Supplementary Methods

BYON4228 antibody development and humanization

First, rabbit antibodies were generated. After immunization of rabbits (MAB Discovery GmbH, Neuried, Germany) with the ECDs of different human SIRPα allelic variants, cell culture supernatants derived from individual B cells were tested in enzyme-linked immunosorbent assays (ELISA) for binding to various SIRP family members from different species (human, mouse, monkey), and for interference with CD47-SIRPα binding. Selected clonal B cell cultures were used to obtain the heavy and light chain sequences for the corresponding antibodies and used to generate rabbit-human chimeric mAbs. After further selection (e.g. in the ADCC assay), the chimeric mAbs were further humanized by replacing amino acids present in antibodies with demonstrated high production levels in mammalian cells and low immunogenicity in humans.

HEFLB, SIRPAB-11-K322A and 1H9 antibody sequences

HEFLB: antibody amino acid sequences identical to those described in patent application WO 2017/178653 HEFLB (1):

>HEFLB_heavy_chain
EVQLVQSGAEVKKPGESLRISCKASGYSFTSYWVHWVRQMPGKGLEWMGNIDPSDSDTHYSPSFQGH
VTLSVDKSISTAYLQLSSLKASDTAMYYCVRGGTGTLAYFAYWGQGTLVTVSSASTKGPSVFPLAPCSRSTS
ESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPS
NTKVDKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVD
GVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYT
LPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG
NVFSCSVMHEALHNHYTQKSLSLSPGK
>HEFLB_light_chain
DVVMTQSPLSLPVTLGQPASISCRSSQSLVHSYGNTYLYWFQQRPGQSPRLLIYRVSNRFSGVPDRFSGS
GSGTDFTLKISRVEAEDVGVYYCFQGTHVPYTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLN
NFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTK
SFNRGEC

SIRPAB-11-K322A: The antibody amino acid sequences of antibody SIRPAB-11 as made at Byondis are identical to those described in patent application WO 2020/068752 SIRPAB-11-K322A (2):

> SIRPAB-11-K322A _heavy_chain
QVQLVQSGAEVKKPGASVKVSCKASGYTFRGYGISWVRQAPGQGLEWMGWISAYGGETNYAQKLQG
RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAREAGSSWYDFDLWGRGTLVTVSSASTKGPSVFPLAPSSK
STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH
KPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKGQPR
EPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
> SIRPAB-11-K322A _light_chain
DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQQKPGKAPKLLIYAASNLQSGVPSRFSGSGSGTD
FTLTISSLQPEDFATYYCQQGASFPITFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE
AKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE
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1H9: The antibody amino acid sequences of antibody 1H9 as made at Byondis are identical to those described in patent application WO 2019/023347 humanized 1H9 (3), with a G1m17,1 allotype:

>1H9_heavy_chain
QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWITWVKQAPGQGLEWIGDIYPGSGSTNHIEKFKSKATL
TVDTSISTAYMELSRLRSDDTAVYYCATGYGSSYGYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGG
TAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT
KVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQ
GNVFSCSVMHEALHNHYTQKSLSLSPGK
>1H9_light_chain
DIQMTQSPSSLSASVGDRVTITCRASENIYSYLAWYQQKPGKAPKLLIYTAKTLAEGVPSRFSGSGSGTDFT
LTISSLQPEDFATYYCQHQYGPPFTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Sequences of WT and domain swap SIRP variants, used for expression on ExpiCHO-S cells and soluble expression

Full-length human SIRPα_1 (CAA71403), human SIRPα_{BIT} (NP_542970), human SIRPβ1v1 (O00241), human SIRPβ1v2 (Q5TFQ8) and human SIRPγ (Q9P1W8), including three ECDs, a transmembrane domain and an intracellular domain were expressed by using their germline signal peptide sequences. Also, domain swap SIRP variants were made and used to identify binding specificity of subjected antibodies to either the first, second or third domain of human SIRPα_1, human SIRPα_{BIT}, or human SIRPγ. The boundaries of each of the domains were based on UniProt annotations. The chimeric SIRP variants were assembled by either replacing human SIRPα_{BIT} or human SIRPα_1 domains by the human SIRPγ domain while retaining the human SIRPα membrane anchor.

Human SIRPα_{BIT} ECD, human SIRPα_1 ECD, human SIRPβ1v1 ECD and human SIRPβ1v2 ECD were expressed as fusion protein, all containing an Avi-tag, a Factor Xa (FXa) cleavage site and an Fc tail for purification. The ECD of human CD47 (Q08722) was expressed as fusion protein using an Fc tail and Avi-tag only and human SIRPγ ECD was fused to the combination of an Avi-tag and a C-tag.

