A CXCR4-targeted nanocarrier achieves highly selective tumor uptake in diffuse large B-cell lymphoma mouse models

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 doi:10.3324/haematol.2018.211490

Received: November 19, 2018.

Accepted: June 26, 2019.

Pre-published: June 27 2019.

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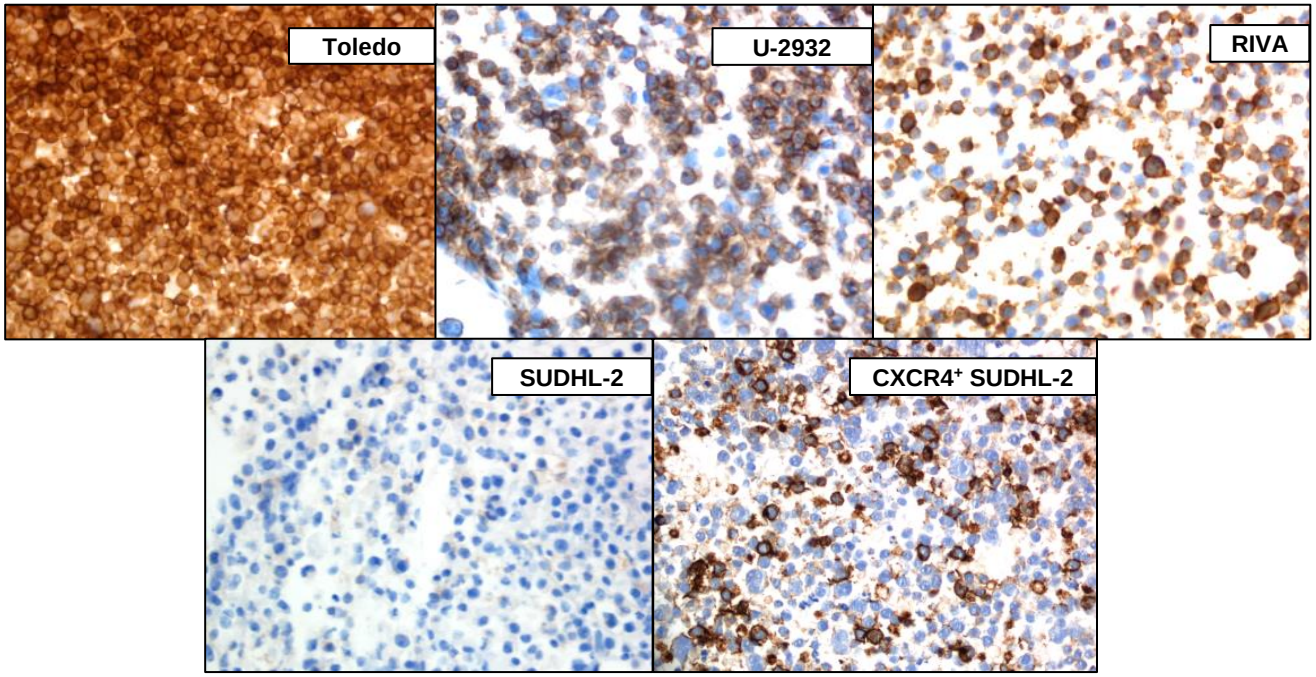
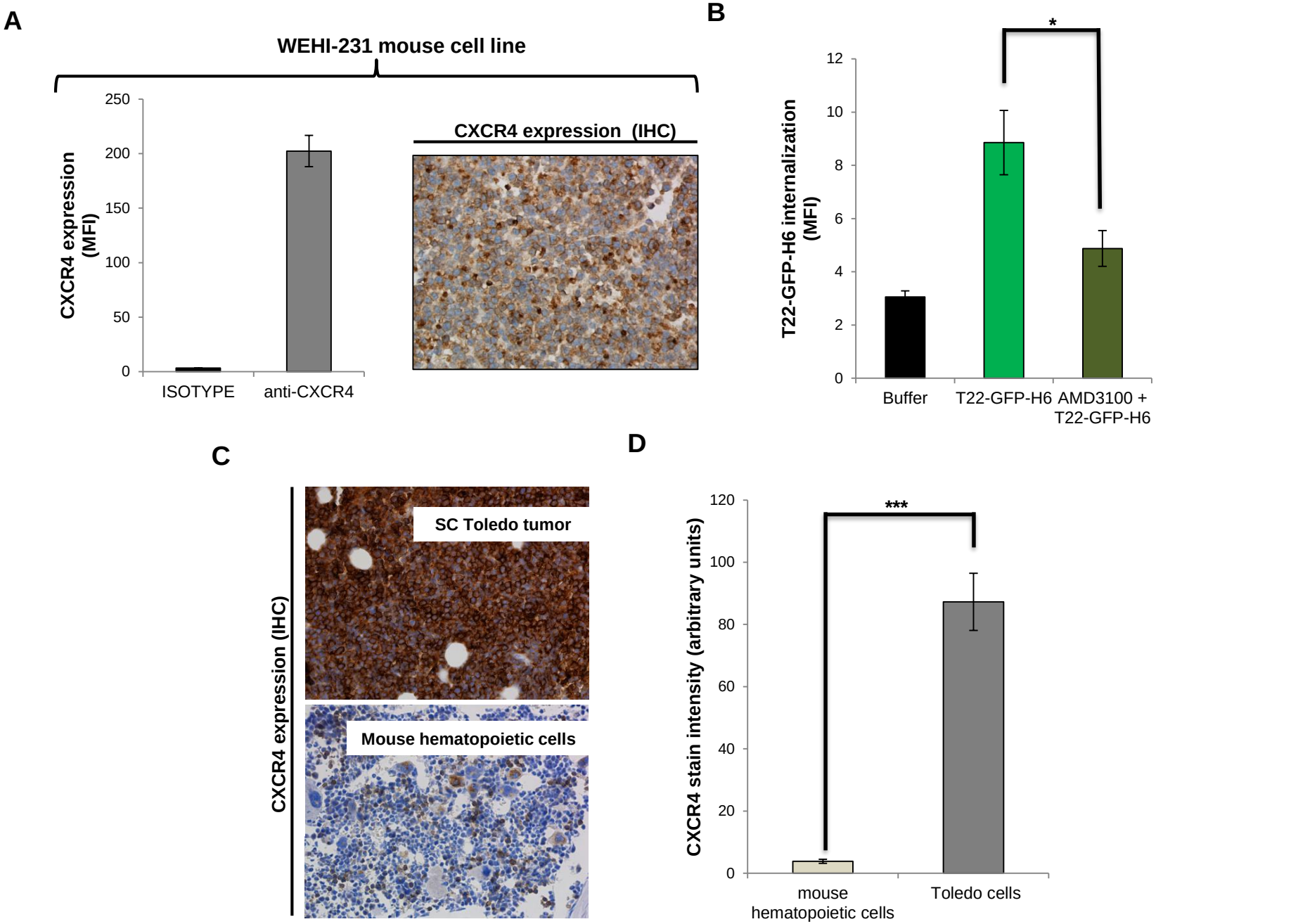
SUPPLEMENTAL DATA

