) or **1** in DMSO (1%) for 1 h and were washed; IgG was added for 4 h. [IgG] = $0.5 \mu\text{M}$. Error bars reflect the standard deviation ($n = 4$).

bloodstream, this cholesterol-laden nanoparticle is rapidly internalized by receptor-mediated endocytosis, and it is known to be efficiently delivered into late endosomes and lysosomes.²³ Thus, treatment of Jurkat cells with the minimalist receptor **1** enabled synthetic receptor-mediated endocytosis of the IgG, mimicking uptake mediated by natural macromolecular FcRs.¹

The uptake of fluorescent IgG promoted by **1** was quantitatively evaluated by flow cytometry (Figure 5). Treatment of Jurkat lymphocytes with **1** at 100 nM enhanced intracellular fluorescence by 57-fold above basal levels. Intracellular fluorescence enhancements of 551-fold ($1 \mu\text{M}$) and 1960-fold ($10 \mu\text{M}$) were observed at higher concentrations, surpassing levels observed with THP-1 cells. At $10 \mu\text{M}$, some cellular aggregation occurred; the bivalent IgG Fc region presumably bridges cells bearing **1** at high surface density. To examine the specificity of recognition, Protein A from *Staphylococcus aureus* was added in a competition experiment. This treatment with a protein that binds the hinge region of human IgG ($K_d \approx 60 \text{ nM}$) blocked the internalization of IgG mediated by **1**, verifying specific binding of **1** to this site.

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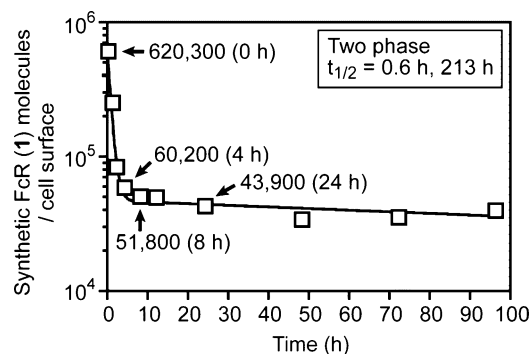


Figure 6. Population and half-life of **1** on the cell surface after washing once with fresh media. Jurkat cells were treated with **1** ($1 \mu\text{M}$, 1 h, 37°C) and washed with media to remove free receptor. Cells were further incubated at 37°C until the times shown, aliquots were cooled to 4°C and treated with excess fluorescent IgG, and cellular fluorescence was analyzed. Receptor molecules per cell surface were calculated from analysis of fluorescent bead standards.

The half-life of **1** on cell surfaces was investigated by flow cytometry (Figure 6). Jurkat lymphocytes were treated with **1** ($1 \mu\text{M}$, 1 h), cooled to 4°C to block dynamic cellular processes such as plasma membrane recycling, and washed with cold media to remove soluble receptors. Treatment with excess fluorescent IgG at 4°C , analysis of cellular fluorescence by flow cytometry, and comparison with beads bearing known numbers of fluorophores revealed an average of $\sim 6.2 \times 10^5$ synthetic receptors (i.e. 110.8 fluorescence units) on each cell surface (Figure 6). After washing the treated cells once, additional incubation at 37°C prior to this analysis revealed a loss of fluorescence that could be fit by a two-phase exponential function. Although a very high surface density of the synthetic receptor is achieved by treatment with **1** at $1 \mu\text{M}$ for 1 h, briefly washing the cells once with fresh media and additional incubation results in rapid shedding of **1** ($t_{1/2} = 0.6$ h) into the media until a surface population of $\sim 5.2 \times 10^4$ receptors per cell is attained. After this equilibration for 8 h, the number of synthetic FcRs per cell surface is comparable to natural FcRs such as $\text{Fc}\gamma\text{RI}$ ($\sim 1.1 \times 10^4$ receptors per cell) and $\text{Fc}\gamma\text{RII}$ ($\sim 3.0 \times 10^4$ receptors per cell) on adult monocyte cells.²⁴ Under these conditions, **1** resides on cell surfaces with remarkable stability ($t_{1/2} = 251$ h). Removal of soluble receptors shed into the media with additional wash steps after 8 h revealed that equilibration with the soluble population of **1** contributes to the long, secondary half-life.

Derivatives of 3β -cholesterylamine can rapidly cycle between the cell surface and early/recycling endosomes.^{9–11,25} To examine whether **1** might traffic similarly, Jurkat lymphocytes treated with **1** were analyzed after cooling to 4°C to block dynamic cellular processes (Figure 7). Cells were washed with media containing 2-hydroxypropyl- β -cyclodextrin (β -CD) to favor solubilization of the cholesterol moiety of **1**, briefly warmed to 22°C , and again cooled to 4°C to remove $\sim 30\%$ more receptors from the cell surface compared with cells washed with media alone. This treatment at relatively low temperature was designed to remove synthetic receptors from the cell surface without affecting the population in intracellular endosomes. Half of the cells subjected to these conditions were warmed to 37°C

