Effective hematopoietic stem cell-based gene therapy in a murine model of hereditary pulmonary alveolar proteinosis

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Supplemental material

Methods

Lentiviral vector generation and production

Generation of the 3rd generation SIN lentiviral vector Lv.Csf2rb was performed as previously described [1]. The murine *Csf2rb* cDNA is driven by an elongation factor 1 α promoter (EF1α). The Csf2rb transgene is followed by an internal ribosomal entry site (IRES) and an enhanced green fluorescent protein (eGFP). The 3rd generation SIN lentiviral vector Lv.GFP that was used as a control is driving eGFP expression by a short version of the EF1α promoter that is missing intron 1 (EFS).

For the production of viral particles, a transient four-vector transfection of HEK293T cells was used, as previously described [2]. Briefly, HEK293T cells were cultured in DMEM (PAA) containing 10% FCS, 100 1 mM penicillin/streptomycin, 20 mM of HEPES (PAA), and 25 μM of chloroquine (Sigma–Aldrich). Cells were transfected using calcium phosphate precipitation in the presence of 8 μg/mL of gag/pol, 5 μg/mL of pRSV-Rev, 5 μg/mL of lentiviral vector plasmid, and 1.5 μg/mL of vesicular stomatitis virus glycoprotein (VSVg). Viral supernatants were harvested 36 and 48 h post transfection, filtered and concentrated by ultracentrifugation (Becton Dickinson) for 16 h at 14,000 *g* and 4°C. Viral titers were determined by several dilutions on SC-1 fibroblasts and flow cytometry analysis.

Clonogenic assay

Lin^- cells were seeded at 4500 cells/ml in basic methylcellulose medium (HSC006) supplemented with 50 ng/ml GM-CSF (Peprotech) or complete methylcellulose medium (HSC007; both R&D systems). After 7 days, colonies composed of >50 cells were counted.

Macrophage (MΦ) differentiation

Lin^- cells were differentiated into MΦ for 7-15 days in RPMI medium supplemented with 10% FCS, 1mM penicillin/streptomycin, and 30% of M-CSF supernatant produced by L929 cells as previously described [3,4]

Cytospins

Approximately 50.000 cells were resuspended in a total volume of 200 µl PBS and centrifuged onto glass slides using a medite Cytofuge® (medite Gesellschaft für Medizintechnik) at 600xg for 7 min. Glass slides were subsequently stained using Pappenheim staining.

STAT5 phosphorylation

500.000 MΦ were seeded in a 6-well culture plate. Cells were cultured overnight (o/n) without cytokines in X-vivo™ 15 medium (Lonza) and subsequently stimulated with 50 ng/ml GM-CSF (Peprotech) for 15 min or continuously cultured without cytokine addition (unstimulated). Cells were harvested and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 30 min, permeabilized by addition of ice-cold methanol (Sigma-Aldrich) and incubated on ice for at least 30 min. Samples were washed using FACS buffer (PBS containing 2% FBS and 20 mM EDTA) and incubated with Phospho-STAT5 (Tyr694) antibody (clone SRBCZX, eBioscience) for 60 min at room temperature. Samples were washed with FACS buffer and analyzed using a BD FACS Calibur.

GM-CSF clearance

Differentiated macrophages were seeded at 100,000 cells per well and kept under cytokine-free conditions in X-vivo™ 15 medium (Lonza) before administration of 1 ng/ml GM-CSF. Supernatant from cells was collected 0, 8, 24, 32, 48 and 72 h post cytokine administration and GM-CSF concentration was measured by ELISA according to manufacturer's protocol (mouse GM-CSF ELISA Ready-SET-Go!, Affymetrix eBioscience).