>hSIRPα_{BIT} ECD_AviTag_FXa_Fc
EEELQVIQPDKSVLVAAGETATLRCTATSLIPVGPIQWFRGAGPGRELIYNQKEGHFPRVTTVSDLTKRNN
MDFSIRIGNITPADAGTYYCVKFRKGSPDDVEFKSGAGTELSVRAKPSAPVVSGPAARATPQHTVSFTCES
HGFSPRDITLKWFKNGNELSDFQTNVDPVGESVSYSIHSTAKVVLTREDVHSQVICEVAHVTLQGDPLRG
TANLSETIRVPPTLEVTQQPVRAENQVNVTCQVRKFYPQRLQLTWLENGNVSRTETASTVTENKDGTYN
WMSWLLVNVSAHRDDVKLTCQVEHDGQPAVSKSHDLKVSAHPKEQGSNTAAENTGSNERNGGGLND
IFEAQKIEWHEIEGRDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

>hSIRPα_1 ECD_AviTag_FXa_Fc

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EEELQVIQPDKSVSVAAGESAILHCTVTSLIPVGPIQWFRGAGPARELIYNQKEGHFPRVTTVSESTKREN
MDFSISISNITPADAGTYYCVKFRKGSPDTEFKSGAGTELSVRAKPSAPVVSGPAARATPQHTVSFTCESH
GFSPRDITLKWFKNGNELSDFQTNVDPVGESVSYSIHSTAKVVLTREDVHSQVICEVAHVTLQGDPLRGT
ANLSETIRVPPTLEVTQQPVRAENQVNVTCQVRKFYPQRLQLTWLENGNVSRTETASTVTENKDGTYN
WMSWLLVNVSAHRDDVKLTCQVEHDGQPAVSKSHDLKVSAHPKEQGSNTAAENTGSNERNGGGLND
IFEAQKIEWHEIEGRDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

>huSIRPβ1v1 ECD AviTag_FXa_Fc
EDELQVIQPEKSVSVAAGESATLRCAMTSLIPVGPIMWFRGAGAGRELIYNQKEGHFPRVTTVSELTKRN
NLDFSISISNITPADAGTYYCVKFRKGSPDDVEFKSGAGTELSVRAKPSAPVVSGPAVRATPEHTVSFTCES
HGFSPRDITLKWFKNGNELSDFQTNVDPAGDSVSYSIHSTARVVLTRGDVHSQVICEIAHITLQGDPLRGT
ANLSEAIRVPPTLEVTQQPMRAENQANVTCQVSNFYPRGLQLTWLENGNVSRTETASTLIENKDGTYN
WMSWLLVNTCAHRDDVVLTCQVEHDGQQAVSKSYALEISAHQKEHGSDITHEAALAPTAPLGGGLNDI
FEAQKIEWHEIEGRDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ
VYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

>huSIRPβ1v2 ECD AviTag_FXa_Fc
EEELQVIQPDKSISVAAGESATLHCTVTSLIPVGPIQWFRGAGPGRELIYNQKEGHFPRVTTVSDLTKRNN
MDFSIRISNITPADAGTYYCVKFRKGSPDHVEFKSGAGTELSVRAKPSAPVVSGPAARATPQHTVSFTCES
HGFSPRDITLKWFKNGNELSDFQTNVDPAGDSVSYSIHSTAKVVLTREDVHSQVICEVAHVTLQGDPLRG
TANLSETIRVPPTLEVTQQPVRAENQVNVTCQVRKFYPQRLQLTWLENGNVSRTETASTLTENKDGTYN
WMSWLLVNVSAHRDDVKLTCQVEHDGQPAVSKSHDLKVSAHPKEQGSNTAPGPALASAAPLGGGLN
DIFEAQKIEWHEIEGRDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

>huSIRPγ ECD AviCtag
EEELQMIQPEKLLLVTVGKTATLHCTVTSLLPVGPVLWFRGVGPGRELIYNQKEGHFPRVTTVSDLTKRNN
MDFSIRISSITPADVGTYYCVKFRKGSPENVEFKSGPGTEMALGAKPSAPVVLGPAARTTPEHTVSFTCES
HGFSPRDITLKWFKNGNELSDFQTNVDPTGQSVAYSIRSTARVVLDPWDVRSQVICEVAHVTLQGDPLR
GTANLSEAIRVPPTLEVTQQPMRVGNQVNVTCQVRKFYPQSLQLTWSENGNVCQRETASTLTENKDGT
YNWTSWFLVNISDQRDDVVLTCQVKHDGQLAVSKRLALEVTVHQKDQSSDATPKGQDNSADIQHSGG
RSSLEGPRFEGKPIPNPLLGLDSTRTGGGGLNDIFEAQKIEWHEACAAADYKPGGGKPGGEPEA

Vector construction and cloning strategy of mAbs and SIRP variants (for soluble expression and ExpiCHO-S cell based expression)

For expression of antibody chains and SIRP variants the mammalian expression vector pcDNA3.4-TOPO (Thermo Fisher Scientific) was used, which contains an expression cassette consisting of a CMV promoter and a BGHpA poly-adenylation site. Antibody chain amino acid sequences and soluble SIRP and CD47 variants were back-translated into a complementary DNA (cDNA) sequence and codon-optimization was performed for expression in human cells.

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Codon optimization was also performed for cell surface expressed SIRP variant sequences for expression in CHO cells. The antibody chain and SIRP variant cDNAs with flanking AscI and NheI cloning sites were synthesized by assembly of synthetic oligos and/or PCR products (Thermo Fisher Scientific) and individually cloned into the single gene vector pcDNA3.4-TOPO. For co-expression of cell surface expressed SIRP variants and green fluorescent protein (GFP) as reporter double gene vectors (DGV) were constructed. First, the DGV was made by excision of the CMV:BGHpA expression cassette from the pcDNA3.1(-) plasmid (Thermo Fisher Scientific) and re-inserted back into the same original vector (still containing an intact CMV:BGHpA expression cassette), to allow expression of two genes from a single plasmid vector. Next, unique multiple cloning sites (MCS) were created to allow cloning of the synthesized GFP gene into the first MCS using BamHI and DraIII restriction sites. Finally, the resulting vector was digested with AscI and NheI restriction enzymes, and ligated with the SIRP variant cDNA fragment, digested with the same restriction enzymes, and thereby introducing the SIRP variant cDNA into the second MCS. After transfer to *E. coli* K12 DH10B T1R and expansion, large-scale production of the final vectors for transfection was performed using the EndoFree Plasmid Maxi kit according to the manufacturer's instructions (Qiagen).

