Blockade of the CD47/SIRPα checkpoint axis potentiates the macrophage-mediated antitumor efficacy of tafasitamab

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Supplement

Materials and Methods

Cell-culture reagents

Cells were cultured in complete medium (referred to as R10), which comprised RPMI 1640 medium (Cat. #11875085, Biochrom, Berlin, Germany) supplemented with glutamine (2 mM; Cat. #G8540, Sigma), 10 mM HEPES (Cat. #P05-0110), 13 mM NaHCO3 (Cat. #P06-22100), 100 µg/ml streptomycin (Cat. #P06-11100P), 60 µg/ml penicillin (Cat. #P06-08100P) (all from Biochrom) and 10% Fetal Calf Serum (C.C.Pro, Charge Nr. C 591).

Cell lines

The DLBCL cell lines Toledo (CRL-2631) and HT (CRL-2260) were purchased from ATCC and U2946 (ACC 795) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cell lines were used for no longer than 16 passages. All cell lines used in this study were monitored for mycoplasma contamination monthly using polymerase chain reaction (PCR) on all cell lines used in this study.

Antibodies and reagents

The following antibodies were used for immunofluorescence, flow cytometry or functional assays: CD19-APC (SJ25-C1, BD Pharmingen), CD19-Pe-Cy7 (SJ25C1, BD Pharmingen), CD19-BV421 (HIB19, BD Pharmingen), CD19-PE (FCM63, Merck/Milipore), CD19-PE (HIB19), CD19-PE (REA675, Miltenyi), CD68 (PG-M1, Dako Cytomation, Hamburg,

Germany), CD163 (10D6, Novocas-tra/Leica, Newcastle Upon Tyne, United Kingdom), CD163-PE (GHI/61, eBioscience, Frankfurt, Germany), CD163-BV421 (GHI/61, Biolegend), CD15-BV510 (W6D3, Biolegend), CD11b-FITC (M1/70.15.11.5, Miltenyi Biotec), CD11b-PeCy7 (ICRF44, BD Pharmingen), CD11b-APC (M1/70.15.11.5, Miltenyi Biotec), Annexin-V-APC (BD), 7-AAD (Sigma), CD47-FITC (B6H12, BD Pharmingen), CD47-PE (CC2C6, Biolegend), CD47-BV421 (B6H12, BD Pharmingen), SIRPα-PE (SE5A5, Biolegend), rabbit IgG F(ab')2 Fragment-488 conjugate, mouse IgG F(ab')2 Fragment Alexa-488, rabbit IgG F(ab')2 Fragment-674 conjugate, and mouse IgG F(ab')2 Fragment-647 conjugate (all from Cell Signaling Technology, Frankfurt, Germany). The Fc-silent antibody CD47-IgGo was generated as described previously[15]. Tafasitamab was provided by MorphoSys (MorphoSys AG, Planegg, Germany). The fluorescent probes were purchased from Essen Bioscience (Cytolight Rapid Green and IncuCyte® pHrodo® Red Cell Labeling Kit), from Sigma (DAPI, CFSE) and from eBioscience (VPD, Cell Proliferation Dye eFluor® 450; CPD, Cell Proliferation Dye eFluor® 670). 123count eBeads were purchased from eBioscience.

Lymph node biopsy and immunohistochemistry

Sections of 9 formalin-fixed and paraffin-embedded DLCBL or 7 reactive tonsil (benign controls) specimens were selected from the archive of the Institute of Pathology, University Hospital Erlangen, Germany. Sections were deparaffinized with xylol and rehydrated with graded ethanol. Antigen retrieval for all staining's was performed in a steam-cooker using a target retrieval solution pH 6 (TRS6; DAKO S1699) for 5 minutes at 120°C. Primary antibodies were incubated over night at room temperature. A triple-staining was performed with primary antibodies against CD68 (1:100, DAKO, monocloal, mouse, M0876), SIRPα (1:50, CellSignalling, monoclonal, rabbit; D6I3M) and CD206 (1:100, Novus, monoclonal, mouse, NBP2-52927) and fluorescence labelled secondary antibodies which were incubated for 30 minutes at room temperature (1:500, Alexa 488 labelled goat anti mouse IgG3, Dianova, 115-