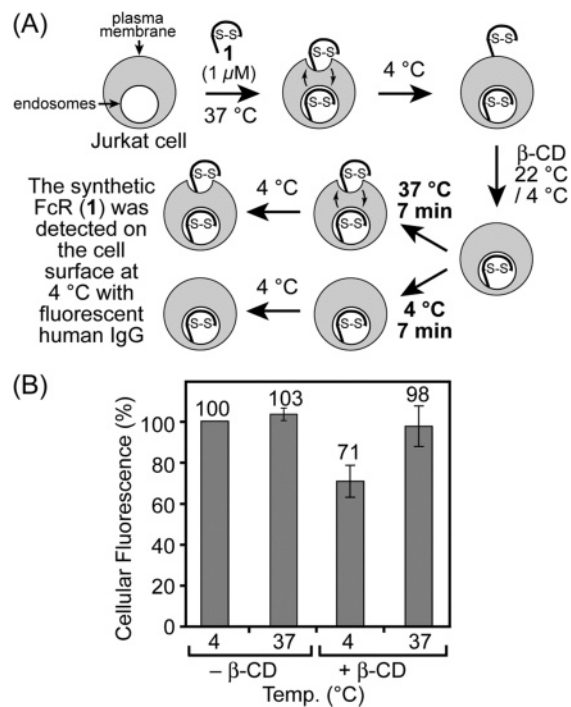


Figure 7. Cycling of the synthetic FcR (**1**) between the cell surface and intracellular endosomes. (Panel A) Illustration of cellular analysis. (Panel B) Quantification of **1** on the cell surface by flow cytometry after the treatment shown in panel A. Cells were washed with media containing or lacking 2-hydroxypropyl- β -cyclodextrin (β -CD, 20 mM) before incubation at 4°C or 37°C as shown in bold in panel A. Error bars reflect the standard deviation ($n = 6$).

$^\circ\text{C}$ for 7 min to activate plasma membrane recycling, allowing return of the receptor to the cell surface, whereas each remaining portion was maintained at 4°C to block this dynamic exchange. Subsequent cooling again to 4°C , addition of excess fluorescent human IgG, and detection of synthetic FcR (**1**) on the cell surface provided the results shown in Figure 7. In these experiments, cells treated with β -CD under conditions that selectively deplete receptors on the plasma membrane, washed with media, and briefly warmed to 37°C showed substantially higher levels of **1** on the cell surface, consistent with rapid plasma membrane recycling of this synthetic receptor.

In certain autoimmune diseases, circulating autoreactive antibodies cause potentially life-threatening tissue damage and inflammation. One therapy successfully applied to the treatment of some of these diseases is immunoadsorption, a method for ex vivo reduction of the concentration of IgG in the circulatory system.^{26–28} In this therapeutic approach, blood is treated by passage over a column modified with bacterial Protein A or other IgG-binding ligands to deplete IgG from circulation. The purified IgG-depleted blood is subsequently reinfused into the patient. As an alternative but related approach, synthetic Fc receptors such as **1** have the potential to enable treated cells to remove antibodies from the extracellular environment such as the bloodstream by synthetic receptor-mediated endocytosis. To examine this hypothesis in a simple model system, we added **1**

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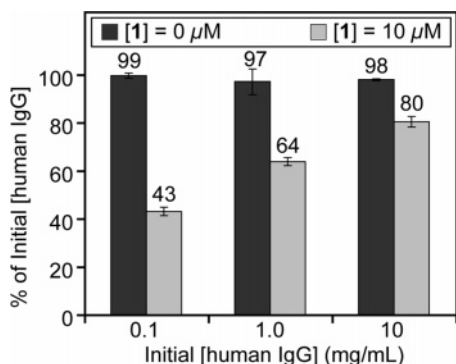


Figure 8. Depletion of human IgG in cell culture by the synthetic Fc receptor (**1**). Jurkat lymphocytes were treated with DMSO alone (1%, dark gray bars on the left) or **1** (10 μ M, light gray bars on the right) and human IgG at the concentrations shown. After 24 h, the concentration of IgG in cell culture media was quantified with a commercial IgG absorbance assay that employs monodisperse polystyrene beads that bind IgG heavy- and light-chain regions. Error bars reflect the standard deviation ($n = 3$).

and human IgG to Jurkat lymphocytes and quantified the extracellular concentration of IgG as a function of time. As shown in Figure 8, addition of **1** resulted in substantial depletion of human IgG from media after 24 h in cell culture. These effects were greatest ($\sim 56\%$ depletion) with subphysiological concentrations of IgG (0.1 mg/mL); however, significant, albeit modest, $\sim 20\%$ depletion was also observed using physiological concentrations of this ligand in blood (10 mg/mL).

Discussion

We constructed a small synthetic mimic of macromolecular Fc receptors by linking a cyclic peptide that binds the Fc region of human IgG to the membrane anchor, *N*-alkyl- β -amino- 5α -cholestane. When treated with this synthetic receptor (**1**), human Jurkat lymphocyte cells that lack natural FcRs gain the ability to actively internalize human IgG. The ability of the synthetic FcR (**1**) to function as a prosthetic molecule on the cell surface presumably relates in part to mimicry of certain properties of cholesterol by the *N*-alkyl- β -amino- 5α -cholestane membrane anchor. This cholesterol mimic likely becomes efficiently incorporated into the plasma membranes of living cells because cholesterol comprises $\sim 30\%$ of the lipid content of the plasma membrane.^{29,30} Membranes of intracellular endosomes, particularly the endocytic recycling compartment (ERC), represent another major cellular reservoir of cholesterol.³¹ Cholesterol cycles rapidly between the plasma membrane and intracellular endosomes,^{32,33} providing a mechanism for the cellular uptake of cell-impermeable compounds that bind cholesterol mimics.

