Supporting Information

An Fc Domain Protein-Small Molecule Conjugate as an Enhanced Immunomodulator

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Experimental Methods

Chemical synthesis

CGS-21680

(‘CGS’) (Figure S1) was synthesized as previously described.1 Briefly, 2,2-dimethoxypropane and a camphorsulfonic acid catalyst were used to protect the 2’,3’-diol of 2-chloroadenosine as an acetonide. Oxidation of the 5’-hydroxyl substituent to the corresponding carboxylic acid was achieved using potassium permanganate and aqueous potassium hydroxide. The carboxylic acid was then converted to the acid chloride with thionyl chloride and a catalytic amount of N,N-dimethylformamide. Gaseous ethylamine was bubbled through a solution of the crude acid chloride in dichloromethane to generate the desired amide intermediate. tert-Butyl 3-[4-(2-aminoethyl)phenyl]propionate was freshly prepared from p-bromophenylacetonitrile and tert-butyl acrylate via a Heck reaction and then immediately reacted with the aforementioned amide intermediate to generate the penultimate ester. Simultaneous hydrolysis of the ketal and the tert-butyl ester in the presence of aqueous hydrochloric acid yielded the final product, CGS, as a hydrochloride salt. CGS produced in this way showed spectroscopic data consistent with those reported previously.1

tert-Butyl (14-amino-5-oxo-1,1,1-triphenyl-9,12-dioxo-2-thia-6-azatetradecan-4-yl) carbamate (I): N-[(1,1-Dimethylethoxy)carbonyl]-S-(triphenylmethyl)-L-cysteine (1.00 g, 2.15 mmol) and carbonyldiimidazole (CDI, 0.40 g, 2.47 mmol) were dissolved in anhydrous THF (20 mL) and allowed to stir at room temperature for 2 hours. This solution was then added dropwise via an addition funnel to 2,2’-[1,2-ethanediylbis(oxy)]bis-ethanamine (6.28 mL, 43.0 mmol) dissolved in anhydrous THF (60 mL) at 0 °C. The flask was purged with argon and the reaction was stirred at 0 °C for 1 hour after which it was allowed to warm to room temperature and stirring was continued overnight (~ 16 hours). After completion as evidenced by thin-layer chromatography, the reaction was concentrated to half the volume in vacuo and then dichloromethane (250 mL) was added and the organic layer was washed with brine (5 x 50 mL), dried over Na₂SO₄, filtered and concentrated in vacuo. The oily residue obtained was dissolved in dichloromethane and purified using flash column chromatography (basic Al₂O₃, 5% methanol/dichloromethane). The title compound was isolated as a pale yellow, viscous oil (0.80 g, 63%). 1H-NMR (500 MHz, CDCl₃): δ 7.42 (m, 6H), 7.29 (m, 6H), 7.22 (m, 3H), 6.75 (br, 1H), 5.37 (d, J
= 8.2 Hz, 1H), 4.00 (br, 1H), 3.47 (m, 11H), 2.85 (t, J = 5.2 Hz, 2H), 2.65 (br, 1H), 2.54 (m, 1H), 1.43 (s, 9H). ESI-MS: [M+H]+ = 593.3 m/z, found: [M+H]+ = 594.3 m/z (Figure S4).

C-CGS synthesis (Figure S3): tert-Butyl (14-amino-5-oxo-1,1,1-triphenyl-9,12-dioxa-2-thia-6-azatetradecan-4-yl)carbamate (1, 0.20 g, 0.33 mmol) was dissolved in anhydrous tetrahydrofuran (3 mL) and added to PAL resin (MidWest Bio-Tech) (0.10 g, 0.09 mmol) suspended in anhydrous THF (2 mL). Glacial acetic acid (0.10 mL) was added to this mixture and the reaction was allowed to stir at room temperature for 1 hour. Then, NaBH(OAc)3 (0.16 g, 0.77 mmol) was added and stirring was continued overnight. The resin was then washed with methanol (5 x 5 mL), dimethylformamide (5 x 5 mL) and dichloromethane (5 x 5 mL) in a Bio-Rad Poly-Prep Chromatography Column.