Blood count

Blood samples were drawn 4, 8 and 12 weeks after HSCT from the retrobulbar venous plexus using a glass capillary. Samples were analyzed on a scil Vet ABC™ Hematology Analyzer (scil animal care company). Afterward, red blood cell lysis was performed by incubating blood samples for 4 min at room temperature with RCL buffer (4,15g NH_4Cl; 2,3g $KHCO_3$; 0,5 ml 0,5 M EDTA; *ad* 500 ml H_2O). Lysis was stopped by adding an excess amount of FACS buffer and subsequent centrifugation for 10 min at 300xg. The pellet was resuspended in 100 µl of MACS buffer and stained for hematopoietic markers to assess hematopoietic reconstitution and engraftment of transduced cells after HSCT.

CT Scans

CT images were acquired on a dedicated small-animal scanner (eXplore CT120, TriFoil Imaging). Animals were continuously warmed and anesthetized with 1.0 to 1.5% isoflurane, maintaining spontaneous breathing in a range of about 50-70 breaths per minute. Maximal inspiration and expiration for gating were set by respiratory monitoring (BioVet, m2m imaging corp.). For CT acquisition, parameters were adjusted to 80 kV, 50mA, 16-ms exposure time, 386 views, and 0.5° increment angle, resulting in scan duration of about 25

minutes. For reconstruction, filtered back-projection with a binning of 2 resulted in isotropic voxel dimensions of 98.3 mm.

Quantitative lung analysis was performed with the software Visage 7 (Visageimaging, Berlin, Germany). Lungs were segmented manually by two radiologists in consensus using a dedicated segmentation tool. The right and the left lung were analyzed separately for inspiration and expiration, and central vessels were excluded. Lung volume and mean lung density were calculated.

Respiratory mechanics

Mice were first anaesthetized with ketamine (80 mg/kg body weight i.p.) and xylazine (5 mg/kg body weight i.p.) and then tracheotomized and mechanically ventilated with a frequency of 100 breaths/min and a tidal volume of 10 ml/kg body weight (flexiVent rodent ventilator Fx1 for mice, SCIREQ). Pulmonary function was assessed by ventilation perturbations at a positive end-expiratory pressure (PEEP) value of 3 cmH_2O after two recruitment maneuvers. Recruitment maneuvers consisted of 2 following deep inflation of the lung up to 30 cmH_2O in 8 s, and inspiratory capacity was measured. After the recruitability maneuver, pressure-volume loops were recorded, and quasi-static compliance (Cst) was calculated according to the Salazar-Knowles equation [5]. Static compliance and inspiratory capacity were normalized by body weight to correct for the differences in animal and lung size.

Organ preparation

Right lung lobes (after lavage), bone marrow, which was flushed from femora and tibiae, spleen and liver were homogenized in FACS buffer using a 100 µm mesh filter. Blood samples were collected after cutting the ventral aorta. All samples were pelleted, and erythrocytes were lysed by incubation with RCL buffer for 3 min at RT. Cell suspensions were resuspended in 200 µl FACS buffer, filtered through a 40 µm mesh filter and divided for flow cytometry staining and genomic DNA (gDNA) isolation.

Lung cryosections

The left lung was filled with 500 µl Tissue-Tek® O.C.T compound (Sakura) diluted 1:2 in PBS, ligated and immediately transferred to dry ice. Cryopreserved lungs were stored at -80°C until they were sectioned into 4µm-thick sequential slides. The slides were separated using systematic uniform random sampling (SURS), and a total of 8-10 slides per lung were selected for Periodic-Acid-Schiff (PAS) positive material quantification. PAS staining was

performed using a PAS staining kit (Merck) and hematoxylin (Sigma-Aldrich) as nuclear staining according to the manufacturer's instructions. Slides were automatically scanned with AxioScanZ.1 (Carl Zeiss Microscopy GmbH), and random subsampling (10% fraction) at 20x magnification was performed using the NewCAST-system software (Viosiopharm A/S). Subsampled images were quantified by superimposing a test system (point grid) using the stereological tool STEPanizer1 [6] and PAS-positive material was quantified as a fraction of total lung parenchyma by point counting. Different subsets of SURS selected slides were used to perform immunofluorescence staining to detect Siglec-F positive cells using a PE-labelled antibody (BDBioscience) and CD45.1 using an APC-labelled antibody (eBioscience).

gDNA isolation

gDNA was isolated from homogenized tissue samples using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's instructions.

