Wiskott-Aldrich syndrome protein-deficient hematopoietic cells can be efficiently mobilized by granulocyte colony-stimulating factor

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Online Supplementary Design and Methods

Transduction of mobilized peripheral blood cells with the GFP lentiviral vector

The LV-PGK-GFP-WPRE vector encoding the green fluorescent protein (GFP) has already been described.[1] Bone marrow cells or mobilized peripheral blood cells from WT or WKO mice were transduced overnight at 37°C in 5% CO_2 with $1x10^8$ i.g./mL of LV-PGK-GFP-WPRE vector in the presence of protamine sulfate (6 μg/mL) in chemically-defined serum-free X-VIVO 20 medium devoid of phenol red and antibiotics (BioWhittaker, Walkersville, NJ, USA) and supplemented with 2% fetal calf serum (FCS), 2 mM L-glutamine, 50 U/L penicillin, 50 ng/mL streptomycin (Invitrogen, Cergy-Pontoise, France). Prior to adding the virus to the culture medium, cells underwent a short (2 h) pre-activation in the presence of murine interleukin-3 (10 ng/mL), human thrombopoietin (25 ng/mL), human Flt-3 ligand (50 ng/mL) (R&D Systems, Lille, France) and murine stem cell factor (50 ng/mL) (Abcys SA, Paris, France). At the end of transduction, fresh medium was added and the following day, cells were washed in VIVO20 and transplanted.

Peripheral blood analysis

One hundred microliters of blood were collected from the mice by retro-orbital puncture using citrate 3.8% as anti-coagulant (1/10 volume). Blood was collected every day for the mobilization study or 2 months after transplantation. Blood samples were analyzed for standard hematologic parameters (white blood cell count, red blood cell count, hematocrit and platelet count) using an MS9.3 counter (Schloessing Melet, Cergy-Pontoise, France), for CFC activity and for cell surface markers as described below.

Colony-forming cell assay

Blood, bone marrow, and spleen cells were harvested from mice using standard techniques. Twenty microliters of blood, $1x10^5$ nucleated spleen cells, or $2x10^4$ nucleated BM cells were plated in duplicate in methylcellulose media supplemented with a cocktail of recombinant cytokines (MethoCult 3434; StemCell Technologies, Vancouver, BC, Canada) and placed in a humidified chamber with 6% CO_2 at 37°C. Colonies were counted after 7 days of incubation.

Immunophenotyping by flow cytometry

Cell suspensions from peripheral blood, thymus, spleen and bone marrow were stained with fluorescently labeled antibodies and analyzed using the dual-laser FACS Calibur cytometer (Becton Dickinson). Briefly, before analysis, the cells were incubated with the antibodies for 30 min and washed twice in phosphate-buffered saline.

Thymic cells were stained with antibodies to CD3-PE (clone 145-2C11), CD4-PerCP-Cy5 (clone H129.19), and CD8-APC (clone 53-6.7) (Pharmingen). Peripheral blood and spleen cells were lysed with ACK (NH_4Cl) and washed before staining. Peripheral blood, spleen and bone marrow cells were stained with antibodies to B220-APC (clone R13-6B2) and CD3-PE or Gr1-PE (cloneRB6-8C5) (Pharmingen). Sca-1^+-c-Kit^+ lineage negative (Lin^-) cells were identified by antibodies to CD3-PE, B220-PE, CD11b-PE (clone M1/70), Gr1-PE, Ter119-PE, Sca-1-FITC (clone E13-161.7) and c-Kit-APC (clone 2B8) (Pharmingen).

Spleen histology

Spleen tissues were delicately removed from euthanized animals and the tissues were fixed in 10% formalin. Paraffin-embedded tissue sections (4 μm) were stained with hematoxylin and eosin for histologic examination by microscopy. The quantification of red and white pulp was done by Image J software. The white pulp region is dense in lymphoid cells and was identified by areas of hematoxylin blue stain. The red pulp corresponding to the vascularized and erythrocyte-rich area was identified by the eosin red stain. The color deconvolution was processed using a imageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, *http://rsb.info.nih.gov/ij/*) plugin which was written by Gabriel Landini (Oral Pathology Unit, School of Dentistry, University of Birmingham, Birmingham, UK. g.landini@bham.ac.uk) and implements stain separation using Ruifrok and Johnston's method.[2] The code is based on a NIH Image macro kindly provided by A.C. Ruifrok.

Quantitative polymerase chain reaction analyses of murine cells

For blood cell analysis, genomic DNA was extracted by a QIAmp DNA blood Mini Kit (Qiagen, Hilden, Germany).

