Supplemental Materials and Methods

Antibodies

Mouse anti-human CD16A (clone 3G8), mouse anti-human CD64 (clone 10.1), BV421-conjugated mouse anti-human CD16A (clone 3G8), BV605-conjugated anti-human CD56 (clone HCD56), AF647-conjuagted anti-c-myc (clone 9E10), FITC-conjugated anti-mouse NK1.1 (clone 517016D), AF488-conjugated anti-mouse/human CD11b (clone M1/70), and APC/Cy7-conjugated anti-mouse Ly-6G (clone 1A8) were purchased from BioLegend (California, USA). PE-conjugated anti-mouse CD19 (clone 1D3) and APC/Cy7-conjugated anti-mouse CD3 (clone 145-2C11) were purchased from BD Pharmingen (New Jersey, USA). Mouse anti-human CD32A/B/C (clone AT10) was purchased from Novus Biologicals Canada (Ontario, Canada). Mouse anti-human CD32A (clone IV3) was purchased from Stemcell Technologies Inc (British Columbia, Canada). Mouse anti human CD16B (clone 2D2G5B9) was purchased from SinoBiological US Inc. (Pennsylvania, USA). Purified mouse IgG1 (clone MOPC-21), IgG2a (clone N/A-CP150), and IgG2b (clone MPC-11) isotype controls were purchased from BioXCell (New Hampshire, USA). Alexa Fluor 647-conjugated mouse anti-human CD42a (clone GRP-P) was purchased from BioRad Laboratories (California, USA). APC-conjugated F(ab)'2 goat anti-mouse IgG-Fcγ specific, AF674-conjugated goat anti-mouse IgG (H+L), and AF674 donkey anti-human IgG (H+L) were purchased from Jackson ImmunoResearch (Baltimore, USA). FITC-conjugated goat anti-human serum albumin was purchased from Bethyl Laboratories (Massachusetts, USA).

BALB/c mice immunization and assessment of the humoral immune response by ELISA

Mice were intraperitoneally injected with seven doses, once weekly (10 µg the first dose and 5 µg each remaining dose) of the recombinant human Fc gamma receptor IIIA (FcγRIIIA) (SinoBiological US Inc, USA) adjuvanted with aluminum hydroxide (alum) (Alhydrogel) (Brenntag Biosector, Denmark) at a final concentration of 4.68 mg/mL and 40 µg/mL of CpG ODN (Integrated DNA Technology, USA) in a volume of 0.2 mL. Animals were bled on weeks 4 and 7, and the anti- FcγRIIIA antibody response from these bleeds was evaluated by ELISA.

Briefly, the antibody response was assessed using polystyrene 96-well plates (Corning, USA) that were coated with 8 µg/mL FcγRIIIA in phosphate-buffered saline (PBS) without calcium and magnesium (Gibco, USA) at 4°C for 16 hours. The plates were blocked with 2% BSA (Millipore Sigma, Canada) in PBS for 1 h at room temperature (RT). After three washes with distilled water, the immune response from each mouse was tested by adding to the plates, serial dilutions of sera (1:400 to 1:6553600) in PBS with 5% sodium azide for 1.5 hours at RT. Normal mouse IgG and antibody 3G8, both at 100 nM, were used as negative and positive controls respectively. Plates were then washed three times with distilled water and incubated with 0.2 µg/mL of goat anti-mouse IgG-peroxidase, Fcγ fragment specific (Jackson ImmunoResearch, USA) in PBS for 1 hour at RT. After that, plates were washed, and incubated with Enhanced K-Blue® Substrate (TMB) (Neogen, USA/Canada) for 30 min at RT. The reaction was stopped with 1M HCl and absorbance at 450 nm was read on Spectramax plate reader (Molecular Devices, USA).

Construction of a single-chain variable fragment (scFv) display phage library, selection strategy, and preparation of individual scFv clones.

FcγRIIIA-immunized mice were splenectomized and the splenocytes were isolated under aseptic conditions, eliminating erythrocytes by lysis in ACK (Ammonium-Chloride-Potassium) lysing buffer (Gibco, USA). Total mRNA was purified using Dynabeads™ mRNA Purification Kit

(Thermo Fisher Scientific, Canada) and cDNA was obtained by reverse transcription essentially as described [1]. Afterwards, universal primers, specific for mouse germline sequences, were used to amplify the genes that encode the heavy and light chain variable regions (V_H and V_L) by PCR essentially as described.[1] PCR products were recovered and further amplified separately to introduce a G4S sequence as a linker for the V_H and V_L. Finally, the V_H- and V_L-linkers were joined by overlap PCR and the final constructions were cloned into the phagemid vector pADL-23c (Antibody Design Laboratories, USA; Cat: PD0111) via Gibson assembly. *Escherichia coli* (TG1 electrocompetent cells; Agilent, USA) were transformed with the phagemid vector to generate the scFv-phage display library essentially as described.[2]

FcγRIIIA blocking scFvs were selected from the phage library following an in-solution selection strategy. Five rounds were performed (SI Appendix Table 1) and all of them concluded with a selection step. Rounds R2B and R3B had a de-selection step before its selection step (SI Appendix Table 1). Briefly, 150 µL of the phage library in 1.5 mL tubes was blocked with 950 µL of blocking buffer (PBS containing 3% skimmed milk) for 1 hour at 4°C. Fifty µL of the blocked phages were stored at -80ºC as "Input phages" until the samples were ready for titration. Concurrently, streptavidin magnetic beads (New England Biolabs, UK) were treated with blocking buffer for 1 hour at 4°C. After that, the blocked streptavidin magnetic beads were mixed with 950 µL of the blocked phages for 1 hour at RT. The mixture was then centrifuged, after which the supernatant was incubated for 2 hours at RT with the concentration of biotinylated FcγR for selection or de-selection as specified in SI Appendix Table 1. Afterwards, the mixture of phage and FcγR was incubated with a fresh set of blocked streptavidin magnetic beads for 15 min at RT, centrifuged, and the supernatant discarded (selection steps) or collected (de-selection steps). For rounds R2B and R3B (SI Appendix Table 1) the collected supernatant was then incubated for 2 hours at RT

with the concentration of biotinylated FcγR for their selection step. Later, the mixture of phage and FcγR was incubated with a fresh set of blocked streptavidin magnetic beads for 15 min at RT, centrifuged, and the supernatant discarded. For all rounds, the elution of phages was performed as follows. Beads were washed 5 times with 1 mL PBS 0.1% Tween-20 and then 5 times more with 1 mL only PBS. Finally, the bound phages were eluted by treating the beads with 1 mL of 100 mM triethylamine (Millipore Sigma, Canada) for 10 min at RT. Eluted phages were transferred to a tube containing 500 µL sterile 1M Tris-HCl, pH 7.5 to neutralize the triethylamine. Fifty µL of eluted phages were stored at -80ºC as "Output phages" until ready for performing titration. Rounds R2A and R2B started with the eluted phages from round R1. Rounds R3A and R3B started with the eluted phages from both (a mixture) of rounds R2A and R2B.

