Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

ValCitGlyPro-Dexamethasone Antibody Conjugates Selectively Suppress the Activation of Human Monocytes

*Justin M. Howe, Siteng Fang, Kelsey A. Watts, Fanny Xu, Samantha R. Benjamin, L. Nathan Tumey**

*Corresponding author: School of Pharmacy and Pharmaceutical Sciences, Binghamton University, P.O. Box 6000, Binghamton NY 13902. Phone: 607-777-5844. Email: NTumey@binghamton.edu.

Supplemental Contents:

Supplemental Figures:

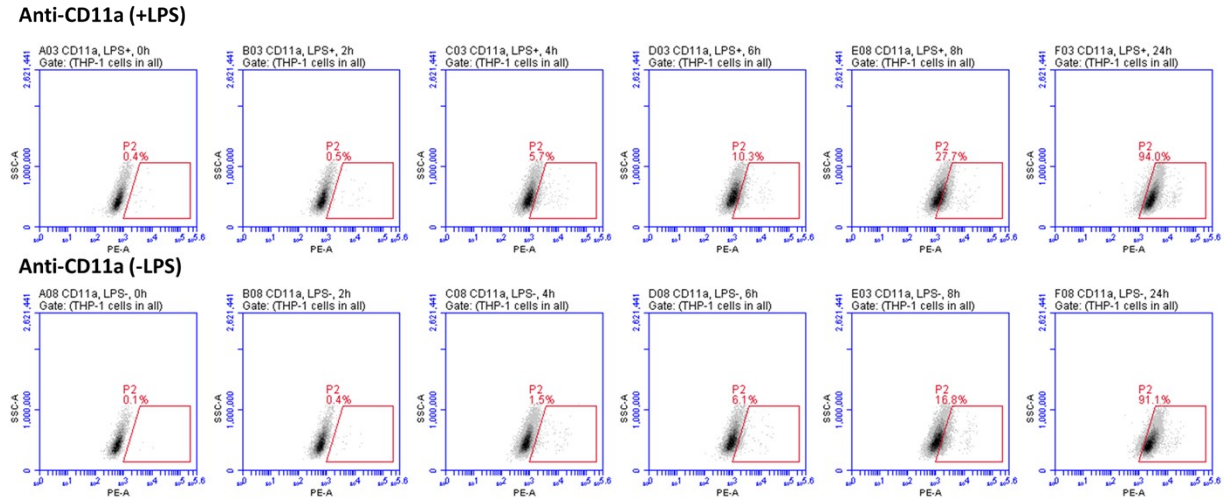