Treatment of Jurkat lymphocytes for 1 h with 1 μ M of **1** installs $\sim 6 \times 10^5$ molecules of **1** per cell surface. At this level of loading of **1**, values recently reported by Maxfield³² suggest that the synthetic receptor comprises $\sim 0.1\%$ of the total lipid and $\sim 0.4\%$ of the total cholesterol²⁹ in the outer leaflet of the cellular plasma membrane. This analysis assumes that lipid molecules possess an average surface area of 6 \AA^2 ,³⁴ the

mammalian cell is 10 μ m in diameter, the plasma membrane is $\sim 2/3$ lipid and $1/3$ protein, and this membrane contains $\sim 10^9$ lipid molecules in both leaflets.

Similar to other cholesterylamine derivatives^{10,11} and many natural cell surface receptors, the synthetic FcR (**1**) cycles between the cell surface and intracellular endosomes. This biological activity mimics macromolecular FcRs found on THP-1 cells, enabling delivery of human IgG into late endosomes and lysosomes for degradation. These results suggest the intriguing possibility that synthetic FcRs administered in vivo might deplete antibodies from circulation by promoting their active cellular uptake and degradation. This approach for controlling the extracellular abundance of IgG could provide a novel strategy for the treatment of certain autoimmune diseases. However, to evaluate the feasibility of this approach, the pharmacokinetics of these types of compounds and the immunogenicity of artificial cell surface receptors must be further investigated.

Minimalistic membrane-anchored mimics of macromolecular cell surface receptors represent novel tools for destroying cell-impermeable ligands by promoting delivery to late endosomes and lysosomes. These compounds also have potential for the delivery of therapeutic molecules to intracellular targets, particularly if they can be combined with agents that disrupt intracellular endosomes and liberate released ligands into the cytosol or nucleus.

Experimental Section

General. Chemical reagents were obtained from Acros, Aldrich, Alfa Aesar, or TCI America. Solvents were from EM Science. Media and antibiotics were purchased from Mediatech. Protected amino acids were from Novabiochem. Commercial grade reagents were used without further purification unless otherwise noted. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). All solution-phase reactions were performed under an atmosphere of dry argon or nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F₂₅₄ (EM Science). TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of phosphomolybdic acid in ethanol (1:5). Flash column chromatography employed ICN SiliTech Silica Gel (32–63 μ m). Analysis by analytical HPLC employed an Agilent 1100 instrument fitted with a Hamilton PRP-1 (polystyrene–divinylbenzene) reverse-phase (RP) column (7 μ m particle size, 4.1 mm \times 25 cm). Purification by preparative RP-HPLC employed an Agilent 1100 preparative pump/gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene–divinylbenzene) RP-column (7 μ m particle size, 21.5 mm \times 25 cm). For preparative HPLC, the flow rate was increased from 10 mL/min ($t = 0$ min) to 20 mL/min ($t = 2$ min) and maintained at 20 mL/min for the remainder of the run unless otherwise noted. Melting points were measured with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were obtained with a Perkin-Elmer 1600 series FTIR. NMR spectra were obtained with Bruker CDPX-300, DPX-300, AMX-360, or DRX-400 instruments with chemical shifts reported in parts per million (ppm, δ) referenced to either CDCl₃ (¹H 7.27 ppm; ¹³C 77.23 ppm) or (CH₃)₄Si (¹H 0 ppm). High-resolution mass spectra were obtained from the University of Texas at Austin and The Pennsylvania State University Mass Spectrometry Facilities (ESI and CI). Peaks are reported as *m/z*.

3 α -Bromo-5 α -cholestane (3). A solution of 3-cholestanol (**2**, 1.17 g, 3.0 mmol) in anhydrous THF (70 mL) was cooled to 4 $^{\circ}$ C by ice–water bath. PPh₃ (1.18 g, 4.5 mmol) was added, the reaction was stirred for 5 min, and a solution of CBr₄ (1.50 g, 4.5 mmol) in anhydrous

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THF (30 mL) was added dropwise. The reaction was slowly warmed to 22 °C and stirred for 16 h. The solvent was removed in vacuo. The resulting residue was resuspended in hexanes (100 mL) and filtered under vacuum to remove insoluble material. The filtrate was concentrated in vacuo to give a yellowish solid. Column chromatography (hexanes) afforded **3** (1.29 g, 95%) as white solid, mp 102–104 °C; ¹H NMR (400 MHz, CDCl₃) δ 4.73 (s, 1H), 1.99–0.81 (m, 40H), 0.79 (s, 3H), 0.65 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 56.4, 56.2, 55.9, 53.9, 42.6, 40.1, 40.0, 39.5, 37.3, 36.2, 36.2, 35.8, 35.4, 32.9, 31.8, 31.0, 28.2, 28.0, 27.9, 24.2, 23.9, 22.8, 22.6, 20.8, 18.7, 12.3, 12.1; IR (film) ν_{\max} 2942, 2906, 2861, 1466, 1444, 1383, 1368, 1247, 1208, 760 cm⁻¹; CI⁺ *m/z* 451.2934 (MH⁺, C₂₇H₄₈Br requires 451.2939).

