Supplementary Methods

Sample preparation for fixed cell microscopy

Cells were plated in glass-bottomed dishes (MatTek Corporation) overnight and treated as indicated in the figure legends. Fixation was carried out using 3.4% paraformaldehyde (Electron Microscopy Sciences) for 10 minutes at 37°C. When necessary, samples were permeabilized with 0.5 mg/ml saponin (Sigma Aldrich). Mouse CD45 was detected by incubating the sample with biotinylated anti-CD45 antibody (BD Biosciences), followed by detection with streptavidin-conjugated quantum dot (QD) 655 (Life Technologies). FITC-labeled anti-mouse CD45 antibody (BD Biosciences) was used in Fig. 2B for CD45 detection in permeabilized cells. Treatment of cells with DiD (Vybrant DiD Cell-Labeling Solution; Life Technologies), Alexa 647-labeled phalloidin (Life Technologies) and Hoechst 33528 (AnaSpec, Inc.) was carried out using protocols recommended by the manufacturer. Macrophages were treated with 25 ng/ml recombinant mouse interferon-γ (IFN-γ; BD Biosciences) for 6 hours prior to use in fixed cell microscopy experiments.

Sample preparation for live cell microscopy

For live cell imaging of trogocytosis using single plane microscopy or MUM, MDA-MB-453 cells were plated in MatTek dishes and labeled with 10 μg/ml Alexa 555-labeled trastuzumab for ten minutes at room temperature. Six hours prior to the experiment, MEM-GFP expressing RAW264.7 macrophages or J774A.1 macrophages labeled with FITC-labeled anti-mouse CD45 antibody were treated with 25 ng/ml IFN-γ. 30 minutes prior to imaging, opsonized cancer cells were added to the macrophages at a 1:1 effector:target cell ratio. CellEvent Caspase-3/7 Green Detection Reagent (Life Technologies) was added to the medium at a concentration of 2 μM throughout assays in which target cell viability was

assessed for up to three hours. RAW264.7 macrophages were transfected with MEM-GFP (Clonetech) using the Amaxa Nucleofection device and Cell Line Nucleofecter Kit V (Lonza).

For imaging WCP events, J774A.1 macrophages were plated overnight and activated with 25 ng/ml IFN-γ six hours prior to addition of target cells. Macrophages were labeled with biotinylated anti-mouse CD45 antibody followed by streptavidin-coated QD655 as described above and Alexa 555-labeled, trastuzumab-opsonized MDA-MB-453 cells added. The cells were imaged using single plane microscopy.

For the long-term imaging of macrophage-cancer cell interactions, macrophages and cancer cells were plated at appropriate ratios in non-vented T25 flasks. The cells were labeled following 24 hours with Alexa 555-labeled trastuzumab and FITC-labeled anti-mouse CD45 antibody. Alexa 488-labeled anti-human CD45 antibody was used when human macrophages were imaged. A pre-marked region of the flask was imaged using the Axiovert 200M fluorescent microscope and a Zeiss 20X Plan Apochromat objective to identify the cancer cells and macrophages. The flask was then gassed by injecting a sterile 95% air /5% CO_2 mixture and subsequently sealed. The same pre-marked region was then imaged at 37°C in the long-term imaging setup.

Microscopy imaging configurations

For fixed cell microscopy, images were acquired using a Zeiss Axiovert 200M inverted fluorescence microscope with a Zeiss 63x/1.4NA Plan Apochromat objective as described previously (1). Single plane imaging of trogocytosis in live cells was performed with the Deltavision epifluorescence microscope (Applied Precision) at the UT Southwestern Live Cell Imaging Core Facility.

The MUM configuration used to image trogocytosis was based on a Zeiss Axio Observer.A1 body and a Zeiss 100x/1.4NA Plan Apochromat objective. The sample was illuminated with a 488 nm solid state laser (Coherent) for GFP excitation and a 543 nm diode laser (Opto Engine LLC) for Alexa 555 excitation. The illumination was directed to the sample and fluorescence filtered back using a polychroic beamsplitter/emission filter combination (488/543/633 RPC and 488/543 M; Chroma Technology Corporation). The emission was split equally between four Andor iXon EM-CCD cameras (Andor Technology Ltd.) using 50:50 beamsplitters (Chroma Technology Corporation). The focal plane imaged by each camera was calibrated and light splitting performed as described previously (2). The cameras were run on conventional gain mode and images were acquired in two colors at a frame rate of 0.67 Hz. Camera acquisition and shuttering of excitation lasers were controlled using custom acquisition software written in LabWindows/CVI (National Instruments Corporation). The acquired images were registered and processed using the Microscopy Image Analysis Tool (MIATool, http://wardoberlab.com/software/miatool) (3) written in MATLAB (MathWorks, Inc).

To image phagocytic events in live cells, the same configuration as above was used but with a Zeiss 40x/1.4NA Plan Apochromat objective and a triband emission filter. The fluorescence signal from excitation at 543 nm was split between two cameras using a 630 DCLP filter, with both cameras positioned at the design focal plane. Images were acquired in two colors simultaneously at 0.9 Hz, registered and processed using MIATool.

The long-term imaging experiments using transmitted light were performed using phase contrast on an Olympus IX70 microscope through an LCPlanFl 20x/0.40 objective. The images were acquired every 30 seconds through an 8.0 Mega Pixel Autofocus Camera module (See3CAM_80, e-con Systems, Inc) that

was attached to the eyepiece of the microscope. The configuration was housed in a 37°C warm room to maintain the temperature. The images were processed with MIATool software.