545-209; 1:500, Alexa 555 labelled donkey anti rabbit, Lifetechnologies, A31572; 1:500 Alexa 647 labelled goat anti mouse IgG2b, Thermo Fischer A21242). Additionally, a double-staining for CD19 (1:50, Zytomed, monoclonal, mouse, MSK043-05) and CD47 (1:50; R&D, monoclonal, sheep, AF4670) was performed with fluorescence labelled secondary antibodies (1:500, Alexa 488 labelled donkey anti mouse, Lifetechnologies, A21202 and 1:500; Alexa 633 labelled donkey anti goat cross-reacting with sheep; Lifetechnologies, A21082). TrueVIEW autofluorescence Quenching Kit (Vector Labs; SP-8400) was applied for quenching in all staining's. Analyses of FFPE samples (9 DLBCL patients and 7 benign controls) were performed in a blinded fashion. After staining, the entire sections were scanned (Zeiss Axio Scan.Z1) and images were analyzed. Regions of Interest (ROIs) matching lymphoma cells/B-cells ($CD19^+$) or macrophages/LAMs ($CD68^+$) were segmented using Zeiss-software (ZEN 2.6) or the open-source software QuPath (https://qupath.github.io), and the mean fluorescence intensity (MFI) of CD47 or SIRPα was assessed for each ROI. The frequency of double-positive B cells ($CD19^+/CD47^+$) on the entire section was analysed using QuPath. Data from 25 lymphoma cells or macrophages from each donor were used for CD47/SIRPα expression levels. For this purpose, five image fields were selected per section and a total of more than 25 lymphoma cells ($CD19^+$) or macrophages ($CD68^+$) were analysed for their respective CD47 or SIRPα expression.

In vitro differentiation of human macrophages

PBMC were isolated by density gradient centrifugation of buffy coat preparations from the peripheral blood of healthy donors (Department of transfusion medicine, University Hospital Erlangen, Germany). Monocytes were isolated by plastic adherence and cultured in the presence of M-CSF (50 ng/ml, R&D) to generate macrophages. Macrophages were detached with EDTA (1mM, Sigma) after 6d of culture. Purity of generated macrophages was greater than 90% and minimally contaminated by CD3+ and CD19+ cells, determined via flow cytometry.

CPD, pHrodo or Cytolight green labelling of cells

Cells were stained in an incubator for 20 min in sterile PBS containing cell proliferation dye (5 µM, CPD) or Cytolight Rapid Green (0.5µM). Cell count was determined after washing the cells three times with R10 supplemented with heat inactivated FCS. To label with pHrodo dye, cells were washed twice with PBS and stained in with100 ng/mL IncuCyte® pHrodo® Red Cell Labeling Dye in an incubator for 1h. Cell count was determined after washing the cells once with IncuCyte® pHrodo® Wash buffer.

CRISPR Cas9

The CRISPR/Cas9 system was used to generate CD47-knockout and CD47-overexpressing Toledo cells. All necessary reagents were purchased from Horizon Discovery, as follows: Dharmacon, Inc. Edit-R™ Inducible Lentiviral Particles hEF1α-Blast-Cas9 (VCAS11227), Dharmacon, Inc. Edit-R™ CRISPRa Lentiviral Particles hCMV-Blast-dCas9-VPR (VCAS11918), Edit-R predesigned lentiviral sgRNA, CD47 human (CD47-Plasmid: GSGH11838-247009754). Target cells were infected with lentivirus particles encoding the Cas9 sequences for at least 24 hours, and subsequently selected with blasticidin (7.5 µg/mL 2 days, 10 µg/mL 3 days). Cells were cultivated for 7 days after removal of blasticidin and single cells were sorted in cell culture plates. Clones for further transduction were selected based on Cas9 expression determined via Western Blot. The CD47-plasmid was transfected with the packing plasmids into 293T cells to generate lentiviruses. Virus containing supernatant was collected 48–72 hours after transfection. To achieve infection, target cells were incubated with lentivirus particles for 48 hours and subsequently selected with puromycin (0.5-1 µg/mL), 48 hours after removal of the virus. The following sgRNA target sequence was used: Human CD47: TTGCTGGGCTCGGCGTGCTG (Exon 1).

Bioinformatical analysis

We searched the gene expression omnibus (GEO) for public transcriptomics data that would elucidate fundamental concepts of our hypothesis concerning DLBCL, the levels of CD47 and SIRPA. Accordingly, we found the study GSE178965 which profiled DLBCL patients and provided diseases status and outcomes. Hence the study presents an ideal fit for our investigation. Subsequently, we downloaded the raw count matrix provided on GEO and performed bioinformatic analysis to gauge possible trends in the expression of the genes. First, raw counts were fed into DESeq2 for differential expression analysis. Subsequent results were investigated of SIRPA or CD47 where both did not show significant differential expression. We further investigated however if there were trends observable over the patient cohort. Since primary patient data is usually marred with heterogeneity, we first performed outlier depletion by calculating the 25th and 75th percentile of expression measured by DESeq2's normalized and setting the upper and lower bound to 1.5 times the interquartile above or below the 25th or 75th percentile respectively. The R code for the analysis can be found under https://github.com/liscruk/cd47_analysis.