Droplet digital VCN PCR

5ng/ul of gDNA was used to perform vector copy number analysis by droplet digital PCR. ddPCR was performed as previously described [7] using primers (HIV Forward: 5′-TACTGACGCTCTCGCACC-3′; HIV Reverse: 5′-TCTCGACGCAGGACTCG-3′) and a probe (FAM 5′-ATCTCTCTCCTTCTAGCCTC-3′) against the primer binding site region of LV and normalized on murine endogenous DNA by a primers/probe set against the murine *sema3a* gene (Sema3A Forward: 5′-ACCGATTCCAGATGATTGGC-3′; Sema3A Reverse: 5′-TCCATATTAATGCAGTGCTTGC-3′; Sema3A probe: HEX 5′-AGAGGCCTGTCCTGCAGCTCATGG-3′ BHQ1). The PCR was performed with each primer (900 nM) and the probe (250 nM) following manufacturer's instructions (Bio-Rad) and read with QX200 reader. Analysis was performed with QuantaSoft Analysis Pro Software (Bio-Rad).

Flow cytometry

Cells were resuspended in FACS buffer (PBS containing 2% FBS and 20 mM EDTA) and incubated with purified Fc blocking CD16/32 antibodies for 20 min at 4 °C. Cells were subsequently stained with primary antibodies (see Supplementary Table 1) for 45 min at 4 °C. Cells were washed with FACS buffer and analyzed on a BD LSR II. Data were analyzed using FlowJo software.

Supplementary Table 1: Antibodies used for flow cytometry

Antigen	Fluorophore	Supplier	Clone	Catalog nr.
CD11c	PE-Cy7	eBioscience	N418	25-0114
CD131	APC	Miltenyi	REA193	130-103-076
CD16/32	purified	eBioscience	93	14-0161-82
CD19	APC	eBioscience	MB19-1	17-0191
CD3e	PE-Cy7	eBioscience	145-2C11	25-0031-81
CD45	eFluor450	eBioscience	30-F11	48-0451
CD45.1	PE-Cy7	eBioscience	A20	25-0453-82
F4/80	APC	eBioscience	BM8	17-4801-80
Gr-1	eFluor450	eBioscience	RB6-8C5	48-5931-82
Siglec-F	PE	BDBioscience	E50-2440	552126

Supplementary figures:

Figure S1: Safety of Lv.Csf2rb-transduction in HSPCs and thereof derived MΦ

(A) Schematic representation of the 3rd generation SIN lentiviral vector Lv.Csf2rb. LTR = long terminal repeat, CMV = cytomegalovirus promoter and enhancer region, R = redundant region, RRE = Rev-responsive element, cPPT = central polypurine tract, EF1α = elongation factor 1 α promoter, IRES = internal ribosomal entry site, eGFP = enhanced green fluorescent protein, wPRE = Woodchuck hepatitis virus posttranscriptional regulatory element, ΔU3 = deletion of promoter and enhancer elements in U3 region. (B) Representative picture of methylcellulose plates containing GM-CSF as the only instructive cytokine. Red arrows indicate some representative colonies that were formed by wildtype (WT) and Lv.Csf2rb-transduced *Csf2rb*$^{-/-}$ cells. (C) Representative higher magnification pictures of colonies formed by wildtype (WT) and Lv.Csf2rb-transduced *Csf2rb*$^{-/-}$ cells in methylcellulose plates containing GM-CSF. Scale bar = 500 µm (D) Total colony number per 1500 input HSPCs in methylcellulose-based colony formation assays containing IL-3, IL-6, SCF, and EPO. WT, *Csf2rb*$^{-/-}$ n=6 in technical duplicates in three independent experiments, Lv.Csf2rb n=4 in technical duplicates in two independent experiments. Bars indicate median ± interquartile range. Statistical analysis was done by one-way ANOVA using Tukey's multiple comparisons testing; ns = not significant. (E) Representative higher magnification pictures of colonies formed by wildtype (WT), *Csf2rb*$^{-/-}$ and Lv.Csf2rb-transduced *Csf2rb*$^{-/-}$ cells in

methylcellulose plates containing IL-3, IL-6, SCF, and EPO. Scale bar = 500 µm (F) Representative pictures of macrophages (MΦ) differentiated from HSPCs showing classical MΦ morphology in Pappenheim-stained cytospins. Scale bar = 20 µm.