Genomic DNA was extracted from murine tissue cells or from human cells using the "Wizard genomic DNA purification

kit" (Promega Corporation Madison WI USA). In murine cells and tissues, VCN was measured by amplifying the WPRE sequence in reference to the murine TTN gene and results were calculated from a standard curve based on dilutions of a plasmid containing both the WPRE and mouse TTN sequences; male and female chimerism was measured by quantification of murine Y sequence as described above. Total RNA was extracted using SV total RNA isolation system (Promega). One microgram of each total RNA sample was reverse transcribed into cDNA with the Super-Script II RT (GIBCO BRL) using random hexamer primers at 42°C for 50 min. The reverse transcription was inactivated by incubation for 15 min at 70°C. Amplification reactions (17 μL) contained 8 μL of sample cDNA and 9 μL of TaqMan buffer (TaqMan Universal CR Master Mix, No AmpErase UNG, Applied Biosystems), 0.2 mM primers (forward and reverse), and 0.1 mM TaqMan probe and consisted of 40 cycles at 95°C (15 s) then 60°C (1 min). Standard amplification curves were obtained by serial dilutions of a known cDNA sample. All PCR measures were performed at least in duplicate. Data were edited using the Primer Express software. Results were expressed as the ratio of the interested gene/mTFIID. Primers and probes are detailed in *Online Supplementary Table S1*.

In vivo *homing assay*

Cells from wild-type 129Sv mice were stained with CSFE (5- and 6-carboxyfluorescent diacetate succinimidyl ester) (Molecular Probes Eugene, OR) (green dye), and cells from WASp-deficient mice were stained with PKH26 (Sigma, St Louis, MO, USA) (red dye) as already described.[3] Then, $10x10^6$ labeled wild-type nucleated cells were mixed with an equal number of labeled WASp-deficient cells, and the 1:1 mixture was injected into lethally irradiated mice. Spleen cells of recipient mice were harvested 24 h after the injection, and the origins of the cells that had migrated in the spleen were analyzed by determining the proportion of either green (CSFE)– or red (PKH26)–labeled cells in total cells, B lymphocytes and granulocytes. Spleen cells were stained with anti-B220-APC or anti-GR1_APC antibody (Pharmingen). The homing index is the percentage of WKO cells compared to the WT cells in WT mice.

References

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3. Lacout C, Haddad E, Sabri S, Svinarchouk F, Garcon L, Capron C, et al. A defect in hematopoietic stem cell migration explains the nonrandom X-chromosome inactivation in carriers of Wiskott-Aldrich syndrome. Blood. 2003;102(4):1282-9.

Online Supplementary Table S1. Sequences of the primers and probes used for real-time quantitative PCR.

Gene name	Gene bank Accession no.	Primer name	Sequence
WPRE	NC_001562	458-WPRE.probe	5'-ACGTCCTTTCCATGGCTGCTCGC-3'
WPRE	NC_001562	422-WPRE.forward	5'-GGCACTGACAATTCCGTGGT-3'
WPRE	NC_001562	529-WPRE.reverse	5'-AGGGACGTAGCAGAAGGACG-3'
mY	AC134433	mZFY-1.probe	5'-TTCTCCAGGACCAGTGACTGGAGAYCA-3'
mY	AC134433	mZFY-1.forward	5'-GTGCTAAGGAGTAGAGCGGAGAA-3'
mY	AC134433	mZFY-1.reverse	5'-CATGGTAACTGCTCAAGCGGT-3'
mTtn	XM130312.3	TitinMex5.probe	5'-TGCACGGAAGCGTCTCGTCTCAGTC-3'
mTtn	XM130312.3	TitinMex5.forward	5'-AAAACGAGCAGTGACGTGAGC-3'
mTtn	XM130312.3	TitinMex5.reverse	5'-TTCAGTCATGCTGCTAGCGC-3'
mTFIID	D01034	654-mTFIID.probe	5'-TGTGCACAGGAGCCAAGAGTGAAGA-3'
mTFIID	D01034	616-mTFIID.foward	5'-ACGGACAACTGCGTTGATTTT-3'
mTFIID	D01034	724-mTFIID.reverse	5'-ACTTAGCTGGGAAGCCCAAC-3'
CXCL12	NM_021704	mCXCL12F	5'-GAGCCAACGTCAAGCATCTG-3'
CXCL12	NM_021704	mCXCL12R	5'-CGGGTCAATGCACACTTGTC-3'
CXCL12	NM_021704	mCXCL12P	5'-TCCAAACTGTGCCCTTCAGATTGTTGC-3'

Online Supplementary Table S2. Phenotype of control and recipient mice 2-4 months after-transplantation. Percentage of $CD3^+$, $B220^+$, $Gr1^+$, $CD4^+CD8^+$ cells in peripheral blood, bone marrow, spleen and thymus. Results are expressed as percentage mean ± SD (range); n= number of mice analyzed.

