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Aged Murine Hematopoietic Stem Cells Drive Aging-Associated Immune Remodeling

Hanna Leins, Medhanie Mulaw, Karina Eiwen, Vadim Sakk, Ying Liang, Michael Denkinger, Hartmut Geiger and Reinhold Schirmbeck

Supplemental Materials and Methods

Mice

B6.129S7-*Rag1*^{tm1Mom}/J (*RAG1*$^{-/-}$), B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (B6.SJL) and C57BL/6J mice were obtained from the animal facility of Ulm University (Ulm, Germany) or from Charles River Laboratories (Wilmington, USA). Studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German Federal Animal Protection Law. The protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Ulm (Tierforschungszentrum Ulm, Oberberghof) and the Regierungspräsidium Tübingen.

Cell Sorting

HSCs were isolated from bone marrow of femur and tibia of young and aged healthy C57BL/6 and B6.SJL mice. Mononuclear cells were separated form total bone marrow by low-density centrifugation (Histopaque 1083, Sigma) and stained with a cocktail of biotinylated rat anti-mouse lineage antibodies: anti-CD11b, anti-B220, anti-CD5, anti-Gr-1, anti-Ter119 and anti-CD8a. Subsequently, lineage depletion was performed by magnetic separation (Dynabeads, Invitrogen) to enrich for lineage negative cells. Thereafter, cells were stained with anti-Sca-1, anti-c-Kit, anti-CD34, anti-Flk2, and Streptavidin (all from e-bioscience). LT-HSCs were then sorted as lin^{-} Sca-1^{+}c-kit^{+} CD34^{-}Flk-2^{-} cells using a BD FACS Aria II or a BD FACS Aria III (BD Bioscience).

Non-competitive HSC transplantation

HSCs were sorted in 1.5 mL reaction tubes and cultured for 16 hrs in HBSS (Lonza) supplemented with 10% FBS (Biochrome) and, where indicated, with 5µM CASIN[1] (Xcessbio) at 37°C (5% CO_2, 3% O_2). Subsequently, HSCs were transplanted into sublethally irradiated *RAG1*$^{-/-}$ mice. Reconstituted mice were maintained on Neomycin solution (1.1 mg/mL, Sigma-Aldrich) in drinking water. Donor contribution in peripheral blood was analyzed by FACS from 4 to 12 weeks post transplantation.

Flow Cytometry

For characterization of the immune system, peripheral blood and spleen immunostaining was performed according to standard protocols. Briefly, erythrocytes from single-cell spleen suspensions and peripheral blood were lysed with a hypotonic buffer. Cell surface was stained for 30 min at 4°C with fluorochrome-conjugated antibodies, cells stained with biotin-conjugated antibodies were stained again for 30 min at 4°C with fluorochrom-tagged streptavidin. Intracellular staining for FoxP3, HELIOS and Ki-67 was performed using the Foxp3 / Transcription Factor Staining Buffer Set (ebioscience) according to the manufacturer's protocol. Samples were collected with LSR-II (BD Bioscience). Flow Cytometer and data was analyzed using FCS express software. All antibodies used are summarized in supplemental Table S1.

RNA Sequencing

12 weeks after transplantation, 50.000 naïve CD4^{+} T cells and CD19^{+} B cells were sorted in Ultra Culture medium (Lonza). RNA was extracted from sorted cells using the RNeasy Micro Kit (Qiagen). cDNA was processed with the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech). Quality and quantity of the cDNA was determined using the 2100 Bioanalyzer (Agilent). cDNA library was prepared using the Nextera XT DNA Library Prep Kit (Illumina). At the end all cDNA libraries contained a unique index barcode and were pooled to one sample. Samples were sequenced on Illumina HiSeq2000 platform. Per sample sequencing depth was 30 million reads.

The raw paired-end reads were adapter trimmed and quality filtered (phred score of > 20) using the cutadapt wrapper trim galore[2]. Filtered sequences were aligned to the mouse genome version mm10 using TopHat and processed further using Cufflinks[3]. All downstream analysis including differential expression analysis and additional statistical tests related to RNA-seq were performed using R and bioconductor packages[4,5] and in-house scripts. The broad MIT GSEA application[6] was used for Gene Set Enrichment Analysis (GSEA).