The following Fmoc-8-amino-3,6-dioxaocctanoic acid (139 mg, 0.35 mmol each reaction) and CGS 21680 (CGS, 22.5 mg, 0.041 mmol prepared as previously described) coupling reactions were based on standard solid phase peptide synthesis methodology. Coupling was carried out at room temperature for 3 hours with 5% diisopropylethylamine (1.8 mL) and O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU, 133 mg, 0.35 mmol). Fluorenylmethoxycarbonyl deprotection was carried out at room temperature for 1 hour with 20% piperidine (3 mL). The solvent used for both reactions was N-methyl-2-pyrrolidone (NMP). The crude product was cleaved from the PAL resin with 4 mL of a 95% TFA (trifluoroacetic acid), 2.5% ddH2O and 2.5% tri-isopropyl-silane solution at room temperature for 1 hour, dried under vacuum, resuspended in 12 mL of ddH2O containing 0.05% TFA and then filtered through a 0.2 µm filter to remove all insoluble particles. The filtrate was further purified by reverse-phase HPLC, Varian Dynamax Microsorb 100-5 C18 column (250 x 21.4 mm), gradient: 5%-60% acetonitrile/H2O,45 min; 60%-100% acetonitrile/H2O,10 min, 100% acetonitrile, 10 min; 100%-5%, 10 minutes, the flow rate was 10 mL per minute. The final purified product, C-CGS, was lyophilized and isolated as a fine white powder (35 mg, 59 %).

Compound characterization was done using matrix assisted laser desorption ionization (MALDI) mass spectrometry (Figure S5).

Protein Purification & Expressed Protein Ligation

The Fc domain of mouse IgG3 gene (aa 104-330), originally from the pFUSE-mIgG3-Fc1 plasmid (InvivoGen), was subcloned in frame into the pTXB1 vector (NEB) by restriction enzymes Ndel and

S3
EcoRI, which contains the GyrA intein from *Mycobacterium xenopi* and the chitin-binding domain (CBD). The Fc-intein-CBD construct was then used as a PCR template for insertion of the secretion signal *Honey Bee Melittin* (HBM: MKFLVNVALVMVVYISIYA )-M2 FLAG-HisX8-TEV cleavage site-KpnI site at the N-terminus. The nested PCR was performed with the following primers:

5’-His-TEV-KpnI site Fc:
TCACCATCACCATCAGACACACCTGTATTTTCAGGTACCTAGAATACCCAAAGCCAG

5’-HBM_FLAGS-His-TEV:
CATCTATGCGATTACAAGGATGACGATGACAAGCATCACCATCACCACATCACATCACGA

5’-HBM-FLAG-His:
TTATGGTGATACATTCTTACATCTGCGATTACAAGGATGACGATGACAAGCAT

5’-BamHI-HBM:
ATAACTGGATCCATGAAATTCATTAGTCACGTTGCCTGTTTTATGGTCGTATAATT

3’-CBD-HindIII:
ATAATTTAAAGCTTTTCATTGAAGCTGCCACAAGG

The resulting PCR product and plasmid pFAST Bac 1 (Invitrogen) were digested with BamHI and HindIII and then ligated together using T4 DNA ligase. The generation of baculovirus was carried out according to manufacturer’s instructions (Bac-to-Bac Baculovirus Expression System, Invitrogen).