Incucyte®-based phagocytosis assay

Lymphoma cells were labeled with IncuCyte® pHrodo® Red Cell Labeling Kit and co-cultured with macrophages (E:T=5:1) in a 96-well flat bottom plate in the presence or absence of tafasitamab (1µg/ml) and/or anti-CD47 antibody (B6H12, 1µg/ml). The plate was placed in Incucyte® S3 Live Cell Imaging Microscope and measured every 15-30 minutes. Recorded images were later analyzed using Incucyte® 2022A software. Phagocytosis was determined by measuring the Total integrated intensity [RCUxµm²/image] of red fluorescent signal.

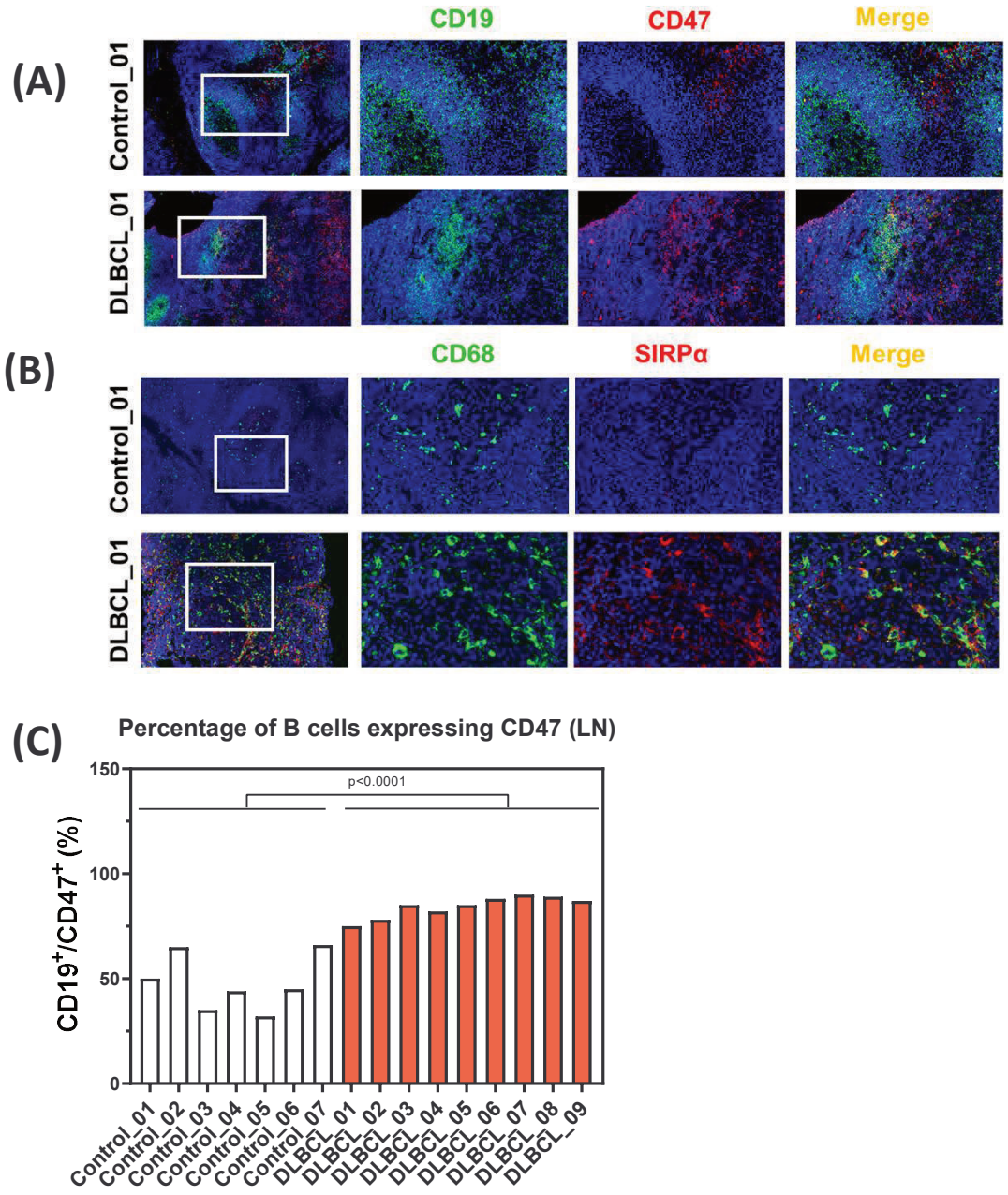
In vivo experiments

Orthotopic tumor model: 1×10^6 Ramos cells were injected into the tail veins of *C.B-17 SCID* mice on study day 0. Two days after cell inoculation, animals were randomized into four treatment groups of 15, based on body weight. On study day 5, treatment with vehicle control (PBS), tafasitamab (3 mg/kg), or anti-CD47 antibody (clone B6H12, BE0019-1, BioXCell; 4 mg/kg) commenced in mono- or a combination regimen. Tafasitamab was administered via an intravenous injection (i.v.), twice a week and anti-CD47 antibody by intraperitoneal injection (i.p.) three times per week for up to three weeks. Treatment volume was calculated and adjusted based on the animal's individual body weight before each dose administration. Clinical signs and body weight loss were monitored until individual ethical endpoint or study termination on day 99 after cell inoculation.