Statistical Analyses

Data were assumed to meet normal distribution. All data are plotted as mean ± 1 (s.e.m.). The s.e.m. is used to indicate the precision of an estimated mean. Such a data representation does not affect the statistical analyses as variance information is used in the test statistics. Data were analyzed using PRISM software (GraphPad). A paired Student's *t*-test was used to determine the significance of the different means among two groups. To compare means of three independent groups, one-way ANOVA. When the overall *P*-value was < 0.05, Bonferroni post-test was applied to compare all pairs of data set. The total number (*n*) of mice, analyzed within a set of experiments, included in the final statistical analyses is indicated. Mice for experiments were randomly chosen from in-house colonies or suppliers. Animals showing signs of sickness, with clear alterations of blood parameter and/or showing signs of major disease involving also non-haematopoietic tissues were excluded from analysis.

References:

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2. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal*. 2011;17.
3. Trapnell C, Williams BA, Pertea G, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol*. 2010;28(5):511-515.
4. R Core Team. R: A language and environment for statistical computing: R Foundation for Statistical Computing, Vienna, Austria, URL http://www.R-project.org/; 2015.
5. Huber W, Carey VJ, Gentleman R, et al. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods*. 2015;12:115-121.
6. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. 2005;102:15545–15550.

Supplemental Tables

Supplemental Table S1. Antibodies used for flow cytometry analyses.

Antibody	Manufacturer	Clone	Catalog No.	Lot No.
anti-CD3 PE-Cy7	ebioscience	145-2C11	25-0031-82	4304567
anti-CD3 FITC	ebioscience	145-2C11	11-0031-85	E00061-165
anti-CD4 APC	ebioscience	RM4-5	17-0042-83	E0741-1632
anti-CD4 biotinylated	ebioscience	RM4-5	13-0042-85	E02363-1630
anti-CD8 Pacific Blue	BD	53-6.7	558106	7144718
anti-CD8 PE-Cy7	ebioscience	53-6.7	25-0081-82	4290706
anti-CD44 FITC	BD	IM7	553133	96243
anti-CD62L biotinylated	ebioscience	MEL-14	13-0621-85	E02581-1631
anti-Gr-1 eFluor 450	ebioscience	RB6-8C5	48-5931-82	4274410
anti-CD11b AlexaFluor700	ebioscience	M1/70	56-0112-82	4319576
anti-CD19 APC	ebioscience	eBio1D3	17-0193-82	4280786
anti-CD244.2 FITC	ebioscience	eBio244F4	11-2441-82	E00538-1633
anti-LAG-3 APC	ebioscience	eBioC9B7W	17-2231-82	E16755-105
anti-TIM-3 AlexaFluor488	R&D Systems	# 215008	FAB1529G	ADJN0315061
anti-PD-1 biotinylated	ebioscience	J43	12-9985-83	E02310-187
anti-FoxP3 FITC	ebioscience	FJK-16s	11-5773-82	E00644-1634
anti-HELIOS Pacific Blue	BioLegend	22F6	137210	B227508
anti-Ki-67 APC	BioLegend	16A8	652406	B223137
anti-CD23 PE-Cy7	ebioscience	B3B4	25-0232-82	E07532-1634
anti-CD21/35 Pacific Blue	BioLegend	7E9	123414	B178057
anti-CD45.1 PE	BD	A20	553776	3303840
anti-CD45.1 APC	BioLegend	A20	110714	B136085
anti-CD45.2 APC-Vio770	Miltenyi Biotec	104-2	130-105-113	5150915279
Streptavidin PerCP-Cy5.5	eBioscience	-	45-4317-82	4295929

Supplemental Table S2. Overlapping genes differentially expressed in naïve CD4$^+$ T cells between recipients of DY versus DO and DO+C versus DO HSCs