3β-Azido-5α-cholestane (4). To a solution of 3α-bromo-5α-cholestane (**3**, 903 mg, 2.0 mmol) in DMSO (20 mL) was added sodium azide (1.3 g, 20.0 mmol). The reaction was heated to 80 °C and stirred for 6 h. The reaction was cooled to 22 °C and poured into deionized H₂O containing ice (50 mL). The product was extracted with diethyl ether (4 × 40 mL), and the organic layers were combined, washed with deionized H₂O (100 mL), and dried over anhydrous sodium sulfate. The solvent was removed in vacuo to obtain the crude product as light-yellow solid. Flash column chromatography (hexanes) afforded **4** (745 mg, 90%) as a white solid, mp 65–67 °C (Lit.²⁰ mp 65–66 °C); ¹H NMR (400 MHz, CDCl₃) δ 3.25 (m, 1H), 1.99–0.85 (m, 40H), 0.80 (s, 3H), 0.65 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 60.6, 56.4, 56.3, 54.2, 45.2, 42.5, 40.0, 39.5, 37.1, 36.2, 35.8, 35.5 (×2), 34.0, 31.9, 28.6, 28.2, 28.0, 27.6, 24.2, 23.9, 22.8, 22.5, 21.1, 18.6, 12.2, 12.0; IR (film) ν_{\max} 2933, 2866, 2091, 1466, 1446, 1382, 1253 cm⁻¹; CI⁺ *m/z* 414.3843 (MH⁺, C₂₇H₄₈N₃ requires 414.3848).

3β-Amino-5α-cholestane (5). To a solution of 3β-azido-5α-cholestane (**4**, 620 mg, 1.5 mmol) in THF (30 mL) and deionized H₂O (3 mL) was added PPh₃ (1.18 g, 4.5 mmol). The reaction was stirred for 24 h at 22 °C. Solvents were removed in vacuo. Flash column chromatography (CH₂Cl₂/MeOH, 10:1 followed by MeOH/Et₃N, 99:1) afforded **5** (552 mg, 95%) as a white solid, mp 118–120 °C (Lit.²⁰ mp 118 °C); ¹H NMR (400 MHz, CDCl₃) δ 2.67 (m, 1H), 1.99–0.85 (m, 40H), 0.77 (s, 3H), 0.64 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 56.4, 56.2, 54.4, 51.1, 45.5, 42.5, 40.0, 39.4, 39.2, 37.6, 36.1, 35.7, 35.4 (×2), 32.4, 32.0, 28.7, 28.2, 27.9, 24.1, 23.8, 22.7, 22.5, 21.1, 18.6, 12.3, 12.0; IR (film) ν_{\max} 3354, 3272, 2933, 2911, 2849, 1466, 1449, 1382 cm⁻¹; CI⁺ *m/z* 388.3939 (MH⁺, C₂₇H₅₀N requires 388.3943).

tert-Butyl(3β)-cholestan-3-yl[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate (6). To a solution of 3β-amino-5α-cholestane (**5**, 388 mg, 1.0 mmol) in DMF (5 mL) was added *N*-(3-bromopropyl)phthalimide (295 mg, 1.1 mmol) and K₂CO₃ (276 mg, 2.0 mmol). The reaction was heated to 60 °C and stirred for 24 h. Solvent was removed in vacuo, and the resulting residue was resuspended in CH₂Cl₂ (10 mL). Insoluble salts were removed by vacuum filtration and washed with CH₂Cl₂ (5 mL). Without further purification, to the filtrate was added (Boc)₂O (327 mg, 1.5 mmol) and DIEA (0.5 mL, 3.0 mmol). The reaction was stirred for 3 h at 22 °C and concentrated in vacuo. Flash column chromatography (hexanes/ethyl acetate, 10:1) afforded **6** (479 mg, 71%) as a white solid, mp 65–68 °C; ¹H NMR (300 MHz, CDCl₃) δ 7.74 (m, 2H), 7.63 (m, 2H), 3.81 (br s, 1H), 3.60 (t, *J* = 6.8 Hz, 2H), 2.79 (br, 2H), 1.91–0.76 (m, 51H), 0.62 (s, 3H), 0.54 (s, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 168.2, 155.2, 133.9, 132.1, 123.2, 79.2, 56.4, 56.2, 55.2, 54.2, 45.9, 42.5, 40.8, 40.0, 39.5, 37.8, 36.2, 35.9, 35.8, 35.5, 35.4, 32.9, 32.0, 29.9, 28.7, 28.4 (Boc Me × 3), 28.3, 28.0, 26.2, 24.2, 23.8, 22.9, 22.6, 21.1, 18.7, 12.3, 12.1; IR (film) ν_{\max} 2931, 2866, 1772, 1716, 1689, 1468, 1395, 1366, 1306, 1242, 1172 cm⁻¹; CI⁺ *m/z* 675.5107 (MH⁺, C₄₃H₆₇N₂O₄ requires 675.5101).