Supplementary figures

Figure S1

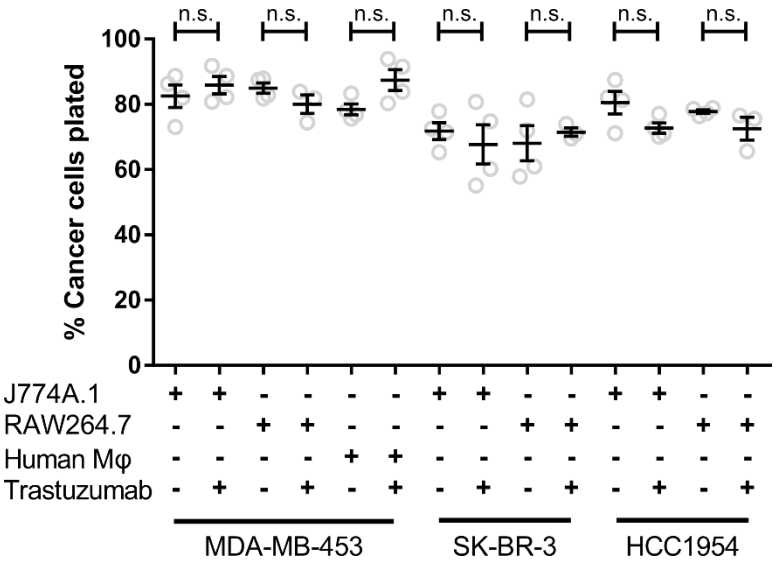

Figure S1. Harvesting efficiency of cancer cells in macrophage:cancer cell co-cultures. To label MDA-MB-453, SK-BR-3 or HCC1954 cells, the cancer cells were incubated with 50 µg/ml Alexa 647-labeled dextran overnight (dextran accumulates by fluid phase uptake within the endolysosomal pathway). Labeled cells were harvested, counted in duplicates using a hemocytometer and mixed with macrophages at a 4:1 ratio and plated in 48 well plates, in the presence or absence of 1 µg/ml trastuzumab. Following a 4 hour incubation at 37°C, the cells were harvested into flow cytometry tubes and 50 µl of a solution containing a defined number of Flow Check beads (counted using a hemocytometer) added to each tube. The samples were analyzed by flow cytometry and the total number of cancer cells harvested from each well was calculated by dividing the number of dextran-positive events by the fraction of added beads counted in each sample. This cell count corresponds to all cancer cells, including those present in macrophage:cancer cell conjugates and in phagosomes inside macrophages. The number of cancer cell events collected was plotted as a fraction of the number of

cancer cells plated in the 48 well plates (Mφ = macrophage). Statistically significant differences between samples were determined using one-way ANOVA analysis followed by a Tukey's multiple comparisons test between all the indicated sample pairs. n.s., no significant difference (95% confidence interval).

Figure S2

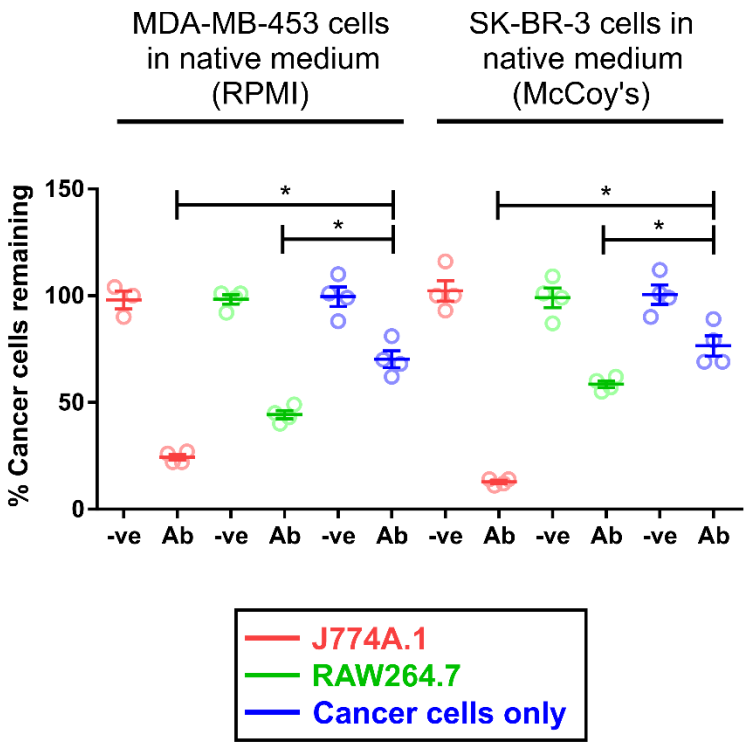

Figure S2. Macrophage-mediated reduction of opsonized target cell numbers occurs in media optimized for cancer cell growth. J774A.1 or RAW264.7 macrophages were plated in 48 well plates with CFSE-labeled MDA-MB-453 or SK-BR-3 cancer cells at a 4:1 effector:target ratio (2.5×10^4:6×10^3 cells) with 1 μg/ml trastuzumab (Ab) or PBS vehicle (-ve) and incubated at 37°C in either RPMI-based or McCoy's-based medium for 72 hours. The cells were then harvested and the remaining number of cancer cells quantitated by flow cytometry. The number of live cancer cells in each sample is shown as a fraction of the control. Error bars represent standard errors. Statistically significant differences between samples were determined using Student's *t*-test. * indicates significant differences between samples ($p < 0.05$).