Gene	Sample 1	Sample 2	log2 fold change	P	Q		Sample 1	Sample 3	log2 fold change	P	Q
Adam19	Aged	Young	-2.99584	0.00005	0.00637929		Aged	Aged_CASIN	-1.33793	0.00005	0.00637929
Ank	Aged	Young	1.10882	0.00005	0.00637929		Aged	Aged_CASIN	1.01384	0.00025	0.0232578
Capg	Aged	Young	-1.8171	0.00005	0.00637929		Aged	Aged_CASIN	-1.0898	0.00005	0.00637929
Cd74	Aged	Young	-1.94069	0.00005	0.00637929		Aged	Aged_CASIN	-1.42174	0.00005	0.00637929
Coro2a	Aged	Young	-2.18448	0.00005	0.00637929		Aged	Aged_CASIN	-1.59865	0.00005	0.00637929
Cox6b1	Aged	Young	-1.36289	0.0005	0.0366025		Aged	Aged_CASIN	-1.31391	0.0004	0.0332316
E2f2	Aged	Young	-1.58993	0.00005	0.00637929		Aged	Aged_CASIN	-1.43616	0.00005	0.00637929
Folr4	Aged	Young	-1.29553	0.00005	0.00637929		Aged	Aged_CASIN	-1.01586	0.0002	0.0201831
Hivep3	Aged	Young	-1.16221	0.00005	0.00637929		Aged	Aged_CASIN	-0.989197	0.0002	0.0201831
Ikzf2	Aged	Young	-1.24203	0.00005	0.00637929		Aged	Aged_CASIN	-1.16622	0.00005	0.00637929
Il2rb	Aged	Young	-1.54187	0.00005	0.00637929		Aged	Aged_CASIN	-1.10026	0.00005	0.00637929
Itgae	Aged	Young	-2.69962	0.00025	0.0232578		Aged	Aged_CASIN	-2.02625	0.00005	0.00637929
Itih5	Aged	Young	-1.02489	0.00015	0.0160437		Aged	Aged_CASIN	-1.32318	0.00005	0.00637929
Lag3	Aged	Young	-2.87494	0.00005	0.00637929		Aged	Aged_CASIN	-1.22154	0.0001	0.0115987
Maf	Aged	Young	-2.84395	0.00005	0.00637929		Aged	Aged_CASIN	-1.61631	0.00005	0.00637929
Mki67	Aged	Young	-2.74954	0.00005	0.00637929		Aged	Aged_CASIN	-1.80689	0.00005	0.00637929
Neb	Aged	Young	-2.64949	0.00005	0.00637929		Aged	Aged_CASIN	-1.53759	0.00005	0.00637929
Penk	Aged	Young	-1.4644	0.00005	0.00637929		Aged	Aged_CASIN	-1.59808	0.00005	0.00637929
Pglyrp1	Aged	Young	-2.80696	0.00015	0.0160437		Aged	Aged_CASIN	-1.52549	0.00025	0.0232578
Ptprs	Aged	Young	-0.855839	0.0007	0.0463089		Aged	Aged_CASIN	-1.18423	0.00005	0.00637929
Rgmb	Aged	Young	1.10331	0.0002	0.0201831		Aged	Aged_CASIN	1.10052	0.0002	0.0201831
Rpl14-ps1	Aged	Young	-0.984562	0.00005	0.00637929		Aged	Aged_CASIN	-1.05092	0.00005	0.00637929
Rplp2-ps1	Aged	Young	-0.848291	0.00005	0.00637929		Aged	Aged_CASIN	-0.78198	0.00025	0.0232578
Rps9	Aged	Young	-0.75268	0.00045	0.0346461		Aged	Aged_CASIN	-0.734776	0.0006	0.0417012
St8sia6	Aged	Young	1.05961	0.00005	0.00637929		Aged	Aged_CASIN	1.00605	0.00005	0.00637929
Tdrp	Aged	Young	1.37976	0.00005	0.00637929		Aged	Aged_CASIN	1.22379	0.00005	0.00637929
Tmsb4x	Aged	Young	-0.727061	0.00025	0.0232578		Aged	Aged_CASIN	-0.919012	0.00005	0.00637929
Tnfrsf1b	Aged	Young	-1.95258	0.00005	0.00637929		Aged	Aged_CASIN	-1.06302	0.00005	0.00637929
Tnfrsf4	Aged	Young	-1.67133	0.00005	0.00637929		Aged	Aged_CASIN	-0.962786	0.0005	0.0366025
Tnfrsf9	Aged	Young	-1.86297	0.00005	0.00637929		Aged	Aged_CASIN	-1.00427	0.00065	0.0439784

Supplemental Table S3. Overlapping genes differentially expressed in CD19$^+$ B cells between recipients of DY versus DO and DO+C versus DO HSCs

