Fc-engineering significantly improves the recruitment of immune effector cells by anti-ICAM-1 antibody MSH-TP15 for myeloma therapy

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Supplementary Information

Supplementary Methods

Generation of MSH-TP15 antibody variants

Variable light (VL) and heavy chain (VH) sequences derived from phage PIII-15 and BI-505 were synthetized to allow cloning into modified pSecTag2/HygroC vectors (Thermo Fisher Scientific) harboring sequences for the constant region of the human kappa light chain (LC) or either wildtype or mutated human IgG1 heavy chain (HC). S239D and I332E mutations were introduced in the Fc domain of the Fc-engineered (Fc-eng.) variant to improve binding to activating FcγR, while the L234F, L235E, and P331S mutations in the Fc knockout (Fc k.o.) variant should dramatically reduce FcγR and C1q binding. Antibodies were termed MSH-TP15 (wildtype IgG1), MSH-TP15 Fc-eng. and MSH-TP15 Fc k.o. All sequences were verified by Sanger sequencing.

Antibody expression and purification

Antibodies were expressed in Lenti-X 293T cells by Ca_2PO_4 transfection and purified by affinity chromatography with CaptureSelect IgG-CH1 beads (Thermo Fisher Scientific). Purity and molecular masses were analyzed by SDS-PAGE and Coomassie staining following standard procedures. Quantification was performed by BCA protein assay kit (Thermo Fisher Scientific). Rituximab (anti-human CD20 IgG1; Roche, Basel, Switzerland) and cetuximab (anti-human EGFR IgG1; Merck) were purchased.

Cloning and expression of human ICAM-1 domains

Full-length human ICAM-1 cDNA was obtained from OriGene Technologies (pCMV-XL5 human ICAM-1 (untagged); NM_000201.1) and was used as template for the generation of truncated ICAM-1 molecules containing of domain 1 (D1) to domain 1-5 (D1-5). To amplify the ICAM-1 domains for cloning into pDisplay vector (Thermo Fisher Scientific) harboring a *myc* tag and a PDGFR transmembrane domain C-terminally of the MCS the specific forward and reverse primers were designed with Primer3 (http://simgene.com/Primer3) and synthetized by Metabion (Planegg/Stein-kirchen, Germany). All primer sequences are listed in Suppl. Tab. 1. PCR reactions were performed with 3 µg pCMV-XL5 human ICAM-1, 0.5 µl *Pwo* SuperYield DNA Polymerase (Roche), 1 µl dNTP mix (10 mM; Thermo Fisher Scientific), 0.5 µl

forward and 0.5 µl respective reverse primer (each 30 µM) in 1x *Pwo* SuperYield DNA Polymerase Buffer (Roche) for 30 cycles (55°C annealing temperature). DNA fragments of 400 bp for D1, 706 bp for D1-2, 976 bp for D1-3, 1231 bp for D1-4, and 1472 bp for D1-5 were amplified and eluted from agarose gel with NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel). Cloning into pDisplay was done using *Sfi*I and *Eco*RI (NEB) restriction sites and T4 DNA ligase (Roche) according to the supplier's instructions. 4 µl of each ligation reaction were transformed into 40 µl of XL1-Blue electroporation-competent *E. coli* (Stratagene) as described by the manufacturer. Single, ampicillin-resistent colonies were picked from LB plates and grown at 37°C overnight with shaking at 250 rpm. Plasmid isolation was done with the NucleoSpin Plasmid Quick Pure Purification kit (Machery-Nagel) prior control digestion with *Sfi*I and *Eco*RI. Clones carrying inserts with the expected sizes were selected and amplified. Sequences of the final constructs named pDisplay huICAM-1 D1 to pDisplay huICAM-1 D1-D4 were finally controlled by Sanger sequencing. To express the human ICAM-1 variants CHO-K1 cells were transiently transfected with plasmids encoding pDisplay huICAM-1 D1 to pDisplay huICAM-1 D1-D5 or the full length ICAM-1 molecule (pCMV-XL5 human ICAM-1; OriGene Technologies) using Lipofectamine LTX and Plus Reagent (Thermo Fisher Scientific). Transfections were performed in 6-well-plates according to manufacturer's instructions. Cells were maintained in medium for 48 h prior flow cytometric analysis.

