Supporting Information

Zhao et al. 10.1073/pnas.1106550108

SI Methods

Cell Lines and Culture. All cell lines were cultured in RPMI medium 1640 (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (i.e., complete RPMI) at 37 °C, 5% CO_2. B16F10 and SKBR-3 cells were passaged and harvested by enzymatic treatment with trypsin (0.05%), EDTA (0.02%), PBS solution. Knockdown of CD47 in SKBR-3 cells was performed using *CD47* shRNA (5′-CCGGGCACAATTACTTGGACTAGTTCTCGAGAACTAGTCCAAGTAATTGTGCTTTTT-3′) that was introduced into the cells by lentiviral transduction according to instructions provided by the manufacturer (Sigma). CHO cells were transfected with full-length human signal-regulatory protein (SIRP)α_1, SIRPα_{BIT}, SIRPβ_1, and SIRPγ constructs and cloned by PCR from appropriate donors into pcDNA3.1 (Invitrogen) using FuGENE transfection reagent (Roche) as described by the manufacturer, and cells were selected with 50 mg/mL G418 (Gibco). Constructs were verified by Sanger sequencing. SIRPα_1 and SIRPα_{BIT} expression was verified by Western blotting using rabbit anti-human SIRPα directed against the invariable C-terminal cytoplasmic region (Abcam8120; Abcam) and mouse anti-human β-actin (clone AC-15; Sigma) as a loading control. IRDye 800CW-conjugated goat anti-rabbit IgG and IRDye 680-conjugated donkey anti-mouse IgG (LI-COR Biosciences) were used as secondary antibodies, and signals were visualized in an Odyssey Infrared Imaging System (LI-COR Biosciences). SIRPβ_1 and SIRPγ expression was verified by flow cytometry using B4B6 (1) and MRC OX119 (2) mAb, respectively.

Antibodies, CD47 Bead Binding Assay, and Flow Cytometry. The following primary antibodies were used: anti-mouse CD47 (Miap301, rat IgG_{2a}) (3), anti-human CD47 (B6H12, mouse IgG_1) (4), either as unconjugated intact antibody, $F(ab')_2$ fragments, or PE-labeled intact antibody (Santa Cruz Biotechnology), anti-human SIRPβ_1 (B4B6, mouse IgG_1) (1), anti-human SIRPγ (MRC OX119, mouse IgG_1) (2), anti-human CD64 (clone 10.1, mouse IgG_1; BD Pharmingen), anti-human CD32 (clone AT10, mouse IgG_1; AbD Serotec), anti-human CD16 (clone 3G8, mouse IgG_{2b}; BD Pharmingen), anti-mouse melanoma antigen gp75 (TA99, mouse IgG_{2a}) (5), and anti-human Her2/Neu (trastuzumab, human IgG_1; Roche). Where applicable, antibodies were produced in protein-free hybridoma medium containing 2% low-IgG FCS and purified by protein G- or protein A- Sepharose affinity chromatography. B6H12 $F(ab')_2$ fragments were generated by pepsin digestion. For flow cytometry, cells were collected as indicated, washed, and incubated in FACS buffer (PBS containing 0.1% BSA) with saturating concentrations of primary antibody for 30 min on ice. After washing, the cells were incubated with either Alexa 633-labeled anti-rat IgG antibody (Molecular Probes), Alexa 633-labeled anti-mouse IgG antibody (Invitrogen, Molecular Probes), or FITC-labeled anti-human IgG (M1325, mouse IgG_{2b}; Sanquin).

