Supplementary Figure Legends and Movie Legend

Figure S1. BYON4228 binds to a conserved epitope on the CD47-binding domain of SIRPα and blocked signaling

(A) BYON4228 (IgG1-L234A/L235A), KWAR23 (1) (IgG1-L234A/L235A) and isotype control (IgG1-L234A/L235A) binding to ExpiCHO-S cells that transiently expressed indicated (chimeric) SIRPα/γ molecules or not. Pictures visualize the composition of the chimeric SIRP molecules; $SIRP\alpha_{BIT}$, $SIRP\alpha_1$ and SIRPγ domains are represented in white, grey and black, respectively. The A/G letter abbreviation depicts the origin of a certain domain (SIRPα or SIRPγ); AAG means domain 1 and 2 from SIRPα, domain 3 from SIRPγ. Results depict the mean relative fluorescence unit +/- SD. (B) Differential heatmaps of differences in deuterium incorporation in antigens $SIRP\alpha_{BIT}$ (top) and $SIRP\alpha_1$ (bottom) alone and in the presence of BYON4228. Darker blue signifies a lower incorporation of deuterium in the presence of BYON4228 and suggests an interaction site between BYON4228 and antigen. (C) The BYON4228 epitope was mapped using hydrogen deuterium exchange mass spectrometry (HDX-MS) on the N-terminal Ig-like CD47-binding domain of $SIRP\alpha_{BIT}$ (top) and $SIRP\alpha_1$ (bottom). Projection of the BYON4228 footprint (orange, left). Indicated are the amino acid differences between $SIRP\alpha_1$ and $SIRP\alpha_{BIT}$ (turquoise blue, middle) and the overlap between the two (brown, right). (D) SIRPα signaling measured using the PathHunter Jurkat SIRPα signaling reporter cell line (DiscoverX), after co-incubation with indicated CD47-expressing or knock-out (KO) cells, in presence of a concentration-range of indicated antibodies. Results are shown as mean +/- SD of N=6 independent experiments.

Figure S2. BYON4228 augmented panitumumab- and cetuximab-induced ADCC of A431 cells

Neutrophil-mediated ADCC measured using the Cr-51 release assay after 4 hours incubation of target cells (A431) and effector cells (primary GM-CSF activated neutrophils) in the presence of a fixed dose of BYON4228 (10 µg/mL), isotype control (10 µg/mL) or nothing and a concentration range of panitumumab or cetuximab. Donors tested: 3 $SIRP\alpha_{BIT}/\alpha_{BIT}$, 3 $SIRP\alpha_{BIT}/\alpha_1$, and 3 $SIRP\alpha_1/\alpha_1$ donors. (A) Results show % killing of A431 cells by activated neutrophils of representative donors with indicated SIRPα genotypes. (B) Summary of ADCC results of all donors by plotting the % killing of A431 cells in presence or absence of BYON4228 or isotype control at 0 or 1 µg/mL anti-TAA (panitumumab or cetuximab). Results from the same donor are connected by lines. (C) Panitumumab (Pmab)- or cetuximab (Cmab)-induced ADCC fold enhancement of all donors (in absence of BYON4228 or isotype control). The fold enhancement ([% killing at 1 µg/mL anti-TAA]/[% killing without anti-TAA]) of anti-TAA-induced ADCC was calculated. The right graph illustrates how the fold enhancement was calculated. The left graph depicts the results of all donors. The lines indicate the mean values. (D) ADCC EC_{50} values of all donors of panitumumab and cetuximab in absence and presence of BYON4228 or isotype control. No EC_{50} values could be calculated from donors with incomplete curve saturation, no response or incorrect curve-fitting as indicated in the graph. (E) BYON4228 and isotype ADCC fold enhancement of all donors. The fold enhancement ([% killing at 1 µg/mL panitumumab or cetuximab + 10 µg/mL anti-SIRPα mAb or isotype]/ [% killing at 1 µg/mL panitumumab or cetuximab]) of panitumumab- or cetuximab-induced ADCC enhancement was calculated. The right graph illustrates how the fold enhancement was calculated. The left graph depicts the results of all donors. The lines

indicate the mean values. For C and E, * *P*<0.05, ** *P*<0.01, *** *P*<0.001; *P*>0.05 is not indicated. *P* values were calculated by a two-tailed t-test (C) and an one-way ANOVA followed by Tukey's multiple comparisons test (E).