Obtaining periplasmic prep (periprep)

For each scFv clone, 5 µL of master glycerol stock was inoculated into 900 µL of 2xYT medium (Millipore Sigma, Canada) containing 0.1% glucose (Millipore Sigma, Canada) and 100 µg/mL carbenicillin (Millipore Sigma, Canada) in 96-well polypropylene DeepWell plates (Thermo Fisher Scientific, Denmark) using Biomek Liquid Handling Automation (Beckman Coulter, Canada). Plates were sealed with breathable seals and incubated in MaxQ8000 incubator (Thermo Fisher Scientific, Canada) at 200 rpm, 37°C for 5 hours. After the initial culture, the expression of scFv was induced with IPTG (Millipore Sigma, Canada) 0.2 mM (final concentration) during 16 hours at 30°C. Plates were then centrifuged at 2500 rpm for 10 min to remove supernatant and cells were lysed with 150 µL periprep buffer 1 (50 mM Tris, 1 mM EDTA, 20% sucrose, pH 8), vigorously mixed, diluted with 150 µL periprep buffer 2 (periprep buffer 1 diluted 1:5) and incubated on ice for 30 min. Plates were centrifuged at 4000 rpm for 15 min at 4°C and 200 µL of

supernatant was collected and non-specific binding was reduced by incubating the plates with 200 µL of 6% skimmed milk, 2% BSA in PBS for 1 hour at RT (blocked periprep).

Expression and purification of scFv clones

For each scFv clone, 2 µL of master glycerol stock was inoculated into 10 mL of 2xYT medium containing 0.1% glucose, 100 µg/mL carbenicillin using loosely capped 50 mL falcon tubes and grown in MaxQ8000 incubator at 200 rpm, 37ºC, for 4 hours. After initial culture, scFv expression was induced with IPTG at final concentration of 0.2 mM in MaxQ8000 incubator at 200 rpm, 30ºC for 16 hours. Five cultures of each clone were combined, and cells pelleted at 2500 rpm for 10 min to remove the supernatant. Cells were lysed with 300 µL periprep buffer 1 (50 mM Tris, 1 mM EDTA, 20% sucrose, pH 8), vigorously mixed, diluted with 300 µL periprep buffer 2 (periprep buffer 1 diluted 1:5) and incubated on ice for 30 min. After the incubation, cell debris was pelleted at 4000 rpm for 15 min at 4ºC, supernatant was collected and spiked with $MgCl_2$ at a final concentration of 10 mM.

His MultiTrap HP plates (GE Healthcare, USA) were initially equilibrated with 500 µL distilled water and two times 500 µL binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole (pH 7.45)). Samples were loaded onto plates, incubated for 3 min, and then centrifuged at 100 x*g* for 4 min. Flow through was re-loaded back onto plates, incubated for 3 min, and centrifuged at 100 x*g* for 4 min. Plates were washed three times with 600 µL binding buffer and centrifuged at 500 x*g* for 2 min between washes. Samples were eluted by two incubations with 200 µL elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole (pH 7.46)) and centrifugation at 500x g for 2 min. Eluted samples were buffer exchanged into PBS with Amicon Ultra Filter Device (10 kDa MWCO) (Millipore Sigma, Canada) according to

manufacturer's protocol and quantified using Thermo Scientific™ Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Canada).

Binding of anti-FcγRIIIA antibodies to human FcγRs and cynomolgus FcγRIIIA by ELISA

MaxiSort 96-well plates (Thermo Fisher Scientific, Canada) were coated with 100 µL/well of FcγRIA (2.5 µg/mL), FcγRIIIA (10 µg/mL), cynoFcγRIIIA (10 µg/mL), or FcγRIIIB (2.5 µg/mL) diluted in PBS at 4°C for 16 hours. For the specific case of FcγRIIA, polystyrene 96-well plates (Corning, USA) were precoated with 10 µg/mL neutravidin protein (Thermo Fisher Scientific, Canada) diluted in PBS at 4°C for 16 hours prior to coating the plate with biotinylated-FcγRIIA (2.5 µg/mL) for an additional hour. All plates were blocked for 1 hour at RT with PBS containing 3% skimmed milk, 1% BSA. The plates were then washed three times with distilled water and incubated with blocked periprep, purified scFv (0.07-700 nM), or controls for 1 hr at RT. Controls for each FcγR are described in SI Appendix Table 2. Afterwards, the plates were washed as described above, except for the periprep samples where plates were washed five times with PBS 0.1% tween-20. The plates were then incubated with the appropriate HRP-conjugated secondary antibody (SI Appendix Table 2) for 1 hour at RT. Plates were washed as described above, followed by a 30-min incubation with Enhanced K-Blue® Substrate (TMB) at RT. The reaction was stopped with 1M HCl and the absorbance was read at 450 nm on a Spectramax plate reader.

Binding of peripreps to human NK cells

For the selection of scFvs which could bind to FcγRIIIA on primary cells, binding to human NK cells was performed. Peripheral blood mononuclear cells (PBMC) were isolated over Ficoll-PaqueTM Plus (Amersham Biosciences AB, Sweden) density gradient centrifugation as previously described [3]. Cells were seeded in a 96-well v bottom plate (Corning, USA) at 50000

cells per well and incubated with peripreps, 70 nM 3G8, 70 nM mIgG1 (isotype control), 700 nM 3G8-scFv or 700 nM Her-scFv for 1 hour on ice. Cells were washed with PBS and incubated for 30 min on ice with a mixture of BV605-conjugated anti-human CD56 (1:100) and propidium iodide (1:1000) in PBS 1% FBS plus AF647-conjuagted anti-c-myc (1:1000) for detection of peripreps or scFv, or 2 µg/mL AF647-conjugated anti-mouse IgG for detection of full-length antibodies. Cells were washed, resuspended in PBS, 1% FBS and analyzed on the Intellicyt iQue ® Screener Plus (Sartorius, Germany) using Forecyt® Software (Sartorius, Germany).

Inhibition of the interaction between FcγRIIIA and human IgG

The capacity of peripreps and scFv to inhibit the interaction between FcγRIIIA and human IgG (hIgG) was evaluated by Homogeneous Time Resolved Fluorescence (HTRF) essentially using a method previously reported.[4] Briefly, equal volumes of 1:62.5 streptavidin-XL665 (CisBio, Canada), 0.25 mg/mL biotinylated-FcγRIIIA, 1:31.25 europium-conjugated anti-hIgG (CisBio, Canada), and 0.8 mg/mL hIgG were mixed in reconstitution buffer (CisBio, Canada). Then, 10 µL of the mixture were plated in each well of 384-well low volume polystyrene microplates (Corning, USA). After that, 10 µL of blocked peripreps, or working stocks (0.06 – 1000 nM) of purified scFvs, Her-scFv, 3G8-HSA or HSA, or 0.6-100 nM 3G8 or mIgG isotype control were added and incubated for 3 hours at RT in the dark. As negative control, 10 µL of reconstitution buffer was used (as a no competition control). After this incubation, samples were analyzed on a Synergy 4 plate reader using Gen5 Software (Filter wheels 330/80 and 620/10).