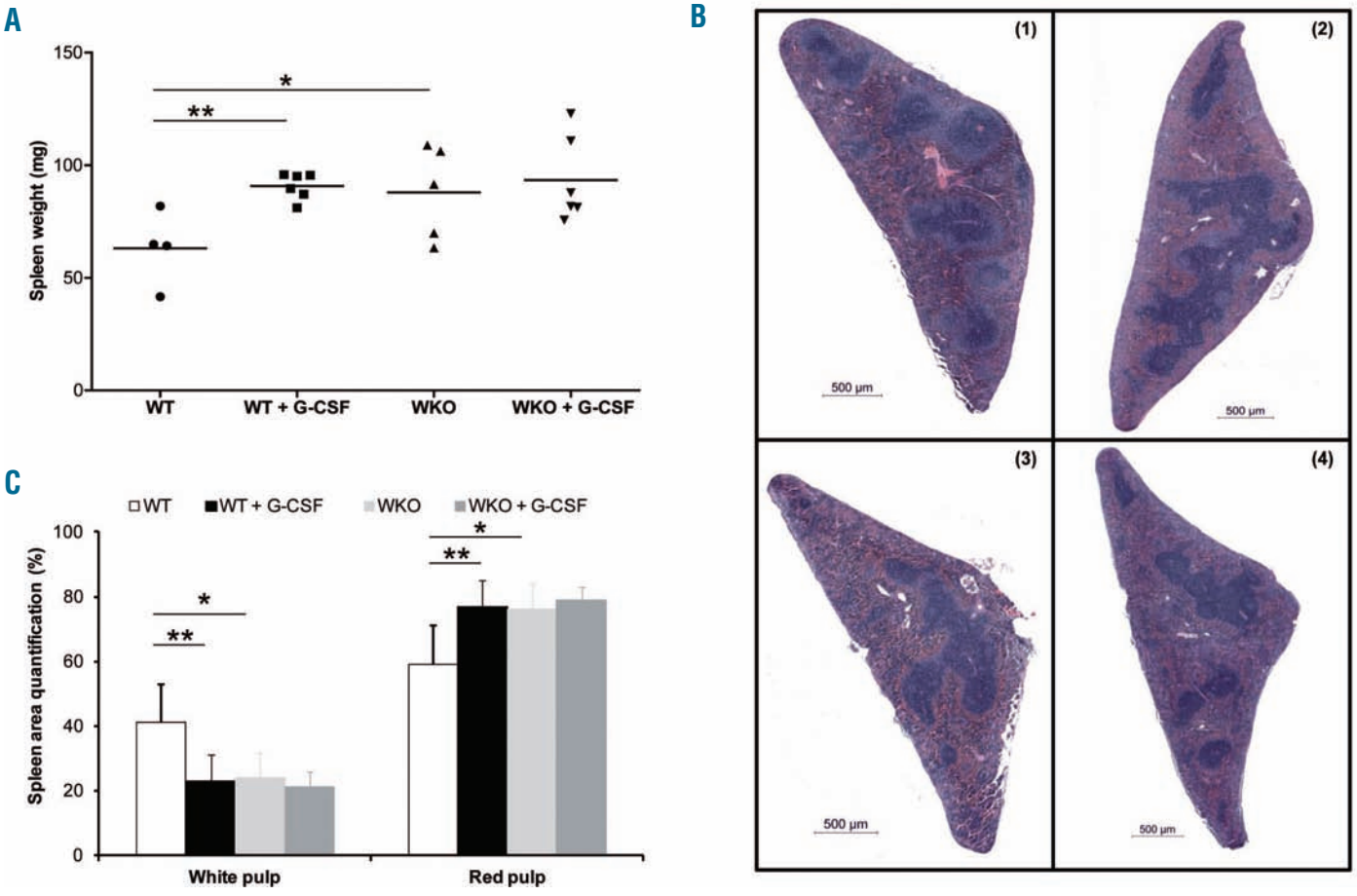
		Peripheral Blood			Bone Marrow			Spleen			Thymus	
Recipient Mice	**Cells engrafted**	**CD3+**	**B220+**	**GR1+**	**CD3+**	**B220+**	**GR1+**	**CD3+**	**B220+**	**GR1+**	**CD3+**	**CD4+CD8+**
non-irradiated WT	None (n=9)	40 ± 14	25 ± 5	19 ± 9	6 ± 2	20 ± 7	43 ± 5	38 ± 10	51 ± 10	3 ± 1	17 ± 6	78 ± 6
non irradiated WKO	None (n=5)	30 ± 9	12 ± 3	41 ± 8	6 ± 2	6 ± 2	73 ± 4	35 ± 13	19 ± 13	21 ± 11	23 ± 7	73 ± 5
WT Lethally irradiated 9.5 Gy	WT BM (n=7)	42 ± 8	24 ± 5	21 ± 9	4 ± 1	10 ± 5	48 ± 6	44 ± 10	44 ± 8	3 ± 1	21 ± 5	76 ± 5
	WKO BM (n=5)	32 ± 13	16 ± 10	36 ± 22	4 ± 1	7 ± 2	46 ± 4	32 ± 9	31 ± 19	11 ± 7	15 ± 2	78 ± 4
	WT MPBC (n=1)	55	21	19	2	4	60	22	56	4	7	36
	WKO MPBC (n=4)	44 ± 4	19 ± 7	35 ± 26	5 ± 2	6 ± 2	49 ± 4	38 ± 10	36 ± 18	4 ± 2	20 ± 7	54 ± 19
WKO Lethally irradiated 9.5 Gy	WT BM (n=12)	37 ± 10	14 ± 8	26 ± 10	5 ± 2	7 ± 3	52 ± 9	35 ± 9	38 ± 10	5 ± 3	19 ± 3	70 ± 12
	WKO BM (n=5)	20 ± 7	8 ± 5	37 ± 10	4 ± 2	3 ± 1	61 ± 11	28 ± 14	14 ± 3	14 ± 5	16 ± 3	42 ± 36
	WT MPBC (n=1)	29	5	46	5	5	45	40	32	2	18	56
	WKO MPBC (n=4)	15 ± 8	5 ± 4	63 ± 11	2 ± 1	2 ± 1	41 ± 7	33 ± 19	11 ± 6	15 ± 3	15 ± 8	39 ± 28