Figure S1a: Flow cytometry / internalization data for anti-CD11a

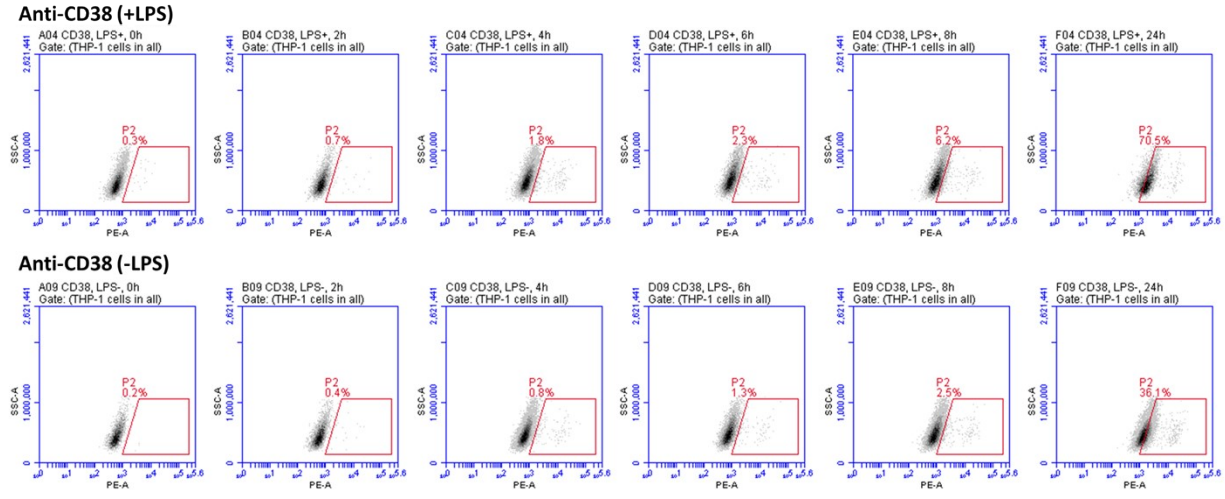

Figure S1b: Flow cytometry / internalization data for anti-CD38

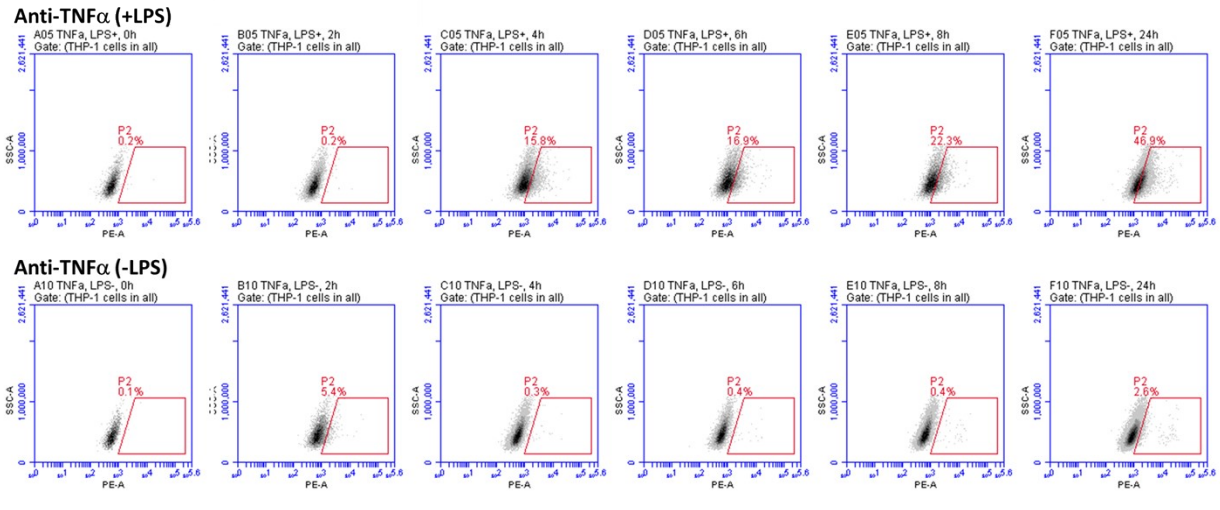

Figure S1c: Flow cytometry / internalization data for anti-TNFα

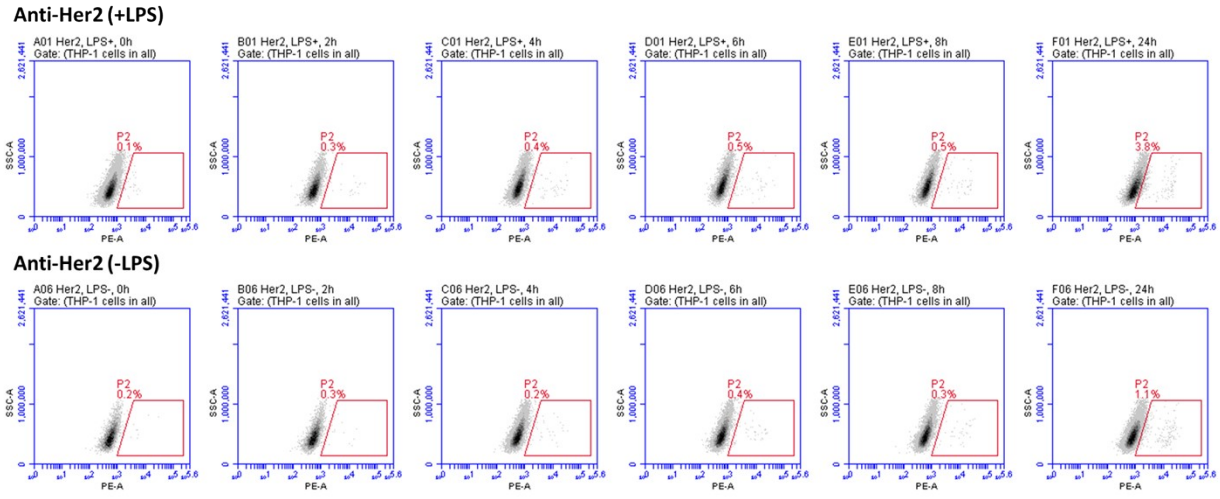

Figure S1d: Flow cytometry / internalization data for anti-Her2

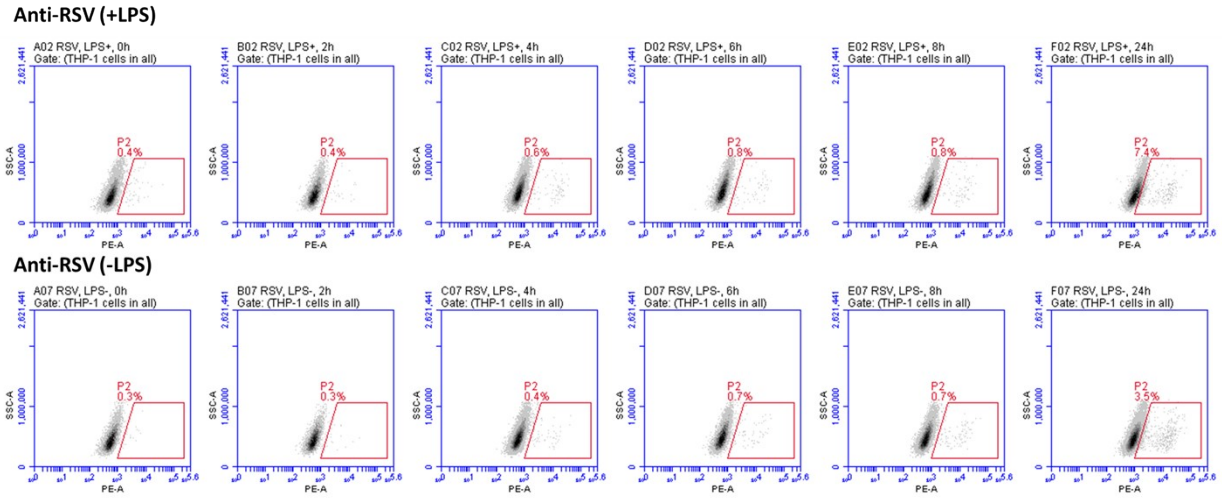

Figure S1e: Flow cytometry / internalization data for anti-RSV

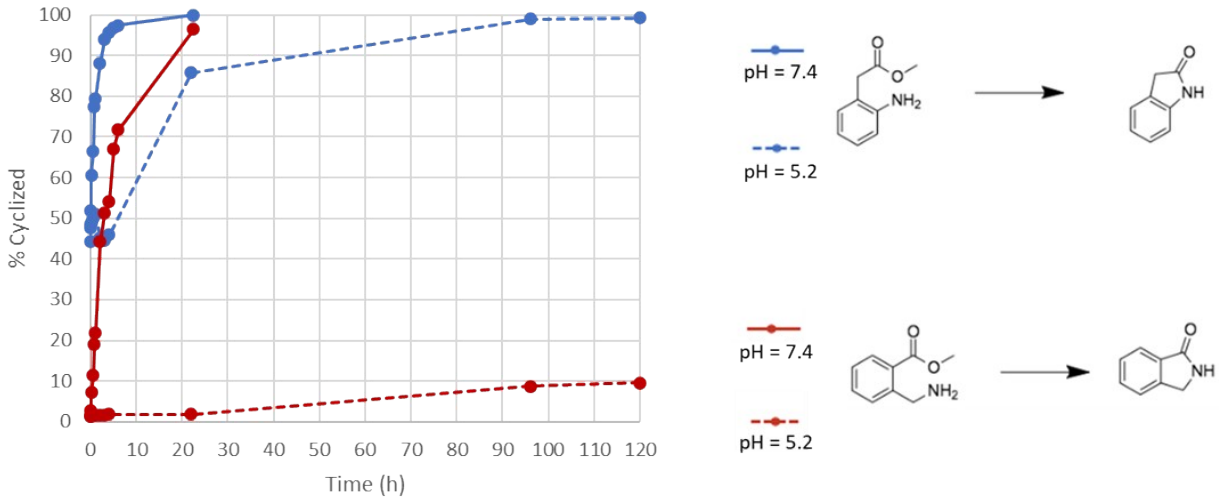