Sf9 insect cells were grown in serum-free media (Sf-900 III, Invitrogen) in suspension culture at 27°C. Fc-Intein-CBD recombinant protein was expressed and secreted by Sf9 insect cells using a multiplicity of infection (MOI) equal to 1 over 72 hours. The supernatant was collected by centrifugation of the insect cell media mixture at 1,500 rpm for 10 minutes to remove the cell pellet. The supernatant containing the fusion protein was then filtered through a 0.2 µm filter to remove all cell debris. EDTA and phenyl-methyl-sulfonyl fluoride (PMSF) were added to the filtrate as protease inhibitors. Fc-intein-CBD fusion protein was purified by passing the supernatant over a bed of chitin beads (NEB, 2 mL bed volume per liter culture) by gravity flow. The beads were washed with 50 mL of phosphate buffered
saline (PBS) 5 times. To initiate the ligation reaction, two column volumes of 400 mM sodium 2-mercaptoethanesulfonate in PBS (pH = 7.2-7.3) containing 500 µM of C-CGS was added to the column. The column was purged with argon and the reaction was carried out at room temperature over 72 hours. The ligated product was followed by coomassie blue stained SDS PAGE electrophoresis, and upon completion (48-72 hours), the product was eluted from the column with one column volume of PBS 5 times, and then dialyzed against 5 liters of PBS with a total of four buffer exchanges. Each buffer exchange lasted at least 8 hours, using 10 kDa molecular weight cutoff (MWCO) SnakeSkin Dialysis Tubing from Thermo Scientific. The Fc-CGS was further concentrated to 0.5-1 mg/mL and dialyzed again in a 10 kDa MWCO dialysis cassette (Slidealyzer) into 2 liters of PBS twice over 24 hours to remove excess unreacted small molecule and reducing agent. Fc-CGS was stored at -80 °C. Typical yield was about 1.5 mg Fc-CGS per liter of Sf9 cell culture.

**ELISA (enzyme-linked immunosorbent assay) measurements of IL-2 and cAMP**

**IL-2 (interleukin-2) measurement**

5C.C7 mice were originally purchased from Taconic (Petersburgh, NY). 5C.C7 mice are B10.A TCR-5C.C7 transgenic. These TCR transgenic mice on a Rag 2 deficient background specifically recognize pigeon cytochrome \( c \) (PCC) peptide. Spleens and inguinal lymph nodes were harvested from 5C.C7 mice and crushed on a cell strainer (BD Bioscience). The red blood cells were then removed using ACK lysis buffer. Cell suspensions were activated with 5 µM PCC peptide in complete medium containing 45% RPMI 1640, 45% Click’s Medium Eagle-Hank’s amino acid, 10% fetal calf serum, 4 mM glutamine, 2.5 µg/ml gentamycin, 100 U of penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol. Total IL-2 secreted from 5C.C7 splenocytes was measured by mouse IL-2 ELISA kit (eBioscience) according to manufacturer’s instructions.

**Intracellular cAMP (cyclic AMP) measurement**

Wild type (C57BL/6) and \( \alpha_2 \beta_2 \)-/- splenocytes were isolated from harvested spleens using a similar protocol to that used for the 5C.C7 cells. Splenocytes were activated by soluble anti-CD3 (1 µg/mL) for
24 hours and this was followed by treatment with fresh medium without anti-CD3 for another 24 hours. To determine the effects of Fc, CGS, C-CGS, and Fc-CGS on A2AR function, the amount of total cAMP produced in wild type (C57BL/6) or A2AR-/ knockoout splenocytes was assayed with the cAMP Biotrak EIA system (GE Healthcare Life Science) according to manufacturer’s instructions.

**Mouse pneumonitis disease model**

C3-HA\textsuperscript{high} transgenic mice expressing hemagglutinin (HA) under rat C3 promoter were used as the recipients.\textsuperscript{3} The C3-HA\textsuperscript{high} line contains 30-50 transgene copies and was established in a B10.D2 genetic background. The donor mice were the TCR-transgenic line 6.5, which expresses a TCR that recognizes an I-E\textsuperscript{d}-restricted HA class II epitope (\textsuperscript{110}SFERFEIFPKE\textsuperscript{120}), that were backcrossed onto the Thy1.1+/+ B10.D2 genetic background.\textsuperscript{4} The mice used for experiments were male, between 7 to 12 weeks old. All experiments involving the mice were performed under the protocols approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