The primers 5′-GAGATCGATATCCCAGCTACTATTTAATAAAACAAAATC-3′ (forward) and 5′-GAGATCAGATCTAAACCATGAAACAACACGATATTTTAG-3′ (reverse) were used to amplify cDNA encoding the extracellular Ig domain of human CD47 and to clone it, using the EcoRV and BglII restriction sites, into the pFUSE IgG_1 Fc vector. The construct was verified by sequencing. The resulting fusion protein was produced by transfecting freestyle HEK293T cells (Invitrogen) according to instructions provided by the manufacturer. The CD47-Fc protein was purified by protein G-Sepharose affinity chromatography from the culture supernatant and tested for reactivity in Western blotting with anti-human CD47 and anti-human IgG antibodies. Fluorescent beads (carboxylate-modified TransFluoSpheres 488/645; Invitrogen) were covalently coated with streptavidin and subsequently used to capture biotinylated goat anti-human Fc (Jackson ImmunoResearch), followed by CD47-Fc. The beads were stored in PBS, 0.5% BSA, 0.02% NaN_3 (stock 1% NaN_3 in H_2O) and used for experiments within 3 mo. Binding to cells was done by incubating 50×10^3 cells with 20 μL of CD47 beads diluted 1:10 in PBS, 0.1% BSA for 45 min at 37 °C followed by a single washing step and flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences) equipped with FlowJo software (Tree Star).

Production of Monoclonal Antibodies. The extracellular domains (ECDs) of human SIRPα_1 corresponding to amino acids 1–364 and 1–365 were cloned using forward TOPO primer 3′-CACCATGGAGCCCGCCGGCC-5′ and reverse primer 3′-GAATAGCAGTAGCAGTCACAAGAGTCGCCGCCATAAAC-5′ into pCDNA 3.1D/V5-HIS-TOPO vector (Invitrogen). The C-terminal HIS-tagged SIRPα_1-ECD proteins were produced in freestyle HEK293T cells as indicated above and purified on Ni columns using an Ni-NTA Fast Start Kit (Qiagen). The HIS tags were removed by enterokinase cleavage using an enterokinase cleavage capture kit (Novagen), and the remaining SIRPα_1-ECD protein was dialyzed against PBS. Removal of the HIS tag was confirmed by Western blotting. Female BALB/c mice were immunized five times with 20 μg of SIRPα_1-ECD protein or five times with SIRPα_1-expressing CHO cells diluted in 50 μL of PBS and 50 μL of montanide adjuvant. The hybridomas 12C4 and 1.23A were generated, respectively, by standard hybridoma technology or by negative and positive panning, respectively, on SIRPβ_1- and SIRPα_1-expressing CHO cells followed by electrofusion technology (6). This was followed by repeated screening by ELISA and flow cytometry for reactive clones and subcloning by limiting dilution. Antibodies were produced by culture in serum-free/low-IgG medium and purified by protein-G-Sepharose affinity chromatography and dialyzed against PBS. Both antibodies were of the IgG_1 isotype.

1. Seiffert M, et al. (2001) Signal-regulatory protein α (SIRPα) but not SIRPβ is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature CD34(+)CD38(–) hematopoietic cells. *Blood* 97:2741–2749.
2. Brooke G, Holbrook JD, Brown MH, Barclay AN (2004) Human lymphocytes interact directly with CD47 through a novel member of the signal regulatory protein (SIRP) family. *J Immunol* 173:2562–2570.
3. Lindberg FP, et al. (1996) Decreased resistance to bacterial infection and granulocyte defects in IAP-deficient mice. *Science* 274:795–798.
4. Latour S, et al. (2001) Bidirectional negative regulation of human T and dendritic cells by CD47 and its cognate receptor signal-regulator protein-α: Down-regulation of IL-12 responsiveness and inhibition of dendritic cell activation. *J Immunol* 167:2547–2554.
5. Hara I, Takechi Y, Houghton AN (1995) Implicating a role for immune recognition of self in tumor rejection: Passive immunization against the brown locus protein. *J Exp Med* 182:1609–1614.
6. Steenbakkers PG, Hubers HA, Rijnders AW (1994) Efficient generation of monoclonal antibodies from preselected antigen-specific B cells. Efficient immortalization of preselected B cells. *Mol Biol Rep* 19:125–134.

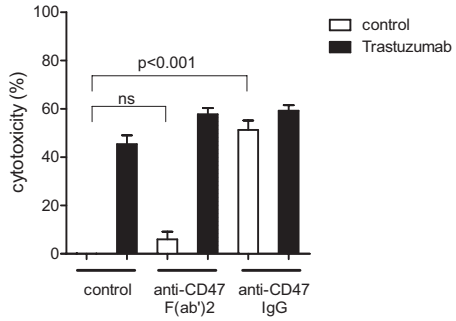

Fig. S1. Antibody-dependent cellular cytotoxicity of human neutrophils toward SKBR-3 breast cancer cells after preincubation with trastuzumab (10 μg/mL) and/or B6H12 (10 μg/mL) anti-CD47 F(ab′)$_2$ or intact IgG. Values shown are means ± SD (n = 3) from a representative experiment out of three. Note that intact anti-CD47 IgG alone but not anti-CD47 F(ab′)$_2$ induces neutrophil-mediated cytotoxicity. P values of statistically significant differences, as determined by Student's t test, are indicated. ns, nonsignificant.