Figure S2:

(A) Immunohistochemistry staining of cryopreserved lung slices showing brightfield (BF), CD45.1- and Siglec-F staining, autofluorescence of lung structures, and merge of all pictures. (B) Representative pseudocolor plots of homogenized lung tissue pregated on $CD45^+/F4/80^+$ cells depicting $CD11c^{high}/Siglec\text{-}F^+$ alveolar macrophages in untreated wildtype (WT) mice, while they are absent in untreated *Csf2rb*$^{-/-}$ mice. (C) Representative pseudocolor plot of bronchoalveolar lavage fluid (BALF) pregated on $CD45^+/F4/80^+$ cells depicting $CD11c^{high}/Siglec\text{-}F^+$ alveolar macrophages in untreated wildtype (WT) mice, while they are absent in untreated *Csf2rb*$^{-/-}$ mice. (D) Representative pseudocolor plot of bronchoalveolar lavage fluid (BALF) showing GFP and CD131 expression in total $CD45^+$ population. (E) Vector copy number (VCN) in bone marrow (BM), peripheral blood (PB), and bronchoalveolar lavage fluid (BALF) in *Csf2rb*$^{-/-}$ mice transplanted with Lv.GFP-transduced cells twelve weeks after HSC-GT in two independent experiments (n=3).

Figure S3: Improved lung function after Lv.Csf2rb HSC-GT

(A) Representative pictures of Periodic Acid-Schiff (PAS) stained lung slices depicting characteristic foci of PAS^+ material in lungs of *Csf2rb*$^{-/-}$ mice either untreated or transplanted with Lv.GFP-transduced cells. (B,C) Static compliance (B) and inspiratory capacity (C) measured during invasive lung function testing. WT n=9, *Csf2rb*$^{-/-}$ n=8, Lv.Csf2rb n=5, CD45.1 n=4, Lv.GFP n=2 in two independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons testing comparing all samples to *Csf2rb*$^{-/-}$ mice; ns = not significant, *p<0.05, **p<0.01, ***p<0.001.

Figure S4: Long-term clinical benefit nine months after Lv.Csf2rb HSC-GT

(A, B) Gating strategy (A) for flow cytometry analysis of AMs in BALF. Living cells were gated according to FSC/SSC. All CD45 positive cells of the living population were gated for Siglec-F and CD11c to evaluate the percentage of $Siglec\text{-}F^+CD11c^+$ alveolar macrophages (AMs) in all mice (B). Lv.Csf2rb n=7, CD45.1 and Lv.GFP n=4 in one experiment. Equal symbols in B-I represent the same mice. (C, D) Representative picture of BALF (C) and BALF turbidity (D) measured as optical density at 600 nm (OD_{600}). Lv.Csf2rb n=7, CD45.1

and Lv.GFP n=4 in one experiment. (E) Total protein concentration [µg/ml] in BALF. Lv.Csf2rb n=7, CD45.1 and Lv.GFP n=4 in one experiment. (F-I) Cholesterol [µg/ml] (F), GM-CSF [pg/ml] (G), M-CSF [pg/ml] (H), and MCP-1 [pg/ml] (I) levels in BALF. Lv.Csf2rb n=7, CD45.1 and Lv.GFP n=4 in one experiment.