The following summarizes final concentrations for HTRF reagents:

- ✓ 1:500 streptavidin-XL665
- ✓ 20 nM biotinylated-FcγRIIIA
- ✓ 1:250 europium-conjugated anti-hIgG
- ✓ 20 nM hIgG
- ✓ Competing reagent: blocked peripreps, 0.03-500 nM scFvs or 3G8-HSA or 0.3-50 nM IgG

For screening, the concentration used were 200 nM scFvs or 3G8-HSA and 20 nM human IgG (hIgG).

The following formulas were used to calculate the percentage of inhibition for each sample (Background = 1:250 europium-conjugated anti-hIgG in reconstitution buffer):

Ratio = (Measurement$_{665nm}$ / Measurement $_{620nm}$) x 10000
Delta Ratio = Sample Ratio - Background Ratio
% Delta F = (Delta Ratio/ Background) x 100%
% Delta F = (Delta Ratio/ Background) x 100%

% Inhibition = [(No competition – sample)/No competition]x100

Binding analysis to FcγRIIIA by Octet

The biomolecular interaction of purified scFv to the human FcγRIIIA was evaluated using streptavidin biosensors (ForteBio, USA) on ForteBio Octet Red96e (ForteBio, USA) with Data Acquisition 11.1 software. Data was analyzed using Data Analysis HT 11.1 software as essentially described.[5] Briefly, the biosensor was dipped into buffer assay (PBS pH 7.4, 0.02% Tween-20, 0.5% BSA) to establish the baseline. For an example of the overview and principle of this see: (https://www.sartorius.com/download/552186/anti-penta-his-biosensors-for-label-free-analysis-of-his-tagged-proteins-technical-note-en-sartorius-data.pdf). Later, the biosensor was dipped into buffer assay containing the biotinylated-FcγRIIIA at 1.5 µg/mL to immobilize the receptor and another baseline step was performed by dipping the biosensor into assay buffer. For the association step, the biosensor containing the Fc receptor of interest, or a control protein was then dipped into assay buffer containing the analyte (e.g., 500 nM purified scFv) and finally the dissociation is allowed by dipping biosensor into assay buffer. Conditions are described in SI Appendix Table 3.

The following molecules were used as controls: 3G8 or mouse IgG1 (mIgG1), 3G8-scFv or Her-scFv, and 3G8-HSA or HSA.

Expression of the selected scFv 17C02 as an albumin fusion molecule, a mouse IgG2a, and a human IgG1 one-armed antibody

The gene that encodes for the selected scFv 17C02 with additional genetic sequences encoding human albumin and linkers as well as a 6xHis tag (Fig. 2A) were cloned into the mammalian expression vector pCEP4 (Thermo Fisher Scientific, Canada).

In addition, the genes that encode for V_L and V_H of the select scFv 17C02 were cloned into the plasmids pFUSE2-CLIg-mK (Catalogue code: pfuse2-mclkm; InvivoGen, USA) and pFUSE-CHIg-mG2a (Catalogue code: pfuse-mchg2a; InVivoGen, USA) respectively, to express the molecule as a full-length mouse IgG2a antibody (Fig. 2C). Both genes were synthesized with IDT (Integrated DNA Technologies, USA) and an IL-2 leading sequence was added to both. Finally, the gene encoding the V_H region from 17C02 was cloned into the plasmid pFUSE-CHIg-mG2a vector using the restriction sites EcoR I and Eco47III. The gene encoding the V_L portion from 17C02 was cloned into the plasmid pFUSE2-CLIg-mK using the restriction sites AgeI and BstAPI.

Finally, the genes encoding the V_L and V_H of the select scFv 17C02 were used to express a human IgG1 one-armed antibody using the "knob into hole" strategy as previously described[6] (Fig. 2B). Briefly, the plasmid pEmiLC (Addgene, USA) was modified to express the heavy chain of the human IgG1 with LALA mutations ($L_{234}A$ and $L_{235}A$) to avoid antibody glycosylation and thus impair Fc region function (e.g., engagement with FcγRs and complement). In addition, the sequence WPRE (Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element) was introduced in the 3′ untranslated region of the coding sequence to enhance the expression of the

gene. The C_H3 region was also modified to create the "hole" site. A second pEmiLC vector was modified to express the kappa light chain of the human antibody. The sequence WPRE was also introduced in the 3′ untranslated region of the coding sequence. A third pEmiLC vector was modified to express the C_H2-C_H3 portion of a human IgG1 with the "knob" site. The WPRE sequence was also incorporated into the 3′ untranslated region of the coding sequence.

For the expression of each construct 40 µg of total DNA were incubated with PEI (Polysciences, USA) in a 1:3 (DNA: PEI) ratio for 20 min at RT. Afterwards, the mixture was added to $40x10^6$ Expi293F cells (Thermo Fisher Scientific, Canada) (at a concentration of 10^6 cells/mL) for a final ratio of 1 µg of DNA per 10^6 cells. Cells were cultured with 0.1% Pluronic F68 (Avantor, Canada) and 0.5 M valproic acid (Sigma Millipore, Canada) for 7 days, after which the supernatant was collected to check for antibody expression and then purified using affinity chromatography.

Enumeration of immune cells from mice treated with 17C02-based molecules

Selected populations of immune cells were enumerated from untreated mice vs mice injected with 540 µM of 17C02-albumin or deglycosylated 17C02-IgG2a for 2 hours. Blood samples, spleens and bone marrow were suspended in PBS 1% BSA, and live cells were identified, and cell numbers quantified using a Guava easyCyte flow cytometer (Luminex Corporation, USA). For flow cytometry, cell concentrations were adjusted to $1x10^6$ cells/mL, and 50 µL of each cell suspension were incubated with specific antibodies for 30 minutes on ice. After washing, cells were analyzed using a BD LSRFortessa X-20 (Beckton Dickson, USA). FlowJo v10 software (Beckton Dickson, USA) was used for subsequent data analysis. Enumeration of B cells as CD19+, T cells as CD3+, NK cells as NK1.1+, and neutrophils as CD11b+ and Ly-6G+ cells was performed using a combination of the absolute cell counts from the Guava coupled with analysis by flow cytometry.