Antibody expression in Expi293F cells and purification

Expi293F cells were cultured in Expi293 Expression medium and maintained in an incubator at 37 °C, 8% CO_2 and 80% humidity on an orbital shaker platform rotating at 100 rpm with a stroke of 50 mm and according to the manufacturer's instructions (Thermo Fisher Scientific). Expi293F cells were transfected using the transfection agent FectoPRO according to the manufacturer's instructions (Polyplus-transfection). Antibodies were transiently expressed by co-expression of heavy and light chain expression vectors at a 1:1 mass ratio. Six days post transfection, the cell culture supernatant was harvested by centrifugation at 4,000 g for 15 minutes and filtering the clarified harvest over MF75 filters (Nalgene). The antibody concentrations in supernatant were determined by ForteBio Octet QK384 using Protein A Biosensors and trastuzumab as calibrator according to manufacturer's instructions (Sartorius). Antibodies were purified from the clarified harvest using protein A (MabSelect SuRe, Cytiva). After elution with 25 mM NaOAc pH 3.0, the antibodies were rebuffered to 4.2 mM histidine, 50 mM trehalose pH 6.0 for storage. The antibody quality was checked by SDS-PAGE using a Criterion TGX Stain Free Precast Gel of 4-20% (Bio-Rad) and using Size Exclusion Chromatography to determine the level of soluble high molecular weights (HMW).

Expression of SIRP variants on ExpiCHO-S cells

ExpiCHO-S cells were cultured in ExpiCHO Expression medium according to the manual provided by Thermo Fisher Scientific and maintained in an incubator at 37 °C, 8% CO_2 and 80% humidity on an orbital shaker platform rotating at 120 rpm with a stroke of 25 mm. For the generation of batches of cell surface-expressed SIRP variants, ExpiCHO-S cells were transfected using ExpiFectamine CHO as transfection agent according to manufacturers' protocols. For co-expression of the SIRP variants and GFP the DGVs were used. Transfected cell cultures were placed at standard culture conditions for 24 hours before being cryopreserved for later use in binding assays.

Production and purification of soluble SIRP variants and soluble CD47

Soluble SIRP variants and soluble CD47 were produced as Fc fusion variants or with a C-tag.

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The soluble Fc fusion variants were purified from the clarified harvest using protein A (MabSelect SuRe, Cytiva), according to the instruction of the supplier. After elution the antigen-Fc variants were rebuffered to 4.2 mM histidine, 50 mM trehalose pH 6.0 for storage. The antigen-Fc quality was checked by SDS-PAGE using a Criterion TGX Stain Free Precast Gel of 4-20% (Bio-Rad) and using Size Exclusion Chromatography to determine the level of soluble HMW.

The soluble antigen-C-tag variants were purified from the clarified harvest using CaptureSelect C-tagXL resin (Thermo Fisher Scientific) according to the instructions of the supplier. Elution was done with 20 mM tris-HCl, 2 M $MgCl_2$, pH 7.0. After elution product containing fractions were pooled and dialyzed to 4.2 mM histidine, 50 mM trehalose pH 6.0 using dialysis membranes with a MWCO of 10 kDa (Thermo Fisher Scientific). The product was then further concentrated using a Vivaspin Turbo 15 (Satorius) with a 30 kDa molecular weight cut-off.

Removal of the Fc tag and biotinylation of soluble SIRP proteins and CD47; coupling of CD47 to fluorescent beads

The Fc tag was removed by digestion with Factor Xa (FXa). First the purified antigen-Fc variants were rebuffered to 20 mM Tris-HCl, 100 mM NaCl, 2 mM $CaCl_2$ pH 8.0 using a PD10 desalting column (Cytiva). The antigen-Fc variants were incubated with FXa (Biolabs) in a 1:50 (w/w) ratio (FXa : antigen-Fc) and incubated for 23 h at 37 °C. The digestion was followed by SDS-PAGE using a 4-20% gradient Stain-Free gel (Bio-Rad) and upon completion the reaction was stopped with a 10-fold molar excess dansyl-glu-gly-arg-chloromethylketone. The Fc part and undigested antigen-Fc was removed from the antigen using protein A (MabSelect SuRe, Cytiva) . The soluble antigen is found in the flow through of the column while the Fc containing parts are eluted with 25 mM NaOAc pH 3.0. The flow through was then rebuffered to 10 mM Tris-HCl, 0.5 M NaCl pH 8.0 using a PD10 desalting column (Cytiva) and loaded on a Benzamidine FF (High Sub) column (Cytiva). The soluble antigen is found in the flow through while the FXa binds to the Benzamidine FF (High Sub) column. The flow through was then rebuffered to 10 mM Tris-HCl, 5 mM NaCl pH 8.0 for biotinylation. The removal of the FXa was followed by SDS-PAGE using a Criterion TGX Stain-Free Precast Gel of 4-20% (Bio-Rad).

CD47-Fc and soluble SIRP proteins were biotinylated using the BirA500 kit (biotin-protein ligase standard reaction kit, Avidity) according to the manufacturer's protocol. CD47-Fc was then conjugated to FluoSpheresNeurtAvidin-labeled microspheres stock (Thermo Fisher Scientific, F8774) at a final concentration of 50 µg/mL according to manufacturer's instructions.