Flank tumor model: $1x10^7$ Ramos cells mixed with 50% (v/v) matrigel (ref. 356237, Corning) were injected into the right flank of *NOD/SCID (NOD.CB17-Prkdcscid/J)* mice. On study day 6, when mean tumor volume reached 100 - 200 mm^3, animals were randomized and treatment with vehicle, tafasitamab (10 mg/kg/dose) or anti-CD47 antibody (clone B6H12, BE0019-1, BioXCell, 4 mg/kg/dose) either alone or in combination commenced. Tafasitamab and the anti-CD47 antibody were administered i.p. two and three times weekly, respectively, for four weeks. Clinical signs and body weight loss were monitored until individual ethical endpoint or study termination on day 55 after cell inoculation. Tumor size was measured twice weekly and tumor volume was calculated using the following formula: $width^2$ x length/2. Kaplan Meier curves depict the time until tumor size reached a volume of 1500 mm^3.

Animal procedures and experimentation were authorized by the French Comité National de Réflexion Ethique sur l'Expérimentation Animale and the Pennsylvania State College of Medicine Institutional Animal Care and Use Committee.

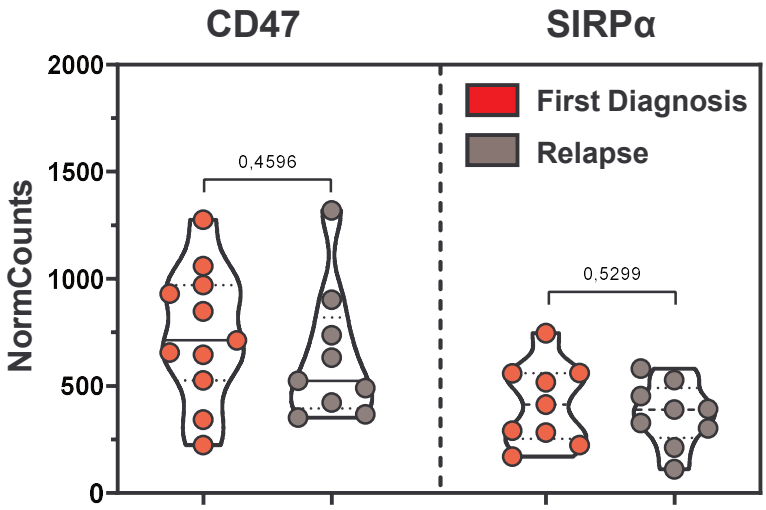
Supplemental Figure 1



Supplementary Figure 1

(**A**) Expression of CD47 (red) on B-cells (CD19, green) (upper panel) and (**B**) SIRPα (red) on macrophages (CD68, green) (lower panel) was quantified using confocal microscopy on tonsils as benign controls (n=7) or DLBCL specimens (n=9). The microscopy images show representative sections of all experiments. (**C**) The percentage of CD47 expressing B-cells was quantified using confocal microscopy on tonsils as benign controls (white bars) (n=7) or DLBCL specimens (red bars) (n=9). QuPath (https://qupath.github.io) was used to segments the cells and calculate the percentage of CD47-expressing $CD19^+$ lymphoma/B cells on the whole sections. Each bar represents a tested donor. Nonparametric Mann-Whitney U-test was performed.