Figure S2. Cyclization of esters of 2-amino phenylacetate and 2-aminomethyl benzoate. Both compounds underwent spontaneous cyclization at pH 7.4, while only 2-amino phenylacetate underwent cyclization at pH 5.2.

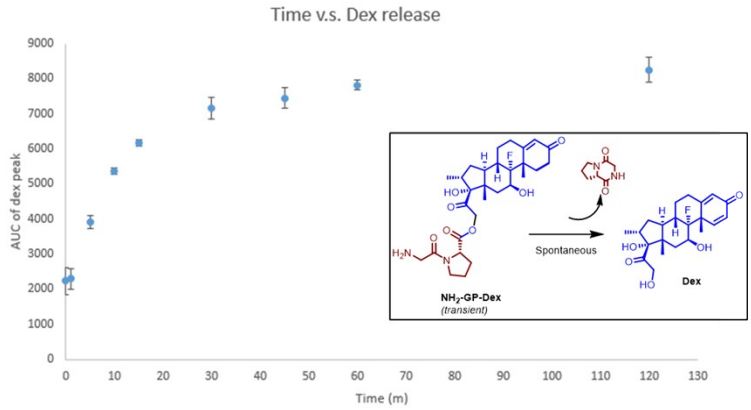

Figure S3. Spontaneous immolation of GlyPro-Dex at pH 7.4 results in the complete release of Dex within 1h.

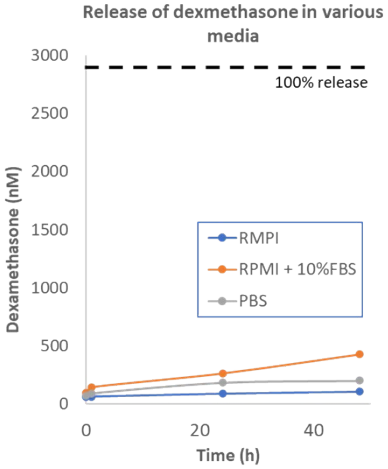

Figure S4. The

Materials and Methods:

Materials: All antibodies were obtained from Syd Labs (www.sydlabs.com) as follows: Anti-CD11a (Efalizumab biosimilar, C045P); Anti-CD38 (Daratumumab biosimilar, C030P); Anti-TNFα (adalimumab biosimilar, C003P); anti-RSV (palivizumab biosimilar, C021P); Trastuzumab biosimilar (C009P). The sequence of the antibodies were confirmed by LCMS prior to use. THP1 cells were obtained from ATCC (TIB-202); THP1-dual™ cells were obtained from Invivogen (thpd-nfis); PBMCs were obtained from iXCells. Human Cathepsin B was obtained from MilliporeSigma (cat# 21936250UG). Rat liver tritosomes were obtained from Xenotech (R0610.LT). Compound 5 (mpValCitGlyPro-OH) was custom synthesized by Genscript.