Gene	Sample 1	Sample 2	log2 fold change	*P*	Q		Sample 1	Sample 3	log2 fold change	*P*	Q
Abl1	Aged	Young	0.880356	0.0001	0.0141471		Aged	Aged_CASIN	0.969329	0.00005	0.00838953
Ada	Aged	Young	-1.06182	0.0001	0.0141471		Aged	Aged_CASIN	-0.986074	0.00035	0.0319652
Aicda	Aged	Young	-2.0379	0.00005	0.00838953		Aged	Aged_CASIN	-1.47907	0.00005	0.00838953
Alyref	Aged	Young	-1.04245	0.00005	0.00838953		Aged	Aged_CASIN	-0.645571	0.0001	0.0141471
Basp1	Aged	Young	-1.89218	0.00005	0.00838953		Aged	Aged_CASIN	-1.39058	0.00005	0.00838953
Ccl5	Aged	Young	-1.5029	0.0007	0.0477965		Aged	Aged_CASIN	-1.90476	0.00005	0.00838953
Ccna2	Aged	Young	-1.33337	0.00005	0.00838953		Aged	Aged_CASIN	-1.02471	0.00015	0.0187673
Ccr7	Aged	Young	0.795555	0.00005	0.00838953		Aged	Aged_CASIN	0.657927	0.0004	0.0343571
Cenpe	Aged	Young	-1.47534	0.00005	0.00838953		Aged	Aged_CASIN	-1.25628	0.00005	0.00838953
Cenpv	Aged	Young	-1.70656	0.00005	0.00838953		Aged	Aged_CASIN	-1.56451	0.00005	0.00838953
Cmtm6	Aged	Young	0.785986	0.0005	0.039212		Aged	Aged_CASIN	0.799921	0.0003	0.0289888
Cnr2	Aged	Young	0.828734	0.00005	0.00838953		Aged	Aged_CASIN	0.722125	0.0001	0.0141471
E2f8	Aged	Young	-1.31291	0.00005	0.00838953		Aged	Aged_CASIN	-1.12067	0.0005	0.039212
Fam43a	Aged	Young	0.938744	0.00005	0.00838953		Aged	Aged_CASIN	0.866558	0.00005	0.00838953
Fosb	Aged	Young	0.778872	0.00005	0.00838953		Aged	Aged_CASIN	0.780242	0.00005	0.00838953
Gstt1	Aged	Young	-2.61658	0.0005	0.039212		Aged	Aged_CASIN	-1.8917	0.00025	0.0263963
H1fx	Aged	Young	-2.14388	0.00005	0.00838953		Aged	Aged_CASIN	-1.3541	0.00005	0.00838953
H2afx	Aged	Young	-1.48817	0.00005	0.00838953		Aged	Aged_CASIN	-1.09089	0.00005	0.00838953
Irf9	Aged	Young	0.888437	0.00005	0.00838953		Aged	Aged_CASIN	0.899991	0.00005	0.00838953
Irs2	Aged	Young	0.738187	0.00015	0.0187673		Aged	Aged_CASIN	0.831347	0.00005	0.00838953
Kif11	Aged	Young	-1.51087	0.00005	0.00838953		Aged	Aged_CASIN	-1.03966	0.00005	0.00838953
Klf4	Aged	Young	0.931962	0.00005	0.00838953		Aged	Aged_CASIN	0.969215	0.00005	0.00838953
Lmnb1	Aged	Young	-1.11865	0.00005	0.00838953		Aged	Aged_CASIN	-0.779146	0.00005	0.00838953
Lmo7	Aged	Young	-1.29653	0.00005	0.00838953		Aged	Aged_CASIN	-1.17151	0.0002	0.0231497
Mef2b	Aged	Young	-1.44234	0.00005	0.00838953		Aged	Aged_CASIN	-0.91891	0.0003	0.0289888
Mki67	Aged	Young	-1.43013	0.00005	0.00838953		Aged	Aged_CASIN	-1.00217	0.00005	0.00838953
Mybl1	Aged	Young	-1.88036	0.00005	0.00838953		Aged	Aged_CASIN	-1.19673	0.00005	0.00838953
Nr4a1	Aged	Young	0.891675	0.00005	0.00838953		Aged	Aged_CASIN	0.795549	0.00005	0.00838953
Pgp	Aged	Young	-0.851248	0.0002	0.0231497		Aged	Aged_CASIN	-0.658618	0.00015	0.0187673
Psat1	Aged	Young	-1.12094	0.00005	0.00838953		Aged	Aged_CASIN	-0.80648	0.00035	0.0319652
Psip1	Aged	Young	-0.828715	0.00005	0.00838953		Aged	Aged_CASIN	-0.718468	0.0004	0.0343571
Racgap1	Aged	Young	-1.42393	0.00005	0.00838953		Aged	Aged_CASIN	-1.07095	0.00045	0.0374625
Rasgef1b	Aged	Young	0.832481	0.0001	0.0141471		Aged	Aged_CASIN	0.671335	0.0004	0.0343571
Rassf6	Aged	Young	-1.52456	0.00025	0.0263963		Aged	Aged_CASIN	-1.98709	0.00015	0.0187673
Rgs13	Aged	Young	-2.53019	0.00055	0.0413359		Aged	Aged_CASIN	-1.63399	0.00045	0.0374625
Rplp2-ps1	Aged	Young	0.750418	0.00005	0.00838953		Aged	Aged_CASIN	0.786135	0.00005	0.00838953
Rrm2	Aged	Young	-1.4238	0.00005	0.00838953		Aged	Aged_CASIN	-1.03132	0.0001	0.0141471
S100a8	Aged	Young	-4.73387	0.00005	0.00838953		Aged	Aged_CASIN	-2.07499	0.00005	0.00838953