Supplementary Figure 1. CXCR4 membrane expression in different DLBCL cell lines. IHC images of CXCR4 expression in Toledo, U-2932, RIVA, SUDHL-2 and SUDHL-2 cells transfected with the CXCR4 plasmid ($CXCR4^+$ SUDHL-2). DLBCL, diffuse large B-cell lymphoma; IHC: immunohistochemistry; Original magnification=x400.


Toledo
U-2932
RIVA
SUDHL-2
CXCR4+ SUDHL-2

Supplementary Figure 2. T22-GFP-H6 internalization in a mouse CXCR4$^+$ cell line and CXCR4 expression in SC Toledo tumor *vs*. mouse hematopoietic cells in BM. (A) CXCR4 membrane expression in the WEHI-231 mouse cell line by flow cytometry and IHC. (B) Internalization of T22-GFP-H6 (250nM, 1h) in WEHI-231 cells and competition assay after 1h pretreatment with the antagonist AMD3100 (2500nM), measured by flow cytometry. (C) Representative image of CXCR4 membrane expression, as assessed by IHC, in SC Toledo tumor and in mouse BM hematopoietic cells. (D) Quantitation of CXCR4 staining intensity in mouse hematopoietic cells resident in BM and Toledo cells in SC tumors. Original magnification=x400; *p<0.05; ***p<0.001; DLBCL, diffuse large B-cell lymphoma; MFI, mean fluorescence intensity; IHC, immunohistochemistry; BM, bone marrow; SC, subcutaneous.


