

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

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Supporting Information

ABSTRACT: Proteins as well as small molecules have demonstrated success as therapeutic agents, but their pharmacologic properties sometimes fall short against particular drug targets. Although the adenosine 2a receptor ($A_{2A}R$) has been identified as a promising target for immunotherapy, small molecule $A_{2A}R$ agonists have suffered from short pharmacokinetic half-lives and the potential for toxicity by modulating nonimmune pathways. To overcome these limitations, we have tethered the $A_{2A}R$ agonist CGS-21680 to the immunoglobulin Fc domain using expressed protein ligation with Sf9 cell secreted protein. The protein small molecule conjugate Fc-CGS retained potent Fc receptor and $A_{2A}R$ interactions and showed superior properties as a therapeutic for the treatment of a mouse model of autoimmune pneumonitis. This approach may provide a general strategy for optimizing small molecule therapeutics.

The increasing number of successful protein based pharmaceuticals clinically approved over the past two decades represents a triumph of molecular biology. Although the 20 genetically encoded amino acids provide considerable structural diversity for generating protein drugs, elaborating proteins by linkage to the nearly infinite chemical space of small molecules offers great potential for novel therapies.^{1–3} The tethering of the antibody drug trastuzumab to the cytotoxic compound maytansine furnished the breast cancer agent TDM1 which was recently approved for the treatment of HER2-positive breast cancer, illustrating the power of protein-small molecule conjugate drugs.¹ A major continuing challenge in this arena is difficulty producing small molecule-modified proteins that are homogeneous and site-specific.^{1–3} Here we apply expressed protein ligation to link the small molecule adenosine receptor

($A_{2A}R$) agonist CGS-21680 (CGS) (Figure S1) to the immunoglobulin Fc region as a therapeutic strategy for immune disorders.

The G-protein coupled receptor $A_{2A}R$ is an attractive target to treat autoimmune and inflammatory diseases,^{4–7} but two obstacles have impeded pharmacologic progress. First, potent and selective small molecule adenosine analog $A_{2A}R$ agonists such as CGS have been observed to have very short pharmacokinetic half-lives (<20 min), limiting modes of administration.^{7,8} Second, in addition to expression in T lymphocytes, $A_{2A}R$ s are also abundant in the brain and heart and thus immunomodulatory $A_{2A}R$ agonists could result in neuro- or cardio-toxicity.⁷ We considered the possibility that attachment of CGS to the immunoglobulin constant region (Fc) might overcome these obstacles.^{9–11} As a stabilizing group in the anti-TNF α drug etanercept, the Fc domain is highly resistant to proteolytic degradation in vivo with a multiday half-life, in part due to interaction with the neonatal Fc receptor isoform (FcRn).^{9–11} Attachment to Fc could thus confer such stability to CGS and prevent CGS internalization by cells where it can be metabolized. In addition, an Fc-CGS conjugate might show enhanced localization to immune complexes given the presence of Fc receptors on antigen presenting cells.^{9–11}

We considered several chemical strategies for tethering Fc to CGS^{1,12–14} but opted for C-terminal attachment via expressed protein ligation (EPL)¹⁵ (Figure 1A). In this method, intein-mediated C-terminal thioester formation in a recombinant protein fragment is used for a chemoselective ligation with an N-Cys containing peptide (Figure 1A).¹⁵ EPL has the advantages of providing for high reaction site specificity and yield and being technically relatively simple compared to other methods.¹⁵ EPL has primarily been used with soluble, bacterially produced