Flow cytometry

All immunofluorescence analyses were performed on a Navios flow cytometer and analyzed with Kaluza software (Beckman Coulter). Cells ($5x10^5$/sample) were incubated with the indicated antibody in PBS supplemented with 1% BSA and 0.1% NaN_3 (PBA buffer) for 30 min on ice. Cell-bound mAbs were detected with goat $F(ab)_2$ anti-human Fcγ (IgG)-FITC (Beckman Coulter; 1:20 in PBA). FcR carrying cells were pre-incubated with PBA buffer supplemented with 500 µg/ml human immunoglobulin (Intratect, Biotest Pharma, Dreieich, Germany) for 15 min to block unwanted Fc-FcγR interactions and directly stained with fluorochrome-labeled antibodies (DyLight 488 and 755 Antibody Labeling Kits; Thermo Fisher Scientific). For cross-blocking experiments, cells were incubated with 50 µg/ml of CD54 antibody RR1/1 (mouse IgG1; Thermo Fisher Scientific), CD54-FITC (clone 84H10; mouse IgG1; Beckman Coulter), BI-505 or MSH-TP15 prior staining with 10 µg/ml of the indicated second CD54

antibody. Detection was done by FITC-labeled, anti-mouse or anti-human IgG secondary antibodies (Merck). Expression of Fcγ R on monocyte-derived macrophages was tested by staining $1x10^6$ cells with commercially available CD16-PE, CD32-PE, CD64-PE and the appropriate isotype control antibody (all Beckman Coulter).

For binding analyses of full-length ICAM-1, encoded by pCMV-XL5 human ICAM-1 (Origene), 10 µg/ml mAb were used and subsequently stained with goat $F(ab)_2$ anti-human Fcγ (IgG)-FITC (Sigma Aldrich; final dilution 1/20 in PBA). To analyze mAb binding to non-transfected or transiently with ICAM-1 D1 to D5 transfected CHO, cells were first incubated with 20 µg/ml 9E10 (mouse anti-*myc*-antibody) for 30 min on ice. After washing with cold PBA, successfully transfected cells were detected with goat anti-mouse IgG (whole molecule) $F(ab')_2$ fragment-FITC (Sigma Aldrich; final dilution 1/20 in PBA) staining the cell surface expressed *myc*-tag and in parallel incubated with Alexa Fluor 755-labeled MSH-TP15 or control IgG1) for additional 30 min on ice. Samples were analyzed after a final washing step. To analyze MSH-TP15 binding to mouse ICAM-1, CHO cells were transiently transfected with pCMV6-AC-GFP plasmid encoding for C-terminal GFP-tagged mouse ICAM-1 (Origene Technologies), and subsequently stained with Alexa Fluor 755-labeled mAbs as described above.

Induction of programmed cell death (PCD)

Ramos cells ($5x10^5$/sample) were incubated with the indicated antibody for 1h on ice prior incubation with 10 µg/ml mouse anti-human IgG Fcγ fragment specific antibody (Jackson ImmunoResearch, Ely, UK) for 6h at 37°C. Cells were stained using annexin V-FITC/7-AAD kit (Beckman Coulter) and measured by flow cytometry.

ADCP experiments

Macrophages were generated from healthy donor monocytes by cultivation in X-VIVO 15 medium (Lonza, Basel, Switzerland) supplemented with 50 ng/ml human M-CSF (PeproTec, Hamburg, Germany) for 7 days. Macrophages were seeded at $1x10^5$/well in 24-well-plates. Raji cells were stained in 100 nM CFSE solution (BioLegend, San Diego, CA, USA) and added together with the indicated mAbs in a 1:3 effector-to-target cell (E:T) ratio to the macrophages. After 2h at 37°C, samples were labeled with CD14-APC and CD19-PE (both Beckman Coulter) prior analyses by flow cytometry. Macrophages that had phagocytosed tumor cells were determined as $CFSE^+/CD14^+/CD19^-$.