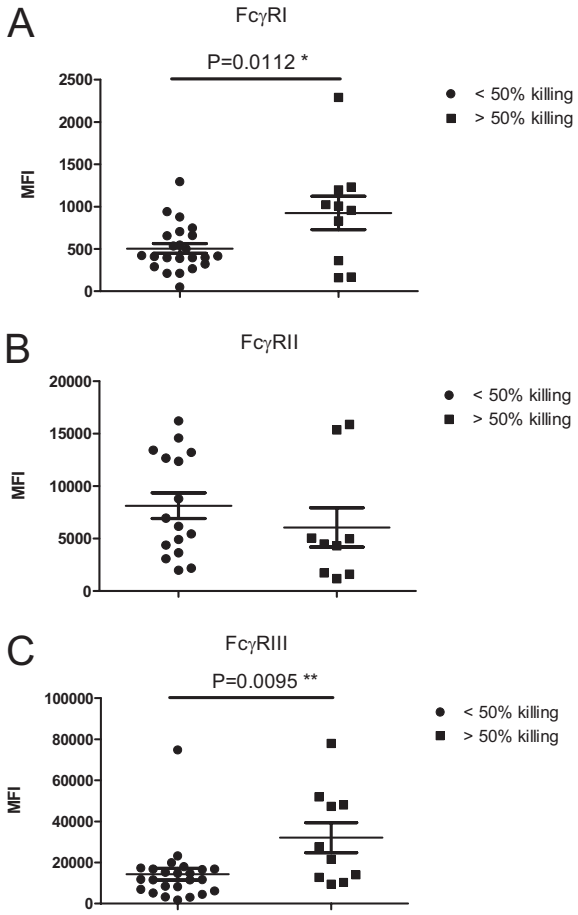

Fig. S2. Relationship between trastuzumab-induced neutrophil-mediated cytotoxicity against SKBR-3 cells and FcR expression. Experiments (n = 25) with individual neutrophil donors were divided into two groups based on high (above 50%) or low (below 50%) trastuzumab-dependent killing capacity. Surface expression levels (MFI) measured by flow cytometry of FcγRI (*A*) (using mAb 10.1, mouse IgG$_1$; BD Pharmingen), FcgRII (*B*) (using mAb AT10, mouse IgG$_1$; AbD Serotec), and FcγRIII (*C*) (clone 3G8, mouse IgG$_{2b}$; BD Pharmingen) combined with Alexa 633-labeled anti-mouse IgG antibody (Invitrogen, Molecular Probes) are shown. Note that statistically higher killing is associated with higher FcγRI and FcγRIII levels. P values of statistically significant differences, as determined by Student's t test, are indicated.