Chemistry:

Small molecule LCMS (normal gradient): Small molecule reactions and product purity were evaluated using a Waters Acquity H-Class UPLC equipped with a TUV Detector and QDa mass spectrometer. The separation was achieved using an Acquity UPLC BEH C18 1.7 um column (2.1 x 50mm) at 80ºC using a gradient from 10% to 90% acetonitrile in water (+0.1% formic acid). Typical injection size was 1 µL. The eluent was monitored by UV (220 and 254 nM) and by mass spec (QDa, ES+/ES-).

Size exclusion chromatography (SEC): SEC analysis was performed on a Biorad NGC chromatography system a UV detector using a TSKGel 3000SW (7.5mmx30cm) column. Analysis was performed at room temperature using an isocratic gradient of Phosphate Buffer (50 mM, pH 7.4) containing 10% acetonitrile at 1 mL/min. The elute was monitored by UV at both 220 and 280 nM. Under these conditions the antibody eluted at ~8.2 minutes and any aggregate eluted at ~7.5 min.

Protein LCMS: Analysis of ADCs was performed using a Water Autopurication system with a 2545 binary gradient module, 2767 sample manager, 2998 UV/PDA and a SQD2-mass spectrometer. Separation was performed using an Agilent Technologies poroshell 300 SB-C8 column (2.1x75 mm, 5 um) at 80 ºC with a gradient of water+0.05% formic acid (Buffer A) and acetonitrile+0.05% formic acid (Buffer B). The gradient elution was performed (flow rate = 1.00 mL/min) from 5%B to 95% B over 5 min. ADC Samples (~20 µL of ~1 mg/mL stock) were treated with 5 µL of 0.5 M TCEP immediately prior to analysis. The eluent was monitored by UV (210 nm – 600 nm) and mass spec (ES+ 700 – 1800 Da. The antibody typically eluted at ~3.9 min. The raw charge envelope was deconvoluted using MaxEnt software.

Synthesis of VCGP-Dex: Peptide **5** (maleimide-propionyl_Val-Cit-Gly-Pro-OH, 10.8 mg, 1.1 Eq) was dissolved in DMA (0.5 mL) and treated with EDC (13 mg, 4 Eq), DIPEA (8.8 µL, 3 Eq), and HOBt (2.6 mg, 1 Eq). After stirring for 30 min, dexamethasone (6.6 mg, 1 Eq) was added and stirring was continued for 14h. An additional 1.1 eq of 5, 4 eq of EDC, and 3 eq of DIPEA was added and stirring was continued 2.5h. The product was purified by preparative LCMS providing 5.8 mg (36%) of the title compound. LCMS rt = 2.70 min (94%); m/z = 976.5 [M+H].

Antibody Conjugation: The IgG1 mAb (1mg) was diluted to a final volume of 500 µL in PBS containing 5 mM ethylenediamine tetraacetic acid (EDTA) and was then treated with 12 equivalents of 5mM tris(2-carboxyethyl) phosphine (TCEP). The reaction was heated at 37ºC for two hours then buffer exchanged into PBS using a centrifuge spin device with a 30KDa filter. The linker-payload (15eq) in DMA was added to the reduced mAb along with sufficient DMA to provide 5-10% organic in PBS. After 1.5 hours, the material was buffer exchanged into 1 mL of PBS using a sephadex column according to the manufacture's protocol. ADCs were filter sterilized and stored at 4°C. An aliquot was reduced using TCEP and evaluated by LCMS to determine loading. The concentration of ADC was assessed by A280 using a Nanodrop. Aggregation was assessed by SEC as detailed in the analytical chemistry section.