**Adoptive transfer**

Clonotypic CD4+ T cells were harvested from 6.5+ transgenic mice (using the same protocol as described for the 5C.C7 cells). The unfractionated population was stained by PE (R-phycoerythrin)-conjugated anti-6.5 and APC (Allophycocyanin)-conjugated anti-CD4 antibodies and checked by flow cytometry. Cells were washed and resuspended into PBS containing 1.5 million 6.5+ T cells in 200 µL PBS for tail vein injection into C3HA mice. Recipient mice were given a single dose intraperitoneally of vehicle (PBS alone), CGS (5 µmol/kg, 2.5 mg/kg), Fc (50 nmol/kg, 1.6 mg/kg), or Fc-CGS (50 nmol/kg, 1.6 mg/kg) on days 1 (same day as adoptive transfer) and 3 after the transfer. The drug injection volume used for all treatments was 200 µL.

**PNGase F digestion of IgG3 Fc for Glycan Analysis**
IgG3 Fc (10 µg) was digested with 500 units of PNGase F (New England Biolabs) in G7 reaction buffer (50 mM sodium phosphate, pH = 7.5) with a total volume of 25 µL at 37 °C for 16 hours. The reaction mixture was dissolved in 1 mL water and the released glycans were purified using a Sep-Pak Vac RC C18 cartridge (500 mg, Waters) that was prewashed 4 times with 2.5 mL of 10% acetic acid, 50% methanol, 100% methanol and then 8 times with 2.5 mL of ddH₂O. After loading the sample, the column was washed 3 times with 1 mL ddH₂O to elute released N-glycans. The elution was then loaded onto a Hypersep Hypercarb PGC column (50 mg, Thermo Scientific) which had been prepared with 3 washes of 1 mL 60% acetonitrile, 1 mL 30% acetonitrile, and 1 mL ddH₂O. After loading, the column was washed with 3 mL of ddH₂O and the glycans were then eluted with 1 mL of 30% acetonitrile, 1 mL of 60% acetonitrile, and 1 mL 100% acetonitrile. The elutions were pooled then dried under reduced pressure and resuspended in ddH₂O for further LC-MS analysis.

*Endoglycosidases (A/D/S) Digestion of IgG3 Fc for Surface Plasmon Resonance (SPR) Binding Experiments*

EndoA from *Arthrobacter protophormiae*, EndoD from *Streptococcus pneumoniae*, and EndoS from *Streptococcus pyogenes* were overexpressed and purified using previously described procedures.⁵,⁶ 1 µg of each Endo A/D/S was added to 25 µg Fc in 50 µL PBS buffer, pH = 7.4 for 1 hour incubation at 37 °C.

*Liquid Chromatography Mass Spectrometry (LC-MS) analysis of Fc and Fc-CGS*

LC-MS was performed on a LXQ system (Thermo Scientific) with a Poroshell 300SB-C8 column (5 um, 75 x 1.0 mm). The Fc samples were treated with 50 mM DTT and heated at 55 °C for 20 minutes then subjected to LC-MS analysis. The LC was performed at 60 °C eluting with a linear gradient of 20-40% acetonitrile:water containing 0.1% formic acid within 10 minutes at a flow rate of 0.25 mL/min.

*High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection*
HPAEC-PAD was performed on a Dionex ICS-5000 system (Fischer Scientific) equipped with an electrochemical detector (ED50 and an anion exchange column (CarboPac PA200, 3 x 250 mm). The mobile phase (flow rate, 0.5 mL/min) was composed of 100 mM NaOH (eluent A) and 100 mM NaOH/250 mM NaOAc (eluent B). The gradient used was as follows: 0 to 20 mM NaOAc in 50 mM NaOH in 20 minutes.

**Surface Plasmon Resonance (SPR) Binding Experiments**

The binding between different forms of mouse IgG3 Fc and mouse Fcγ receptors type I (from Creative BioMart) was measured on a Biacore T100 instrument (GE Healthcare, USA). Protein A was immobilized on a CM5 biosensor chip (GE Healthcare) using standard (ethyl-dimethylamino-carbodiimide/N-hydroxy-succinimidyl ester) amine coupling reactions at pH of 4.5 to achieve a level of 1200-1500 RU. Each individual mouse IgG3 Fc in HBS-P buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 0.05% v/v surfactant P20) was captured onto the protein A surface until reached the capture level of 150 RU. A 2-fold serial dilution of mouse FcγRI was flown through for 3 min at a flow rate of at 10 µL/min and allowed to dissociate for another 3 min. After each cycle, the surface was regenerated by injecting 20 mM HCl at 10 µL/min for 30 seconds. The carrier buffer system in the Biacore T100 instrument is HBS-P buffer. Data was evaluated using Biacore T100 evaluation software. Replicate experiments showed agreement within 20%.