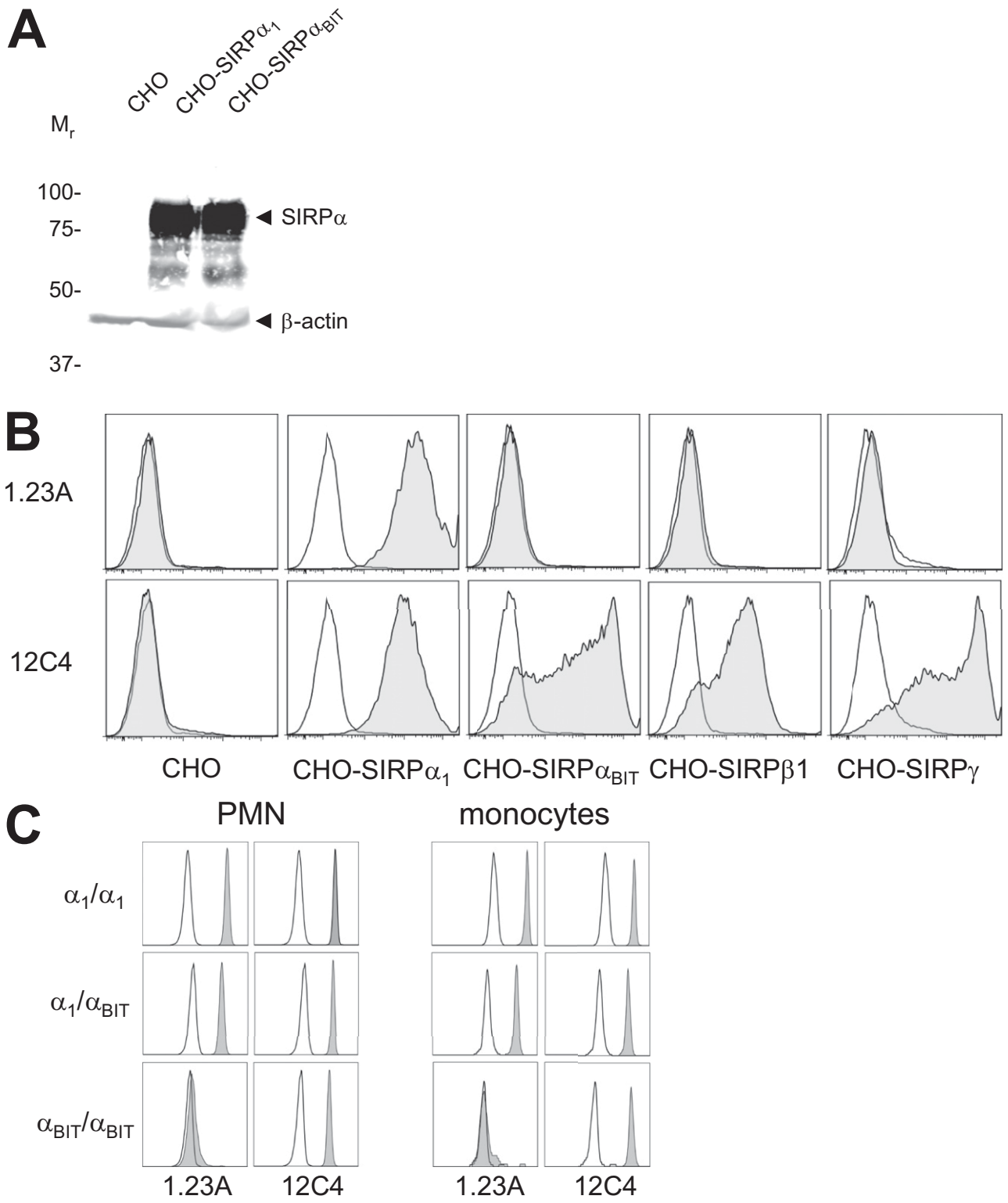

Fig. S3. Characterization of mAb against SIRPα. (*A*) Expression of the two SIRPα variants was verified by Western blotting using a polyclonal antibody against the invariant C terminus. (*B*) The 1.23A and 12C4 mAbs were generated as described in *SI Methods* and tested for binding to CHO cells transfected with the full-length SIRP family members SIRPα (including the two polymorphic variants SIRPα_1 and SIRPα_{BIT}), SIRPβ1, and SIRPγ (filled histograms). The open histogram represents the isotype control. Note that the 1.23A mAb selectively recognizes SIRPα_1, whereas 12C4 recognizes all of the indicated family members. (*C*) Flow cytometric surface staining of phagocytes, namely freshly isolated neutrophils (PMN) and monocytes, from individuals with α_1/α_1 or $\alpha_{BIT}/\alpha_{BIT}$ homozygotes or α_1/α_{BIT} heterozygotes using mAbs 1.23A and 12C4 (filled histograms). The open histogram represents the isotype control. Note that whereas mAb 12C4 recognizes both SIRPα alleles (and perhaps also SIRPβ_1), mAb 1.23A specifically recognizes the α_1 allele, with higher levels in the homozygote than in the heterozygote individual. Stainings shown are a representative example of 12 experiments.