Supplementary Figures

Figure 1

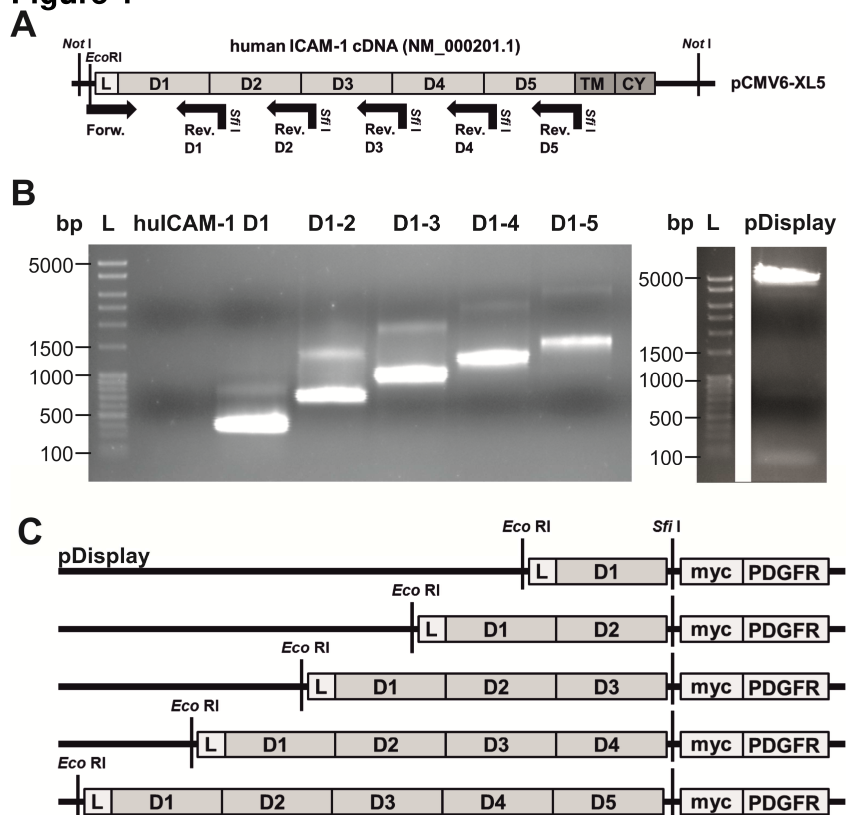

Fig. S1: Generation of truncated human ICAM-1 domains for transient expression in CHO cells. (A) Schematic view of the forward (Forw.) and reverse (Rev.) PCR primer locations (for primer sequences refer to Suppl. Tab. 1) on the human ICAM-1 cDNA in the pCMV6-XL5 vector from OriGene used to generate plasmids encoding for truncated ICAM-1 molecules. L = human ICAM-1 leader sequence; D = domain; TM = transmembrane domain; CY = cytoplasmic domain. **(B)** Agarose gel electrophoresis of the PCR amplified and *Eco*RI/*Sfi*I double digested ICAM-1 domains (left) and pDisplay expression vector (right) for cloning. L = ladder, bp = basepair(s). **(C)** Schemes of the constructs for expression of human ICAM-1 domains cloned N-terminally of a *myc* tag and a PDGFR transmembrane domain in pDisplay vectors. L = human ICAM-1 leader sequence; D = domain.

Figure 2

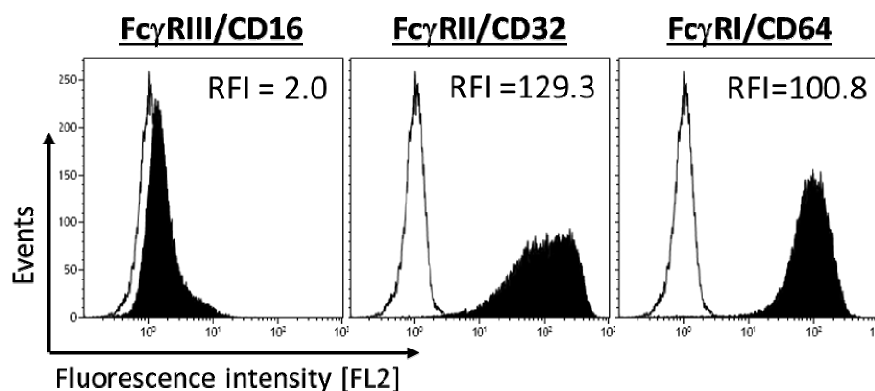

Fig. S2: FcγR expression of macrophages used for ADCP experiments. Flow cytometric analyses of monocyte-derived macrophages to analyze the FcγR expression profile was done by staining $1x10^6$ cells with commercially available, PE-labeled CD16, CD32, CD64 antibodies (black histograms), and the IgG-PE control (white histograms). RFI = relative fluorescence intensity.

Supplementary Tables

Suppl. Tab. 1: Primer for the amplification of truncated ICAM-1 variants

Primer Name	Nucleotide Sequence (5' - 3')
Forw. huICAM-1 *Eco*RI	TACGTAGAATTCGGCTTGGGGATATCCACCATGGCTCCCAGCAGCCCCCGGCCCGCGCTGCCCGC
Rev. huICAM-1 D1 *Sfi*I	TACGTAGGCCGGCTGGGCCTTCCACCCGTTCTGGAGTCCAGTACACGGTGAGGAAGG
Rev. huICAM-1 D2 *Sfi*I	TACGTAGGCCGGCTGGGCCTGGGGGAGTCGCTGGCAGGACAAAGGTCTGGAGCTGG
Rev. huICAM-1 D3 *Sfi*I	TACGTAGGCCGGCTGGGCCCACGTTGGGCGCCGGAAAGCTGTAGATGGTCACTGTCTGC
Rev. huICAM-1 D4 *Sfi*I	TACGTAGGCCGGCTGGGCCCTCGTCCAGTCGGGGGCCATACAGGACACGAAGCTCC
Rev. huICAM-1 D5 *Sfi*I	TACGTAGGCCGGCTGGGCCCTCATACCGGGGGGAGAGCACATTCACGGTCACCTAGG

Forw. = forward, Rev. = reverse; restriction sites are underlined