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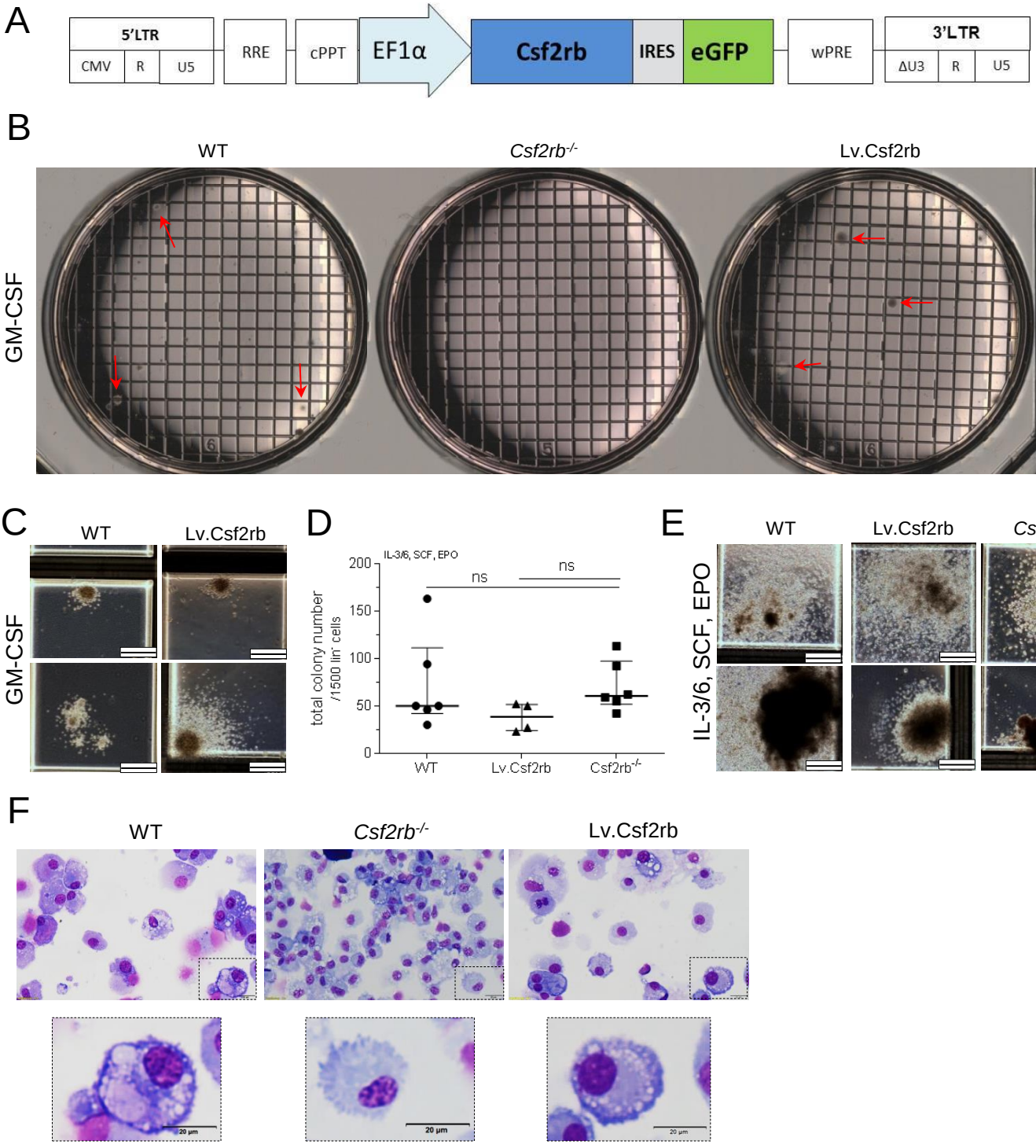
Sup. Fig. 1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B
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Lv.Csf2rb
GM-CSF
C
WT
Lv.Csf2rb
GM-CSF
D