Received: January 21, 2014

Published: February 17, 2014

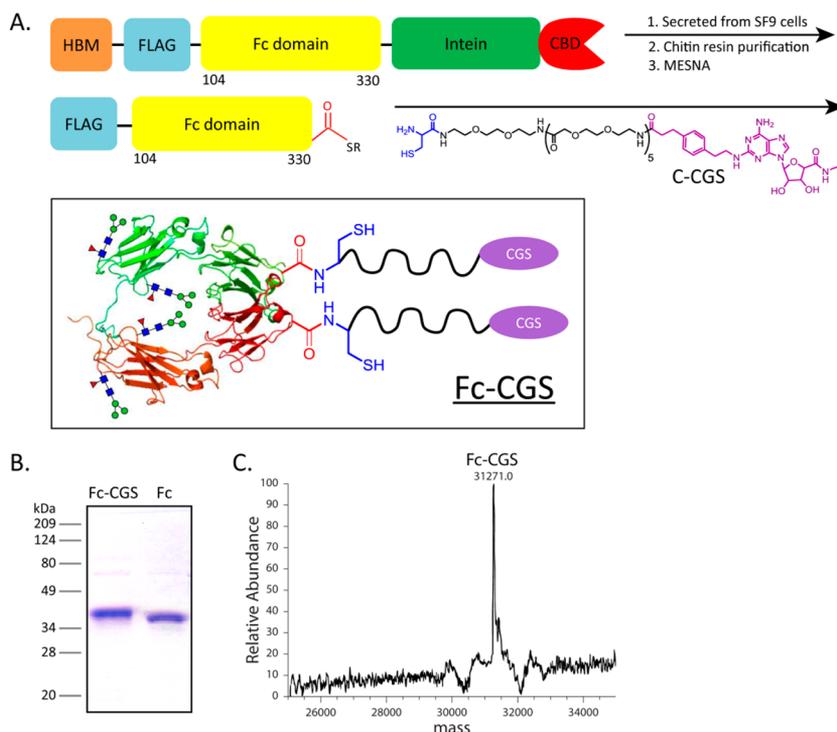


Figure 1. Fc-CGS production and mass spectrometry analysis. (A) Fc-CGS semisynthesis by expressed protein ligation (EPL). The Fc domain is cloned into an intein-fusion vector and secreted from Sf9 cells. The carboxy-terminal chitin-binding domain (CBD) is for purification purposes. The addition of sodium 2-sulfanylethanesulfonate (MESNA) generates the Fc-thioester intermediate by intein-mediated transthioesterification, and then C-CGS reacts with the Fc-thioester via the native chemical ligation reaction to form the amide linkage in Fc-CGS. (B) SDS-PAGE analysis (coomassie blue) of Fc-CGS (Left) and Fc (Right). (C) Mass spectrometric analysis of Fc-CGS. Calculated (M^+) $m/z = 31271$. Yield of purified Fc-CGS is about 1.5 mg per liter of Sf9 culture.