Generation of F(ab')$_2$ fragments

Protein A purified antibody was rebuffered to 0.2 M NaOAc pH 4.0 using PD10 desalting columns (Cytiva). Pepsin from porcine gastric mucose (Sigma) was dissolved in 10 mM HCl and diluted to 0.1 mg/mL. Pepsin was added to the antibody in a 1:5 molar ratio (pepsin:antibody) and incubated at 37 °C. After 4 hours the digestion was stopped by adding 2 M Tris until a neutral pH. Remaining undigested antibody was removed using protein A (MabSelect SuRe, Cytiva) were the F(ab')$_2$ fragment was found in the flow through of the column. Pepsin was removed from the F(ab')$_2$ fragment and further purified using protein L

(Capto L, Cytiva) in bind and elute mode, where the $F(ab')_2$ fragment was eluted with 0.1 M acetic acid pH 3.0. The $F(ab')_2$ fragment was rebuffered to 4.2 mM histidine, 50 mM trehalose pH 6.0. The quality of the $F(ab')_2$ fragment was checked by SDS-PAGE using a Criterion TGX Stain-Free Precast Gel of 4-20% (Bio-Rad), a single band of about 100 kDa was found.

Cell lines and cell culture

An overview of the cell lines used in this study can be found in Table 4. The generation of SK-BR-3, A431 and Raji CD47-KO cells was described earlier (4-6).

Table 4: Overview of cell lines used and their culture conditions

Cell line	Origin	Cell culture medium
U937	ATCC CRL-1593.2	RPMI-1640 (Gibco)/10% FBS/penicillin-streptomycin (Lonza)
Daudi	DSMZ ACC78	RPMI-1640 (Gibco)/10% FBS/penicillin-streptomycin (Lonza)/GlutaMAX (Gibco
PathHunter cells	DiscoverX	Assay complete cell culture reagent (DiscoverX)
SK-BR-3	ATCC HTB-30	IMDM (Gibco)/20% FBS/2mM L-glutamine/penicillin-streptomycin
A431	DSMZ ACC 91	RPMI-1640 (Gibco)/10% FBS/penicillin-streptomycin (Lonza)
SW48 (WT) = SW48 (006) SW48 BRAF V600E, SW48 KRAS G12D, SW48 KRAS G13D	Horizon Discovery	RPMI-1640 (Gibco)/10% FBS/penicillin-streptomycin (Lonza)
Raji	DSMZ ACC319	RPMI-1640 (Gibco)/10% FBS/penicillin-streptomycin (Lonza)
HT-29	ATCC HTB-38	McCoys 5A (Lonza)/10% FBS/penicillin-streptomycin (Lonza)
OCI-AML2	DSMZ ACC 99	MEM Alpha Eagle (Lonza)/20% FBS/penicillin-streptomycin (Lonza)
MOLM-13	DSMZ ACC 554	RPMI-1640 (Gibco)/10% FBS/penicillin-streptomycin (Lonza)

Origin of healthy donor blood, SIRPα genotyping and phenotyping

SIRPα variant expression (i.e. $SIRP\alpha_{BIT}$ and/or $SIRP\alpha_1$) of donor immune effector cells was determined by either DNA sequencing and/or phenotyping using flow cytometry. SIRPα genotypes of individual donors were determined by Sanger sequencing using genomic DNA isolated from PBMCs of healthy Caucasian donors with the QIAamp kit (Qiagen). SIRPα polymorphic variants, namely $SIRP\alpha_1$ or $SIRP\alpha_{BIT}$, were identified by sequencing the V-Ig domain encoded by the third exon. For PCR amplification and sequencing the following primers were used, located in the introns surrounding exon 3, thus amplifying the whole exon. Forward PCR primer: AACACTTGAGGAAACACAGAG. Reverse PCR primer: CACCTACCACCACACCTGA. Primer used for Sanger sequencing: AAAAAATGACTGCTTTGTGCTCCTTTCC. Phenotyping of donors was performed using a

fluorescent-labeled antibody against SIRPα_{BIT} (clone 4G5, internal production) and a fluorescent-labeled antibody that has preference for binding to SIRPα_1 (internal production).

Affinity measurements

Two different assay setups were used for SIRP ECD binding analysis by surface plasmon resonance (SPR). Data were analyzed using double reference subtraction (DRS): Responses of a sample cycle (Flow $cell_{specific}$ – Flow $cell_{background}$) subtracted with response of a blank cycle (Flow $cell_{specific}$ – Flow $cell_{background}$). All assays were performed at 25 °C.

Antigen on surface

C-terminal Avi-tag biotinylated human SIRPα_1, SIRPα_{BIT}, SIRPβ1v1, SIRPβ1v2 and SIRPγ ECD was captured on a streptavidin surface (Biacore) to a capture level allowing the maximum binding (Rmax) < 50 RU. Responses of a concentration series were used to estimate the K_D-obs by single cycle kinetics (SCK). After DRS, a 1:1 Langmuir model was used to fit the kinetic data.

Antibody on surface

Antibodies were captured on an anti-human-IgG (Fc) surface to a capture level allowing the maximum binding (Rmax) < 50 RU. Responses of a concentration series of human SIRPα_1, SIRPα_{BIT}, SIRPβ1v1, SIRPβ1v2 or SIRPγ ECD was used to estimate the K_D-obs by SCK. After DRS, the data were fitted with a 1:1 Langmuir model.

