Memory CD8+ T cells support the maintenance of hematopoietic stem cells in the bone marrow

Sulima Geerman,[1,2] Giso Brasser,[1,2] Sudeep Bhushal,[1,2] Fiamma Salerno,[1,2] Natasja A. Kragten,[1,2] Mark Hoogenboezem,[2,3] Gerald de Haan,[4] Monika C. Wolkers,[1,2] María Fernanda Pascutti[1,2] and Martijn A. Nolte[1,2,3*]*

[1]Department of Hematopoiesis, Sanquin Research, Amsterdam; [2]Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam; [3]Department of Research Facilities, Sanquin Research, Amsterdam and [4]European Research Institute for the Biology of Ageing, University Medical Center Groningen, University of Groningen, the Netherlands

**Currently working at: Model Systems for Infection and Immunity, Helmholtz Centre for Infection Research, Braunschweig, Germany*

Correspondence: m.nolte@sanquin.nl
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Content:

METHODS

Mice

Wild-type (WT) C57BL/6J, TCRα$^{-/-}$, OT-I and Rag2$^{-/-}$-OT-I mice were kept under specific pathogen-free conditions in the animal facility of the Academic Medical Center (Amsterdam, The Netherlands) or Netherlands Cancer Institute (Amsterdam, The Netherlands). WT mice were bred in house or bought from Charles River Laboratories or Janvier Labs. TCRα$^{-/-}$ mice were bought from The Jackson Laboratories. Both female and male mice were used, ranging in age between 8-24 weeks, unless indicated otherwise. For LCMV experiments, mice were injected intraperitoneally with 200 µl PBS containing 2.0 × 10^5 PFU of the Armstrong or Clone13 strain, kindly provided by Ramon Arens (LUMC, Leiden, The Netherlands). Mice were sacrificed during memory phase (>27 days post injection). Mice received chow and acidified drinking water *ad libitum*. For transplantation experiments mice were lethally irradiated twice with 5.0 Gray (4 hours interval) and received soft food and sterile water supplemented with antibiotic the day before and 2 weeks after transplantation. Animal experiments were performed in accordance with the institutional and national guidelines and approved by the Experimental Animal Committees of both animal facilities.

Sample Collection and Preparation

Mice were euthanized using CO_2. Bones were harvested, cleaned and crushed in MACS buffer (PBS + 1% FCS + 2 mM EDTA) using a mortar and pestle. BM cell suspensions were filtered through a 70 µm cell strainer (BD) to remove bone debris. Single splenocyte suspensions were prepared by crushing the spleen through a 70 µm cell strainer with the plunger of a syringe. Erythrocytes were lysed with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 127 mM EDTA). Whole BM and spleen cells were enriched for $CD8^+$ T cells with CD8α microbeads (Miltenyi Biotec) and MACS LS columns (Miltenyi Biotec). To enrich for HSCs, cells were stained with biotin-conjugated antibodies directed against lineage (CD4, CD8, B220, Gr-1, CD11b and Ter119) and positively selected with streptavidin microbeads (Miltenyi Biotec) and MACS LS columns (Miltenyi Biotec).

Flow cytometry and cell sorting

The following antibodies were used for T cell stainings; TCRβ-APC (H57-597, eBioscience), CD4-Qdot 605 (RM4-5, Life Technologies), CD4-eFluor 450 (GK1.5, eBioscience), CD4-FITC (GK1.5, eBioscience), CD8α-FITC (53-6.7, eBioscience), CD8α-BUV395 (53-6.7, BD Biosciences), CD8α-eFluor 450 (53-6.7, eBioscience) CD8β-PE-Cy7 (53-6.7, eBioscience) CD44-BV785 (IM7, BioLegend), CD44-PE (IM7, eBioscience), CD44-PE-Cy7 (IM7, BioLegend), CD62L-BV510 (MEL-14, BioLegend), CD62L-APC (MEL-14, eBioscience), CD69-FITC (H1.2F3, BD Biosciences). The MHC class I tetramers H2-D^b $GP_{33–41}$ PE or APC and H2-D^b $NP_{396–404}$ PE or APC were kind gifts from Ramon Arens (LUMC, Leiden, The Netherlands). H2-D^b $GP_{276–286}$ BV421 was kindly provided by the NIH Tetramer Core Facility, (Emory University, USA).