Metabolism studies:

Cathepsin B cleavage of model linkers: Cathepsin B was prepared as a 0.5 mg/mL solution in sodium acetate buffer, pH 5.2. A 2µg aliquot was treated with 4µL of 50mM dithiothreitol in water. After allowing the mixture to stand at rt for 30 min, 90µL of sodium acetate buffer (pH=5.2) was added followed by 2 µL of the appropriate model linker (**1**-**4**). The reaction was incubated at 37°C. At each time point, a 10 µL aliquot was withdrawn and quenched with 10 µL of ACN. The ratio of the 3 major products was determined by LCMS and plotted as a function of time.

Lysosomal Catabolism of ADCs. 30ug of ADCs were buffer exchanged into NaOAc buffer (pH 4.7) with a final volume of 60uL (0.5mg/mL). Separately, rat liver tritosomes (xenotech) (7uL, 2.5mg/mL, 17.5ug) were activated by addition to 77uL of 2mM DTT and 56uL NaOAc buffer (pH 4.7). After incubation at 37ºC for 15 minutes, 40uL of the activated tritosomes (5.0ug) were added to the 60uL of each ADC. Aliquots (10µL) were taken at 1, 5, 10, 15, 30, 60, 120, 180, and 240 minutes. Immediately after removal of the aliquot, 90uL ACN was added and the samples were immediately vortexed and stored at -80ºC till the time of analysis. At the time of analysis, all samples were thawed and centrifuged for 5 minutes. 80uL was carefully removed from the supernatant of each sample and transferred into a LCMS vial with 80uL of PBS (pH=7.4). Samples were incubated at 37ºC for 2 hours to allow for cyclization to take place. After 2 hours, 10uL of 5% formic acid in water was added to each vial to halt and further cyclization. 10uL aliquots were analyzed by LCMS using a single ion resonance (SIR) method tuned for 393.24 m/z. The AUC was compared to a standard curve generated immediately prior to the analysis.

THP1 and PBMC assays:

Antibody internalization assay: Anti-TNFα, anti-CD11a, anti-CD38, anti-Her2, and anti-RSV were conjugated to pHAb thiol reactive dye (Promega, G9831) via endogenous cysteine residues following the manufacturer's protocol. THP-1 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS). On the day of the assay, THP-1 cells were collected via centrifugation and re-suspended to 0.5 million cells/mL. Cells (2 mL per well) were added to a 24-well plate. LPS from Escherichia coli O55:B5 (Sigma-Aldrich, L2880) was diluted in DI water and applied to the appropriate wells to give a final concentration of 1 ng/mL. The cells were incubated with LPS for 4 hours before both LPS positive and negative groups were dosed with the corresponding pHAb conjugates. The final concentration for the pHAb conjugates was 10 ug/mL. At 0, 2, 4, 6, and 24 hours timepoints, 300 uL of the suspension was collected from each well. The cells were rinsed and fixed with 4% paraformaldehyde (PFA) before performing a flow cytometry analysis using a BD accuri C6 flow cytometer. Antibody internalization was evaluated based on the mean fluorescence intensity (MFI) of the PE channel.

Evaluation of cytotoxicity of MMAE ADCs: THP1 cells were maintained in RPMI-1640 media supplemented with 10% FBS. Cells were diluted to 0.2 million cells/mL in fresh media and gently agitated, and 90 µL was placed into each well of a clear 96-well plate (Corning). For experiments that included LPS, only 80 µL of cells were added alongside 10 µL of a 100 ng/mL solution of LPS. Plates were incubated at 37 °C for 3 hours before adding ADCs. Anti-CD11a_vcMMAE, Anti-TNFα_vcMMAE, Anti-CD38_vcMMAE, and Anti-HER2_vcMMAE were added to the cells to reach the final concentrations of 30, 10, 3.3, 1.1, 0.37, 0.12, 0.04, 0.01, and 0 µg/mL. After incubation at 37 °C for 72 hrs, the viability was assessed using XTT (Biotium) following the manufacturer's procedure. Data were plotted and analyzed using GraphPad Prism software.