The assay to estimate the observed binding affinity (K_D-obs) between human FcγRI (Sino Biological, 10256-H27H-B) and BYON4228 consisted of capture of recombinant FcγRI receptor containing a C-terminal biotinylated Avi-tag to a streptavidin sensor surface (Biacore). Binding of 5 increasing concentration of BYON4228 was measured by SPR using SCK. The K_D-obs were estimated from a 1:1 Langmuir interaction model fitted to the measured data.

The assay to estimate a K_D-obs between human FcγRIIIa, FcγRIIIb, FcγRIIa (or FcγRIIb (all from ACRO biosystems) and BYON4228 consisted of capture of each Fcγ receptor containing a C-terminal biotinylated Avi-tag to streptavidin sensor tips. Response of a concentration series of BYON4228 was measured simultaneously on eight sensor tips by bio-layer interferometry (BLI, Octet). The data was evaluated by a steady state affinity model.

Cellular binding to SIRP-expressing ExpiCHO-S cells and primary granulocytes

For cellular binding to ExpiCHO-S cells, transiently transfected ExpiCHO-S cells were rapidly thawed at 37 °C in a water bath. Cells were transferred to a tube containing RPMI-1640 with 10% HI FBS, centrifuged at 250-300xg to remove DMSO and the pellet was resuspended to a concentration of $1x10^6$ cells/mL in ice-cold FACS buffer (PBS + 0.1% v/w BSA + 0.02% v/v Sodium Azide (NaN_3)). Then, $1x10^5$ cells/well were added to 96-well V-shaped plates (100 µL/well), centrifuged at 300xg for 3 minutes, and the supernatant was discarded.

For staining of primary cells, heparinized whole blood samples were obtained from Vietnamese cynomolgus monkeys (Labcorp Drug Development, formerly Covance, Germany) and healthy human donors. One mL whole blood was transferred to a 15 mL tube and samples were lysed with ice-cold 1X BD Pharm Lyse lysing solution (555899), until a color change occurred from opaque red to dark/transparent red. After centrifugation, the pellet was again incubated with 5 mL BD Pharm Lyse lysing solution for 5 minutes, washed twice with 5 mL IMDM (Lonza, BE12-722Q) + 10% HI FBS (10100-147, Gibco), and finally the pellet was resuspended in 5 mL IMDM + 10% FBS. Subsequently, $2x10^5$ cells per well were stained in 96-well round-bottom microtiter plates.

For staining, cells were incubated for 30 minutes with 50 µL serial diluted antibodies in ice-cold FACS buffer, washed three times by centrifugation at 300xg for 3 minutes and resuspended in 50 µL APC-conjugated secondary $F(ab')_2$ goat anti-human IgG (Fc fragment specific, Jackson ImmunoResearch, 109-136-098, 1:500, diluted in FACS buffer), and incubated on ice. After 30 minutes of incubation, the cells were washed twice in FACS buffer and then resuspended in 50 µL ice-cold FACS buffer which contained DAPI (diluted to 0.1 µg/mL) for the ExpiCHO-S binding and analysis was performed using flow cytometry (FACSymphony or FACSVerse, BD Bioscience). For ExpiCHO-S binding, the APC-700 MFI was determined after gating on GFP-positive live, single cells. For the mock transfected cells, gating on GFP-positive cells was not possible and therefore gating was performed on viable, single cells. For primary binding to granulocytes, granulocytes gating was based on FSC-A/SSC-A.

HDX-MS

HDX-MS was performed by the Chemical Proteomics Core Facility of the Karolinska Institutet, Sweden. For this, $hSIRP\alpha_{BIT}$ or $hSIRP\alpha_1$ (Fc tail removed with Factor Xa cleavage) were combined with 1:1 molar ratio mAb BYON4228. The volume of the antigen/mAb and control samples were equalized using a 10 kDa protein concentrator. Samples were analyzed in an automated HDX-MS system (CTC Pal/Biomotif HDX) where samples are automatically labeled, quenched, digested, cleaned and separated at 2 °C. Control and antigen/mAb samples were deuterium labeled in triplicate for 4, 10 and 60 minutes. The labeling reaction was quenched by decreasing the pH to ~2.3 and temperature to ~4 °C by adding a solution containing 6 M Urea, 100 mM tris(2-carboxyethyl)phosphine and 0.5% trifluoroacetic acid. Digestion was performed using an immobilized pepsin column (2.1 × 30 mm) at 60 µL /minute, for 2 minutes followed by an on-line desalting step using a 2 mm I.D x 10 mm length C-18 pre-column (ACE HPLC Columns, Aberdeen, UK) using 0.1% formic acid at 400 µl/minutes for 1 minute. Peptic peptides were then separated by a 18 minutes 8-55 % linear gradient of acetonitrile in 0.1% formic acid using a 2 mm I.D x 50 mm length HALO C18/1.8 µm analytical column operated at 60 µL/minute. Peptides were sequenced using an LTQ Elite Orbitrap mass spectrometer (Thermo Fisher Scientific) operated at 120,000 resolution at m/z 400. All HDX MS data was processed by a HDExaminer Version 2.5.1. Mascot was used for peptide identification using a dedicated database, using a 10 ppm precursor tolerance, 0.05 Da MS/MS mass error. Visualizations of 3D protein structures and overlaying binding sites were rendered by PyMol 2.5.2 (Schrödinger). Protein data bank numbers 4CMM ($SIRP\alpha_1$) and 2JJS ($SIRP\alpha_{BIT}$) were used (7, 8).