References

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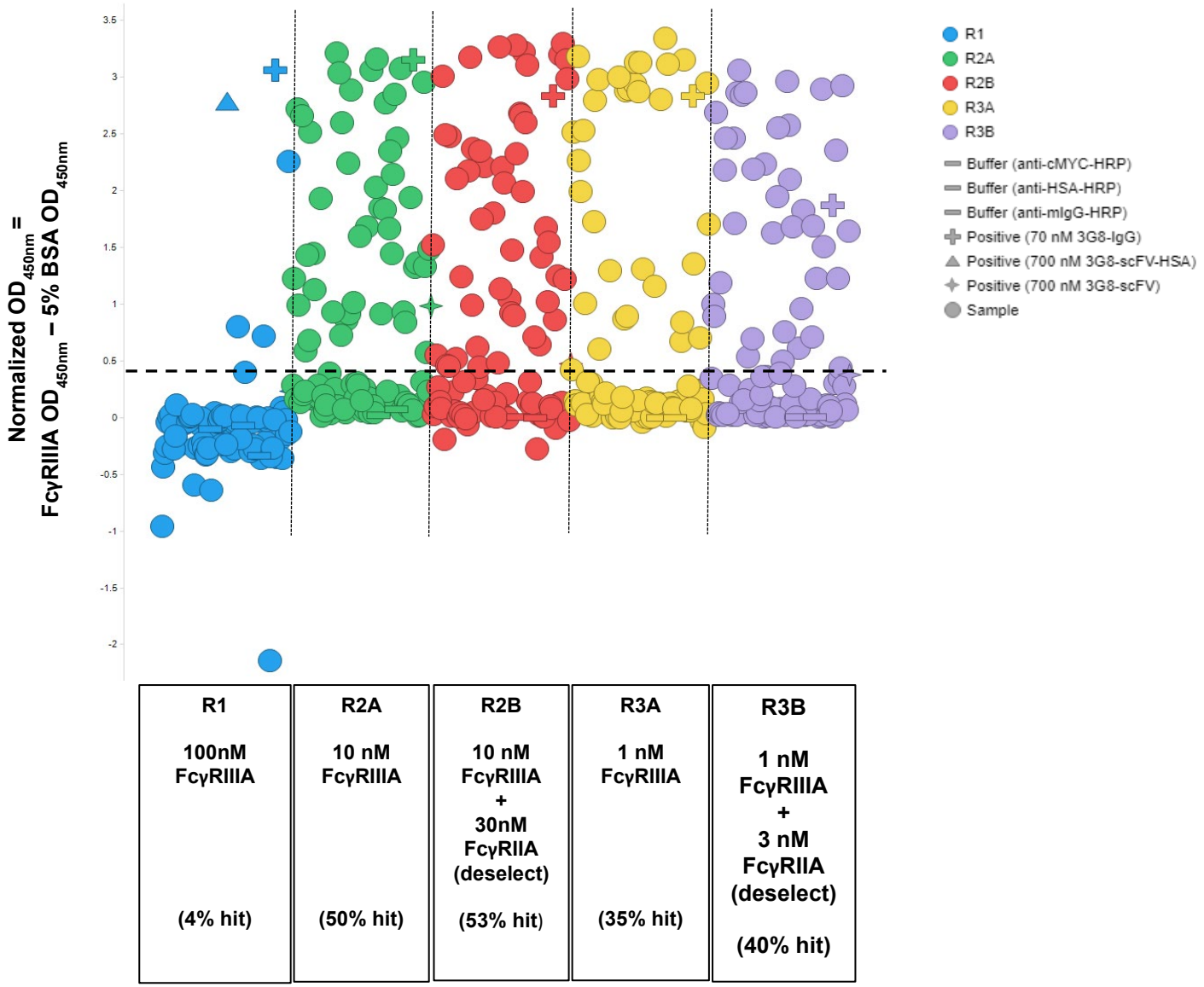
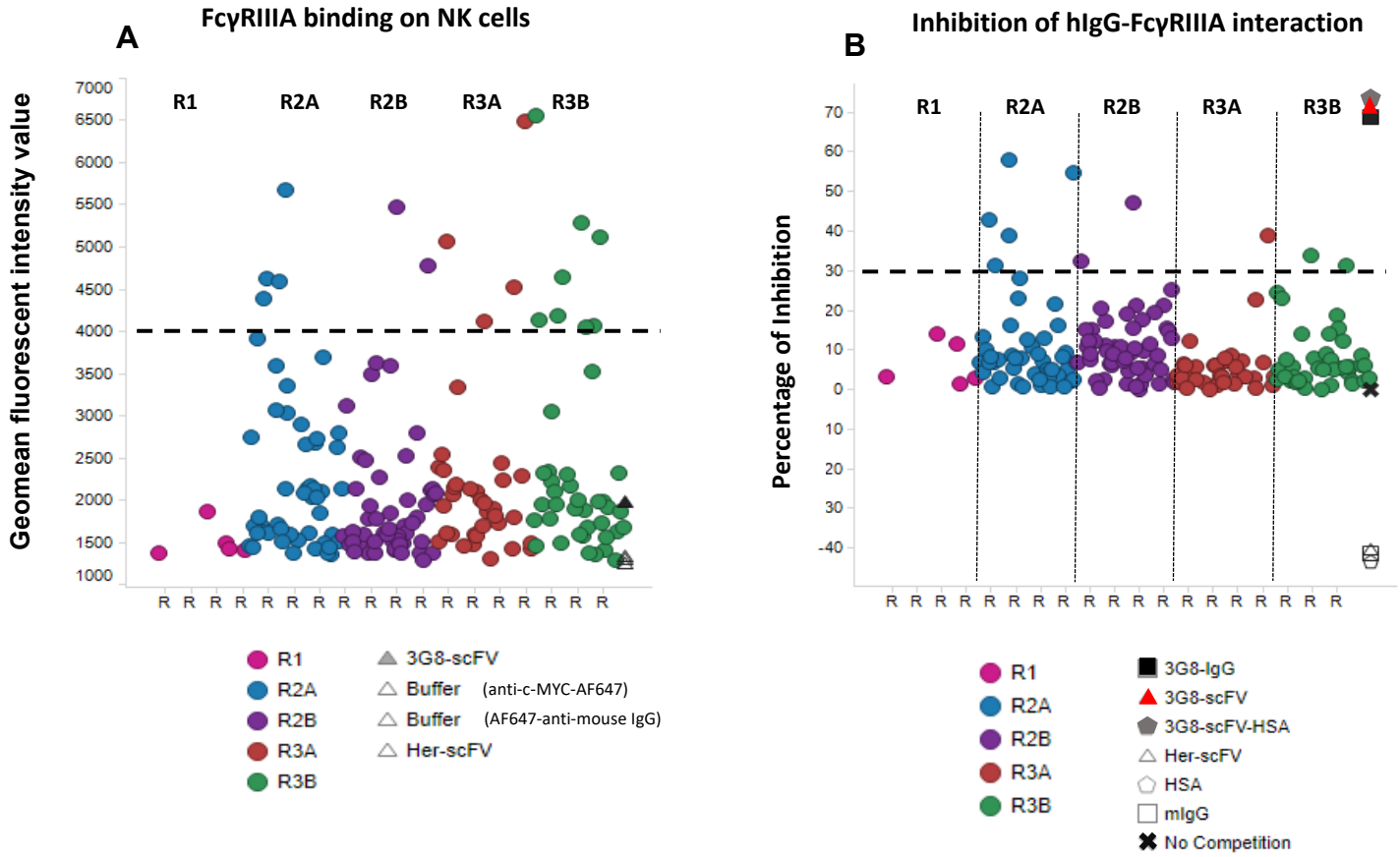
Supplemental Figure 1