Figure S3

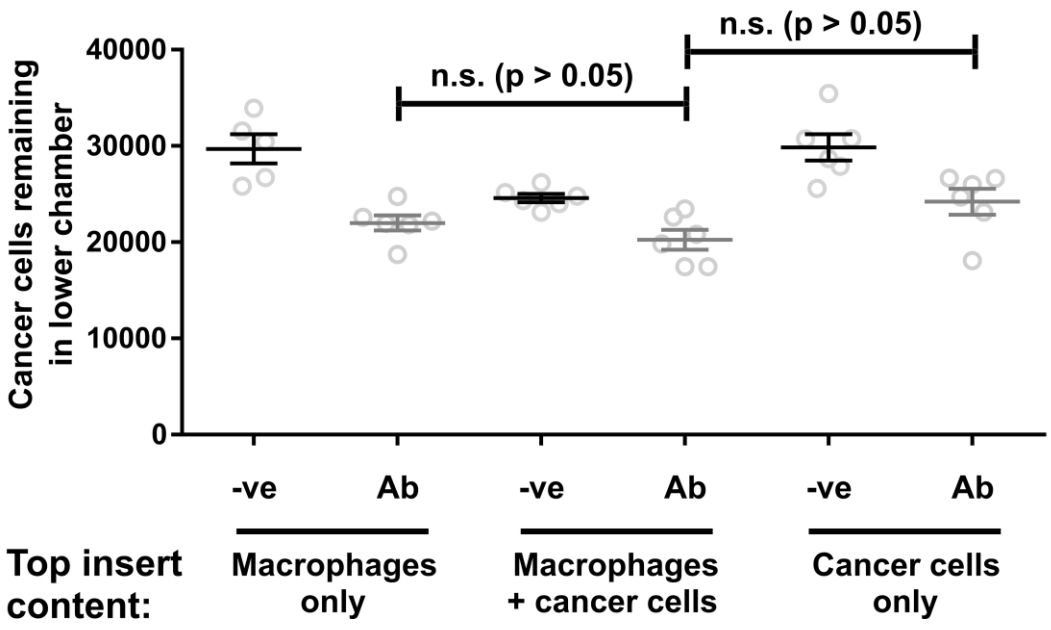

Figure S3. Macrophage-mediated killing of opsonized target cells is contact-dependent. Different combinations of 1×10^5 RAW264.7 cells, $2.5x10^4$ MDA-MB-453 cells and 1 µg/ml trastuzumab were mixed in the inserts of transwell plates (0.4 µm pores; Corning) and $1x10^5$ MDA-MB-453 cells were cultured in the lower chambers. Following incubation at 37°C for 72 hours, the cancer cells in the lower chambers were harvested and live cells quantitated by flow cytometry. Error bars represent standard errors. Statistically significant differences between samples were determined using Student's *t*-test. n.s., no significant difference ($p > 0.05$).

Figure S4

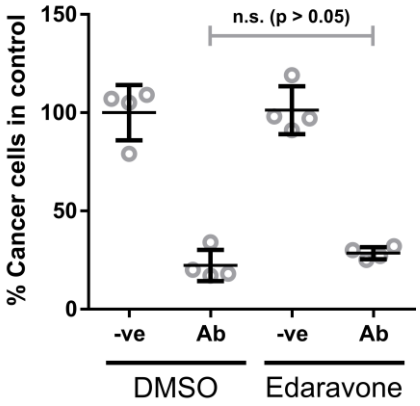

Figure S4. Macrophage-mediated killing of opsonized target cells occurs in the presence of the reactive oxygen species scavenger, edaravone. RAW264.7 macrophages were co-cultured with CFSE-labeled MDA-MB-453 cancer cells at 4:1 effector:target ratio (2.5×10^4:6×10^3 cells) and incubated at 37°C for 72 hours in the presence of 1 μg/ml trastuzumab (Ab) or PBS vehicle (-ve). Edaravone (1 μM) or vehicle (DMSO) were added to the cultures. Live cancer cell numbers were determined using flow cytometry and expressed as % cancer cell levels in control. Statistically significant differences between samples were determined using Student's *t*-test. n.s., no significant difference ($p > 0.05$).

Figure S5

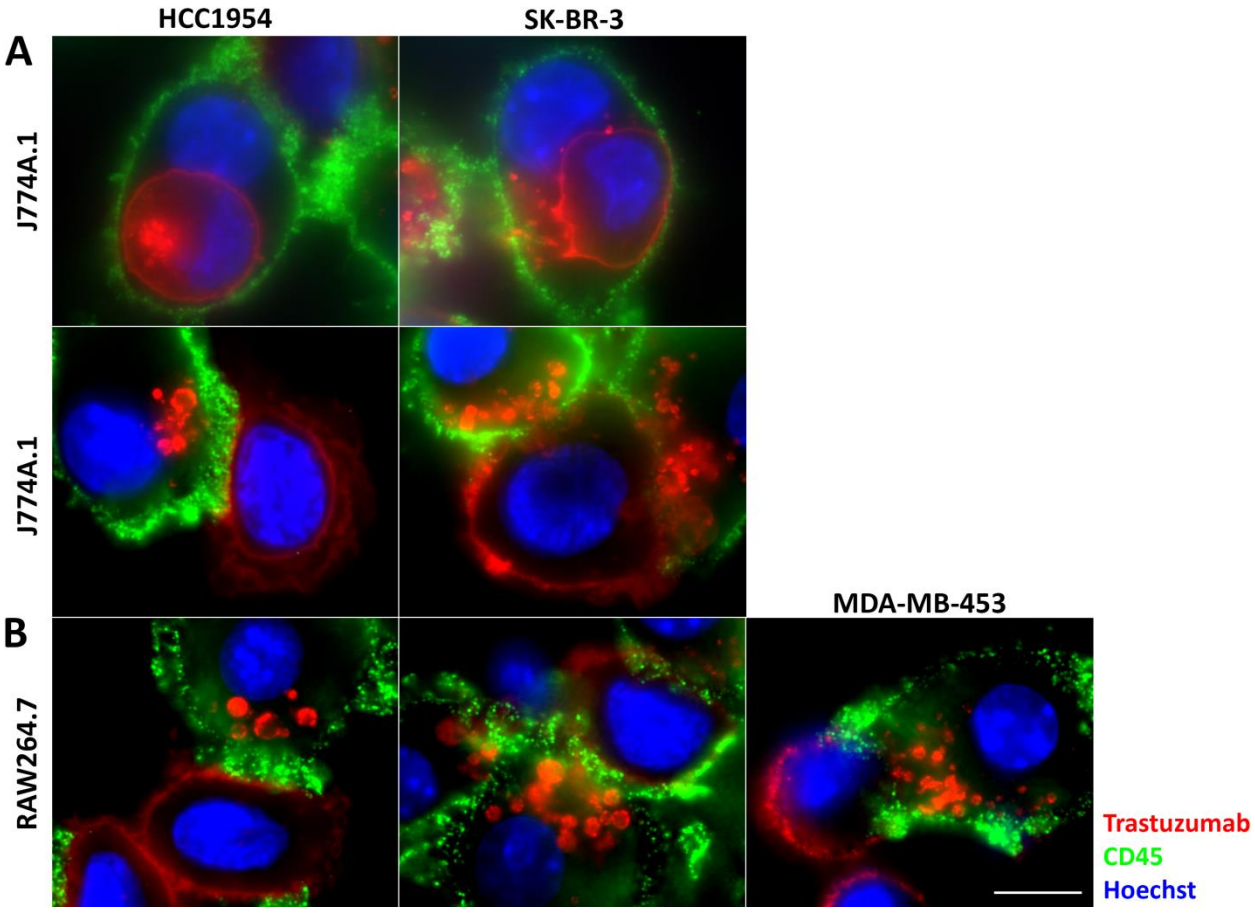

Figure S5. Representative images of conjugates formed between two macrophage cell lines and three cancer cell lines. SK-BR-3, HCC1954 or MDA-MB-453 cells were harvested and opsonized by incubation with 10 µg/ml Alexa 555-labeled trastuzumab at room temperature for ten minutes followed by washing. The opsonized cancer cells ($2.5x10^4$ cells/imaging dish) were added to adhered, IFNγ-activated J774A.1 macrophages for 30 minutes and the samples fixed and stained for CD45 using a mouse CD45-specific antibody (pseudocolored green). Nuclei were stained with Hoechst dye (pseudocolored blue). The upper row shows examples of WCP for J774A.1 macrophages, and the second and third rows show examples of trogocytosis for J774A.1 and RAW264.7 macrophages as indicated. Scale bar = 20 µm.