A
WEHI-231 mouse cell line
CXCR4 expression (MFI)
250
200
150
100
50
0
ISOTYPE
anti-CXCR4
CXCR4 expression (IHC)
B
T22-GFP-H6 internalization (MFI)
12
10
8
6
4
2
0
*
Buffer
T22-GFP-H6
AMD3100 + T22-GFP-H6
C
CXCR4 expression (IHC)
SC Toledo tumor
Mouse hematopoietic cells
D
CXCR4 stain intensity (arbitrary units)
120
100
80
60
40
20
0
***
mouse hematopoietic cells
Toledo cells

Supplementary Table 1. Comparison of the kinetics of T22-GFP-H6 emitted fluorescence between SC Toledo tumors and non-tumor organs.

	FLI RATIO*		
	2h	**5h**	**24h**
Spleen	0.17 (±0.10)	0.00 (±0.00)	0.15 (±0.10)
Liver	0.27 (±0.27)	0.00 (±0.00)	2.41 (±1.21)
Kidneys	0.51 (±0.48)	0.50 (±0.32)	0.00 (±0.00)
Heart	0.37 (±0.22)	0.37 (±0.37)	0.16 (±0.09)
Lungs	1.00 (±0.44)	1.00 (±0.48)	1.00 (±0.38)
BM	0.53 (±0.31)	0.00 (±0.00)	0.16 (±0.16)
Tumors	10.49 (±1.25)	35.85 (±4.22)	9.36 (±1.25)

*FLI ratios are described for the different time points (2, 5 and 24h) after a 200µg T22-GFP-H6 single intravenous injection in mouse bearing SC Toledo tumors. FLI ratio from experimental mice was calculated subtracting the FLI auto-fluorescence of control mice and dividing the FLI signal of each tumor/tissue by the FLI signal of the lungs. Values represent mean ± SE.

FLI, fluorescence intensity (expressed as average radiant efficiency); BM, bone marrow; SC, subcutaneous; SE, standard error.

Supplementary Table 2. Comparison of AUC, uptake ratio and percentage of uptake between SC Toledo tumors and non-tumor organs after T22-GFP-H6 administration.

	AUC[a]	UPTAKE RATIO[b]	% UPTAKE[c]
Spleen	0.28E+07 (±0.17E+07)	0.10 (± 0.06)	0.46 (±0.27)
Liver	3.64E+07 (±1.73E+07)	1.31 (± 0.62)	5.96 (±2.83)
Kidneys	0.66E+07 (±0.28E+07)	0.24 (± 0.10)	1.07 (±0.45)
Heart	0.71+07 (±0.50E+07)	0.26 (± 0.18)	1.17 (±0.81)
Lungs	2.78E+07 (±0.73E+07)	1.00 (± 0.26)	4.55 (±1.20)
BM	0.41E+07 (±0.31E+07)	0.15 (± 0.11)	0.67 (±0.51)
Tumors	52.60E+07 (±2.47E+07)	18.93 (± 0.89)	86.13 (±4.04)

[a]AUC of emitted FLI along time (2-24h) registered from tumors and non-tumor organs in mouse bearing SC Toledo tumors treated with 200µg of T22-GFP-H6.

[b]Uptake ratio (relative units) was obtained by dividing the AUC from each tumor or non-tumor organ by the AUC obtained for the lungs.

[c]% uptake was calculated using the AUC for each tumor or non-tumor organ and its relation to the total AUC (tumors+non-tumor organs).

[a,b,c]Values represent mean ± SE.

FLI, fluorescence intensity (expressed as average radiant efficiency); AUC; area under the curve; BM, bone marrow; SC, subcutaneous; SE, standard error.

Supplementary Table 3. Comparison of T22-GFP-H6 emitted fluorescence between lymphoma affected and non-affected organs in the Toledo DLBCL disseminated mouse model.