Supplemental Figure 1. Periprep selection showing FcγRIIIA binding capacity of selected clones. One hundred peripreps were sampled from each round of selection (R1, R2A, R2B, R3A, and R3B) and evaluated for their binding capacity to FcγRIIIA by ELISA. The monoclonal antibody 3G8, a single chain of this antibody (3G8-scFv), and the single chain fused to human albumin (3G8-scFv-albumin) were included as positive controls. The graph plots the normalized optical density at 450 nm ($OD_{450\ nm}$) for each sample calculated as the subtraction of the $OD_{450\ nm}$ measured for a solution of PBS 5% BSA used as a negative control to the $OD_{450\ nm}$ measured for peripreps. The dashed line represents the cut-off value for the peripreps that were considered a binder or "hit" ($OD_{450\ nm} = 0.4$). The chart demonstrates the percentage of "hits" identified after each round of selection, which also necessitated that the periprep have an $OD_{450\ nm}$ equal or higher than that detected for 3G8-scFv or 3G8-scFv-albumin. Data are representative of two independent assays.

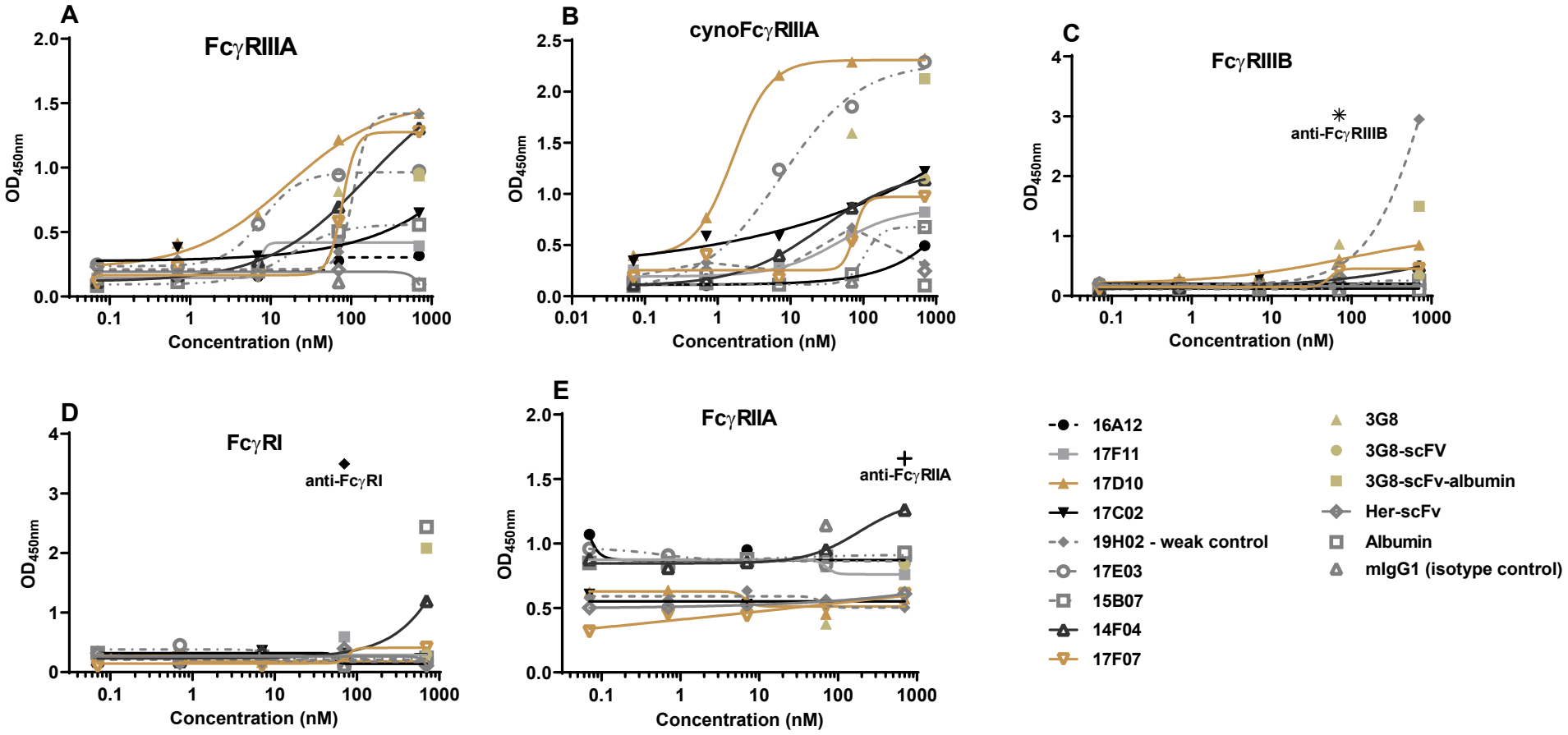
Supplemental Figure 2



Supplemental Figure 2. Binding of peripreps to FcγRIIIA expressed on human NK cells and inhibition of hIgG-FcγRIIIA interaction. Peripreps from each round of selection (R1, R2A, R2B, R3A, and R3B), which had positive binding to FcγRIIIA by ELISA,