tert-Butyl(3-aminopropyl)cholestan-3-ylcarbamate (7). To a solution of *tert*-butyl(3β)-cholestan-3-yl[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate (**6**, 405 mg, 0.6 mmol) in absolute EtOH (10 mL) was added anhydrous hydrazine (57 μL, 1.2 mmol). The reaction was heated to 45 °C and stirred for 4 h. A white precipitate was removed

by filtration, and the filtrate was concentrated in vacuo. The resulting residue was resuspended in CHCl₃ (15 mL), and an insoluble substance was removed by vacuum filtration and washed with CHCl₃ (2 mL). The filtrate was evaporated in vacuo to give **7** (326 mg, 99%) as a white solid, mp 112.5–115 °C; ¹H NMR (300 MHz, CDCl₃) δ 3.61 (br, 1H), 3.06 (br, 2H), 2.63 (t, *J* = 6.8 Hz, 2H), 1.91–0.80 (m, 51H), 0.73 (s, 3H), 0.58 (s, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 155.5, 79.2, 56.5, 56.3, 55.5, 54.3, 46.2, 42.6, 41.2, 40.8, 40.1, 39.6, 38.0, 36.2, 35.9, 35.6 (×2), 35.3, 33.1, 32.1, 28.9, 28.6 (Boc Me × 3), 28.3, 28.1, 26.4, 24.3, 23.9, 22.9, 22.7, 21.2, 18.8, 12.5, 12.2; IR (film) ν_{\max} 3376, 2930, 2866, 1689, 1468, 1411, 1384, 1366, 1302, 1244, 1162 cm⁻¹; CI⁺ *m/z* 545.5049 (MH⁺, C₃₅H₆₅N₂O₂ requires 545.5046).

Ethyl *N*-{[(3-[(*tert*-Butoxycarbonyl)(cholestan-3-yl)amino]propylamino]carbonyl}-β-alaninate (8). A solution of *tert*-butyl(3-aminopropyl)cholestan-3-ylcarbamate (**7**, 930 mg, 1.7 mmol) in CH₂Cl₂ (30 mL) was cooled to 4 °C by an ice–water bath. Ethyl 3-isocyanatopropionate (0.45 mL, 3.4 mmol) and DIEA (1.45 mL, 8.5 mmol) were added via syringe. The reaction was stirred at 4 °C for 2 h and subsequently at 22 °C for 16 h. The reaction solution was diluted with CH₂Cl₂ (30 mL) and washed with aqueous citric acid (10% w/v, 50 mL). The organic extracts were combined and dried over anhydrous Na₂SO₄, and solvent was removed in vacuo. Flash column chromatography (hexanes/ethyl acetate, 2:1) afforded **9** (1.15 g, 98%) as a white solid, mp 66–68 °C; ¹H NMR (300 MHz, CDCl₃) δ 5.67 (m, 1H), 4.90 (m, 1H), 4.13 (q, *J* = 5.35 Hz, 2H), 3.60–2.90 (m, 7H), 2.51 (t, *J* = 6.00 Hz, 2H), 2.20–1.48 (m, 12H), 1.45 (s, 9H), 1.43–0.81 (m, 33H), 0.78 (s, 3H), 0.64 (s, 3H); ¹³C NMR (75.41 MHz, CDCl₃) δ 173.0, 158.3, 156.6, 79.7, 60.7, 57.8, 56.6, 56.4, 54.4, 46.5, 42.8, 41.6, 40.2, 39.7, 38.1, 36.3, 36.1, 36.0, 35.7, 35.6, 35.1, 33.3, 32.2, 31.8, 29.0, 28.7 (Boc Me × 3), 28.4, 28.2, 26.7, 24.4, 24.0, 23.0, 22.7, 21.3, 18.8, 14.4, 14.3, 12.6, 12.2; IR (film) ν_{\max} 3360, 2932, 2867, 1733, 1689, 1644, 1573, 1170 cm⁻¹; FAB⁻ *m/z* 686.5497 (M – H⁻, C₄₁H₇₂N₃O₅ requires 686.5472).

***N*-{[(3-[(*tert*-Butoxycarbonyl)(cholestan-3-yl)amino]propylamino]carbonyl}-β-alanine (9).** In a 250-mL round-bottom flask, a solution of ethyl-*N*-{[(3-[(*tert*-butoxycarbonyl)(cholestan-3-yl)amino]propylamino]carbonyl}-β-alaninate (**8**, 1.15 g, 1.67 mmol) in THF (20 mL) and MeOH (60 mL) was cooled to 4 °C. A solution of LiOH monohydrate (351 mg, 8.35 mmol) in distilled water (20 mL) was added. The reaction was stirred at 22 °C for 16 h. The reaction mixture was concentrated in vacuo to remove ~80% of the organic solvents and acidified with aqueous HCl (1 M) to pH = 1. The precipitated product was filtered, washed with ice-cold deionized H₂O (10 mL), and dried in vacuo to afford **9** (1.04 g, 94%) as a white solid, mp 118–121 °C; ¹H NMR (300 MHz, CDCl₃) δ 6.31 (br s, 2H), 3.60–2.90 (m, 6H), 2.55 (br s, 2H), 2.10–1.47 (m, 10H), 1.44 (s, 9H), 1.41–0.81 (m, 33H), 0.79 (s, 3 H), 0.64 (s, 3H); ¹³C NMR (75.41 MHz, MeOD-*d*₄) δ 177.0, 159.8, 156.5, 80.5, 58.1, 56.7, 56.5, 54.4, 46.5, 42.8, 40.2, 39.7, 38.1, 38.1, 36.4, 36.0, 35.9, 35.8, 35.7, 35.4, 33.3, 32.2, 30.5, 30.3, 29.9, 29.0, 28.7 (Boc Me × 3), 28.5, 28.2, 24.4, 24.1, 23.0, 22.8, 21.4, 18.9, 12.7, 12.3; IR (film) ν_{\max} 3391, 2932, 2867, 1715, 1668, 1651, 1574, 1368, 1165 cm⁻¹; FAB⁻ *m/z* 658.5179 (M – H⁻, C₃₉H₆₈N₃O₅ requires 658.5159).