Figure S6

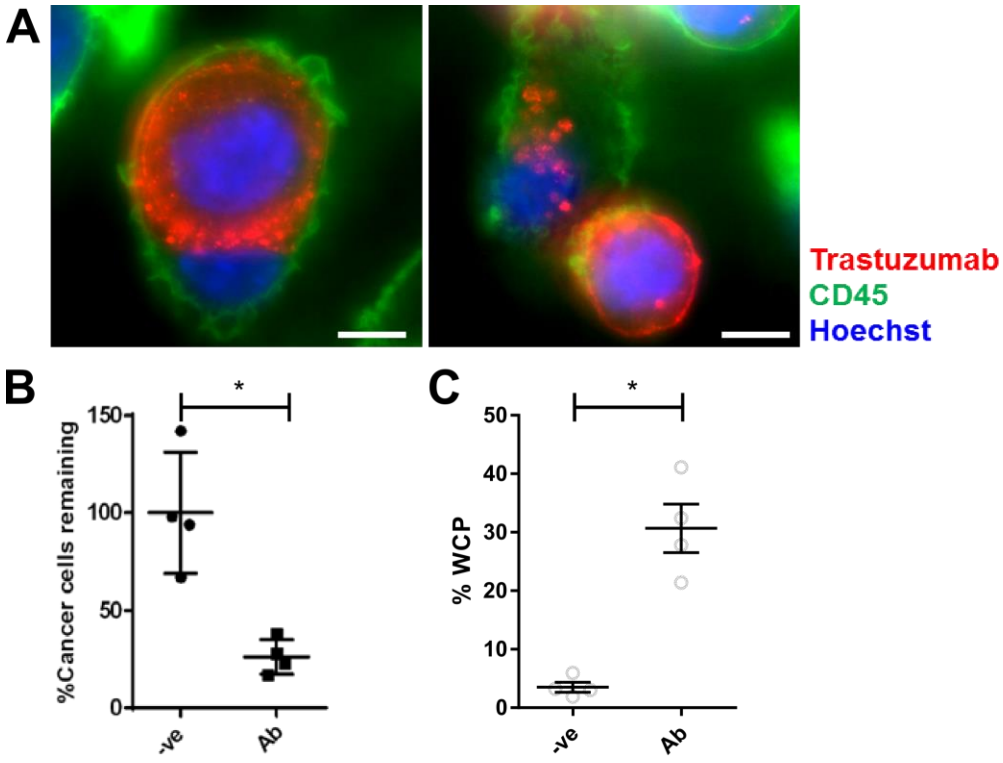

Figure S6. Human macrophages perform both phagocytosis and trogocytosis of antibody-opsonized target cells and reduce cancer cell numbers. **A**, 4×10^4 human monocyte-derived macrophages were cultured in MatTek dishes for six days and treated with 25 ng/ml IFN-γ for six hours, followed by the addition of 2.5×10^4 MDA-MB-453 cells opsonized with Alexa 555-labeled trastuzumab. The samples were incubated for 30 minutes, fixed and stained with a human CD45-specific antibody and Hoechst dye to detect macrophages and nuclei, respectively. Representative images show examples of WCP and trogocytosis performed by the human macrophages. **B**, CFSE-labeled MDA-MB-453 cells were added to monocyte-derived macrophages cultured in 48-well plates at a 4:1 effector:target cell ratio, with 1 µg/ml trastuzumab (Ab) or PBS vehicle (-ve) for 72 hours. The cells were harvested and the remaining CFSE-positive live cancer cell fraction was quantitated by flow cytometry. **C,** EdU-labeled MDA-MB-

453 cells were added to monocyte-derived macrophages cultured in 48-well plates at a 10:1 effector:target cell ratio with 1 μg/ml trastuzumab (Ab) or PBS vehicle (-ve) for 6 hours. The cells were harvested and stained for human CD45. Cancer cells accessible to the medium (i.e. not phagocytosed) were detected using fluorescently labeled pertuzumab, followed by fixation and staining for EdU. Stained samples were analyzed by flow cytometry. % WCP was calculated as the fraction of EdU-positive cells that were CD45-positive and pertuzumab-negative. Error bars represent standard errors. All experiments were performed using macrophages differentiated from monocytes isolated from fresh PBMCs, and similar results were obtained using purified monocytes from frozen stocks. Statistically significant differences between samples were determined using Student's *t*-test. * indicates significant difference between samples ($p < 0.05$).

Figure S7

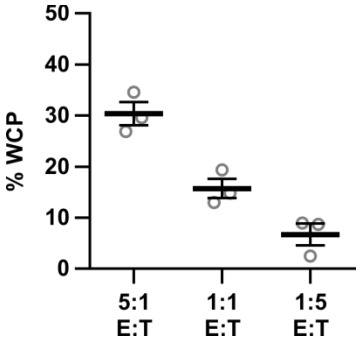

Figure S7. Effect of effector:target ratio on WCP activity. J774A.1 macrophages were plated for 18 hours, followed by addition of EdU-treated MDA-MB-453 cancer cells at different effector:target cell ratios in the presence of 1 µg/ml trastuzumab or PBS vehicle for 6 hours (3×10^5:6×10^4 cells for 5:1, 1.5×10^5:1.5×10^5 cells for 1:1 and 6×10^4:3×10^5 cells for 1:5 ratios, respectively). The samples were then harvested and stained for mouse CD45 and cancer cells accessible to the medium detected using labeled-pertuzumab, followed by fixation and staining for EdU. The samples were then analyzed by flow cytometry and % WCP was calculated as the fraction of EdU-positive cells that were CD45-positive and pertuzumab-negative. Error bars represent standard errors.