THP-1 dual reporter assay and cytokine measurement. A 5-fold serial dilution of each ADC and mAb was performed in PBS resulting in concentrations 10x higher than the intended test concentration. THP-1 Dual Cells (InvivoGen, cat# thpd-nfis) were cultured using high glucose RPMI media supplied with 10% fetal bovine serum and 25mM HEPES according to the manufacturer guidelines. The media was supplemented with 50ug/mL penicillin, 50ug/mL streptomycin, 100 ug/mL normocin, 500ug/mL blasticidin, and 500ug/mL zeomycin to prevent bacterial contamination. Cells were seeded at a density of 0.2x10^6 cells/mL in media supplemented with 10% human serum. The cell suspension (120 µL) was added to each well followed 15 µLthe appropriate ADC (or PBS control). All experiments were performed in triplicate. Plates were incubated at 37ºC under 5% CO_2 for 4 hours. A freshly prepared 10 ng/mL solution of lipopolysaccharides (LPS) was added to each well (15 µL) resulting in a final LPS concentration of 1ng/mL. After 16 hours of incubation, the plate was centrifuged at 1990rpm for 10 minutes. SEAP production (NFκB activation) was assessed by adding and the 40uL of supernatant was added to 160uL of Quanti-Blue solution (InVivogen cat# rep-qbs) and incubated at 37ºC. UV readings at 630 nm were made at 4 hours and 24 hours. IRF activation was assessed by adding 20uL of the cell supernatant to 50uL of Quanti-Luc solution (invivogen) in an opaque bottom 96-well plate. Luminescence was immediately analyzed at 100ms and 500ms. Cytokine levels in the cell supernatant were assessed using an automated ELISA system (Ella, ProteinSimple) with a multiplex human cytokine cartridge (part# ST01E-PS-005711)

THP-1 TNFα assay: THP-1 cells were maintained in RPMI 1640 media supplemented with 10% FBS. On the day of the assay, the cells were collected and re-suspended to a 0.2 million per mL suspension. 100 uL of the suspension was added to a 96-well plate with a seeding volume of 100 uL per well. Two ADCs: anti-CD11a_VCGP-Dex and anti-Her2_VCGP-Dex, as well as the naked anti-CD11a mAb, were serial diluted and added to corresponding wells to reach a final concentration gradient of 20, 4, 0.8, 0.16, 0.032, and 0 ug/mL. The cells were incubated under 5% CO_2 for 24 hours before being challenged with 10 ng/mL LPS (final). After 8h of incucation, the cells were centrifuged and the supernatant was isolated. TNFα levels were quantified using a DuoSet TNFα ELISA kit (R&D systems, DY210-05). Data was analyzed and plotted using the GraphPad Prism software.

Suppression of IL6 in human PBMCs: Working solutions of all test ADCs and mAbs were prepared at 20x the final test concentration in PBS. (200, 66, 22 µg/mL) A 20 ng/mL stock solution of LPS was prepared in DI water. PBMCs were thawed and suspended at a density of 1 million cells/mL in RPMI1640/10% FBS/10% human serum containing 1x pen/strep. Cells were plated (90µL/well) and treated with 5 µL of the appropriate ADC, mAb, or PBS control. After 2h, the wells were further treated with either 5 µL LPS or DI water. After 24h incubation at 37 °C under 5% CO2, the plates were centrifuged and the supernatant was collected and stored in the -80 °C until ELISA analysis. IL-6 levels were quantified using a DuoSet IL-6 ELISA kit (R&D systems, DY20605). Data was analyzed and plotted using the GraphPad Prism software.