	FLI RATIO*
Spleen	0.00 (±0.00)
Liver	0.00 (±0.00)
Kidneys	0.00 (±0.00)
Heart	0.62 (±0.48)
Lungs	1.00 (±0.58)
BM	31.05 (±13.14)
LN	12.98 (±6.08)

*FLI ratio is described 5h after a 400µg T22-GFP-H6 single intravenous injection in the Toledo DLBCL disseminated mouse model. FLI ratio from experimental mice was calculated subtracting the FLI auto-fluorescence of control mice and dividing the FLI signal of each tumor/tissue by the FLI signal of the lungs. Values represent mean ± SE.

Lymphoma affected organs (BM and LN); Non-affected organs (spleen, liver, kidneys, heart and lungs); DLBCL, diffuse large B-cell lymphoma; FLI, fluorescence intensity (expressed as average radiant efficiency); BM, bone marrow; LN, lymph nodes; SE, standard error.

ONLINE SUPPLEMENTAL METHODS

Cell culture

Toledo, U-2932, RIVA and SUDHL-2 are human DLBCL cell lines. WEHI-231 is a B-cell lymphoma mouse cell line. RIVA and SUDHL-2 were cultured in IMDM medium, whereas Toledo and U-2932 in RPMI 1640. WEHI-231 was cultured with DMEM and 2-mercaptoethanol was added to a final concentration of 0.05mM. Culture media for all cell lines were supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 100U/ml penicillin-streptomycin (Life Technologies). All cells were incubated at 37ºC and 5% CO_2 in a humidified atmosphere. RIVA and SUDHL-2 were kindly provided by Dr L. Pasqualucci (Columbia University, NY, USA). U-2932 cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) whereas Toledo and WEHI-231 cells from the American Type Culture Collection (ATCC).

Transfection with Luciferase and CXCR4 plasmids

The Luciferase plasmid (pPK-CMV-F3, Promokine) and the CXCR4 plasmid (provided by Dr. Jun Komano, Japan) were transfected by electroporation (Nucleofector TM 2b Device, Lonza) into Toledo and SUDHL-2 cells, respectively.

Expression of the Luciferase gene in Toledo transfected cells was detected by the IVIS Spectrum 200 (Xenogen) Imaging System. SUDHL-2 cells transfected with the CXCR4 plasmid ($CXCR4^+$ SUDHL-2) were isolated by FACSAria cell sorter (BD Biosciences) using PE-Cy5 mouse anti-human CXCR4 monoclonal antibody (BD Biosciences). Both stable clones were obtained by selection in medium containing 0.2mg/ml of geneticin (G418, Life Technologies) for a period of 5-10 weeks.

Flow cytometry

To determine CXCR4 expression in the membrane, human DLBCL cells were washed with PBS 0.5% BSA and incubated either with PE-Cy5 mouse anti-human CXCR4 monoclonal antibody (BD Biosciences) or PE-Cy5 Mouse IdG2a isotype (BD Biosciences) as negative control. CXCR4 expression in WEHI-231 cells was done using PE anti-mouse CD184 (CXCR4) antibody (Biolegend) and its respective isotype PE Rat IgG2b (Biolegend) as negative control.

Quantitation of T22-GFP-H6 internalization (GFP+) was performed by FACS after 1 hour of cell exposure to different nanocarrier concentrations (0.1nM 1nM, 5nM, 50nM, 125nM and 250nM). Then, cells were washed with PBS and trypsinized (1mg/ml trypsin, Life Technologies) in order to remove nonspecific binding of nanocarriers to the cell membrane. The competitive assays were done by preincubating the cells with AMD3100 (ratio 1-T22-GFP-H6:10-AMD3100)

Results of fluorescence emission were analyzed with software Cell Quest Pro and expressed as mean fluorescence intensity (MFI).

Cell Proliferation

Cell viability was evaluated measuring metabolic capacity using the colorimetric cell proliferation kit II (XTT, Roche Diagnostics). First of all, DLBCL cells were seeded in 96 well plates and incubated O/N at 37ºC. Next day, T22-GFP-H6 was added at different concentrations for 48h and absorbance was read at 490nm (FLUOstar OPTIMA, BMG Labtech). Data were shown as percentage of cell viability of nanocarrier-treated cells in relation to buffer-treated cell viability.