Figure S8

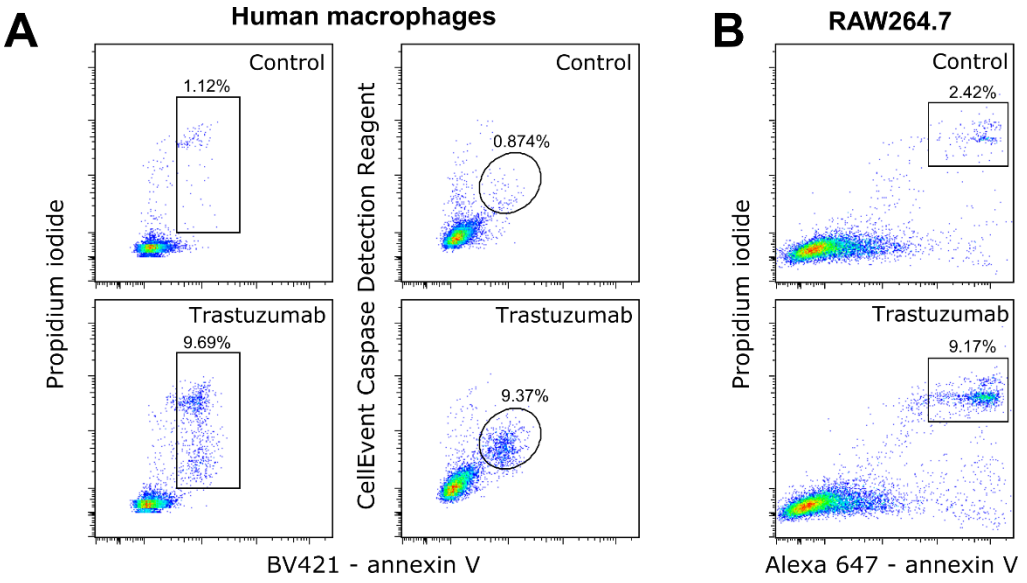

Figure S8. Macrophages induce apoptosis in opsonized cancer cells. MDA-MB-453 cells were co-incubated with human monocyte-derived macrophages or RAW264.7 macrophages at a 4:1 effector:target cell ratio in the presence of 1 µg/ml trastuzumab or PBS vehicle (control). Following 36 hours, cells were harvested and stained with fluorescently labeled annexin V and propidium iodide as indicated. CellEvent caspase-3/7 Green Detection Reagent was added to human macrophage:cancer cell co-cultures for the last 4 hours of culture prior to harvesting. Representative dot-plots for cancer cell populations following co-incubation with human macrophages (**A**) or RAW264.7 macrophages (**B**) are shown. Cell populations that are positive for cell death/apoptotic markers and their percentage (of total cancer cells) are indicated.

Figure S9

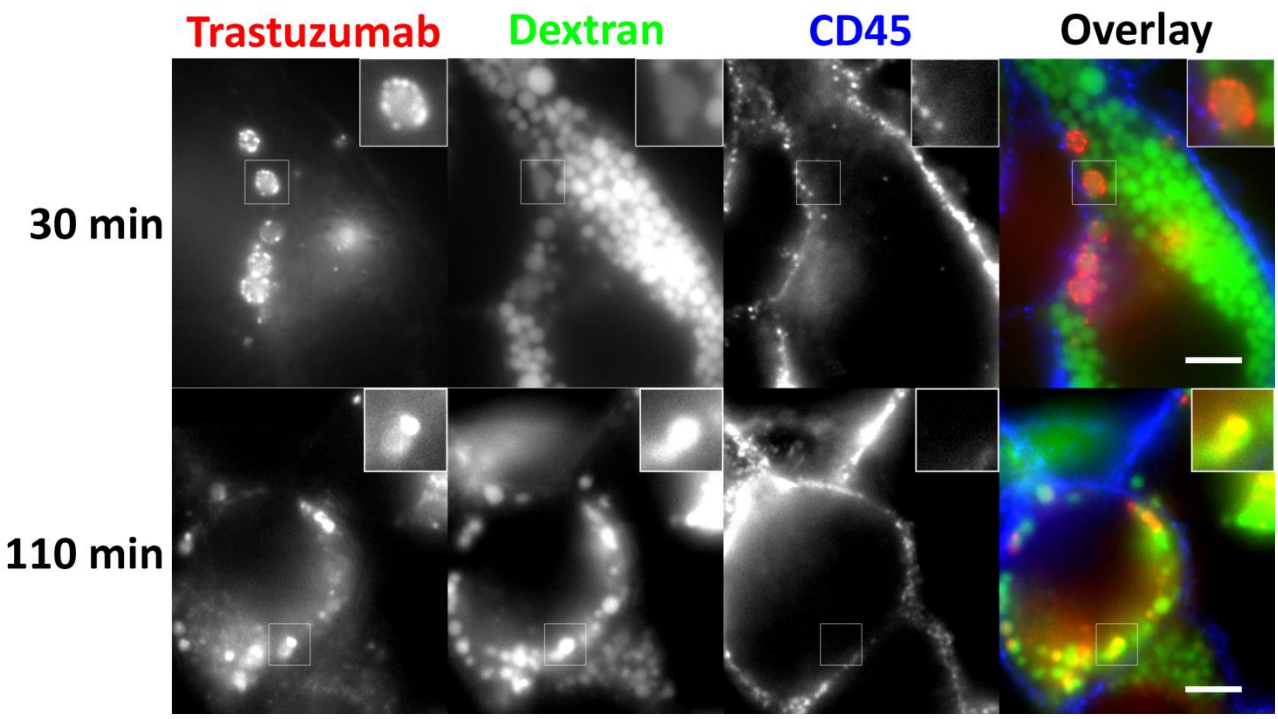

Figure S9. Trogosomes are rapidly targeted to lysosomes. MDA-MB-453 cells were harvested and opsonized by incubation with 10 µg/ml Alexa 555-labeled trastuzumab for ten minutes at room temperature followed by washing. J774A.1 macrophages (4×10^4 cells/dish) were incubated with the opsonized cancer cells (2.5×10^4 cells) for 30 minutes or 110 minutes, stained for mouse CD45 at room temperature (without fixation) followed by imaging. Prior to the addition of the cancer cells, lysosomes in J774A.1 macrophages were labeled by incubating cells with 100 µg/ml Alexa 488-labeled dextran for 1 hour followed by washing and incubating the cells in medium containing 25 ng/ml IFN-γ for 6 hours. Cells were imaged as live cells without fixation. Boxed regions are enlarged in the upper right-hand corners of each panel. Scale bars = 5 µm.