IL-3/6, SCF, EPO
total colony number /1500 lin- cells
200
150
100
50
0
ns
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WT
Lv.Csf2rb
Csf2rb-/-
E
WT
Lv.Csf2rb
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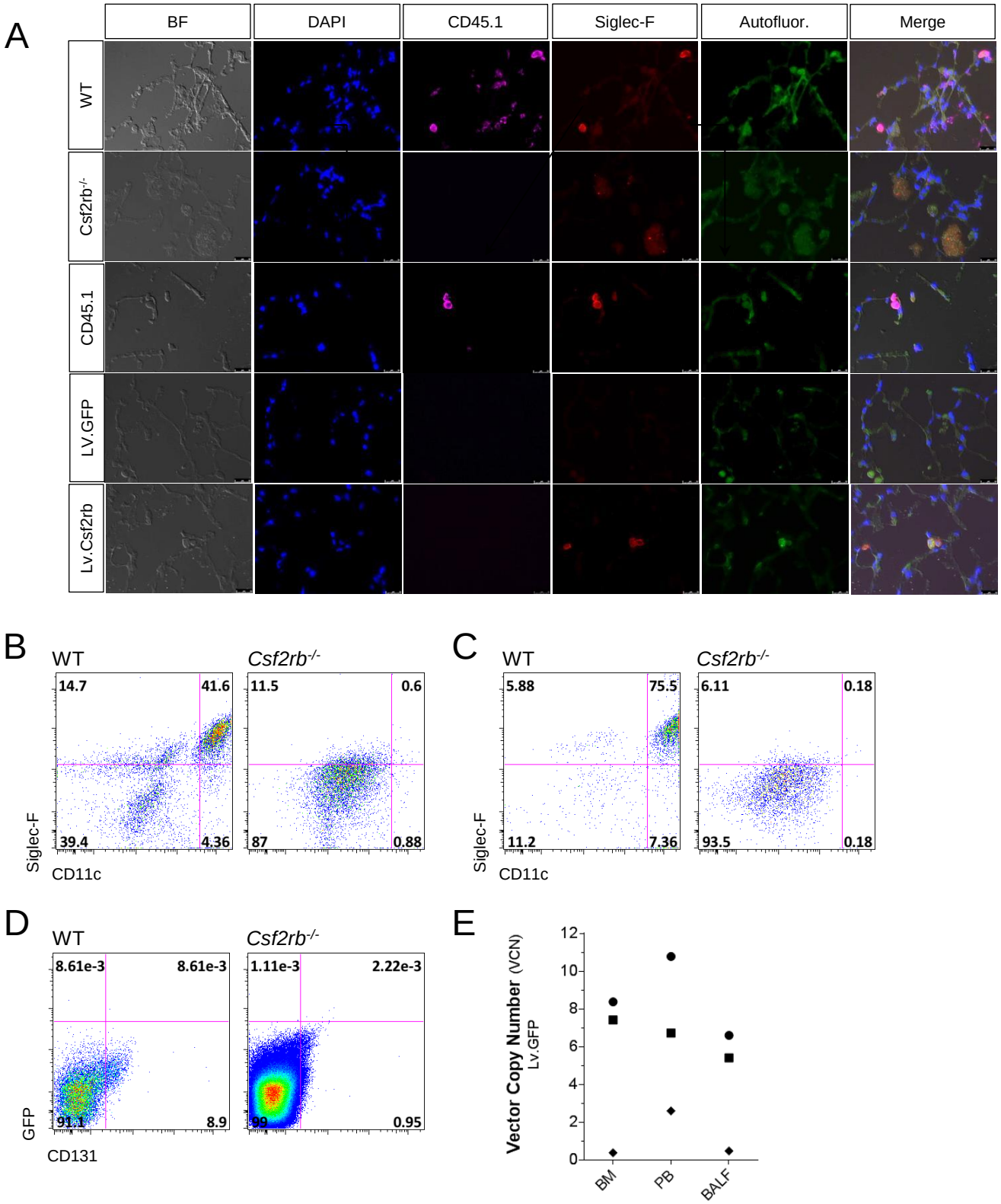
20 µm