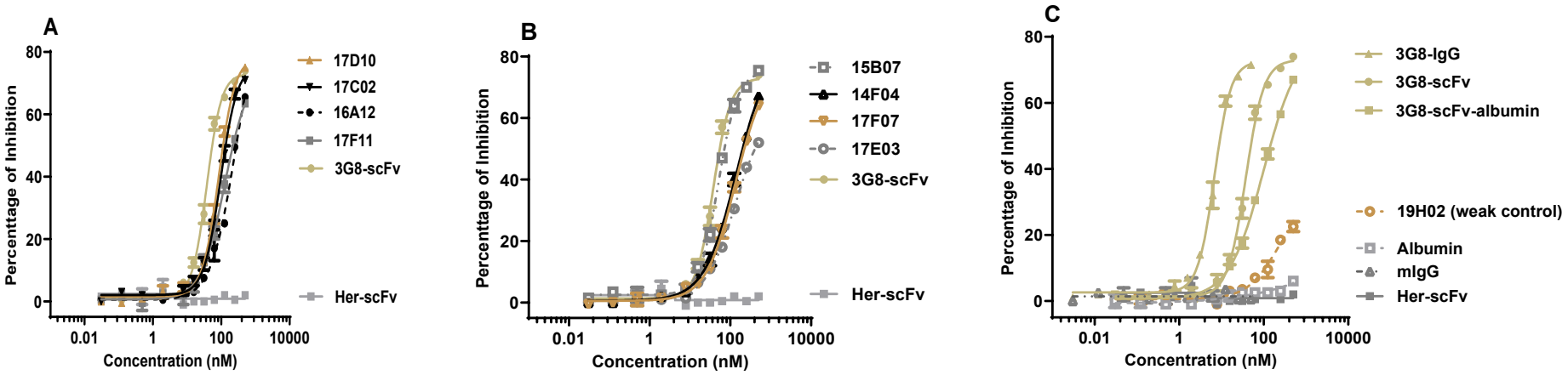
were then screened for binding to NK cells and inhibition of hIgG-FcγRIIIA interaction. Panel A: NK cell binding. PBMC from human donors were incubated with selected peripreps or 3G8-scFv, 3G8, Her-scFv or mIgG1 (isotype control) as controls. After washing, cells were incubated with a mixture of BV605-conjugated anti-human CD56 and propidium iodide plus AF647-conjugated anti-c-myc for the detection of either peripreps or scFv, or AF647-conjugated anti-mouse IgG for the detection of full-length antibodies. The cells were then washed and analyzed on the Intellicyt iQue ® Screener Plus using Forecyt® Software. The graph plots the geometric mean (geomean) of the fluorescent intensity of live CD56+ population for each sample. Data are representative of two independent determinations. The dashed line represents the cutoff value defined as twice the geomean of 3G8-scFv. Panel B: Capacity of peripreps to inhibit hIgG-FcγRIIIA interaction, as measured by Homogeneous Time Resolved Fluorescence. Equal volumes of streptavidin-XL665, biotinylated-FcγRIIIA, europium-conjugated anti-hIgG, and hIgG were incubated with either the peripreps, 3G8, 3G8-scFv, 3G8-scFv-albumin (3G8-scFv-HSA), Her-scFv, albumin (HSA), or mIgG1 (isotype control). Reconstitution buffer (CisBio, Canada) was used as a “no competition” negative control. Samples were then analyzed on a Synergy 4 plate reader using the Gen5 Software (Filter wheels 330/80 and 620/10). The graph plots the percentage of inhibition for each sample as described in the Methods section. Data are representative of two independent assays. The dashed lines represent the cut-off values used for scFv selection.

Supplemental Figure 3



Supplemental Figure 3. Binding of purified scFv to FcγRIIIA, cynoFcγRIIIA, FcγRIIIB, FcγRI, and FcγRIIA, as measured by ELISA. Plates were coated with FcγRIA (2.5 μg/mL), FcγRIIIA (10 μg/mL), cynoFcγRIIIA (10 μg/mL), or FcγRIIIB (2.5 μg/mL). For the specific case of FcγRIIA, plates were precoated with 10 μg/mL neutravidin protein prior to coating with biotinylated-FcγRIIA (2.5 μg/mL). Plates were then incubated with selected purified scFv candidates (0.07-700 nM), or controls (3G8-scFv, 3G8-scFv-albumin, Her-scFv, albumin, 3G8 or mIgG1 (isotype control)), after which the optical density at 450 nm ($OD_{450\ nm}$) was assessed for each scFv concentration. Antibodies against FcγRI (♦ clone 10.1), FcγRIIA (+ clone IV.3) and FcγRIIIB (* clone 2D2G5B9) were used as positive controls in the analysis of binding to these specific FcγRs. Data are representative of two independent determinations.