**Fc-CGS Stability Test in Blood**

Blood was collected from C3HA mice by performing a cardiac puncture. EDTA was added into 3 mL blood as an anticoagulant to a final concentration of 50 mM. Fc-CGS was then diluted in mouse blood to a final concentration of 100 nM. After 24, 48 and 72 hours incubation at 37 °C, 100 µL of serum was isolated from 200 µL of blood by centrifugation (1,500 rpm, 5 minutes) then incubated with 10 µL of anti-FLAG agarose beads to immunoprecipitate the Fc-CGS. The binding was performed by rotating at 4 °C for 2 hours. Fc-CGS bound to the beads was then washed with 1 mL PBS twice, further eluted by using 20 µL of SDS denaturing buffer and boiled at 95 °C for 10 minutes prior to being run on an 10%
SDS PAGE gel and visualized by western blotting. The primary antibody (anti-FLAG M2 antibody, Sigma-Aldrich F3165) was used at a 1:1,000 dilution and the secondary antibody (anti-mouse IgG, GE Healthcare NXA931) was employed at a 1:5,000 dilution.

Size exclusion chromatography

25 µg of Fc-CGS in 50 µL phosphate-buffered saline (PBS) was injected onto a Superdex 75 column (10x300 mm) through a 125 µL injection loop on an AKTA FPLC. The flow rate = 0.5 mL/min, total elution volume was 1.5 column volumes with fraction volumes of 0.5 mL.

Immunohistochemistry Staining

Mouse organs (brain, heart, lung) were harvested after systemic perfusion. Briefly, mice were anesthesized with 200 µL of sodium pentobarbital (20 mg/mL) intraperitoneally. The left ventricle was perfused with 30 mL of 1X PBS then 30 mL of formalin for tissue fixation. Paraffin-embedded tissue slides were first dewaxed by soaking into propar (Anatech, #511) 3 times, then soaked in 100% EtOH, 95% EtOH, 70% EtOH and H2O for 5 minutes in each solution. Antigen retrieval was performed by soaking slides in 1X Dako Target Retrieval Solution (Citrate buffer, pH6.0, Dako #S1699) in a high pressure cooker with heating at 121 °C for 20 minutes. Slides were cooled down to room temperature for 20 minutes, then rinsed with deionized water followed by 1x Dako Wash Buffer (Dako # S3006). After rinsing with Dako Wash Buffer, slides were then blocked with goat serum (Vector, PK6101) for 20 minutes. The slides were incubated with anti FLAG tag primary antibody (DYKDDDDK Tag, Cell Signaling #2368 diluted in 1:500 in Dako Antibody Diluent. Dako # S0809) for 2 hours at room temperature. Slides were then washed with 1x Dako Wash Buffer three times, 5 minutes each. Slides were incubated with secondary antibody (Alexa Fl 555 Goat anti-Rabbit IgG H+L. Invitrogen #A21429 diluted 1:1000 in TBS) for one hour, then washed with 1x Dako Wash Buffer 3 times, 5 minutes each. DAPI stain (Vector #H1500) was added according to manufacturer’s instructions. All slides were air-dried before imaging. Each image is representative of stained tissues from at least two mice under each treatment condition.
**Figure S1**
Chemical structure of the small molecule adenosine 2A receptor agonist, CGS-21680 (‘CGS’).
**Figure S2**