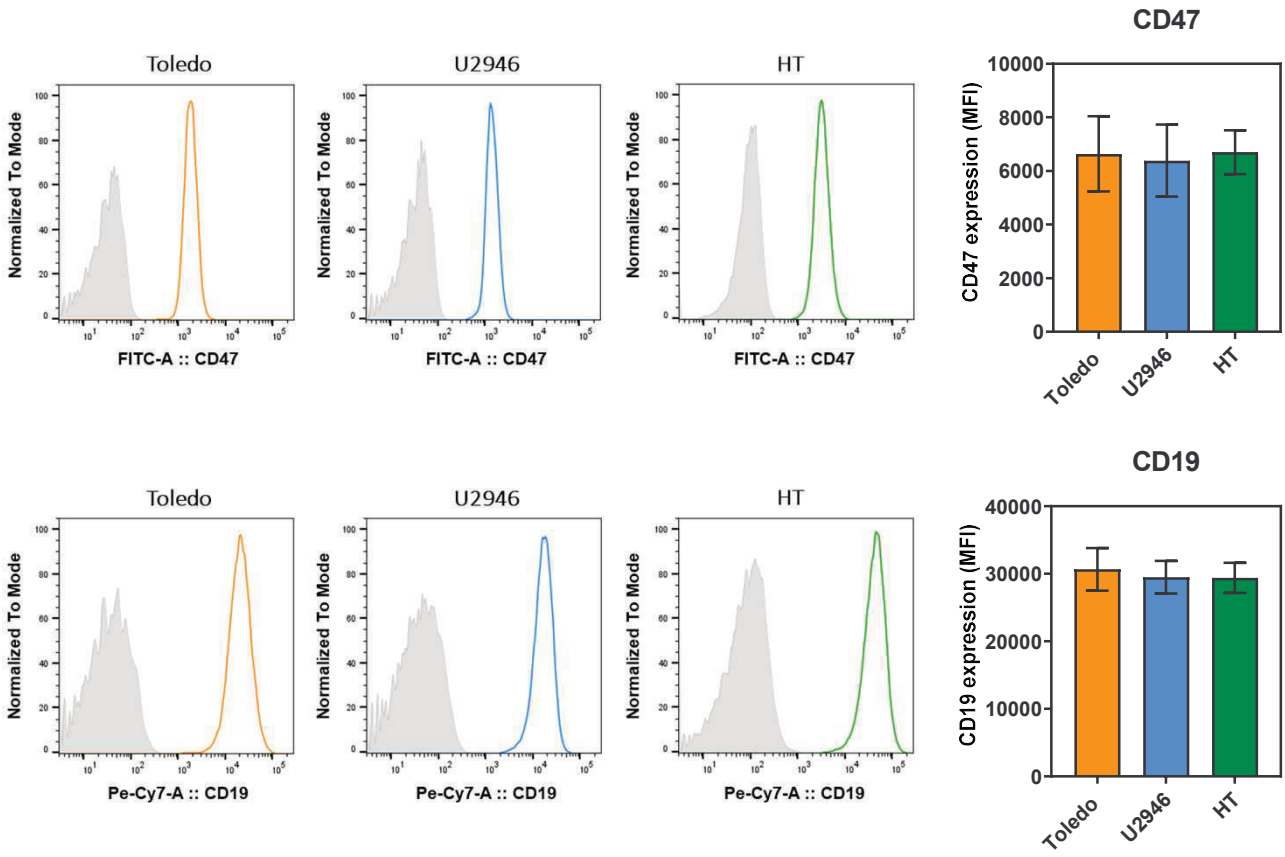
Supplemental Figure 2



Supplementary Figure 2

Expression of CD47 and SIRPα in lymph node biopsies of newly diagnosed (red dots, n=11) or relapsed (grey dots, n=9) DLBCL patients from publicly available transcriptomics data are shown as normalized counts. Normalized counts were extracted after running DESeq2. Shown are the expression values in the analyzed samples after removal of strong outliers, defined by removal of all samples lying outside the 1.5-fold interquartile range in both directions. Two tailed paired Students t test was performed.