Scimp	Aged	Young	-2.41945	0.00005	0.00838953		Aged	Aged_CASIN	-1.38356	0.00005	0.00838953
Sh2b2	Aged	Young	-1.34978	0.00005	0.00838953		Aged	Aged_CASIN	-0.787783	0.0005	0.039212
Sik1	Aged	Young	0.84603	0.00005	0.00838953		Aged	Aged_CASIN	0.729784	0.00015	0.0187673
Ssbp3	Aged	Young	-1.31069	0.00005	0.00838953		Aged	Aged_CASIN	-0.690089	0.0005	0.039212
Tbx21	Aged	Young	-1.61984	0.00005	0.00838953		Aged	Aged_CASIN	-0.71298	0.00065	0.0459779
Top2a	Aged	Young	-0.965513	0.00005	0.00838953		Aged	Aged_CASIN	-0.697743	0.0006	0.043875
Zbtb10	Aged	Young	0.679227	0.0007	0.0477965		Aged	Aged_CASIN	0.700107	0.0004	0.0343571
Zbtb38	Aged	Young	-0.92191	0.00005	0.00838953		Aged	Aged_CASIN	-0.878069	0.00055	0.0413359

Supplemental Figures

Figure S1

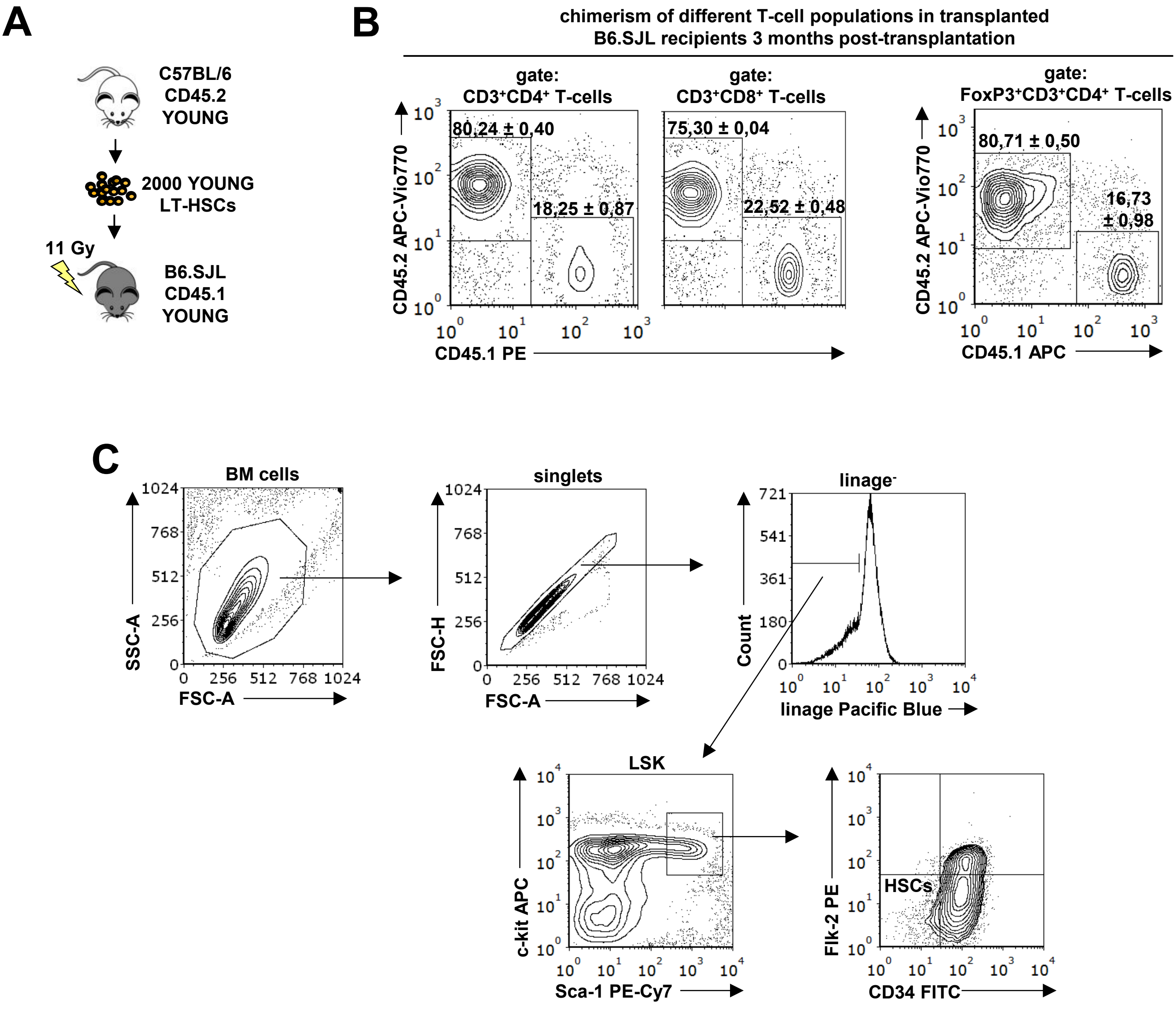

Figure S1. Limitations of previous transplantation schemes in immune competent recipients. (A) HSC transplantation in lethally irradiated B6.SJL mice. Schematic representation of the experimental setup: 2000 HSCs were isolated from young C57BL/6 (CD45.2) mice and were subsequently transplanted into lethally irradiated young B6.SJL (CD45.1) recipients. (B) 3 months after transplantation, chimerism of different splenic T-cell populations was determined in recipient mice (*n* = *2*). Representative graphs of individual stains and quantification (± SD) of donor- and recipient-derived cells are shown. (C) Representative flow cytometry gating to isolate HSCs. Panels were gated as shown after exclusion of dead cells and doublets. LT-HSCs were sorted out as lin$^-$ c-kit$^+$Sca-1$^+$ Flk-2$^-$CD34$^-$ cells using a BD FACS Aria II or a BD FACS Aria III (BD Bioscience). (B) mean ±SD

Figure S2

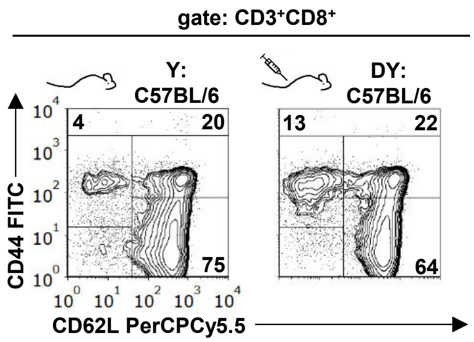

Figure S2. Generation of a youthful naïve CD8$^+$ T-cell pattern is independent of the congenic CD45.1 interval in CD45.1$^+$ B6.SJL animals. Representative flow cytometry plots from splenic CD3$^+$CD8$^+$ T-cells isolated from non-transplanted young C57BL/6 mice and RAG1$^{-/-}$ recipients transplanted with young HSCs isolated from C57BL/6 donors. Depicted are surface expression profiles of CD44 and CD62L within the CD3$^+$CD8$^+$ T-cell population. Data is representative of two experiments with *n = 3* animals each.