Figure S3. BYON4228 augmented panitumumab-induced ADCC of the CRC cell line SW48 regardless of mutations downstream the EGFR signaling pathway

(A-E) Neutrophil-mediated ADCC measured using the Cr-51 release assay after 20 hours incubation of target cells (SW48) and effector cells (primary GM-CSF activated neutrophils) in the presence of a fixed dose of BYON4228 (10 µg/mL), isotype control (10 µg/mL) or nothing and a concentration range of panitumumab or cetuximab. Donors tested: 5 SIRP$\alpha_{BIT}/\alpha_{BIT}$, 4 SIRP$\alpha_{BIT}/\alpha_1$, and 3 SIRP$\alpha_1/\alpha_1$ donors. (A) Results show % killing of SW48 cells by activated neutrophils of representative donors with indicated SIRPα genotypes. (B) Summary of ADCC results of all donors by plotting the % killing of SW48 cells in presence or absence of BYON4228 or isotype control at 0 or 1 µg/mL panitumumab or cetuximab. Results from the same donor are connected by lines. (C) Panitumumab (Pmab)- or cetuximab (Cmab)-induced ADCC fold enhancement of all donors (in absence of BYON4228 or isotype control). The fold enhancement ([% killing at 1 µg/mL anti-TAA]/ [% killing without anti-TAA]) of anti-TAA-induced ADCC was calculated. The lines indicate the mean values. (D) ADCC EC_{50} values of panitumumab and cetuximab of all donors in absence and presence of BYON4228 or isotype control. No EC_{50} values could be calculated from donors with incomplete curve saturation, no response or incorrect curve-fitting as indicated in the graph. (E) BYON4228 and isotype ADCC fold enhancement of all donors. The fold enhancement ([% killing at 1 µg/mL anti-TAA + anti-SIRPα mAb or isotype]/ [% killing at 1 µg/mL anti-TAA]) of panitumumab- or cetuximab-induced ADCC enhancement was calculated. The lines indicate the mean values. (F-I) Neutrophil-mediated ADCC measured using the Cr-51 assay after 20 hours incubation of target cells (SW48 cells and isogenic SW48 mutant cells) and effector cells (primary GM-CSF activated neutrophils) in the presence of a fixed dose of BYON4228 (10 µg/mL) or nothing and a concentration range of panitumumab. Cell lines tested are SW48 (WT) (= parental cell line), SW48 KRAS exon 2 G12D, SW48 KRAS exon 2 G13D and SW48 BRAF V600E. Donors tested: 2 SIRP$\alpha_{BIT}/\alpha_{BIT}$, 2 SIRP$\alpha_{BIT}/\alpha_1$, and 2 SIRP$\alpha_1/\alpha_1$ donors. (F) Summary of ADCC results of all donors by plotting the % killing of SW48 cells in presence of BYON4228 at 0 or 10 µg/mL with or without 1 µg/mL panitumumab. Results from the same donor are connected by lines. (G) ADCC EC_{50} values of panitumumab for all donors in presence of absence of BYON4228. Results from the same donor are connected by a line. No EC_{50} values could be calculated from donors with incomplete curve saturation, no response or incorrect curve-fitting as indicated in the graph. (H) BYON4228 or isotype ADCC fold enhancements of all donors. The fold enhancement ([% killing at 1 µg/mL anti-TAA + anti-SIRPα mAb or isotype]/ [% killing at 1 µg/mL anti-TAA]) of panitumumab-induced ADCC enhancement was calculated. The lines indicate the mean values. For C, E and H, * *P*<0.05, ** *P*<0.01, *** *P*<0.001; *P*>0.05 is not indicated. *P* values were calculated by two-tailed t-tests (C: panitumumab vs cetuximab, H: for each cell line panitumumab + BYON4228 versus panitumumab + isotype) and one-way ANOVA followed by Tukey's multiple comparisons test (E).