Sup. Fig. 2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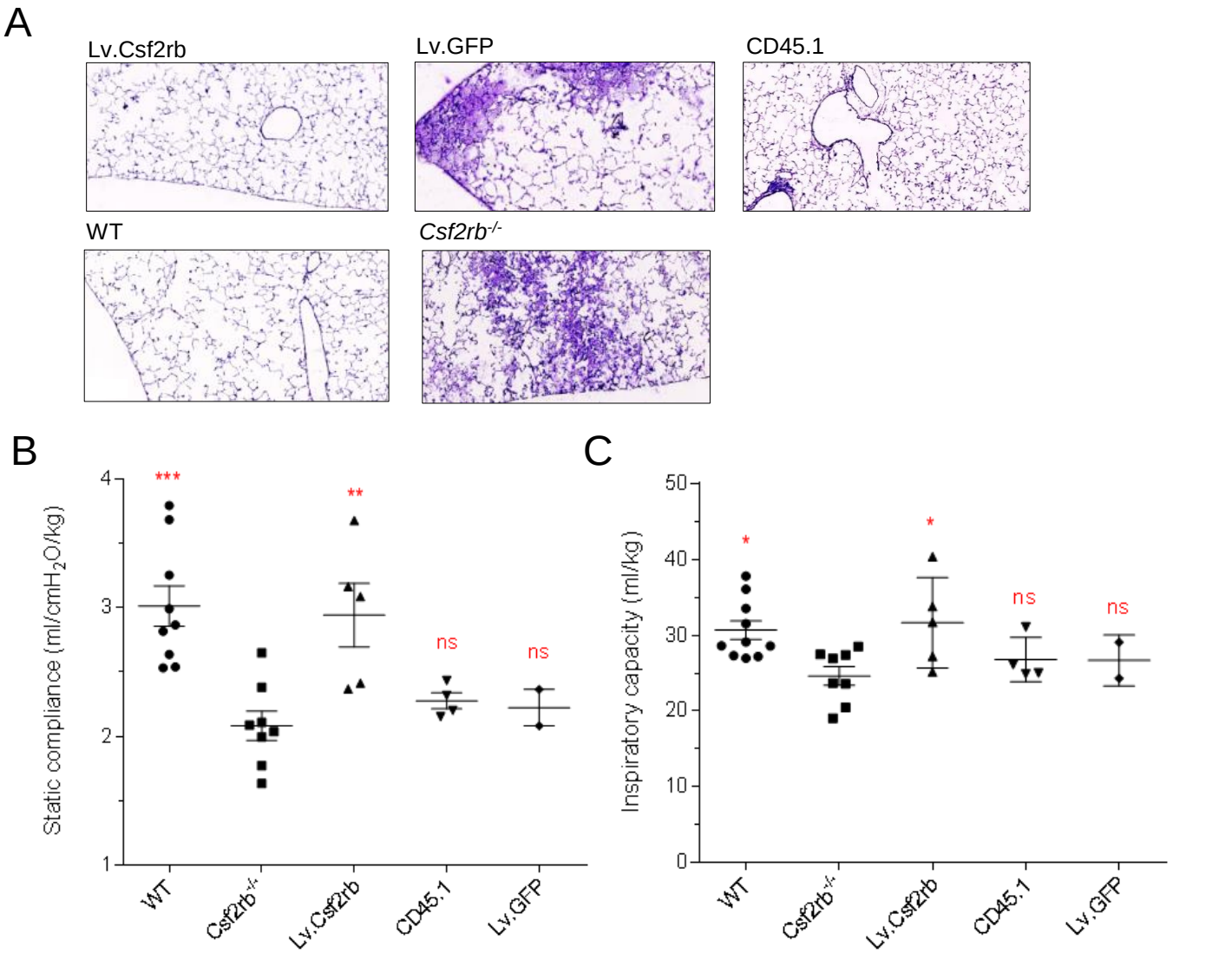
BALF