CD47-bead and mAb binding to U937 cells and primary cells

Thawed PBMCs (125.000 cells/well) or cultured U937 (50,000 cells/well) cells were stained in a 96-well U-bottom plate with 50 µl diluted AF647-labeled antibodies at the indicated final concentration (Invitrogen, A20186) plus Fc block (Miltenyi, 130-059-901, used for PBMCs only) for 30 minutes at 4 °C and subsequently, 45 µL cell solution (U937) or 40 µL cell solution (PBMCs) was transferred to a new 96-well U-bottom plate containing 5 µL 10% CD47-bead solution or 10 µL 5% CD47-bead solution plus anti-CD3 APC-H7 (BD Pharmingen, 560176, clone SK3, 20x diluted) and anti-CD14 PerCp-Cy5.5 (BD Pharmingen, 550787, clone M5E2, 20x diluted), respectively. This plate was incubated for 30 minutes at 4 °C, the cells were washed twice, resuspended in 100 µL ice-cold FACS buffer containing DAPI (0.1 µg/mL) and analyzed using flow cytometry (FACSVerse, BD Biosciences). MFIs (FITC and AF647) were

determined on DAPI-negative single cells (U937) and DAPI-negative CD3-positive or CD14-positive single cells (PMBCs).

Blocking of CD47-induced signaling (PathHunter)

Jurkat SIRPα_{BIT} signaling cells (DiscoverX) were incubated with a concentration range of anti-SIRP antibody in combination with CD47 ligand cells. Ratio of Jurkat SIRPα_{BIT}:ligand cells used were 1:2 for Raji cells, 1:1.5 for SK-BR-3 cells and 1:2.5 for A431 cells. The assay was performed in a 384-wells plate. 12.5 µL SIRPα_{BIT} signaling cell suspension ($0.8x10^6$ cells/mL) was added to each well followed by 2.5 µL of a 11x concentrated anti-SIRPα antibody solution in PBS-0.1% BSA. The assay was started by adding 12.5 µL CD47 ligand cell suspension ($1.6x10^6$ Raji cells/mL, $1.2x10^6$ SK-BR-3 cells/mL, and $2.0x10^6$ A431 cells/mL). Plates were incubated for 5 hours at 37 °C. After incubation, 2 µL of reagents A (DiscoverX detection kit) in PBS-BSA 0.1% solution was added. Plates were incubated for 30 minutes on a shaker (300 rpm), in the dark, at room temperature (RT). Then, 10 µL of reagents B (DiscoverX detection kit) in PBS-BSA 0.1% solution was added. Plates were incubated for 1 hour on a shaker (300 rpm) in the dark at RT and luminescence was measured (Envision, PerkinElmer).

Complement dependent cytotoxicity (CDC) assay

Cells were incubated with diluted antibodies (final concentration 10 µg/mL) for 15 minutes at RT in a volume of 87.5 µL in a 96-wells plate. Then, 12.5 µL baby rabbit complement serum (BRC; Bio-Rad) or HI BRC (30 minutes at 56 °C) was added and incubated for 1 hour at 37 °C and live cells were determined using CTG (Promega) by measuring luminescence (Envision, PerkinElmer). Percentage survival was calculated by dividing the measured luminescence for each mAb by the measured luminescence of the corresponding no mAb control cells (0% BRC, 12.5% HI BRC or 12.5% BRC) multiplied by 100.

NK cell-based antibody dependent cellular cytotoxicity (NK-ADCC)

NK cells were isolated from buffy coats using the human NK cell isolation kit (Biolegend, 480054) and were used freshly or thawed to determine NK-ADCC. NK cells (effector cells, E) were incubated for 24 hours with 15,000 MOLM-13 cells (target cells, T) and antibodies in 96-well plates in 150 µL at an effector:target ratio of 4:1. Cytotoxicity was determined using the Cytotoxicity detection kit PLUS (LDH, 4744934001, Roche/Merck Life Science) and absorbance was measured (Envision, PerkinElmer). For each sample, the absorbance (A) was calculated as the absorbance at 490 nm minus the background absorbance at 630 nm. The maximally-observed absorbance ($A_{(max\ T)}$) was obtained by chemical lysis of target cells. The % killing = $(A_{(sample)} - A_{(E\ only)} - A_{(T\ only)}) / (A_{(max\ T)} - A_{(T\ only)}) \times 100$. The % antibody-induced killing = $\%\ killing_{(E+T+Ab)} - \%\ killing_{(E+T)}$.

Neutrophil-ADCC

Human polymorphonuclear cells, which are mainly neutrophils, were isolated by density gradient centrifugation and RBC lysis and activated at a concentration of $5x10^6$ cells/mL at 37 °C for 30 minutes with 10 ng/mL of human GM-CSF (Peprotech). Target cells were labeled with 100 µCi Cr-51 (PerkinElmer) for 90 minutes at 37 °C. Then after washing, target cells, effector cells and antibodies were co-cultured for 4 hours or 20 hours (SW48 cells) at 37 °C and 5% CO_2 at an effector: target ratio of 50:1. The supernatant was harvested and analyzed

for radioactivity in a MicroBeta^2 Microplate Counter (PerkinElmer). The percentage of killing was calculated as [(experimental release - spontaneous release)/(total release - spontaneous release)] *x* 100%. The spontaneous release is defined as the release from target cells in absence of neutrophils. The total release is defined as the release from target cells in presence of 0.05% Triton-X100. All conditions were measured in triplicate. Normalization was calculated using the bottom and top values of the fitted curves as '(value –bottom)/(top – bottom) *x* 100%'.