Figure S4. BYON4228 enhanced daratumumab-induced ADCP of Daudi cells in a pan-allelic fashion

(A-C) Macrophage mediated ADCP measured using confocal microscopy after 3 hours incubation of target cells (Daudi) and effector cells (macrophages) in the presence of a fixed dose of daratumumab (5 ng/mL) and a concentration range of indicated antibodies or respective isotype controls. Donors tested: 4 SIRP$\alpha_{BIT}/_{BIT}$, 4 SIRPα_{BIT}/α_1, and 4 SIRPα_1/α_1 donors. (A) Results show phagocytosis index (PI) of macrophages from representative donors with indicated SIPRα genotypes. (B) SIRP-mAb induced ADCP EC_{50} values of all donors. No EC_{50} values could be calculated from donors with incomplete curve saturation, no response or incorrect curve-fitting as indicated in the graph. (C) SIRP-mAb induced ADCP fold enhancement of all donors. The means are depicted. The fold enhancement= [PI at 5 ng/mL daratumumab + 10 µg/mL anti-SIRP mAb]/ [PI at 5 ng/mL daratumumab]. (D-F) Macrophage mediated ADCP measured using the live-cell imaging pHrodo ADCP assay after up to 8 hours incubation of target cells (Daudi) and effector cells (macrophages) in the presence of a fixed dose of daratumumab (5 ng/mL) and a concentration range of indicated antibodies or respective isotype controls. Donors tested: 7 SIRP$\alpha_{BIT}/_{BIT}$, 3 SIRPα_{BIT}/α_1, and 6 SIRPα_1/α_1 donors. (D) Results show % phagocytosed tumor cells by macrophages from representative donors with indicated SIPRα genotypes. (E) SIRP-mAb induced ADCP EC_{50} values of all donors. No EC_{50} values could be calculated from donors with incomplete curve saturation, no response or incorrect curve-fitting as indicated in the graph. (F) SIRP-mAb induced ADCP fold enhancement of all donors. The means are depicted. The fold enhancement= [PI at 5 ng/mL daratumumab + 10 µg/mL anti-SIRP mAb]/ [PI at 5 ng/mL daratumumab]. For C and F, * P<0.05, ** P<0.01, *** P<0.001; P>0.05 is not indicated. P values were calculated by one-way ANOVA for each genotype (comparing the different mAbs) and for each mAb (comparing the different genotypes) followed by Tukey's multiple comparisons test (C) and an one-way ANOVA followed by Tukey's multiple comparisons test (F).

Figure S5. BYON4228 enhanced panitumumab and cetuximab-induced ADCP of HT-29 CRC cells in a pan-allelic fashion

Macrophage mediated ADCP measured using the live-cell imaging pHrodo ADCP assay after up to 8 hours incubation of target cells (HT-29) and effector cells (macrophages) in the presence of a fixed dose of panitumumab (A-C) or cetuximab (D-F) (both fixed at 40 ng/mL) and a concentration range of indicated antibodies or respective isotype controls. Donors tested (A-C): 7 SIRP$\alpha_{BIT}/_{BIT}$, 3 SIRPα_{BIT}/α_1, and 9 SIRPα_1/α_1 donors. Donors tested (D-F): 8 SIRP$\alpha_{BIT}/_{BIT}$, 2 SIRPα_{BIT}/α_1, and 6 SIRPα_1/α_1 donors. (A, D) Results show % phagocytosed tumor cells by macrophages from representative donors with indicated SIPRα genotypes. (B, E) SIRP-mAb induced ADCP EC_{50} values of all donors. No EC_{50} values could be calculated from donors with incomplete curve saturation, no response or incorrect curve-fitting as indicated in the graph. (C, F) SIRP-mAb induced ADCP fold enhancement of all donors. The means are depicted. The fold enhancement= [% phagocytosed cells at 40 ng/mL panitumumab or cetuximab + 10 µg/mL anti-SIRP mAb]/ [% phagocytosed cells at 40 ng/mL panitumumab or cetuximab]. For C and F, * P<0.05, ** P<0.01, *** P<0.001; P>0.05 is not indicated. P values were calculated by one-way ANOVA followed by Tukey's multiple comparisons test.