Online Supplementary Table S3. Number of progenitor cells in bone marrow cells (BMC) or mobilized peripheral blood cells (MPBC) of WT or WKO mice. A fraction of the BMC or MPBC destined for transplantation was either plated in methyl-cellulose or analyzed by flow cytometry to measure the hematopoietic progenitor cell content. CFC=colony-forming cells, $c\text{-}Kit^+$, $SK=Sca^+c\text{-}Kit^+$; Lin^-=lineage negative; $LK= Lin^-C\text{-}Kit^+$; $LSK =Lin^-Sca\text{-}1^+c\text{-}Kit^+$ cells. n.s. non-significative.

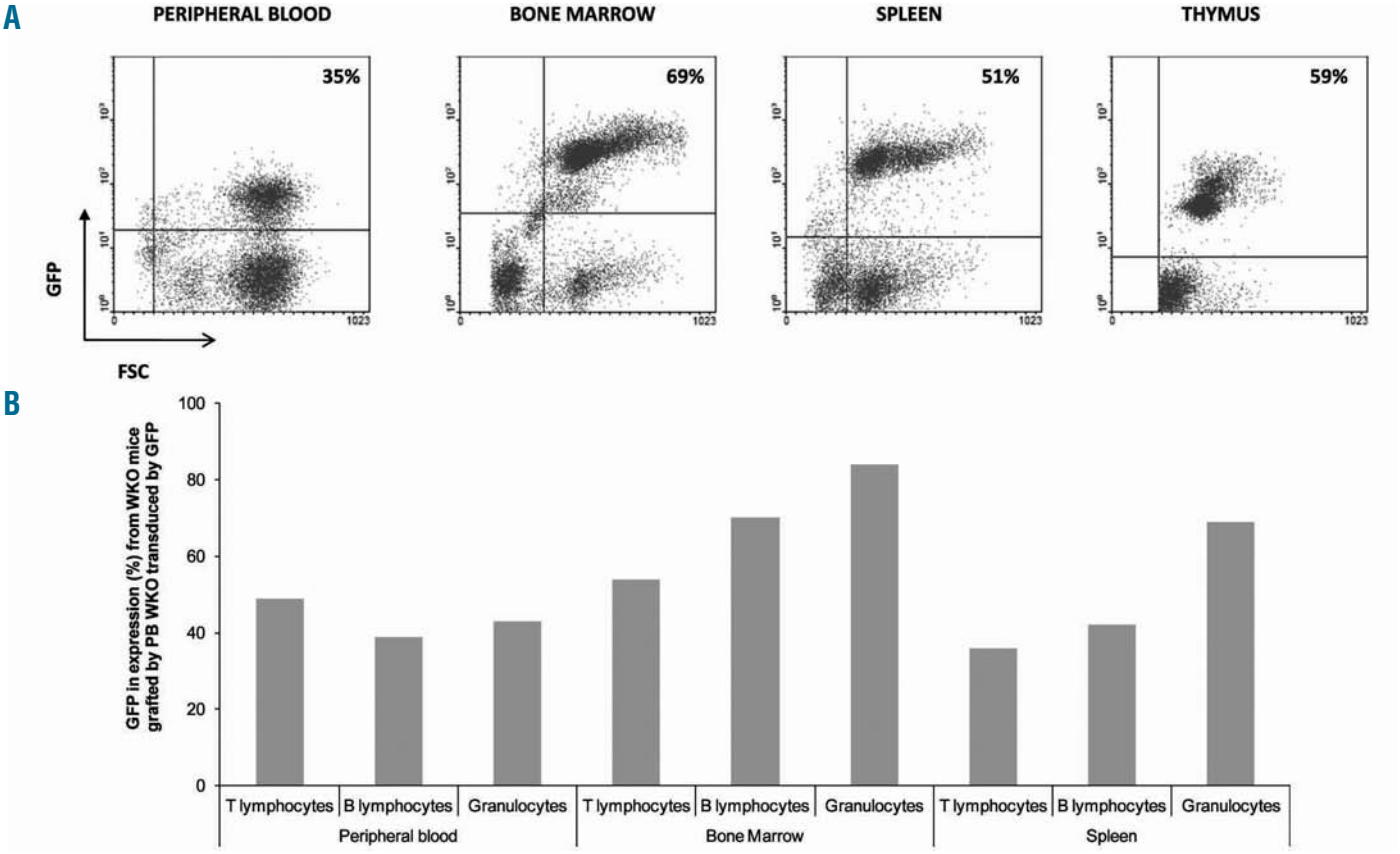
Number of cells		**WT**	**WKO**	***P value***
For 10^6 BMC	CFC	1910 ± 870	3230 ± 240	< 0.05
	C-Kit+	44 240 ± 9064	52460 ± 9734	*n.s.*
	SK	8460 ± 1718	9780 ± 2742	*n.s.*
	Lin-	55 560 ± 7600	41 100 ± 2800	*< 0.01*
	LK	5952 ± 1036	9053 ± 2039	*< 0.01*
	LSK	840 ± 209	2551 ± 916	*< 0.01*
For 10^6 MPBC	CFC	579	1600 ± 1200	*N.A.*
	C-Kit+	47 700 ± 7200	60 500 ± 3500	*< 0.05*
	SK	4300 ± 600	4000 ± 1000	*n.s.*

Online Supplementary Table S4. Transduction efficiency of mobilized progenitor cells after 7 days of *in vitro* culture. Transduction was measured by expression of GFP by FACS as a percentage of the cell population and by vector copy number per cell (VCN) measured by qPCR amplification of HIV sequences normalized to mouse TTN gene. Results are expressed as the mean value ± SD (range); n: number of experiments.