ADCP using confocal microscopy

ADCP using confocal microscopy was performed with CD20-positive target cell lines. Monocytes were isolated from buffy coats using the RosetteSep human monocyte enrichment cocktail (STEMCELL technologies, 15028) according to manufacturer's instructions and frozen. Thawed monocytes were seeded at 30,000 cells/96-well in 100 µL and differentiated to macrophages using 50 ng/mL M-CSF (MACS Miltenyi Biotec) in medium (IMDM without phenol red, + 8.5% HI FBS + penicillin/streptomycin + glutaMAX) for 7 days. On day 3 or 4, the medium was aspirated and fresh medium containing 50 ng/mL M-CSF was added. For the phagocytosis assay, target cells were labeled with 5 µM CellTrace Far Red (CTFR, Live Technologies) for 20 minutes at 37 °C. After washing, target cells were resuspended to 0.6x10^6 cells/mL in assay medium (IMDM without phenol red + 0.5% HI FBS + penicillin/streptomycin + 100 µg/mL Privigen-IVIg). A master plate was generated with assay medium containing anti-TAA (e.g. rituximab or daratumumab) plus serial diluted anti-SIRP-mAb or isotype controls + target cells (30,000/100µL). After aspiration of medium from the macrophages, 100 µL/well of the master plate was directly transferred to the macrophage plate. After 3 hours incubation at 37 °C + 5% CO_2, non-adherent cells were removed by washing twice with PBS and 65 µL fixation solution was added (BD cytofix, BD biosciences) and incubated for 10 minutes on ice and 10 minutes at RT. Cells were washed with PBS, and 100 µl FACS buffer (PBS + 0.1% v/w BSA + 0.02% v/v Sodium Azide (NaN_3) was added. For CD19 staining, FACS buffer was removed and 50 µL FACS buffer containing anti-CD19 PE (1:200, clone REA675, MACS Miltenyi Biotec) + Hoechst 33342 (10 µg/mL) was added and incubated for 30 minutes in the dark at RT. Cells were washed twice with FACS buffer and measured on the ImageXpress (Molecular Devices). Full well single plane fluorescent images were acquired. Data was analyzed using MetaXpress, and the PI was determined. PI = number of phagocytosed tumor cells (CTFR-positive CD19-negative)/total number of macrophages (Hoechst-positive CTFR-negative) *x* 100. 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].

ADCP using live-cell imaging and pHrodo

ADCP using live-cell imaging and pHrodo was performed for CD20-negative cell lines and Daudi cells. Macrophages were differentiated from monocytes as described in 'ADCP using confocal microscopy'. Target cells were labeled with pHrodo (Sartorius, 0039) for 60 minutes in a 37 °C waterbath. Cells were washed twice and resuspended at 0.6x10^6 cells/mL in assay medium (IMDM without phenol red + 0.5% HI FBS + penicillin/streptomycin + 100µg/mL Privigen IvIg). A master plate was generated with assay medium containing anti-TAA plus serial diluted or a fixed concentration of BYON4228 or isotype controls + target cells (30,000 cells/well). After aspiration of medium from the macrophages, 100 µL/well of the master plate was directly transferred to the macrophage plate. The plate was placed in the IncuCyte

(37 °C + 5% CO_2) for 8 hours. Images were taken every 30 or 60 minutes. Data was analyzed using IncuCyte 2019B rev2 software and phagocytosis was determined by selection of pHrodo bright objects. % phagocytosed tumor cells = number of phagocytosed tumor cells (pHrodo bright)/total number of tumor cells (pHrodo low) *x* 100. ADCP fold enhancement = [% phagocytosed tumor cells at 10 µg/mL anti-SIRP mAb in combination with panitumumab, cetuximab or daratumumab]/ [% phagocytosed tumor cells with panitumumab, cetuximab or daratumumab only or isotype control mAb] or = [% phagocytosed cells at 31.6 µg/mL anti-SIRP]/ [% phagocytosed cells at effector + target].

Generation and characterization of huSIRPα$_{BIT}$-transgenic mice

Mice were generated that expressed human SIRPα$_{BIT}$. A full-length human SIRPα$_{BIT}$ cDNA sequence was inserted into the *Gt(ROSA)26Sor* locus and crossed to *Cebpa*$^{Cre/+}$ mice (9), creating human SIRPα$_{BIT}$-transgenic mice with a selective expression on myeloid cells (Rosa26-stop^{flox}human SIRPα$_{BIT}$ x *Cebpa*$^{Cre/+}$ (9, 10), named huSIRPα$_{BIT}$ mice). These mice where then crossed with scid mice to yield huSIRPα$_{BIT}$-scid mice. Western blotting of bone marrow-derived macrophage samples was performed after cultured for 7 days with CSF-1. Endogenous mouse SIRPα and transgenic human SIRPα were detected simultaneously using rabbit antibodies directed against the SIRPα cytoplasmic domain (Abcam #AB8120). For FACS analysis whole blood was lysed with lysis buffer and blocked with CD16/CD32 blocking antibody clone 2.4G2 for 30 minutes on ice. Cells stained with anti-human SIRPα in 20% normal goat serum containing PBS, washed 1x in PBS + 3% albumin and stained with the mix of directly labelled antibodies F4/80 APC-Cy7, CD19 PerCP-Cy5.5, CD3 Pacific Blue, mouse SIRPα APC, CD11b AF488 and a secondary antibody anti-human IgG AF568. For ADCC assays, bone marrow isolated neutrophils were overnight stimulated with human G-CSF (10 ng/mL) and IFNγ (50 ng/mL). SK-BR-3 target cells were labelled with 100 mCi Cr-51 (PerkinElmer), incubated in RPMI culture medium supplemented with 10% (v/v) fetal calf serum together with stimulated neutrophils in an effector:target ratio of 50:1 in the presence of trastuzumab and anti-SIRPα antibody 12C4 (4). After incubation of target cells and effector cells for 4 hours, supernatant was harvested and analyzed for radioactivity in a gamma counter (Wallac). The percentage of cytotoxicity was calculated as [(experimental cpm-spontaneous cpm)/(total cpm- spontaneous cpm)] *x* 100%. All conditions were measured in triplicate.