***N*-[(2S)-2-[(*N*-{[(2S)-2-[(2R)-3-(*tert*-Butyldithio)-2-[(2S)-3-carboxylato-2-[(*N*-{[(3-[(β)-cholestan-3-ylammonio]propyl]amino]carbonyl}-β-alanyl-β-alanyl-β-alanyl-β-alanyl]amino]propanoyl]-amino]propanoyl]amino]propanoyl]-L-tryptophyl]amino]-3-(1*H*-imidazol-3-ium-5-yl)propanoyl]-L-leucylglycyl-5-oxidanidyl-5-oxidanylidene-L-norvalyl-L-leucyl-L-valyl-*N*-{[(1R)-2-[(1S,2R)-1-(aminocarbonyl)-2-hydroxypropyl]amino]-1-[(*tert*-butyldithio)methyl]-2-oxoethyl]-L-tryptophanamide (10).** Peptide synthesis employed an Advanced Chemtech FBS-357 automated batch-mode synthesizer, *N*α-Fmoc methodology, and Rink amide Novagel resin (123 mg, 0.62 mmol/g, 0.0763 mmol). The following protected amino acids (AA) were used to construct the peptide: Fmoc-Thr(*t*-Bu)-OH, Fmoc-β-Ala-OH, Fmoc-Trp(Boc)-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Glu(*t*-Bu)-

OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ala-OH, Fmoc-Cys(*t*-Buthio)-OH, and Fmoc-Asp(*Or*-Bu)-OH. Deprotection of Fmoc carbamates of AA on the resin was effected by addition of piperidine (30%) in DMF (0.8 mL for 5 min followed by 0.8 mL for 10 min followed by an additional 0.8 mL for 20 min). AA were consecutively coupled to the treated resin by sequential addition of the following reagents: AA + HOBT in DMF (400 μ L, 0.5 M of each component), followed by DIEA (800 μ L, 0.5 M) and HBTU (400 μ L, 0.5 mM). The resin was shaken automatically at 22 °C for 45 min. Acylation of unreacted amines after each AA coupling employed addition of acetic anhydride (30%) in DMF (600 μ L) and DIEA (400 μ L of 0.5 M in DMF) with shaking for 20 min. After removal of the *N* α -Fmoc group of the *N*-terminal AA, the resin was transferred to a glass reaction vessel, and the *N*-terminus was capped by addition of DIEA (80 μ L, 0.458 mmol, in 0.5 mL of DMF), HOBT (20 mg, 0.153 mmol), *N*-{[(3-[(*tert*-butoxycarbonyl)(cholestan-3-yl)amino]propyl)amino]carbonyl}- β -alanine (**9**, 105 mg, 0.153 mmol, 0.5 mL of DMF) and HBTU (57 mg, 0.150 mmol, 0.5 mL of DMF). The resin was shaken at 22 °C by wrist shaker for 24 h followed by washing with DMF (5 \times 10 mL), MeOH (3 \times 10 mL), and CH₂Cl₂ (5 \times 2 mL). The product was cleaved from the resin with concomitant removal of Boc, *t*-Bu, and Trt protecting groups by treatment with TFA containing triisopropyl silane (TIS, 2.5%) and deionized H₂O (2.5%). After gently shaking for 1 h, the resin was removed by filtration and washed twice with TFA (4 mL). The filtrates were collected and solvents removed in vacuo (16 h). The crude, brown, oily product was dissolved in DMSO (4 mL) and purified by preparative RP-HPLC (gradient: 39.9% MeCN, 60% H₂O, and 0.1% TFA to 69.9% MeCN, 30% H₂O, and 0.1% TFA over 40 min; retention time = 31.4 min (215 nm)). HPLC solvents were removed by lyophilization to afford **10**, the uncyclized Cys (*t*-Buthio) precursor to **1**, (4 mg, >95% homogeneity by HPLC) as a white solid (2.1% overall yield). FAB⁺ *m/z* 2462.3120 (MH⁺, C₁₂₀H₁₈₉N₂₄O₂₃S₄ requires 2462.3235).