The following antibodies were used for mature blood cells and HSC stainings; CD3ε-APC (145-2C11, eBioscience) CD3ε-eFluor 450 (145-2C11, eBioscience) CD4-biotin (GK1.5, eBioscience), CD8α-biotin (53-6.7, eBioscience), CD19-PE-Cy7 (1D3, eBioscience), B220-biotin (RA3-6B2, eBioscience), B220-PE-Cy7 (RA3-6B2, eBioscience), CD11b-biotin (M1/70, eBioscience), CD11b-eFluor 450 (M1/70, eBioscience), CD11b-APC (M1/70, eBioscience), CD11b APC-Cy7 (M1/70, eBioscience), Gr-1-biotin (RB6-8C5, eBioscience), Gr-1-FITC (RB6-8C5, eBioscience), Gr-1-eFluor 450 (RB6-8C5, eBioscience), Ter119-biotin (Ly76, eBioscience), streptavidin-PerCpCy5.5 (BD Pharmingen), streptavidin-eFluor 450 (eBioscience), CD48-APC (HM-48-1, eBioscience), sca-1-PE (D7, eBioscience), sca-1-PerCPCy5.5 (D7, eBioscience) c-kit-Alexa Fluor 780 (2B8, eBioscience), CD150-PE-Cy7 (TCF15-12F12.2, BioLegend). CD45.1-FITC (A20, eBioscience), CD45.1-PE (A20, eBioscience), CD45.2-PerCpCy5.5 (104, eBioscience), CD45.1-eFlour 450 (104, eBioscience), CellTrace Violet (Thermo Fisher Scientific). Cells were fixed with Foxp3/Transcription Factor Staining buffer set (eBioscience) and stained with Ki-67 PE (B56, BD Biosciences). Dead cells were excluded with propidium iodide (Sigma-Aldrich) or LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific). Samples were acquired with an LSRFortessa Analyzer (BD) or CANTO II (BD) and sorted on FACSAria II (BD) or FACSAria III (BD) and analyzed with FlowJo software (Tree Star).

Liquid co-culture HSC assay

SLAM HSCs ($Lin^{-/lo}Sca\text{-}1^{+}cKit^{+}CD150^{+}CD48^{-}$)[22] were sorted (100 cells) and cultured in 100 µl X-VIVO 15 medium (Lonza) for 7 days in U-bottom 96 wells plate (Corning Incorporated) at 37° C (5% CO_2) in the presence of Gentamycin (10 µg/ml, Gibco), β-mercaptoethanol (50 µM, Sigma-Aldrich), SCF (10 ng/ml, PeproTech), TPO (2 ng/ml, PeproTech) and Flt3L (5 ng/ml, PeproTech). For co-culture experiments, 100 HSCs were cultured with 1000 T cells including T cell survival factors *i.e.* IL-7 (5 ng/ml, PeproTech) and IL-15 (10 ng/ml, PeproTech) or control medium, which lacked T cells but included T cell survival factors. Experiments with supernatants were performed by supplementing the HSC culture medium with 25% control medium or medium harvested from T cells after a culture period *(see Production of supernatant)*. For all experiments, 7 days after the start of the co-culture assay, cells were harvested and stained with antibodies against myeloid lineage cells (Gr-1 and Mac-1) and HSCs. To assess cell proliferation, 1×10^6 - 1×10^7 BM cells were labeled with Celltrace Violet (1.25 nM) according to manufacturer instructions (Thermo Fisher Scientific) followed by staining with antibodies against myeloid lineage cells and HSCs. Subsequently, CTV labeled HSC were sorted and cultured (3000 cells per well) with 25% control medium or T cell derived supernatant.