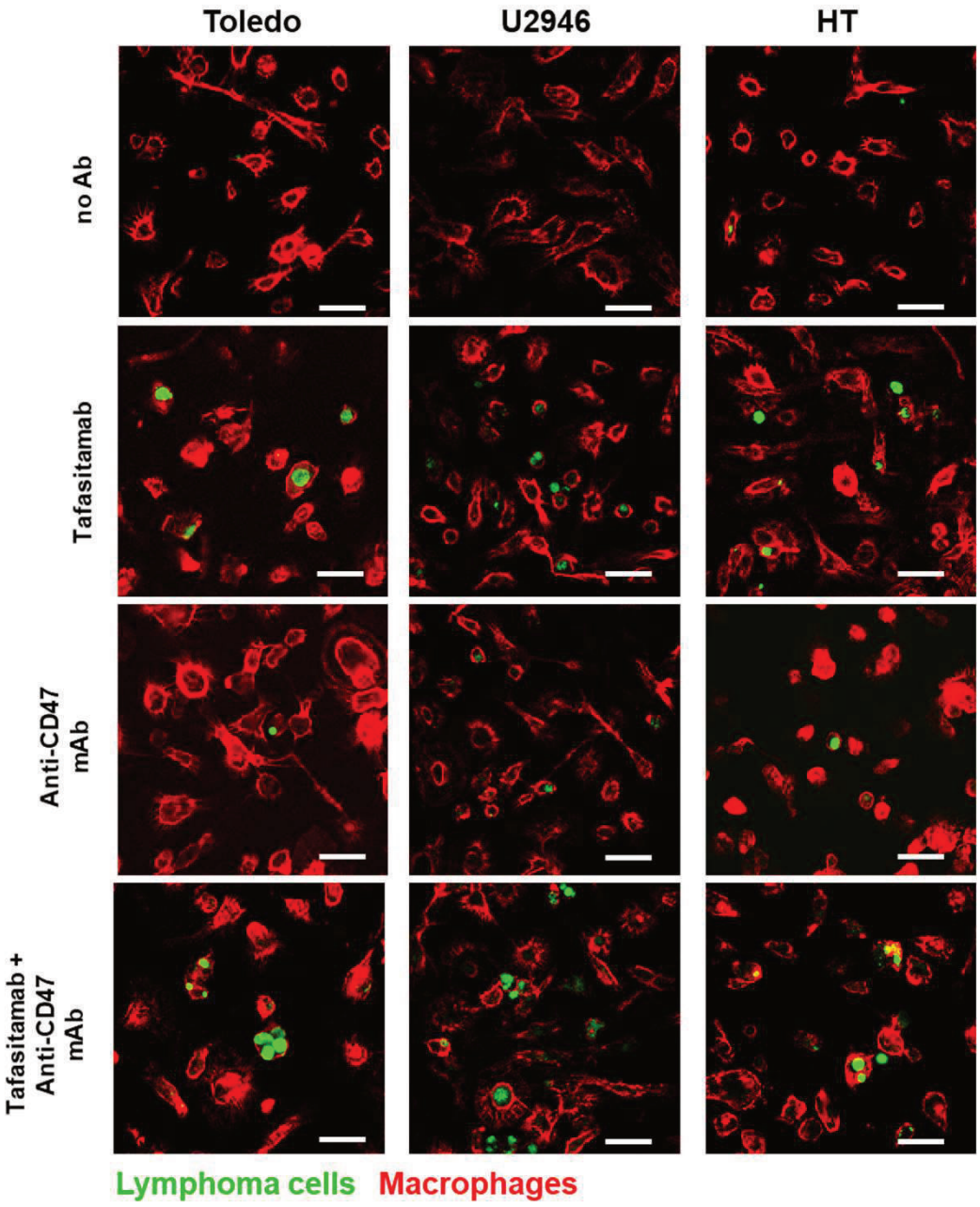
Supplemental Figure 3



Supplementary Figure 3

CD47 (upper panel) and CD19 (lower panel) expression in DLBCL cell lines. Cells (Toledo, orange; U2946, blue and HT, green) were stained for CD47 and CD19 and surface expression was measured by flow cytometry. Grey histogram represents the isotype control. Surface expression was calculated as Mean Fluorescence Intensity (MFI) of cells stained with anti-CD19 or anti-CD47 antibodies. The graph shows the average result of four independent experiments. Error bars show SEM.