Figure S10

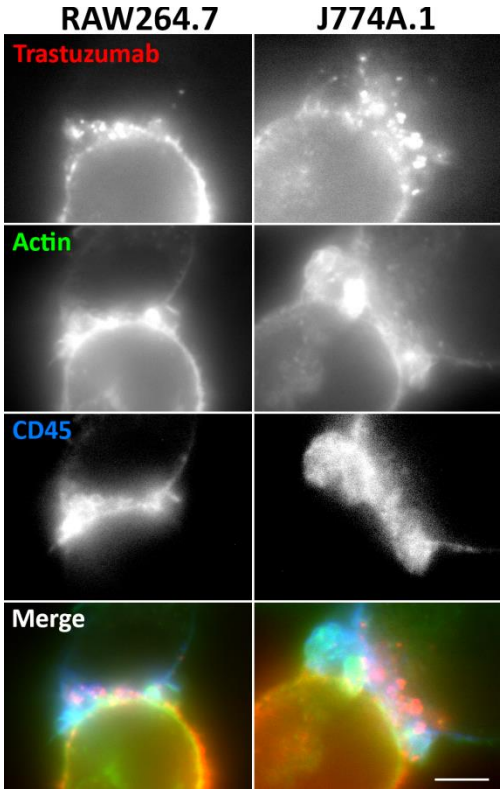

Figure S10. The macrophage:cancer cell synapse is similar for both RAW264.7 and J774A.1 macrophages. MDA-MB-453 cells were harvested and opsonized by incubation with 10 µg/ml Alexa 555-labeled trastuzumab for ten minutes at room temperature followed by washing. The opsonized cancer cells were then added to RAW264.7 or J774A.1 macrophages plated in a dish (2.5×10^4 cancer cells and 4×10^4 macrophages) and allowed to settle at room temperature for ten minutes. The dishes were subsequently transferred to 37°C for 2 minutes, fixed and stained for mouse CD45 and actin (using fluorescently labeled phalloidin). Scale bar = 5 µm.

Figure S11

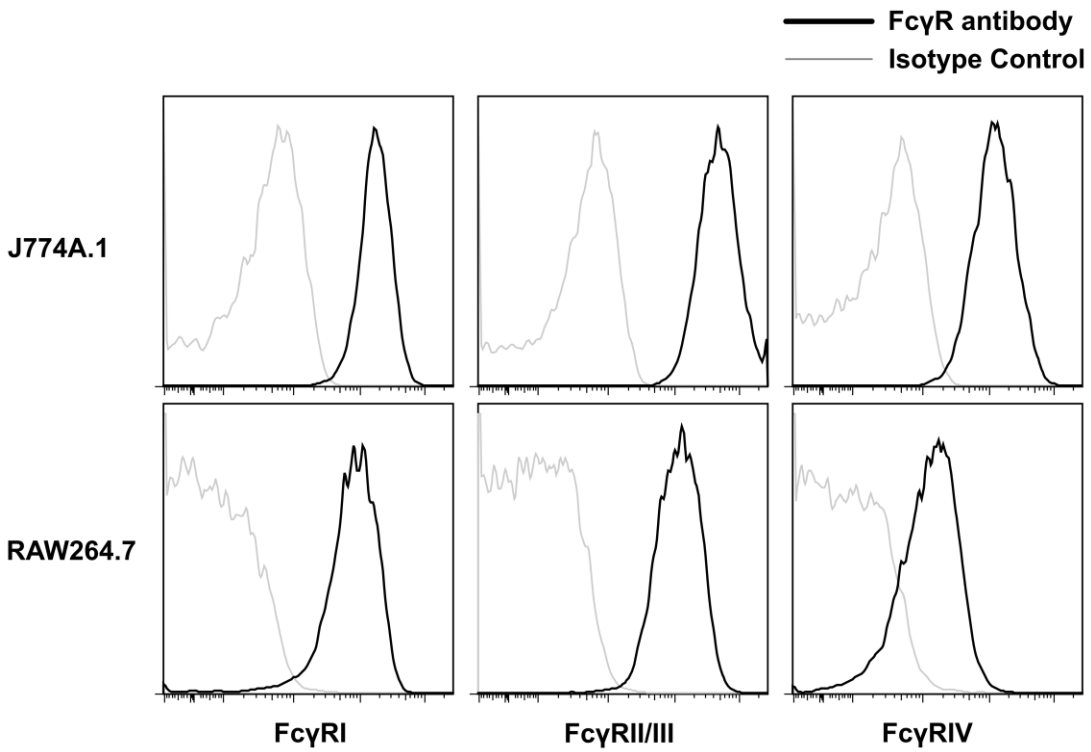

Figure S11. J774A.1 and RAW264.7 macrophages express all of the mouse FcγRs. The FcγR expression profile of J774A.1 and RAW264.7 macrophages was analyzed by incubation with phycoerythrin-labeled antibodies (1 µg/ml) specific for mouse FcγRs for 1 hour at 37°C. Cells were then harvested and analyzed by flow cytometry. Isotype controls for the respective FcγR-specific antibodies are shown in each histogram plot.