Figure S3

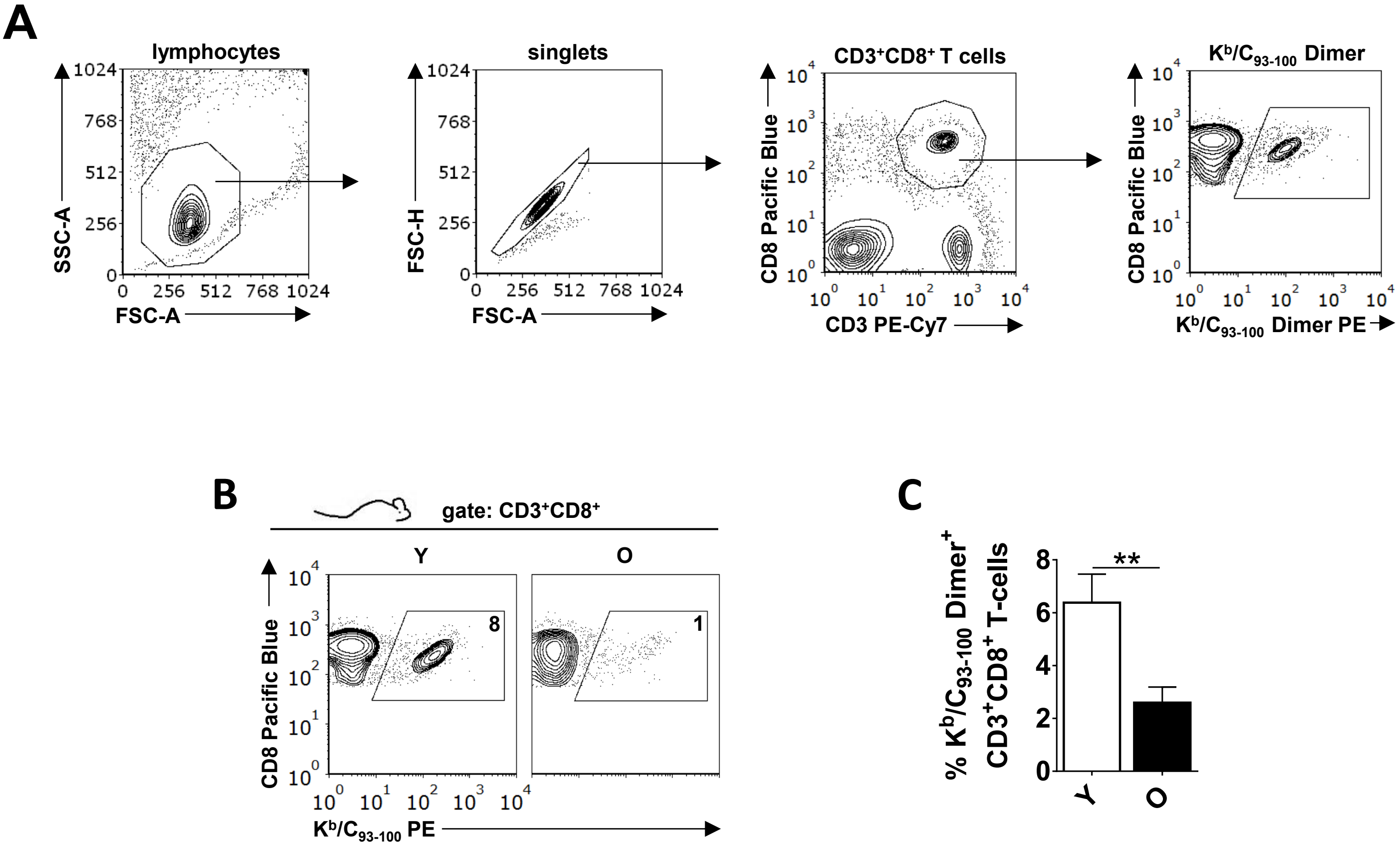

Figure S3. Functional changes in CD8$^+$ T-cells with age. Young and aged mice were immunized with 100 µg pCI/C i.m. 13 days postvaccination, antigen specific CD8$^+$ T-cell frequencies were determined flow cytometrically in the spleen by *ex vivo* staining of CD8$^+$ T-cells with K^b/C$_{93-100}$-specific dimers. (A) General gating strategy to identify for antigen-specific K^b/C$_{93-100}$$^+$ CD3$^+$CD8$^+$ T-cells: CD3$^+$CD8$^+$ T-cells were gated as mononucleated cells in the lymphocyte population. K^b/C$_{93-100}$$^+$ cells were quantitatively determined within the CD3$^+$CD8$^+$ T-cell population. Representative graphs of individual stains and quantification of antigen-specific CD3$^+$CD8$^+$ T-cells are shown in (B) and (C), respectively (Y *n = 7*, O *n = 7*). ** $p < 0.01$, two-tailed unpaired student's t-test, mean + SEM.