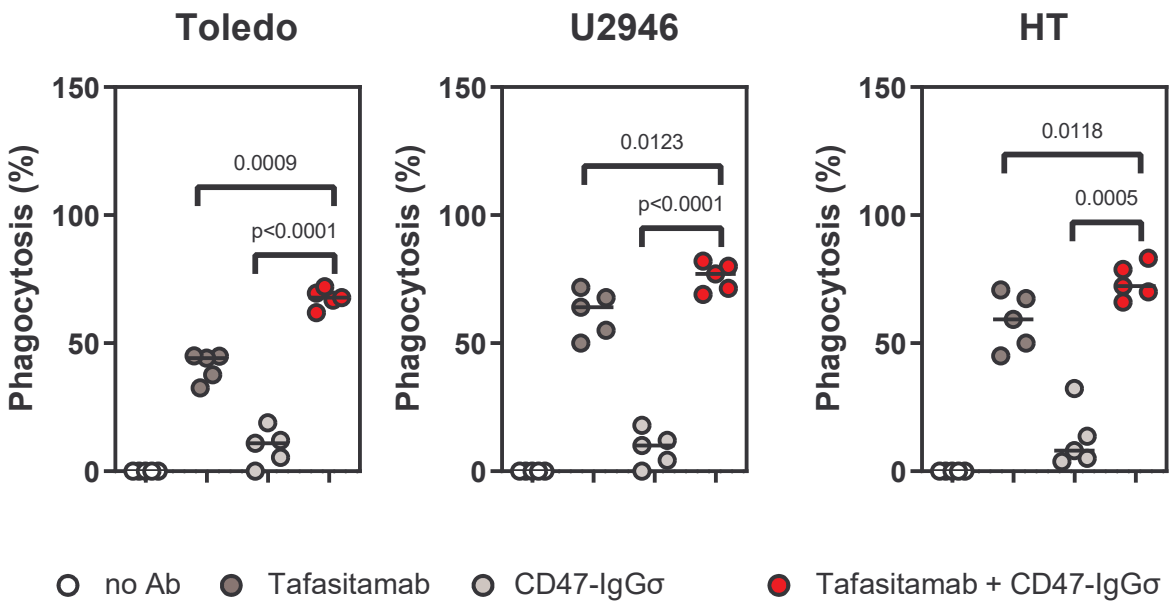
Supplemental Figure 4



Supplementary Figure 4

Immunofluorescence of *in vitro* differentiated macrophages (red) co-cultivated with indicated cell lines (Toledo, U2946, HT) (green) and tafasitamab +/- anti CD47-mAb (1µg/ml) for three hours to detect phagocytosis. Images were taken after washing steps, removing non-adherent and/or non-phagocytosed cells. The microscopy images show representative sections of five performed experiments. Scale bar: 20µm.

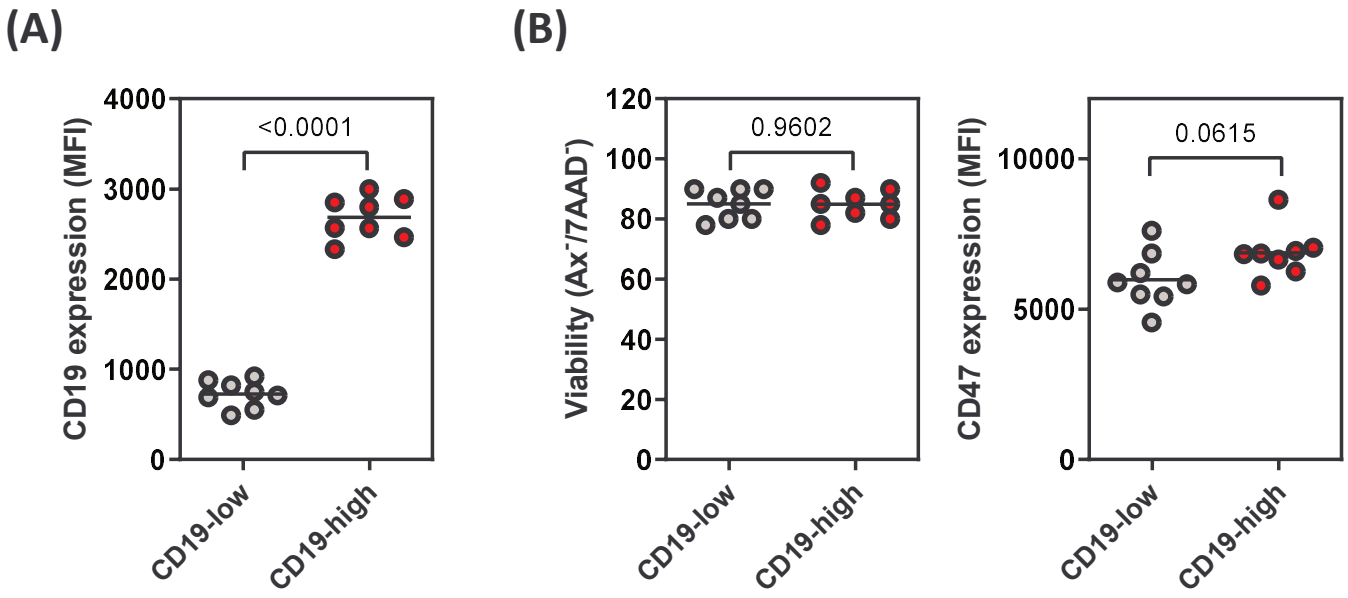
Supplemental Figure 5



Supplementary Figure 5

Quantification of phagocytosis by Incucyte microscopy. Macrophages were co-incubated with fluorescently labeled lymphoma cells (as indicated) in the absence (white circles) or presence of tafasitamab (dark grey circles) or anti-CD47 (CD47-IgGσ, light grey circles) or a combination of both (red circles). After 3 hours, phagocytosis was analyzed and quantified by Incucyte microscopy and Incucyte® 2022A software. The graphs show the result of five independent experiments. Lines show the mean value. Two tailed paired Students t test was performed.