Fc-Intein-CBD protein expression. The Fc-Intein-CBD fusion protein is expressed in Sf9 insect cells with a Honey Bee Mellitin (HBM) secretion signal, followed by a FLAG and 8X His tag, TEV cleavage site and a spacer sequence N-terminal to the start of the Fc-Intein-CBD fusion protein. The HBM directs the protein into secretory pathway where the Fc portion is glycosylated.
Figure S3
Synthetic scheme for C-CGS.
Figure S4
The \( ^1\text{H}-\text{NMR} \) (500 MHz, CDCl\(_3\)) of C-CGS intermediate: tert-Butyl (14-amino-5-oxo-1,1,1-triphenyl-9,12-dioxa-2-thia-6-azatetradecan-4-yl) carbamate.
Figure S5
Chemical structure of C-CGS. CGS synthetically linked to a hexa-ethylene glycol spacer followed by a cysteine. The MALDI mass spectrum of the purified compound is shown (matrix: 2,5-dihydroxybenzoic acid).
LC-MS analysis of Fc. Fc was treated with PNGase F at 37 °C for 18 hours, followed by treatment with 50 mM DTT at 55 °C for 20 minutes to reduce disulfide bonds prior to LC-MS analysis.

Figure S6

Calculated M.W.=29933
Figure S7
Glycan analysis of Fc. Glycan was released from Fc by PNGase F digestion and purified by Waters Sep-Pak Vac RC C\textsubscript{18} column and then Hypersep Hypercarb PGC column for LC-MS analysis. Asterisk peaks are \([\text{M+Na}]^{+}\) and the insert shows the HPAEC-PAD analysis of the glycan for semi-quantification purposes.
Figure S8
Gel filtration analysis of Fc-CGS. 25 µg Fc-CGS was injected onto a Superdex 75 column on an AKTA FPLC (flow rate: 0.5mL/min; Buffer: phosphate-buffered saline (PBS). The insert shows the anti-FLAG western blot from fraction 17 to 22 (Elution volume 8.5 mL to 11 mL).
Figure S9
Surface-plasmon resonance binding assay. Commercial full length Fc (A) and endoglycosidase mixture (EndoA, EndoD, EndoS) treated Sf9 expressed Fc (B) were captured with Protein A on the surface of a CM5 chip. Mouse Fcγ receptor I was passed through as the analyte. The surface-plasmon resonance sensograms were recorded with 2-fold serial dilutions, starting at the highest concentration of 2 µM (A) or 2.5 µM (B) Fc receptor.
**Figure S10**
Intracellular cAMP levels after incubation with different CGS forms (1 μM) after anti-CD3 stimulation of wild type C57BL6 splenocytes for 6 hours (The asterisks represent p<0.05).
Fc-CGS stability in blood. Fc-CGS was diluted in mouse blood ex vivo to a final concentration of 100 nM. At 24, 48 and 72 hour time points, serum was incubated with anti-FLAG agarose beads to isolate the Fc-CGS protein. Fc-CGS bound to the beads was then eluted using SDS denaturing buffer and boiled at 95°C for 10 minutes prior to being run on SDS-PAGE and visualized by western blotting using an anti-FLAG antibody.
Figure S12
Immunohistochemistry staining with anti-FLAG of hearts and brains from C3HA mice. Left, an untreated healthy C3HA mouse; middle, 8 days post adoptive transfer of the 1.2 million CD4+ 6.5+ cells with single doses of Fc-CGS (50 nmol/kg) intraperitoneal injection on days 1 and 3; right, a healthy C3HA mouse with single doses of Fc-CGS (50 nmol/kg) intraperitoneal injection on days 1 and 3. All 3 groups were harvested on the same day.
Figure S13
Immunohistochemistry staining with anti-FLAG of lungs from C3HA mice. Left, 8 days post adoptive transfer of the 1.2 million CD4+ 6.5+ cells into mice treated with single doses of Fc-CGS (50 nmol/kg) by intraperitoneal injection on days 1 and 3; right, a healthy C3HA mouse treated with single doses of Fc-CGS (50 nmol/kg) by intraperitoneal injection on days 1 and 3. Both groups were harvested on the same day.
Supplementary References

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