Figure S4

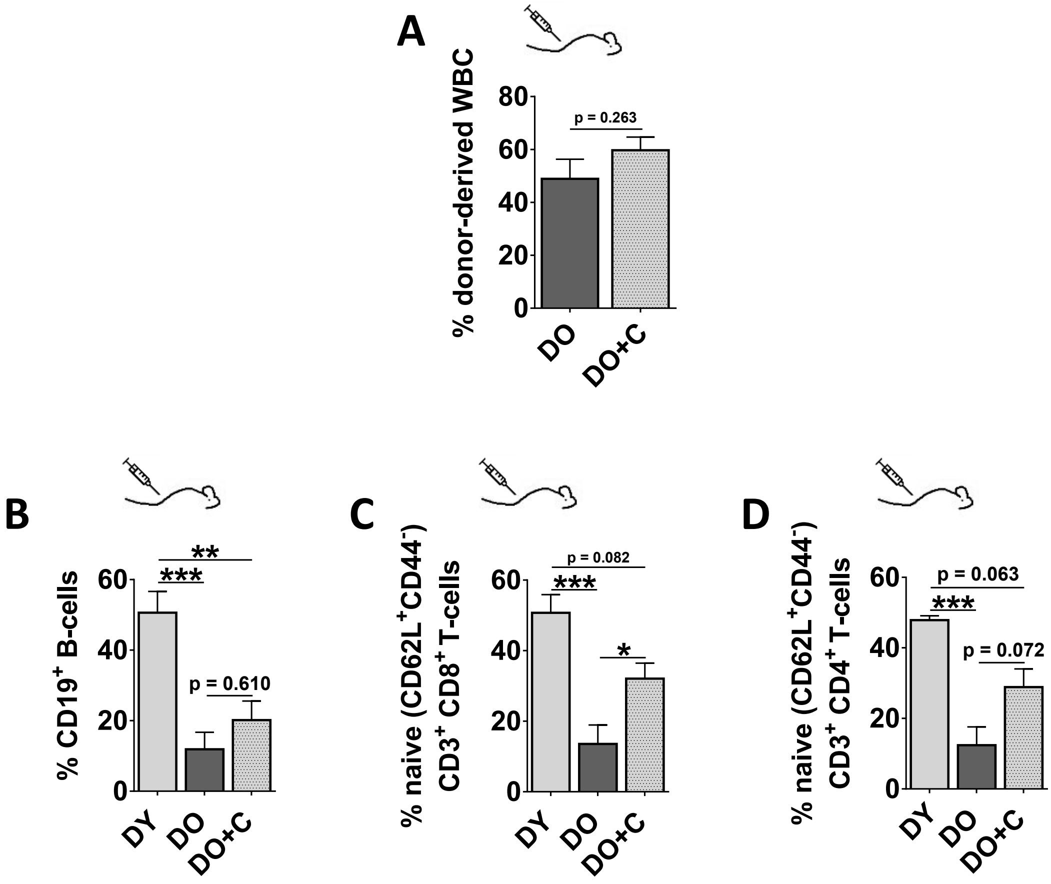

Figure S4 WBC chimerism and frequency of naïve T- and B-cells in HSC-transplanted RAG1$^{-/-}$ recipients. (A) Contribution of DO and DO+C HSCs to WBC was analyzed in spleen 12 weeks post-transplantation (DO *n = 11*, DO+C *n = 9*). To identify for B-cells as well as naïve CD4$^+$ and CD8$^+$ T-cellls, splenocytes of recipients transplanted with DY, DO and DO+C HSCs were stained for CD3, CD4, CD8, CD62L, CD44 and CD19 expression. (B) B-cells were gated as CD19$^+$ mononucelated cells within the lymphocyte population. The proportion of CD19$^+$ B-cells is depicted. (C) Quantification of naïve (CD44$^-$CD62L$^+$) CD8$^+$ T-cells within the total CD3$^+$CD8$^+$ T-cell population. (C) Frequency of naïve (CD44$^-$CD62L$^+$) CD4$^+$ T-cells within the CD3$^+$CD4$^+$ T-cell population. (DY *n = 4*, DO *n = 6* and DO+C *n = 6*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ordinary one-way ANOVA, mean + SEM.

Figure S5

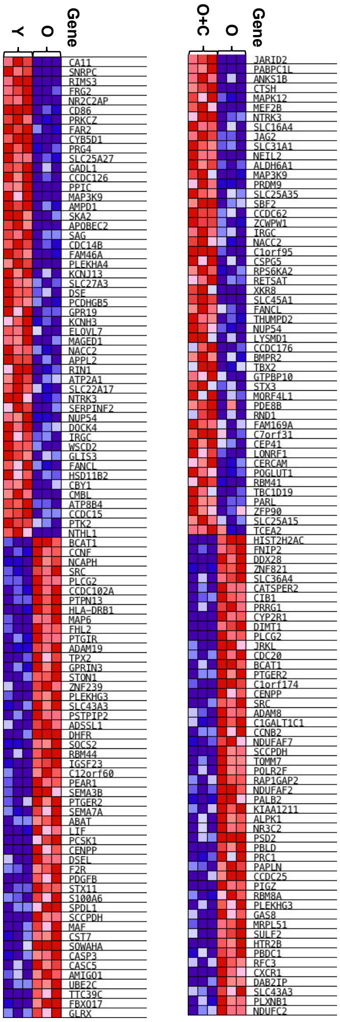

Figure S5 GSEA on genes previously identified by Mirza *et al.* (2011). Heat map of the GSEA depicting significantly enriched gene sets previously identified by Mirza et al. (2011) to be differentially expressed in naïve CD4^{+} T-cells from non-transplanted young and aged mice compared to differentially expressed genes between DY vs. DO as well as DO+C vs. DO HSC recipients. DY, DO and DO+C *n = 3*.