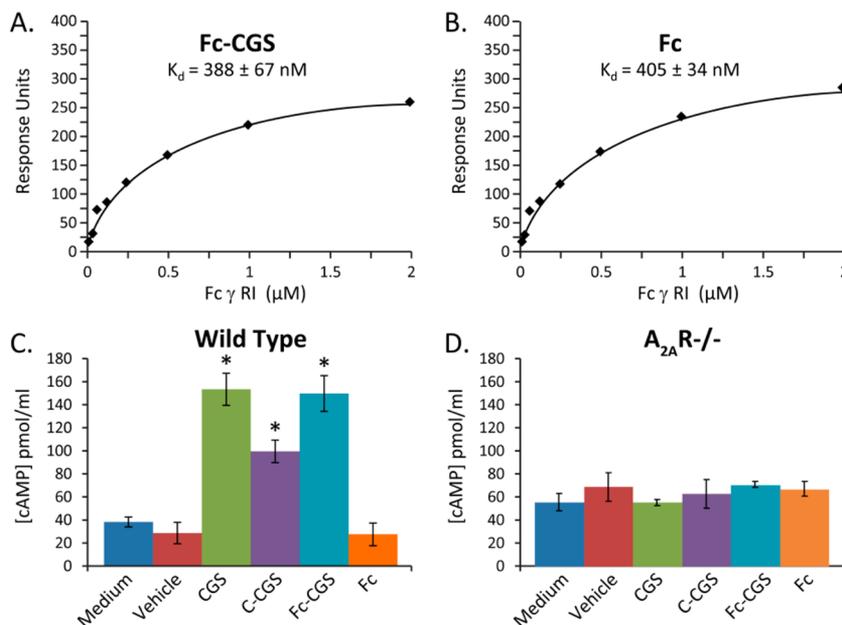


Figure 2. Fc Receptor binding assays and cAMP measurements with Fc-CGS. (A, B) Surface plasmon resonance binding assay. Fc-CGS (A) and Fc (B). Mouse Fc receptor I was flowed through as the analyte. (C, D) Intracellular cAMP levels after incubation with different CGS forms ($5 \mu\text{M}$; concentration of Fc-CGS based on monomeric form determined from SDS PAGE) after anti-CD3 stimulation of wild type C57BL/6 (C) splenocytes or $A_{2A}R^{-/-}$ (D) for 6 h. cAMP levels were measured using a competitive enzyme-linked immunosorbent assay (ELISA). All data are representative of three independent experiments.

proteins, but our goal was to generate the Fc domain in a glycosylated, disulfide-linked form which requires a eukaryotic expression system.¹⁶ Hence, we used a baculovirus expression

system and Sf9 cells¹⁶ to express an Fc-intein-chitin binding domain (CBD) construct containing the mouse IgG3 Fc domain and an N-terminal secretion signal sequence (honey bee mellitin,

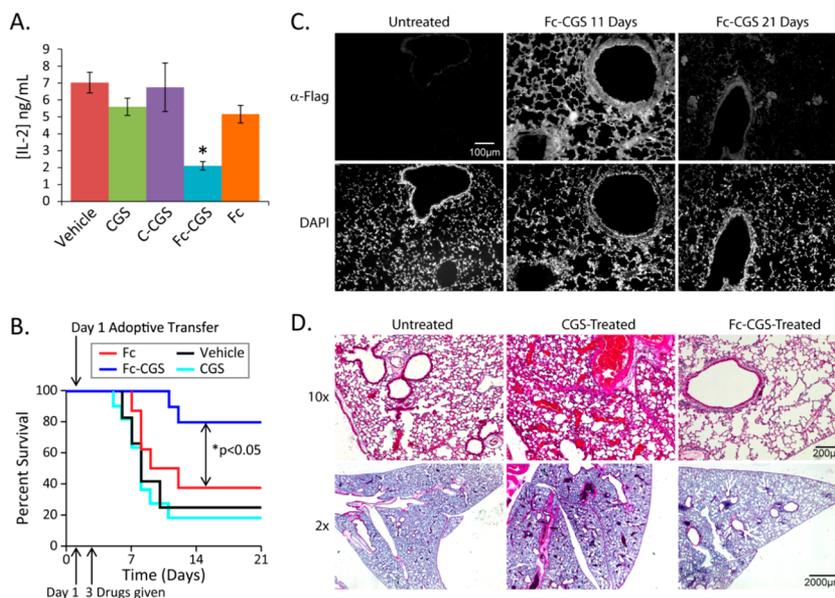


Figure 3. Fc-CGS as an immunomodulator. (A) Naïve 5 cc7 (TCR transgenic, specific to Pigeon Cytochrome C, PCC) CD4+ T cells were stimulated with PCC in the presence of different CGS forms (30 nM) as indicated. (B) Survival curve of C3HA mice given 1.5 million CD4+ 6.5+ cells and two doses of vehicle, CGS, Fc-CGS, or Fc. Vehicle ($n = 12$), CGS ($n = 11$), Fc-CGS ($n = 10$), and Fc ($n = 8$). (C) Immunohistochemistry staining with anti-FLAG of lungs from C3HA mice treated as in B. Left, an untreated healthy C3HA mouse; middle, 11 days post-adoptive transfer; right, 21 days post-adoptive transfer. (D) Hematoxylin and eosin staining of the pulmonary tissue from (left) an untreated healthy mouse, (middle) a CGS treated mouse (died on day 6), and (right) an Fc-CGS treated mouse (survived over 3 weeks) after the 1.5 million CD4+ 6.5+ cells and the drugs were given.