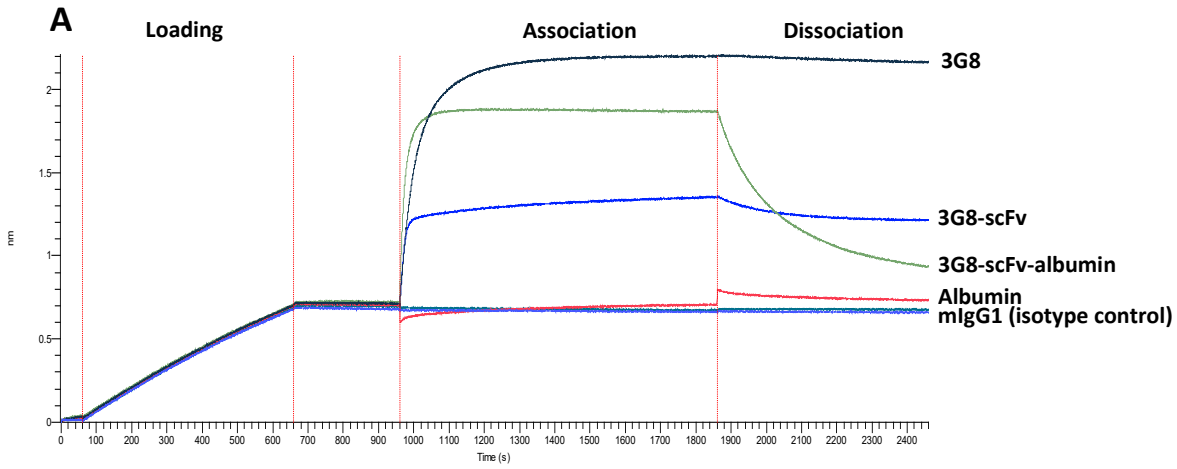
Supplemental Figure 4

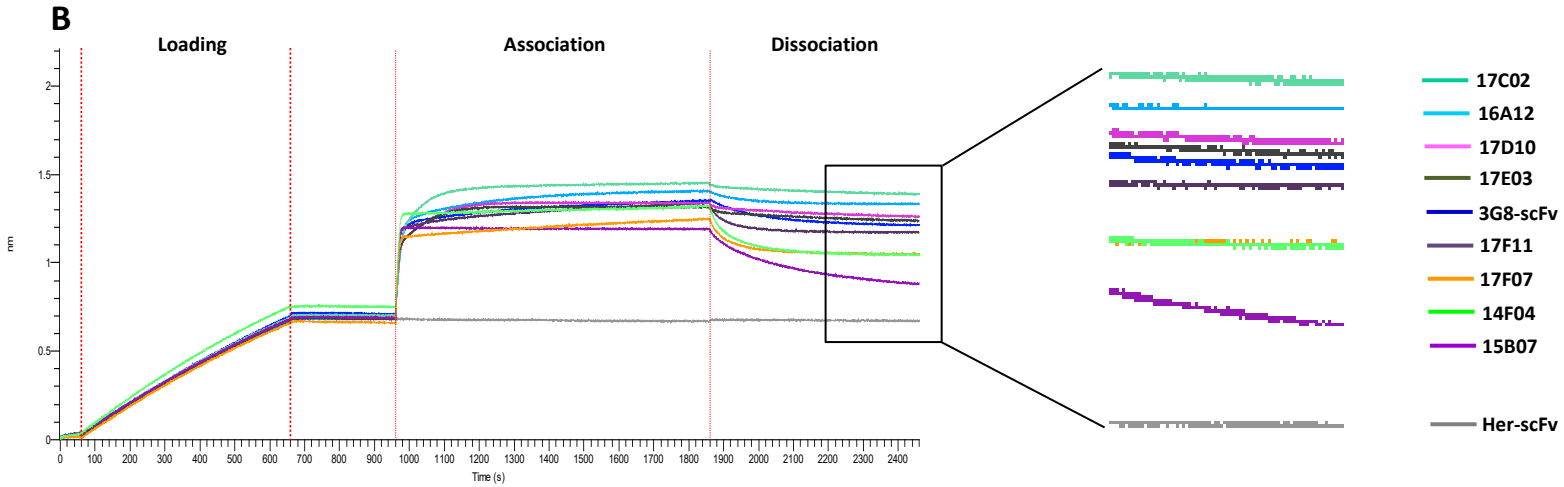


Supplemental Figure 4. Inhibition curves of hIgG-FcγRIIIA interaction. The ability of human IgG to bind to FcγRIIIA in the presence of different concentrations of each purified scFv is shown (measured by Homogeneous Time Resolved Fluorescence). Equal volumes of streptavidin-XL665, biotinylated-FcγRIIIA, europium-conjugated anti-hIgG, and hIgG were mixed and incubated with a purified scFv candidate (0.03-500 nM), or 3G8, 3G8-scFv, 3G8-scFv-albumin, Her-scFV, albumin, or mIgG1 (isotype control) for 3 hours at room temperature in the dark. Incubation with reconstitution buffer (CisBio, Canada) was used as a "no competition" negative control. Samples were then analyzed on a Synergy 4 plate reader using the Gen5 Software (Filter wheels 330/80 and 620/10) and the data were plotted as the percentage of hIgG-FcγRIIIA interaction inhibition. Panels A and B show the capacity of scFv to inhibit hIgG-

FcγRIIIA interaction. Panel C shows the positive and negative controls. Data is presented as the mean ± standard deviation of two independent experiments.