Western Blot

Proteins from tumor, liver or kidney were extracted with lysis buffer (1M Tris/acetate, 1M sucrose, 100mM EDTA, 100mM EGTA, 10% Tritó X-100, 100mM Naorto, 100mM Naβglycerol, 0.5M NaF, 100mM Napyro, β-mercapto, 100mM Benzamidine, 1.74mg/ml PMSF and 2mg/ml leupeptin). Samples were sonicated and then centrifuged at 14000min^{-1} for 10 mins at 4ºC. Protein concentrations in supernatant were determined using the Bradford protein assay, according to the manufacturer's instructions (BioRad). Cell lysates (50µg) were separated using 15% sodium dodecyl sulfate-polyacrilamide gel (SDS-PAGE) and transferred to a nitrocellulose blotting membrane (GE Healthcare life sciences). The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 2h at room temperature, and then incubated 1 hour with primary antibodies, GFP rabbit polyclonal (1:500, Santa Cruz Biotechnology) or mouse monoclonal-GAPDH (1/10000, Millipore). Membranes were washed with TBST and then incubated with the appropriate secondary antibody (1:10000, Jackson Immune Research) for 1 hour at room temperature. After TBST washing, the signals were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and the G:BOX iChemi XT Imaging System (Syngene).

Histopathology, immunohistochemistry and DAPI staining of paraffin sections

Tumor and organ sections of paraffin-embedded samples were analyzed histopathologically (H&E staining) by two independent observers to assess antitumor activity or possible toxicity on normal organs. The anti-CD20 (Dako) antibody was used, in IHC assays, to detect the presence of human DLBCL cells, in all DLBCL-infiltrated organs, including the mouse BM. Moreover, CXCR4 (1:300, Abcam) expression was evaluated in cellular blocks obtained for all cell lines, SC DLBCL tumors, DLBCL-infiltrated organs and mouse BM. CXCR4 staining intensity was quantified by measuring the mean intensity (255 minus "mean gray value") after selectively visualizing CXCR4 staining, using the ImageJ software per 12 high power fields (magnification x400). All pictures were taken with the same light exposition (1.25ms).

Apoptosis detection was evaluated by IHC to detect the presence of cleaved PARP, anti-PARP p85 fragment (1:300, Promega), and nuclear condensation after DAPI staining (Thermo Scientific). The stained area of cleaved PARP cells was quantified using the Olympus Cell^{D} Imaging 3.3 software in 12-fields (magnification x100). DAPI staining was performed, firstly, deparaffining the sections in xylene, rehydrating them in descending alcohols and permeabilizing the sections with Triton X-100 (0.5%). Slides were stained with DAPI mounting medium and analyzed under

fluorescence microscope. The number of apoptotic bodies was quantified recording the number of condensed and/or defragmented nuclei per 12 high-power fields (magnification x400).

All IHC stainings were performed in a DAKO Autostainer Link48 following the manufacturer's instructions. Representative pictures were taken using an Olympus DP73 digital camera and processed with the Olympus Cell^{D} Imaging 3.3 software at x400 or x1000 magnifications.

Confocal laser scanning microscopy

Simultaneous immunodetection of GFP and CXCR4 was performed by IF. Paraffin sections were heated 15 mins at 60ºC, dewaxed and rehydrated. Antigen retrieval was performed using pH=9 citrate buffer with DeCloaking Chamber. For the immunodetection, sections were washed 3 times with TBS for 5 mins and blocking buffer was added 1 hour at RT. The GFP chicken Ig4 1:250 (AVES) and the CXCR4 rabbit IgG 1:250 (Abcam) primary antibodies were incubated diluting them in TBS++ over night at 4ºC and 2h at RT. Tissue sections were washed and the anti-chicken Ig4-Cy2 1:50 antibody together with the anti-rabbit IgG-Cy5 1:200 antibody were added for the secondary antibody incubation diluting them in TBS++ 2h at 37ºC. After three additional TBS washes, the sections were stained with DAPI 1:10000 for 10 mins at RT. Finally, the mounting medium was added and immunofluorescent pictures were taken with the zoom of x63 (20.5065 distance in pixels) in a Confocal TCS SPE microscope (Leica), using LAS AF software (Leica). The percentage of CXCR4+/GFP+ cells was quantified recording as positive the cells stained for GFP or CXCR4 around or within the cell in 12-high power pictures (HCX PL APO lambda blue 63.0x1.40 OIL UV).