HBM; Figure S2). The secreted Fc-intein-CBD protein was isolated and purified with chitin resin and then the Fc thioester was formed by treatment with MESNA, which released the Fc moiety from the chitin resin. The Fc thioester was subsequently reacted with C-CGS (CGS synthetically fused to a hexa-ethylene glycol spacer followed by a Cys, Figure S3–5), to generate Fc-CGS. The length of the spacer, about 100 Å in an extended conformation, and its connection site on CGS, were designed based in part on the $A_{2A}R$ X-ray structure as well as agonist studies on prior synthetic CGS analogs.^{7,17} This arrangement was intended to provide for adequate separation of the Fc and CGS to allow for dual receptor occupancy by the Fc-CGS conjugate. As a control, Fc protein was prepared in a similar fashion but cysteine was used in place of C-CGS during the ligation step.

Fc-CGS was more than 90% pure based on Coomassie stained SDS-PAGE (Figure 1B), contained expected levels of N-linked glycosylation, and showed the correct molecular weight by mass spectrometry (Figures 1C and S6, S7). Size exclusion chromatography confirmed that Fc-CGS exists in the expected dimeric state based on the known properties⁹ of the Fc domain (Figure S8). Surface plasmon resonance was used to measure the affinity of Fc-CGS and Fc with the mouse Fc receptor (FcR gamma I), showing K_d 's of 388 ± 67 nM (Figure 2A) and 405 ± 34 nM (Figure 2B), respectively, equivalent to that of commercial immunoglobulin containing Fc (K_d of 384 ± 69 nM) (Figure S9A). These data show that the EPL-produced Fc interacts normally with the Fc receptor and that the CGS and linker do not interfere with this interaction. As expected, partial deglycosylation by a mixture of endoglycosidases led to the reduced affinity of Fc for the mouse FcR gamma I (K_d of 1330 ± 50 nM, Figure S9B), indicating a role for N-linked glycosylation in mediating interactions with this receptor subtype.¹⁸

To explore the potential of Fc-CGS to serve as an $A_{2A}R$ agonist, we exposed activated splenocytes (anti-CD3 treated) to

Fc-CGS and control compounds for 6 h and measured subsequent cyclic AMP (cAMP) production, a known second messenger response to $A_{2A}R$ activation.³ These experiments revealed that 5 μ M Fc-CGS treatment resulted in a 5-fold increase in cAMP production, similar to that of free CGS but slightly greater than that of C-CGS (Figure 2C). Under these conditions, Fc alone had no effect. With $A_{2A}R^{-/-}$ splenocytes, the effect of CGS-containing compounds on cAMP production was abolished (Figure 2D). 1 μ M free CGS showed a somewhat greater cAMP stimulation effect than Fc-CGS on wt splenocytes, whereas C-CGS was less effective under these conditions (Figure S10). These results suggest that substitution of the side chain of CGS with a linker somewhat reduces its strength as an $A_{2A}R$ agonist in this short-term assay, but conjugation to Fc helps restore agonist activity. Based on these findings, we envisioned that Fc-CGS could show promise as an immunomodulator.

To investigate the functional effects of Fc-CGS on immune response, we stimulated T cells (5C.C7 splenocytes treated with 5 μ M pigeon cytochrome C peptide as antigen) and measured interleukin 2 (IL-2) production by ELISA.⁵ These experiments revealed a sharp, 70% reduction in IL-2 72 h after treatment with 30 nM Fc-CGS (Figure 3A). In contrast, treatment with free CGS, C-CGS, and Fc induced less than a 30% reduction of IL-2 under these conditions. Moreover, in an ex vivo experiment, Western blots with anti-FLAG antibody revealed that Fc-CGS was quite stable in blood for at least 72 h (Figure S11). These results suggest that the stability of Fc-CGS might contribute to its 72 h effects on IL-2 production, and its relatively greater potency than CGS compared with the cAMP responses in the 6 h assays (Figures 2D, S10). Encouraged by these ex vivo results with Fc-CGS, we investigated the effects of Fc-CGS in a mouse autoimmune model of pneumonitis.⁵ C3HA transgenic recipient mice, which express the hemagglutinin (HA) antigen under the control of the C3 (lung-selective) promoter, were injected with CD4+ T cells from HA-specific TCR (6.5) and experience