Figure S12

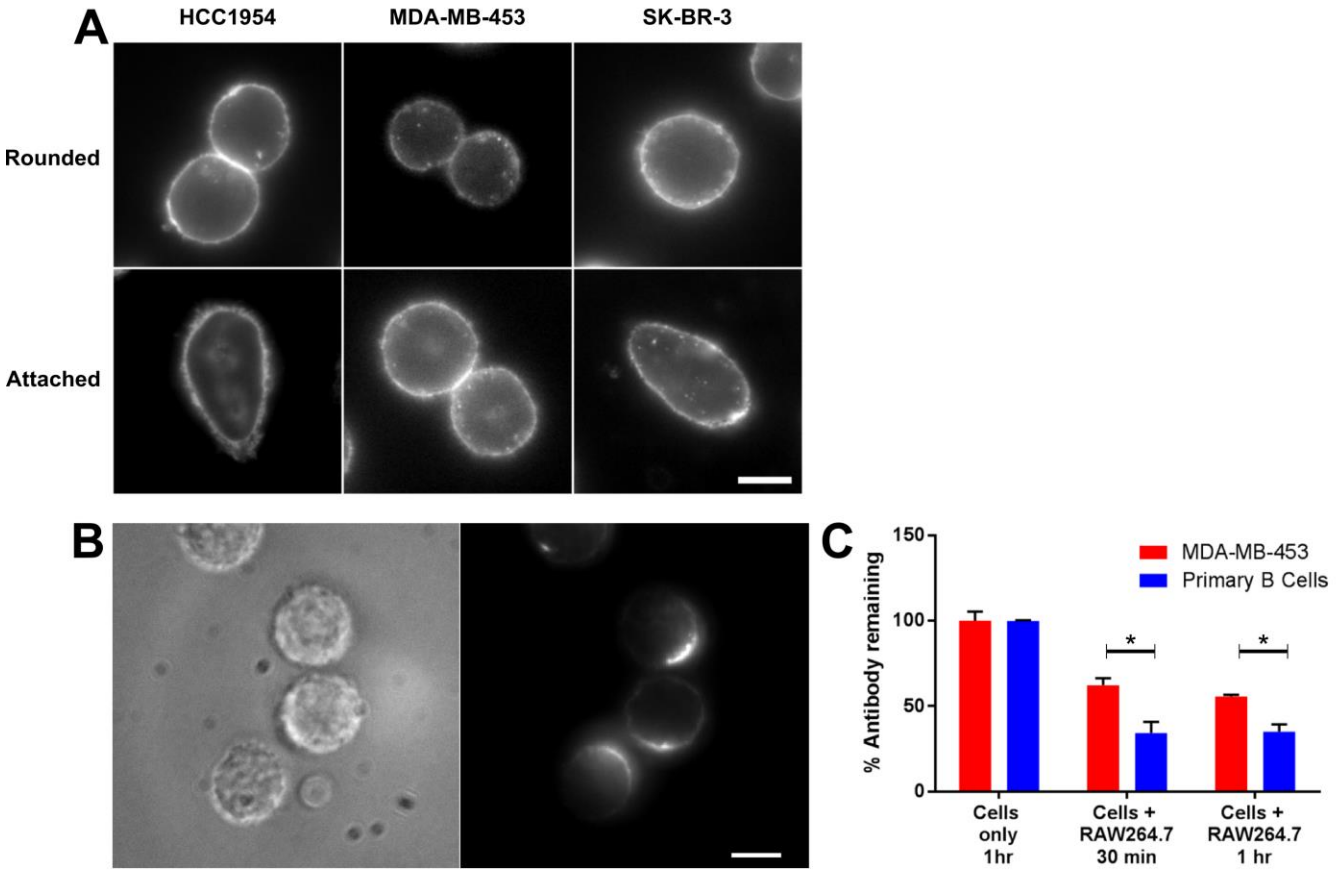

Figure S12. Breast cancer cell lines and B cells exhibit different capping behavior in response to antibody opsonization. **A,** HCC1954, MDA-MB-453 or SK-BR-3 cells were plated in glass-bottomed dishes overnight, followed by treatment with medium containing 10 µg/ml Alexa 555-labeled trastuzumab for 30 minutes at 37°C. Unbound trastuzumab was washed out and cells were fixed directly, or induced to round up by treatment with trypsin for 5 minutes at 37°C and then fixed, followed by microscopy analyses. **B,** primary B cells were opsonized with 10 µg/ml Alexa 555-labeled rituximab at room temperature for ten minutes, followed by washing and incubation at 37°C for 30 minutes. The cells were then transferred to MatTek dishes and analyzed as live cells by microscopy. **C,** primary B cells or MDA-MB-453 cells were opsonized with 10 µg/ml Alexa 555-labeled rituximab or 10 µg/ml

Alexa 555-labeled trastuzumab, respectively. The cells were then added to RAW264.7 macrophages in a 48-well plate for 30 minutes or 1 hour at a 4:1 effector:target cell ratio, harvested and the fluorescent antibody levels associated with the cancer cell populations quantitated using flow cytometry. Scale bars = 10 μm. Statistically significant differences between samples were determined using Student's *t*-test. * indicates significant differences between samples ($p < 0.05$).

Figure S13

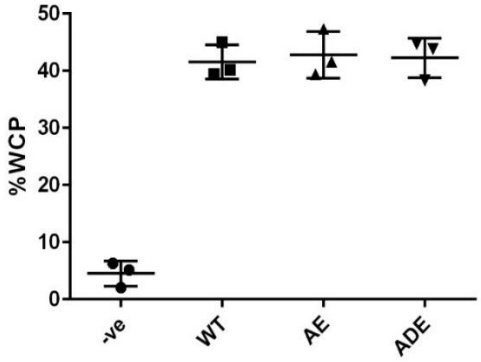

Figure S13. Engineered antibodies with enhanced FcγR binding affinities do not increase WCP activity during a 3 hour incubation. The WCP assay was performed as in Fig. 5B using thioglycollate-elicited macrophages from mice expressing human FcγRs and opsonized MDA-MB-453 cells as targets following a 3 hour incubation period. Samples were treated with PBS vehicle (-ve) or 10 μg/ml WT, AE or ADE variants of trastuzumab as indicated.