Figure S6. BYON4228 does not induce Fc dependent immune effector activation and Fc tail variants all potently enhance cetuximab-induced ADCC

(A) NK-mediated ADCC: Purified NK cells were incubated for 24 hours with indicated antibodies and MOLM-13 cells (effector: target = 4:1) and antibody-induced killing was determined. BYON5664 contains the variable domains of BYON4228 but has a wildtype IgG constant domain with wildtype effector functions. Left: representative donor. Right: results of N=4 donors using 20 µg/mL antibody. (B) CDC: U937 or MOLM-13 cells were opsonized with indicated antibodies and then treated with 0% baby rabbit serum, 12.5% heat inactivated (HI) baby rabbit serum or 12.5% baby rabbit serum. % survival was measured using CellTiter-Glo (CTG) luminescent assay. Data show the percentage of survival +/- SD of three independent experiments. (C) Blocking of CD47-induced SIRPα-mediated signal transduction: SIRPα signaling measured using the PathHunter Jurkat SIRPα signaling reporter cell line (DiscoverX), after co-incubation with CD47-expressing or knock-out (KO) Raji cells, in presence of a concentration-range of BYON4228 (IgG1-L234A/L235A) or BYON5306 which is a $F(ab')_2$ of BYON4228. Results are shown as mean +/- SD of N=6 independent experiments. (D-F) Neutrophil-mediated ADCC measured using the Cr-51 release assay after 4 hours incubation of target cells (A431) and effector cells (primary GM-CSF activated neutrophils) in the presence of a fixed dose of cetuximab (10 µg/mL) and a concentration range of indicated antibodies or respective isotype controls. Donors tested: 5 $SIRP\alpha_{BIT}/\alpha_{BIT}$, 4 $SIRP\alpha_{BIT}/\alpha_1$, and 5 $SIRP\alpha_1/\alpha_1$ donors. (D) Results show % killing of A431 cells by activated neutrophils of representative donors with indicated SIRPα genotypes. (E) SIRP-mAb induced ADCC EC_{50} values of all donors. No EC_{50} values could be calculated from donors with incomplete curve saturation, no response or incorrect curve-fitting as indicated in the graph. (F) SIRP-mAb induced ADCC fold enhancement of all donors. The means are depicted. The fold enhancement = [% killing at 10 µg/mL cetuximab + 67 nM anti-SIRPα mAb]/ [% killing at 10 µg/mL cetuximab]. For A, B and F, * $P<0.05$, ** $P<0.01$, *** $P<0.001$; $P>0.05$ is not indicated. *P* values were calculated by one-way ANOVA for each mAb (comparing the different treatment conditions) followed by Tukey's multiple comparisons test (B) and an one-way ANOVA followed by Tukey's multiple comparisons test (A, F).

Figure S7. BYON4228 and BYON4228-$F(ab')_2$ induce potent enhancement of rituximab and daratumumab-induced ADCP

Macrophage mediated ADCP was measured using confocal microscopy after 3 hours incubation of target cells (Raji, A-C or Daudi, D-F) and effector cells (macrophages) in the presence of a fixed dose of rituximab (80 ng/mL, A-C) or daratumumab (5 ng/mL, D-F) and a concentration range of indicated antibodies or respective isotype controls. BYON5306 is a $F(ab')_2$ fragment of BYON4228. Donors tested (A-C): 5 $SIRP\alpha_{BIT}/_{BIT}$, 6 $SIRP\alpha_{BIT}/\alpha_1$, and 6 $SIRP\alpha_1/\alpha_1$ donors, (D-F): 4 $SIRP\alpha_{BIT}/_{BIT}$, 4 $SIRP\alpha_{BIT}/\alpha_1$, and 4 $SIRP\alpha_1/\alpha_1$ donors. (A, D) Results show phagocytosis index (PI) of macrophages from representative donors with indicated SIPRα genotypes. (B, E) SIRP-mAb or -$F(ab')_2$ induced ADCP EC_{50} values of all donors. No EC_{50} values could be calculated from donors with incomplete curve saturation, no response or incorrect curve-fitting as indicated in the graph. (C, F) SIRP-mAb or -$F(ab')_2$ induced ADCP fold enhancement of all donors. The means are depicted. The fold enhancement= [PI at 80 ng/mL rituximab or 5 ng/mL daratumumab + maximum concentration of anti-SIRP mAb/$F(ab')_2$]/ [PI at 80 ng/mL rituximab or 5 ng/mL daratumumab]. For C and F, * $P<0.05$, ** $P<0.01$, *** $P<0.001$; $P>0.05$ is not indicated. *P* values were calculated by one-way