PK mouse

BYON4228 was administered by injection into the caudal vein (IV), or injection into the peritoneal cavity (IP) of huSIRPα$_{BIT}$ transgenic mice or C57BL/6mice at doses; 3, 10 or 30 mg/kg. The administration volume was 10 mL/kg. For repeat IP dosing BYON4228 was administered every 3 days for 6 times (Q3Dx6). Blood was collected into collection tubes with anticoagulant (K2 EDTA) and plasma was used for PK analysis. BYON4228 plasma levels were determined as explained in the supplementary method section ‘generic antibody assay for determination of BYON4228 levels in mice and cynomolgus monkeys’.

Raji xenograft in huSIRPα$_{BIT}$ transgenic mice

Tumors were induced by subcutaneous injection of $2x10^7$ Raji cells in RPMI 1640 medium into the flank of female huSIRPα$_{BIT}$-scid transgenic animals. Raji tumor cell implantation was performed 24 to 72 hours after a whole body irradiation with a γ-source (1.44 Gy, 60Co).

BYON4228 was administered IP 3 times a week for 4 weeks ((Q2Dx3) x 4) at a dose of 5 mg/kg. Rituximab was administered at 1 mg/kg IP.

Safety evaluation of BYON4228

BYON4228 was studied *in vitro* for its propensity of hemolysis and RBC clumping in human whole blood as well as precipitate formation in plasma.

A cytokine release assay was performed using heparinized-preserved whole blood obtained from 18 healthy donors who provided informed consent (Immunomonitoring services, Sanquin Pharma & Biotech services). For this, BYON4228 (10 µL) was added to round-bottom 96-well plates, whole blood was added (190 µL) and the plates were incubated overnight at 37 °C in 5% CO_2. The supernatants were assayed for 18 different cytokines and chemokines using The Human ProcartaPlex™ Inflammation Panel on a Luminex FlexMap 3D.

A single dose range toxicity and PK study was conducted in naïve cynomolgus monkeys (*Macaca fascicularis*, 1 animal/sex/group) at 0, 1, 3, 10, 30 and 100 mg/kg BYON4228 via a 30 or 100 minutes IV infusion. All animals were euthanized at day 22 for necropsies.

A 5-cycle toxicity study was performed in which four groups of naïve cynomolgus monkeys (*Macaca fascicularis*, 5 animals/sex/group) received 5 IV, 30 minutes infusions of 0, 3, 10, 30 mg/kg, each 1 week apart. Two animals/sex in the control group and three animals per sex per dose group were sacrificed one week after the last dose, whereas 2 animals per sex per group were allowed an additional recovery period of 7 weeks and were then sacrificed. In-life evaluations included mortality/moribundity, clinical observations, body weight, food consumption, standard neurologic and respiratory/cardiovascular safety, clinical pathology, urinalysis (including urine chemistry) and pharmacokinetics (including ADAs). Upon terminal sacrifice, macroscopic and microscopic evaluation of selected tissues was performed.

Generic antibody assay for determination of BYON4228 levels in mice and cynomologus monkeys

A biotinylated camel single chain domain specifically directed to the constant domains of human IgG (CaptureSelect human IgG-Fc PK Biotin Conjugate, Thermo Fisher Scientific) was used for capturing the analyte. This antibody was coated to a streptavidin-coated ELISA plate (EvenCoat Plate, R&D Systems). An horseradish peroxidase HRP-labeled antibody directed against the constant domains of human IgG (sheep anti-human immunoglobulins (pre-adsorbed with monkey IgG) – peroxidase conjugate, The Binding Site) was used for detection.

Specific antibody assay for determination of BYON4228 levels in cynomologus monkeys A biotinylated anti-idiotype camel single chain domain directed towards the complementarity determining region (CDR) of BYON4228 was used for capturing the analyte. This antibody was coated to a streptavidin-coated ELISA plate (EvenCoat Plate, R&D Systems). A second HRP labeled anti-idiotype camel single chain domain also directed towards the CDR of BYON4228 was used for detection, followed by the addition of 3,3',5,5'-Tetramethylbenzidine (TMB) (TeBu-Bio, Cat. No. TMB100). The color reaction was stopped with H2SO4 and the plate was read at 450 and 630 nm with a plate reader (Varioskan 3001). Each analytical run included appropriate calibrators and quality-control samples.

PK evaluation

PK was evaluated using non-compartmental analysis for IV bolus injection or extravascular injection in Phoenix WinNonlin version 8.2 or higher. Derived PK parameters were rounded to three significant figures. PK figures were made in Graphpad Prism.

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