Sup. Fig. 3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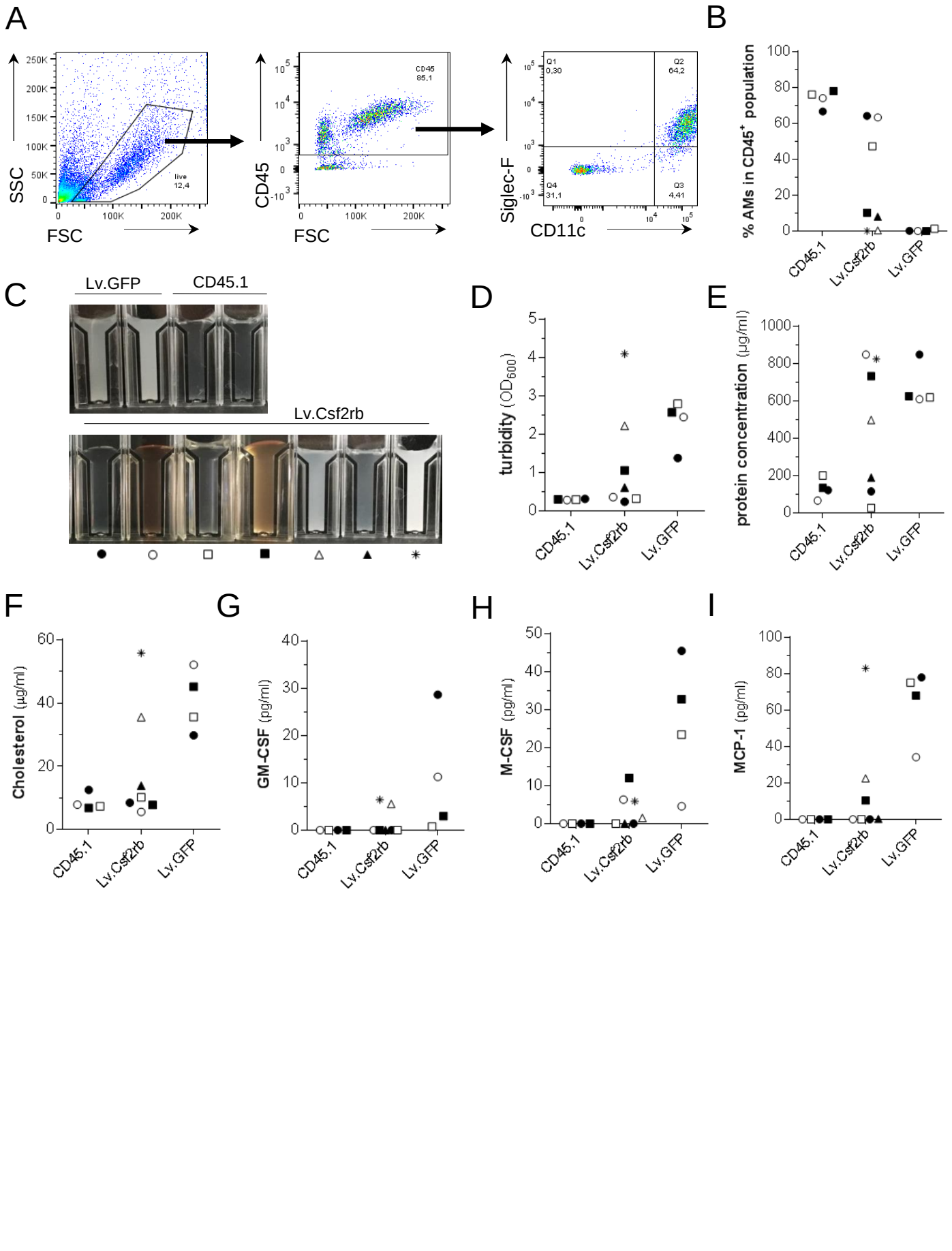
Sup. Fig. 4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Q3
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% AMs in CD45+ population
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Lv.Csf2rb
Lv.GFP
C
Lv.GFP
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D
turbidity (OD600)
E
protein concentration (µg/ml)
F
Cholesterol (µg/ml)
G
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H
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I
MCP-1 (pg/ml)