ANOVA for each mAb (comparing the different genotypes) followed by Tukey's multiple comparisons test and two-tailed t-tests for each genotype (comparing the different mAbs).

Figure S8. Characterization of huSIRPα$_{BIT}$ transgenic mice and BYON4228 pharmacokinetic studies in mice

(A) Western blotting of bone marrow-derived macrophages (BMDM) samples from *Cebpa*$^{+/+}$huSIRPα$_{BIT}$$^{Tg/Tg}$ or *Cebpa*$^{Cre/+}$huSIRPα$_{BIT}$$^{Tg/WT}$ or *Cebpa*$^{Cre/+}$huSIRPα$_{BIT}$$^{Tg/Tg}$ mice as indicated, or of human primary neutrophils (N). Indicated is murine (mu) SIRPα and human (hu) SIRPα. (B, C) FACS analysis for SIRPα expression on blood monocytes (B) and neutrophils (C) of indicated mice. FMO is fluorescence minus one. (D, E) ADCC (normalized to trastuzumab) by neutrophils of trastuzumab opsonized SK-BR-3 cells in absence or presence of an anti-SIRPα blocking mAb in *Cebpa*$^{+/+}$huSIRPα$_{BIT}$$^{Tg/Tg}$ (D) and *Cebpa*$^{Cre/+}$huSIRPα$_{BIT}$$^{Tg/Tg}$ (E) mice. * P<0.05 , ** P<0.01 Tukey's multiple comparison test, ns; not significant. (F-H) Pharmacokinetic (PK) of BYON4228 in C57Bl/6 mice (purple line) and huSIRPα$_{BIT}$ mice (*Cebpa*$^{Cre/+}$huSIRPα$_{BIT}$$^{Tg/Tg}$ or *Cebpa*$^{Cre/Cre}$huSIRPα$_{BIT}$$^{Tg/Tg}$) (orange/yellow lines) after single IV (F, H, solid line) or IP (G) dosing, or repeated IP dosing every 3 days for 6 times (H, dashed line). Concentration mean +/- SD, N=3 per time-point. Arrows in (H) indicate timepoints of dosing.

Figure S9. BYON4228 administration to cynomolgus monkeys does not induce anemia or thrombocytopenia

(A,B) Cellular binding of BYON4228 to human or cynomolgus granulocytes and the EC_{50} summary (N=65). (C-E) Measurement of hemoglobin levels, red blood cells and platelets after repeated administration of BYON4228 or vehicle to cynomolgus monkeys as indicated with the dotted lines at indicated doses (N=5, +/- SEM).

Supplementary Movies Legend. Visualization of real-time phagocytosis using live-cell imaging

Panitumumab-opsonized (40 ng/mL) HT-29 tumor cells were labeled with pHrodo and co-incubated with unlabeled macrophages and BYON4228 (10 µg/mL). Images were taken every 3 minutes for 4 hours. The light-red tumor cells become bright red upon phagocytosis by macrophages. Two representative movies are shown.

References

1. Ring NG, Herndler-Brandstetter D, Weiskopf K, Shan L, Volkmer JP, George BM, et al. Anti-SIRPalpha antibody immunotherapy enhances neutrophil and macrophage antitumor activity. Proc Natl Acad Sci U S A. 2017;114(49):E10578-E85.