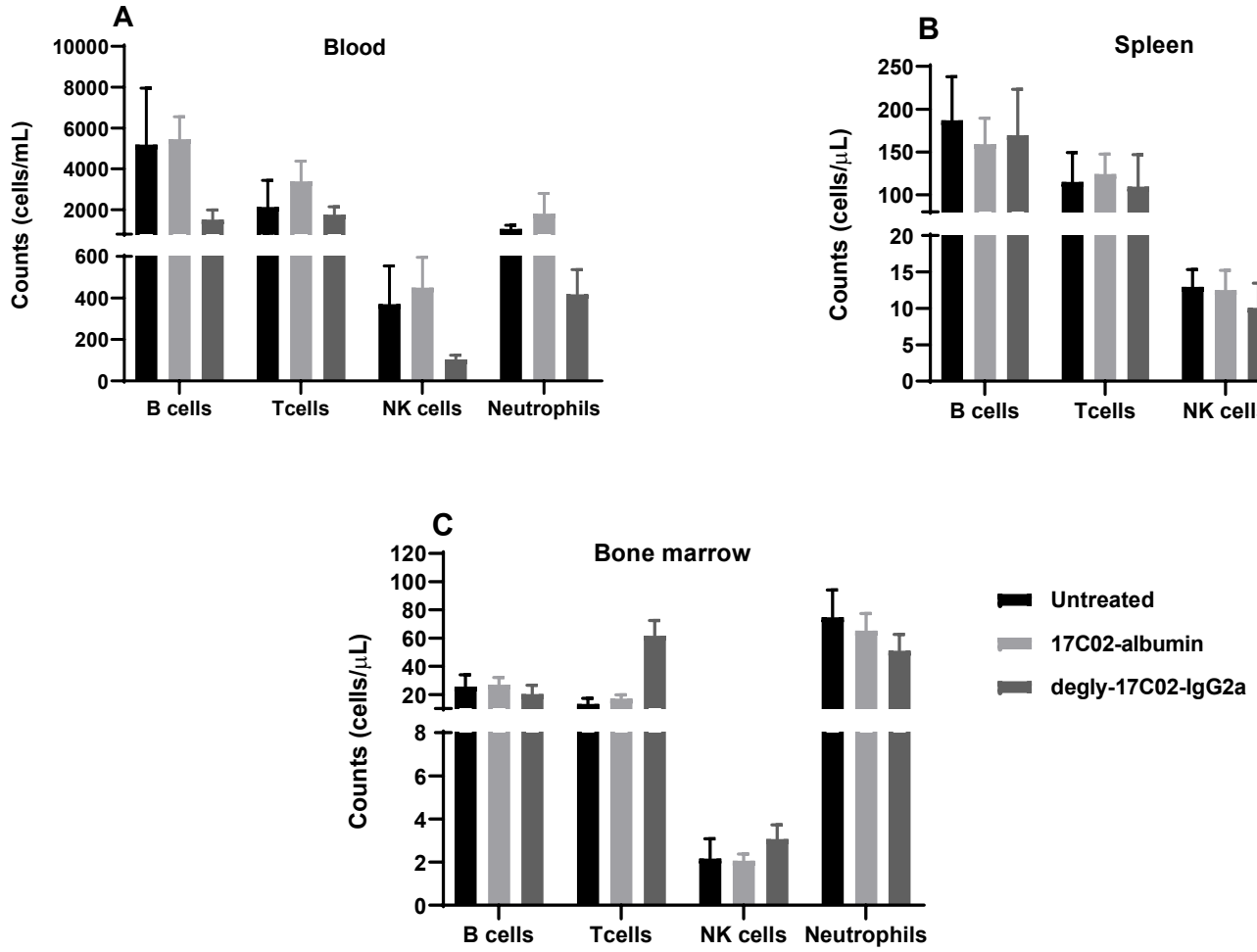
Supplemental Figure 5





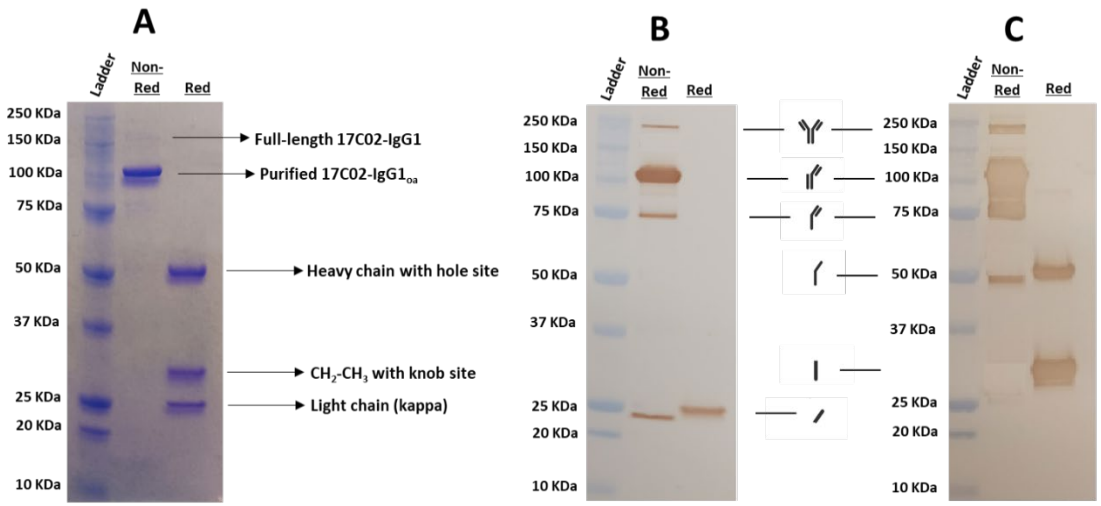
Supplemental Figure 5. Sensograms demonstrating the binding capacity of antibodies and scFv to the human FcγRIIIA. Biotin-FcγRIIIA (3 μg/mL) was loaded onto Streptavidin Biosensors, followed by association and dissociation of the molecules of interest. Panel A: Association with 50 nM of 3G8 or mIgG1 isotype control or 500 nM of 3G8-scFv, 3G8-scFv-albumin, or albumin, and subsequent dissociation. Panel B: In addition to 3G8-scFv (dark blue line), the association and dissociation of the Herceptin single chain (Her-scFv; negative control) and other scFv antibodies obtained from the scFv phage display library were assessed. 17C02-scFv is shown as the dark green line (slowest dissociation). Binding was performed using Streptavidin Biosensors on ForteBio Octet Red96e with the Data Acquisition 11.1 Software and data analyzed using the Data Analysis HT 11.1 Software.

Supplemental Figure 6



Supplemental Figure 6: Enumeration of immune cells in FcγR-humanized mice treated with 17C02-albumin and deglycosylated 17C02-IgG2a. Immune cells were enumerated from blood, spleen, and bone marrow from mice treated with either 540 μM/mouse of 17C02-albumin or deglycosylated 17C02-IgG2a (degly-17C02-IgG2a). Control data was obtained from untreated animals. Live cells were quantified, and the frequencies of B cells (CD19+), T cells (CD3+), NK cells (NK1.1+), and neutrophils (CD11b+/Ly-6G+) were assessed using a combination of absolute cell counts from a Guava cytometer coupled with flow cytometry. Panels show the enumeration of immune cells in the blood (A), in the spleen (B), and in the bone marrow (C). The data is presented as the mean ± standard deviation (n=3).

Supplemental Figure 7



Supplemental Figure 7. SDS-PAGE and western-blotting analysis of 17C02-IgG1$_{oa}$ under reducing and non-reducing conditions. (A) Three micrograms of total antibody under reducing and non-reducing conditions were applied into each lane. (B) Immuno-identification using a mouse anti-human kappa light chain antibody conjugated to HRP (Millipore Sigma, Canada). (C) Immuno-identification using a mouse anti-human IgG (Fc-specific) antibody conjugated to horseradish peroxidase (HRP) (Millipore Sigma, Canada). (B-C interval) Cartoon depicting the logical 17C02-IgG1$_{oa}$ species corresponding to each position in the western blot.

Supplemental Table 1. Rounds of selection and its steps

Round	Steps	Number of phage selected
R1	Selection with 100 nM of FcγRIIIA	$9.3x10^4$ CFU
R2A	Selection with 10 nM of FcγRIIIA	$3.22x10^6$ CFU
R2B	De-selection with 30 nM of FcγRIIA ; Selection with 10 nM of FcγRIIIA	$6.1x10^6$ CFU
R3A	Selection with1 nM of FcγRIIIA	$3.45x10^7$ CFU
R3B	De-selection with 3 nM of FcγRIIA ; Selection with1 nM of FcγRIIIA	$2.07x10^7$ CFU

Selection refers to the process of retaining phages that were bound to the FcγR specified while de-selection refers to the process of collecting the phages that were not bound to the FcγR.

Supplemental Table 2. Controls and secondary antibodies for FcγR binding by peripreps

	FcγRI	FcγRIIA	FcγRIIIA cynoFcγRIIIA	FcγRIIIB
Positive controls	70 nM mouse anti-human FcγRI	1:500 mouse anti-human FcγRIIA	70 nM 3G8 700 nM 3G8-scFv 700 nM 3G8-HSA	70 nM mouse anti-human FcγRIIIB
Negative controls	70 nM mIgG1 (isotype control)	1:500 mIgG1 (isotype control)	70 nM mIgG1 (isotype control) 700 nM Her-scFv 700 nM HSA	70 nM mIgG2a (isotype control)
Secondary antibody	0.2 µg/mL goat anti-mouse IgG (Fcγ fragment specific)-peroxidase (detection of full-length antibodies) 1:1000 anti-HSA antibody-peroxidase (Abcam, Canada) (detection of albumin and albumin constructs) 1:1000 mouse anti-c-myc-peroxidase (Millipore Sigma, Canada) (detection of blocked peripreps and purified scFv bound to FcγRI, FcγRIIIA, cynoFcγRIIIA, or FcγRIIIB) 1:10000 goat anti-c-myc-peroxidase (Jackson ImmunoResearch, USA) (detection of blocked periprep and purified scFv bound to FcγRIIA)			

mIgG1: mouse IgG1; mIgG2a: mouse IgG2a; HSA: human serum albumin (Abcam, Canada)

Supplemental Table 3. Conditions for the binding of purified scFv to FcγRIIIA by Octet

Step	Reagent	Time (s)
Baseline	Kinetic buffer	60
Loading	1.5 µg/mL biotinylated-FcγRIIIA	600
Baseline	Kinetic buffer	300
Association	500 nM purified scFv 50 nM 3G8 or mIgG1 500 nM 3G8-scFv or Her-scFv 500 nM 3G8-HSA or HSA	900
Dissociation	Kinetic buffer	600