Figure S14

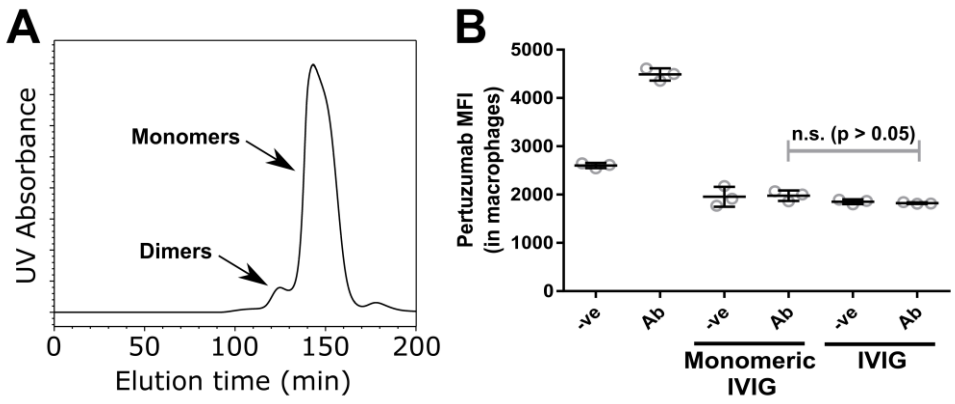

Figure S14. The inhibitory effect of IVIG on trogocytosis is not due to the effect of aggregated IgG or IgM. Size exclusion chromatography (HiLoad 16/600 Superdex 200 pg column) was used to separate the IgG monomers (150 kDa), IgG dimers, aggregates and IgM in IVIG. **A,** elution profile of IVIG from the size exclusion column showing monomeric and dimeric IgG peaks. **B,** the effect of the monomeric IgG fraction on trogocytosis was compared with that of IVIG. Thioglycollate-elicited macrophages isolated from C57BL/6 mice transgenically expressing human FcγRs (4) were plated with MDA-MB-453 cells at an effector:target ratio of 0.4:1 (4×10^4:1×10^5cells). 18 hours later, 1 µg/ml wild type (WT) trastuzumab, together with 0.5 µg/ml Alexa 488-labeled Fab fragments derived from pertuzumab, were added to the co-cultures for 60 minutes with monomeric IVIG or unfractionated IVIG at a final concentration of 10 mg/ml. As controls, cells were co-cultured without trastuzumab (-ve). The mean fluorescence intensity (MFI) values for pertuzumab Fab staining in the macrophage population are shown. Statistically significant differences between samples were determined using Student's *t*-test. n.s., no significant difference ($p > 0.05$).

Figure S15

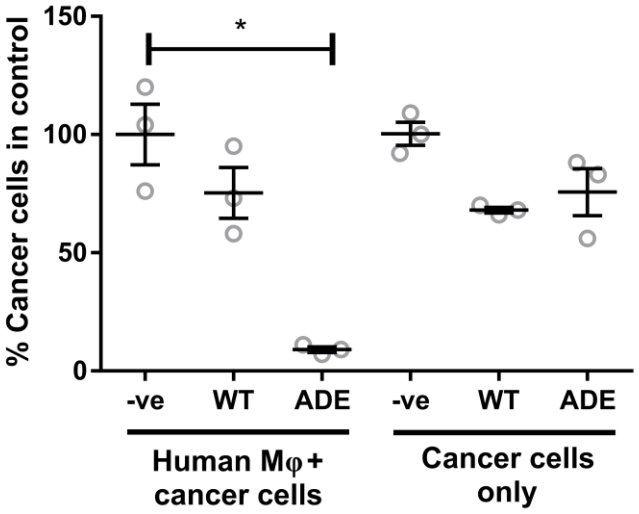

Figure S15. Human macrophage (Mφ) effectors can deplete cancer cells in the presence of IVIG and antibodies with enhanced binding affinities for FcγRs. 2×10^5 purified human monocytes were plated in 48 well plates with M-CSF for six days, followed by the addition of 5×10^4 MDA-MB-453 cancer cells. 24 hours later, mixtures of 10 μg/ml WT or ADE variants of trastuzumab were added with 10 mg/ml IVIG. PBS vehicle (-ve) and cancer cells plated without macrophages were used as controls. The medium was replaced by fresh medium containing the same additions after 3 days. Cells were harvested after 5 days and the remaining numbers of cancer cells quantitated. Error bars represent standard errors. Statistically significant differences between samples were determined using Student's *t*-test. * indicates significant difference between samples ($p < 0.05$).

Movie legends

Movie S1. Two-color live cell imaging of a J774A.1 macrophage phagocytosing MDA-MB-453 cancer cells. Macrophages with surface-labeled CD45 (green) engulf three trastuzumab-opsonized MDA-MB-453 cells (red). Time is indicated at the top left in minutes:seconds format. The movie plays at a speed of 340x real-time.

Movie S2. Transmitted-light imaging of the fate of an SK-BR-3 cell in a J774A.1:SK-BR-3 co-culture in the presence of trastuzumab. The left panel in the movie tracks an SK-BR-3 cell (yellow box) amongst J774A.1 macrophages in the presence of 1 μg/ml trastuzumab over 3 days. The right panel traces the absolute displacement of the tracked SK-BR-3 cell in the flask over time. The title on the right panel shows the time (days) and the speed at which the movie is being played (2700x real-time until 00:42 and 900x real-time after 00:42).

Movie S3. Transmitted-light imaging of the fate of an SK-BR-3 cell in a RAW264.7:SK-BR-3 co-culture in the presence of trastuzumab. Movie shows the fate of an SK-BR-3 cell as in Movie S2, but with RAW264.7 macrophages as the effector cells. The title on the right panel shows the time in days – hours:minutes format. The movie plays at a speed of 1400x real-time.

Movie S4. Transmitted-light imaging of an SK-BR-3 cell in a human macrophage co-culture in the presence of trastuzumab. Movie shows the fate of an SK-BR-3 cell as in Movie S2, but with human macrophages as effector cells. Purified human monocytes were plated six days prior to the addition of cancer cells. The title on the panel indicates the time progression in days – hours:minutes format. The movie plays at a speed of 3600x real-time.

Movie S5. Live cell imaging of macrophage-mediated trogocytosis. The movie shows trastuzumab from live, opsonized MDA-MB-453 cells being trogocytosed by CD45-labeled J774A.1 macrophages. Upper left panel shows signal from Alexa 555-labeled trastuzumab, upper right panel shows CD45 signal, lower left panel shows the transmitted light image and the lower right image shows the overlay of the upper two panels. The sample was imaged in the presence of caspase 3/7 detection reagent for which the signal is also detected in the CD45 channel. The movie plays at a speed of 340x real-time. Scale bar = 5 μm.

Movie S6. MUM images of trogocytic tubules. The movie corresponds to Figure 4B. Images of trastuzumab (left panels) or trastuzumab and MEM-GFP (red and green, respectively; right panels) from the cameras set at focal planes of ~ 1.2 μm and 1.8 μm in the MUM configuration are displayed. The cyan line in the left panels indicates the approximate edge of the macrophage, obtained by averaging and thresholding the MEM-GFP images. The movie pauses for 2 seconds at different timepoints to indicate the same features of the tubulation process (yellow arrows) presented as individual frames in Figure 4B. Time on the upper left is shown in minutes:seconds format. The movie plays at a speed of 27x real-time. Scale bar = 5 μm.

References

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4. Smith P, DiLillo DJ, Bournazos S, Li F, Ravetch JV. Mouse model recapitulating human Fcγ receptor structural and functional diversity. Proc Natl Acad Sci USA. 2012;109:6181-6.