	Transduced BM cells (n=3)	Transduced Mobilized PB cells (n=5)
GFP expression (%)	48 ± 20	47 ± 27
VCN	1.4 ± 0.7	1.4 ± 1.2



Online Supplementary Figure S1. Spleen characteristics in the steady state and after 4 days of G-CSF administration in WT and WKO mice. (A) The weight of spleen. (B) Hematoxylin-eosin staining of spleen sections (1) WT spleen; (2) WT spleen after 4 days of G-CSF; (3) WKO spleen; (4) WKO spleen after 4 days of G-CSF. (C) Quantification of white and red pulp of spleen in the following numbers of mice: (WT=4; WKO=5; WT+GCSF=6; WKO+G-CSF=6). Lines indicate mean values. *P value < 0.05, **P value < 0.01.



Online Supplementary Figure S2. Gene-modified cells are found in mice transplanted with GFP-transduced mobilized peripheral blood cells (MPBC) from WKO mice. Lethally-irradiated WKO mice were transplanted with GFP-transduced WKO MPBC and GFP expression was measured by FACS at 2 months in peripheral blood and at 4 months in bone marrow, spleen and thymus. (A) Representation of GFP levels in the different compartments. (B) The quantification of GFP in different cell subsets defined as $B220^+$ B lymphocytes, $CD3^+$ T lymphocytes, and $Gr1^+$ granulocytes.