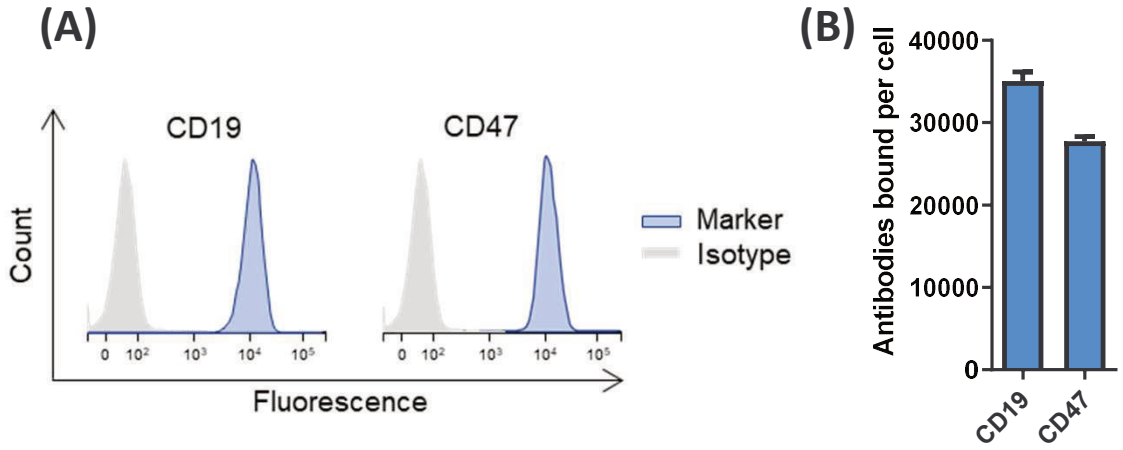
Supplemental Figure 6



Supplementary Figure 6

Phenotype of sorted Toledo cells. The cell line Toledo was stained with an anti-CD19 antibody and separated in CD19 low expressing (CD19-low, grey circles) or CD19 high expressing (CD19-high, red circles) cells by flow cytometry. Isolated cells were subsequently analyzed for their (**A**) CD19 surface expression or for their (**B**) viability and CD47 expression. Surface expression was calculated as Mean Fluorescence Intensity (MFI) of cells stained with anti-CD19 or anti-CD47 antibodies. Viability of isolated cells was measured by Annexin-V and 7-AAD by flow cytometry. The graphs shows the average result of eight independent experiments. Lines show the average value. Two tailed paired Students t test was performed.

Supplemental Figure 7



Supplementary Figure 7

Flow cytometric analysis of CD19 and CD47 expression on Ramos cells. (**A**) Ramos cells were stained for CD47 and CD19 (blue histograms) or isotype control (grey histograms) and surface expression was measured by flow cytometry. (**B**) Surface expression of CD19 and CD47 receptors was analyzed using the Quantibrite system (BD Biosciences), according to manufacturer's instructions. Mean fluorescence intensity (MFI) values were measured upon staining with PE-labeled anti-CD19 or anti-CD47 antibodies, and the MFI values of reference beads were used to correlate MFI to the number of antibodies bound per cell via linear regression. The graph shows the average result of three independent experiments. Error bars show SEM.

Supplemental Table 1: Patients' characteristics (FFPE)					
Case No.	Sex [m/f]	Age [years]	Cell-of-origin subtype Hans criteria	IPI risk group	Figure
1	m	53	GCB	Low-intermediate	1A, 1B, Suppl.1
2	m	77	GCB	high-intermediate	1A, 1B, Suppl.1
3	f	68	non-GCB	high	1A, 1B, Suppl.1
4	m	75	non-GCB	Low-intermediate	1A, 1B, Suppl.1
5	f	81	non-GCB	high	1A, 1B, Suppl.1
6	f	59	non-GCB	Low-intermediate	1A, 1B, Suppl.1
7	f	80	GCB	Unknown	1A, 1B, Suppl.1
8	m	87	non-GCB (not clear)	Low-intermediate	1A, 1B, Suppl.1
9	f	81	non-GCB	Low-intermediate	1A, 1B, Suppl.1

Supplemental Table 2: Patients' characteristics (viable cell data)				
Case No.	Sex [m/f]	Age [years]	Cell-of-origin subtype Hans criteria	Figure
1	f	47	non-GCB	1C, 1D, 4C
2	f	57	non-GCB	1C, 1D, 4C
3	f	61	non-GCB	1C, 1D, 4C
4	m	48	GCB	1C, 1D, 4C
5	m	53	non-GCB	1C, 1D, 4C
6	f	28	non-GCB	1C, 1D, 4C
7	m	67	non-GCB	4C
8	m	75	GCB	1C, 1D, 4C
9	m	54	non-GCB	4C
10	m	61	non-GCB	4C
11	f	75	non-GCB	4C
12	m	39	non-GCB	4C
13	f	62	non-GCB	4C
14	m	65	GCB	1C, 1D, 4C
15	m	60	GCB	1C, 1D, 4C
16	m	79	non-GCB	4C
17	f	63	GCB	4C

FFPE: formalin-fixed paraffin-embedded; m: male; f: female; GCB: germinal center B-cell like