N-{[(3-[(3 β)-Cholestan-3-ylammonio]propyl)amino]carbonyl}- β -alanyl- β -alanyl- β -alanyl-*N*-[(1*S*)-2-[(4*R*,7*S*,10*S*,13*S*,16*S*,22*S*,25*S*,28*S*,31*S*,34*R*)-4-[(1*S*,2*R*)-1-(aminocarbonyl)-2-hydroxypropyl]-amino]carbonyl]-16-(2-carboxylatoethyl)-25-(1*H*-imidazol-3-ium-5-ylmethyl)-7,28-bis(1*H*-indol-3-ylmethyl)-13,22-diisobutyl-10-isopropyl-31-methyl-6,9,12,15,18,21,24,27,30,33-decaoxo-1,2-dithia-5,8,11,14,17,20,23,26,29,32-decaazacyclopentatriacontan-34-yl]amino]-1-(carboxylatomethyl)-2-oxoethyl]- β -alaninamide (**1**). A solution of **10** (3.69 mg, 1.5 μ mol), dithiothreitol (DTT, 12 mg, 77.8 μ mol), and NH₄HCO₃ (0.1 M) in DMF (1 mL) was stirred at 22 °C for 12 h. DMSO (3 mL) was added, and the reaction mixture was stirred at 22 °C for 12 h. The reaction was monitored by analytical RP-HPLC (gradient: 39.9% MeCN, 60% H₂O, and 0.1% TFA to 69.9% MeCN, 30% H₂O, and 0.1% TFA over 40 min; retention time of the desired cyclized product = 27.5 min (215 nm) using this gradient). HPLC revealed partial conversion of the starting material (**10**, retention time = 35.2 min) to an intermediate (retention time = 31.42 min) and **1** (analyzed by mass spectrometry, retention time = 27.5 min). The majority of the starting material and the intermediate disappeared only after DMSO was added for at least 4 h. The synthetic FcR (**1**) was purified by preparative RP-HPLC (gradient: 44.9% MeCN, 55% H₂O, and 0.1% TFA to 67.4% MeCN, 32.5% H₂O, and 0.1% TFA over 30 min; retention time = 19.6 min (215 nm)). Lyophilization afforded **1** at >95% homogeneity by HPLC as a white solid (3.4 mg, 99%), FAB⁺ *m/z* 2284.2328 (MH⁺, C₁₁₂H₁₇₁N₂₄O₂₃S₄ requires 2284.2385).

Cell Culture. Jurkat (human acute T-cell leukemia, ATCC #TIB-152) and THP-1 (human monocytes, ATCC #TIB-202) cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with fetal bovine serum (FBS, 10%), penicillin (100 units/mL), and streptomycin (100 μ g/mL). Unless otherwise noted, this cell culture media and wash steps with this media included antibiotics and FBS.

Microscopy. A Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat objective (63 \times) was employed. Alexa Fluor-488 was excited by argon ion laser (488 nm), and emitted photons were collected through a 505-nm LP filter. DiI-LDL was excited with a 543-nm HeNe laser, and emitted photons were collected through a 560-nm LP filter.

Flow Cytometry. Analyses were performed with a Beckman-Coulter XL-MCL benchtop flow cytometer. Forward-scatter (FS) and side-scatter (SSC) dot plots afforded cellular physical properties of size and granularity that allowed gating of live cells. After gating, 10⁴ cells were analyzed. For studies of uptake of human IgG Alexa Fluor-488, the fluorophore was excited at 488 nm with a 15 mW air-cooled argon-ion laser, the emission was split with a 550-nm dichroic and filtered through a 510-nm long pass filter and 530/30-nm band-pass filter. The PMT voltage for this instrument was set to 724. Calibration with Sphero Rainbow Calibration particles (Spherotech) bearing 330,000 molecules of fluorescein/particle provided a fluorescence of 16.7 (calibration for the data shown in Figure 5) or 19.6 (calibration for the data shown in Figure 6) at this voltage.

Assays of Uptake of Human IgG Alexa Fluor-488. To Jurkat or THP-1 cells (7 \times 10⁵) in media (0.5 mL) was added **1** (typical final concentration = 1 μ M) in DMSO (final [DMSO] = 1%). These cells were incubated at 37 °C for 1 h to load the receptor into cellular plasma membranes. The cells were washed with media (0.5 mL) to remove unincorporated receptor/DMSO and resuspended in fresh media (95 μ L). To these cells was added human IgG conjugated to Alexa Fluor-488 (5 μ L of 1.40 mg/mL, final concentration = 0.47 μ M). Cells were incubated at 37 °C for 4 h to promote synthetic receptor-mediated endocytosis. Prior to analysis, treated cells were washed with RPMI media and further incubated in media containing nonlabeled human IgG (2.7 μ M, 2 \times 100 μ L \times 20 min) to remove any noninternalized fluorescent IgG. Cells were centrifuged, the supernatant was discarded, and the cells were resuspended in media (0.5 mL) for analysis by confocal microscopy and flow cytometry. For the competitive inhibition of uptake experiment, bacterial Protein A from *S. aureus* (5 μ M, Sigma) was included with the fluorescent IgG during the incubation for 4 h.

Labeling of Human IgG. IgG from human serum (1 mg, Sigma) was labeled using an Alexa Fluor-488 antibody labeling kit (Invitrogen). As analyzed by UV spectroscopy, the concentration of human IgG Alexa Fluor-488 was determined to be 1.4 mg/mL (9.3 μ M) with an average of three Alexa Fluor-488 molecules per IgG molecule.