pulmonary inflammation resulting in death within about two weeks, but this outcome can be prevented by dosing twice daily with 5 $\mu\text{mol/kg}$ intraperitoneal CGS for four days after adoptive transfer.³ Anticipating enhanced pharmacokinetic stability of Fc-CGS vs CGS, we designed a related pneumonitis therapeutic trial in which treatment with Fc-CGS involved two intraperitoneal injections total (day 1 and day 3) of 50 nmol/kg. Control arms of the study involved treating mice with vehicle, 50 nmol/kg Fc, or 5000 nmol/kg CGS, also on days 1 and 3. As shown in Figure 3B, mice treated with Fc-CGS showed significantly enhanced survival over animals injected with vehicle, CGS, or Fc. Necropsy of the animals that succumbed showed a lymphocytic infiltrate in the lungs that appeared less pronounced in surviving mice treated with Fc-CGS (Figure 3D). Immunocytochemistry revealed that Fc-CGS could be detected in the pulmonary tissue on day 11 and at a lower level on day 21 of the experiment, 8 and 18 days after the Fc-CGS second treatment (Figure 3C). These images establish the tremendous stabilization of the Fc-containing conjugate at the critical site of action versus that previously established for the untethered small molecule CGS. It is noteworthy that mice receiving Fc-CGS showed an improved outcome relative to CGS, even though a 100-fold lower dose of the protein–small molecule conjugate was administered.

It is not yet determined the extent to which the various FcR isoforms (FcRn, Fc gamma receptor I) are important for Fc-CGS pharmacology, nor the relative importance of pharmacokinetic stabilization versus immune cellular targeting conferred by the Fc domain. FcRn would be expected to be more important to the Fc stabilizing functions whereas Fc gamma receptor I might have more influence on immuno-targeting.^{9–11} Fc-CGS was readily detected in the heart by immunohistochemistry but was barely detectable in the brain of mice 5 days after treatment (Figure S12). Low detection of Fc-CGS in the brain may be related to the presence of the blood-brain barrier to large molecules.¹⁹ Interestingly, the level of Fc-CGS observed in cardiac tissue appeared to be reduced in the mice with pneumonitis compared with healthy controls (Figure S12). In contrast, Fc-CGS appeared to be more abundant in the lung tissue of mice with pneumonitis compared with healthy controls (Figure S13). Taken together, these data suggest that the lung immune response facilitated recruitment of Fc-CGS to the site of inflammation, although further studies will be needed to fully explore these mechanisms.

In summary, we have successfully generated an Fc-small molecule conjugate that retains the agonist properties of the attached A_{2A}R small molecule agonist but shows enhanced pharmacokinetic and pharmacodynamic performance in a mouse model of inflammatory pneumonitis. Conjugating a small molecule to the immunologically relevant Fc domain may prove to be a general method to enhance small molecule delivery to areas of inflammation. The bivalency of such Fc conjugates may also be beneficial for receptor binding. Expressed protein ligation with Sf9 cell secreted proteins thus offers a straightforward and efficient technique to generate such Fc conjugates in functional, glycosylated form, placing the chemical modification at the terminus of the natural antibody domain. This approach may be broadly applicable for improving the pharmacokinetic properties of small molecule therapeutics and the production of next generation bivalent protein-based drugs.

■ ASSOCIATED CONTENT

📄 Supporting Information

Experimental methods and supplementary figures are included in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the NIH and FAMRI Foundation for financial support. We thank T. Jackson, X. Kuo, H. Weng, C. Patel, Y. Lo, Y. Li, J. Kavran, M. Ward, C. Nirschl, C. Drake, and J. Zhang for helpful advice and/or experimental assistance.

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