Colocalization Studies with DiI-LDL. To Jurkat lymphocytes (7 \times 10⁵) in media (0.5 mL) was added **1** (final concentration = 1 μ M) in DMSO (final [DMSO] = 1%). These cells were incubated at 37 °C for 1 h, washed with media (0.5 mL) to remove unincorporated receptor/DMSO, and were resuspended in fresh media (85 μ L). Human IgG–Alexa Fluor-488 (final [IgG] = 0.47 μ M) and DiI-LDL (Invitrogen, final concentration = 40 nM) were added to THP-1 or Jurkat cells. Cells were maintained at 37 °C for 4 h, washed, and resuspended in media (150 μ L) for analysis by confocal microscopy.

Quantification of the Stability of Fc Receptors on the Cell Surface. Jurkat lymphocytes (7 \times 10⁶) in media (5 mL) were incubated with **1** (1 μ M, 1% DMSO) for 1 h at 37 °C in an aerated centrifuge tube (15 mL). Cells were washed once with media (5 mL) to remove unincorporated receptor, resuspended in media (5 mL), transferred to a tissue culture flask (25 cm²), and maintained at 37 °C. At fixed time points, an aliquot of this culture (300 μ L) was removed, and cells were isolated by centrifugation at 4 °C. These cells were resuspended in ice-cold media (50 μ L) containing human IgG–Alexa Fluor-488 conjugate (2 μ M) and incubated on ice for 20 min. Treated cells were centrifuged (1300 rcf, 2 min, 4 °C), the supernatant was removed, cells were resuspended in ice-cold media (0.5 mL), and cellular fluorescence was analyzed by flow cytometry. The loss of **1** from the cell surface was analyzed with a two-phase exponential decay model (GraphPad Prism version 3.0 software, GraphPad Software, San Diego, CA) to calculate cell surface half-lives of 0.6 and 251 h ($\chi^2 = 0.996$).

Analysis of Cycling of the Synthetic FcR (1) between Endosomes and the Cell Surface. Jurkat lymphocytes (3.6×10^6) in media (1 mL) were treated with **1** ($1 \mu\text{M}$ final concentration) in DMSO (1% final concentration) for 1 h, washed with media (1 mL) to remove unincorporated receptor/DMSO, resuspended in media ($500 \mu\text{L}$), and incubated at 37°C for 90 min to equilibrate. Cells were partitioned equally into two 1.5-mL Eppendorf tubes designated #1 (cells to be washed with media only) and #2 (cells to be washed with media containing (2-hydroxypropyl)- β -cyclodextrin to remove **1** from the cell surface). Cells in both tubes were isolated by centrifugation (2 min at 1300 rcf) at 4°C to block plasma membrane recycling. The synthetic FcR (**1**) was removed from the surface of cells in tube #2 by washing with media (lacking FBS) containing (2-hydroxypropyl)- β -cyclodextrin (20 mM, $3 \times 800 \mu\text{L} \times 1 \text{ min}$ at 22°C) followed by immediate washing again with ice-cold media (lacking FBS, $2 \times 1 \text{ mL}$). Cells in tube #1 were treated identically, but the (2-hydroxypropyl)- β -cyclodextrin was omitted from the wash media. Cells were isolated by centrifugation at 4°C , the supernatant was removed, ice-cold media ($40 \mu\text{L}$) was added to each tube, and the samples were maintained at 4°C . Cells in tubes #1 and #2 were partitioned equally into two Eppendorf tubes labeled 4°C and 37°C . The tubes labeled 37°C was warmed to this temperature in a 5% CO_2 incubator for 7 min, to allow **1** trapped in endosomes to cycle back to the cell surface, followed by cooling again to 4°C (5 min). The tubes labeled 4°C were maintained on ice to inhibit plasma membrane recycling. Ice-cold human IgG–Alexa Fluor-488 conjugate was added to each tube ($5 \mu\text{L}$ of 1.4 mg/mL) followed by incubation at 4°C for 25 min to allow binding of the IgG to **1** on the cell surface. Cells were washed with ice-cold media ($500 \mu\text{L}$) and

isolated by centrifugation (1300 rcf at 4°C). Cell pellets were resuspended in ice-cold media ($400 \mu\text{L}$), and the fluorescence of Alexa Fluor-488 bound to the cell surface was analyzed by flow cytometry.

Estimation of Number of Synthetic Receptor Molecules on the Surface of Jurkat Lymphocytes. Sphero Rainbow Calibration particles (Spherotech) were analyzed by flow cytometry. Linear regression analysis of known molecules of equivalent fluorescein (MEFL) plotted versus fluorescence yielded $y = 16820x - 2804$ ($r^2 = 0.9987$). One molecule of Alexa Fluor-488³⁵ was assumed to be equivalent to one molecule of fluorescein in these experiments. For the calculation, the cellular fluorescence was divided by a factor of 3 to compensate for the extent of conjugation of Alexa Fluor-488 per IgG.

Depletion of Human IgG from Media Promoted by 1. To Jurkat lymphocytes (1.6×10^6) in media ($100 \mu\text{L}$) containing human IgG (0.1, 1.0, or 10 mg/mL) was added **1** (final concentration = $10 \mu\text{M}$) in DMSO (final [DMSO] = 1%). These cells were incubated at 37°C for 24 h. Cells were isolated by centrifugation, and the supernatant was collected for quantitative analysis of human IgG. The percentage of initial human IgG was determined using an Easy-Titer IgG (H+L) Assay kit (Pierce).

Acknowledgment. We thank the NIH (R01-CA83831) for financial support.

JA062377W

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