Production of supernatant

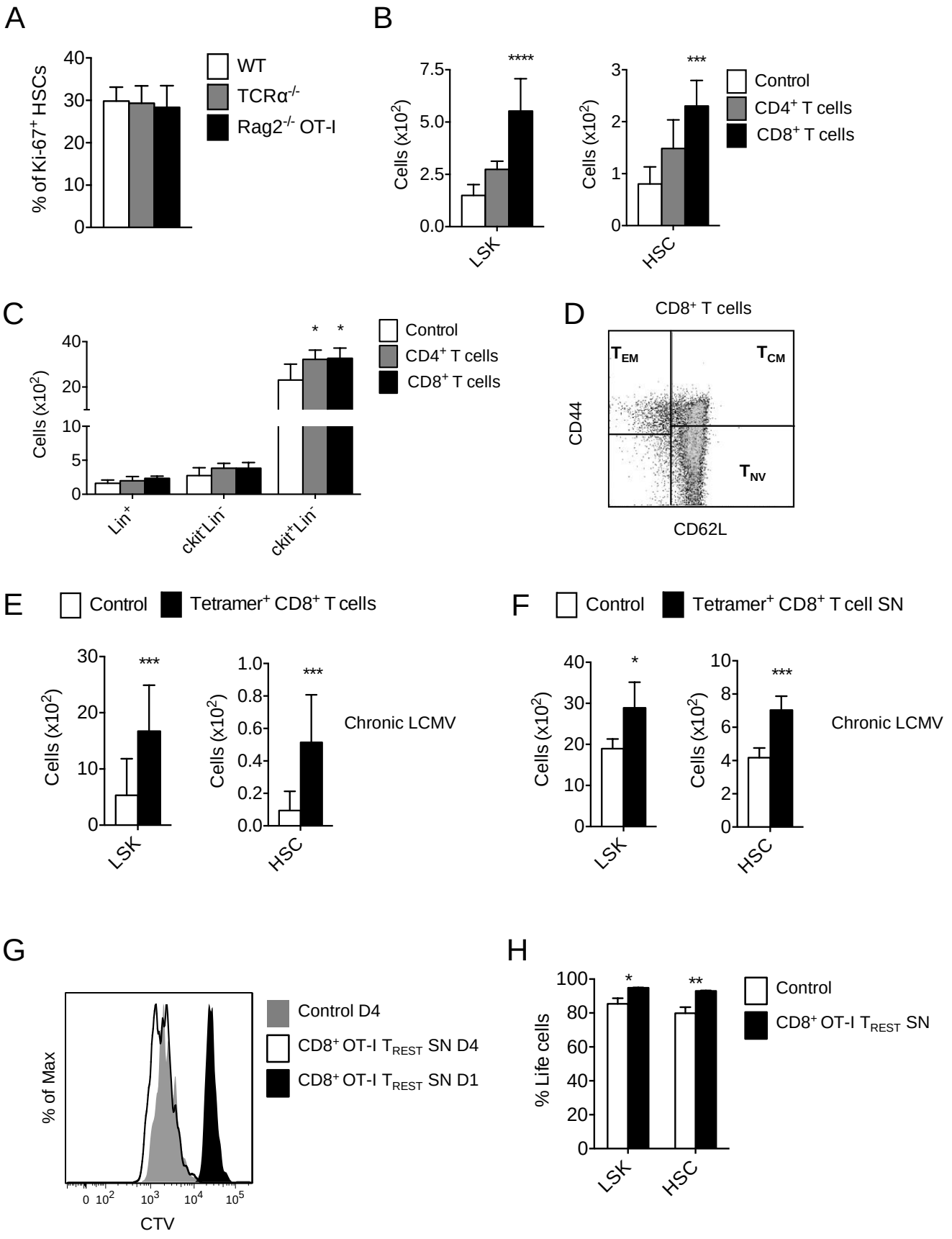
Supernatant was produced by two different approaches. 1) $CD8^{+}$ T cells were sorted and cultured in U-bottom 96 wells plate (Corning Incorporated) at 37° C (5% CO_2) in the presence of β-mercaptoethanol (50 µM, Sigma-Aldrich), IL-7 (5 ng/ml, PeproTech) and IL-15 (10 ng/ml, PeproTech). After 4 days, the medium was harvested and spun-down to remove any cellular debris cells. 2) A 24 wells plate (Falcon) was pre-seeded with 1.0×10^5 MEC.B7.SigOVA (SAMBOK) cells and left to adhere for a minimum of 6 hours. After removal of unattached cells, 3×10^6 total OT-I splenocytes were added[23]. T cells were activated overnight, and subsequently removed and left to rest in X-vivo 15 (Lonza) or serum-free IMDM without phenol red (Gibco) supplemented with β-mercaptoethanol (50 µM, Sigma-Aldrich), IL-7 (5 ng/ml, PeproTech) and IL-15 (10 ng/ml, PeproTech) for 2 days. The medium was then refreshed with cytokines and the T cells continued to rest for another 4 days. Medium from the second resting period was used as supernatant in our experiments.

Transplantation and transfer experiments

SLAM HSCs ($Lin^{-/lo}Sca\text{-}1^{+}cKit^{+}CD150^{+}CD48^{-}$) were sorted and cultured for 3 or 7 days with control medium or T cell derived supernatant. Cultured HSCs were co-injected with 2×10^5 whole BM cells in 200 µl PBS. Mice were bled (vena saphena) every 4-5 weeks and the contribution to hematopoiesis was measured by examining the CD45 variant of the mature blood cells. Secondary transplantations were performed by injecting irradiated recipients with 200 µl PBS containing 10×10^6 whole BM cells harvested from the femur of primary recipients.

Statistical Analysis

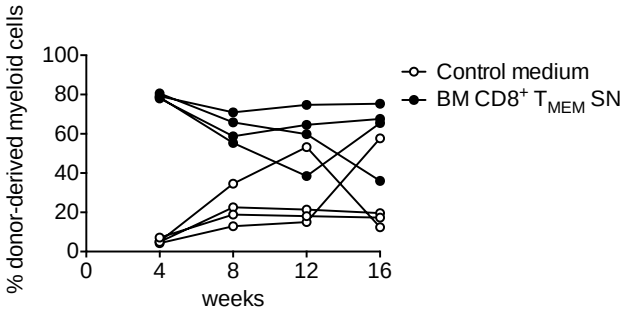
Statistical analyses were performed with Prism (GraphPad Software, Inc.) using unpaired *t* test followed by Welch's correction or one-way ANOVA followed by Tukey's correction. Significance is indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $**** p < 0.0001$.


A
% of Ki-67+ HSCs
WT
TCRα-/-
Rag2-/- OT-I
B
Cells (x10^2)
LSK
HSC
Control
CD4+ T cells
CD8+ T cells
C
Cells (x10^2)
Lin+
ckit+Lin-
ckit+Lin-
D
CD8+ T cells
TEM
TCM
TNV
CD44
CD62L
E
Control
Tetramer+ CD8+ T cells
Chronic LCMV
F
Control
Tetramer+ CD8+ T cell SN
Chronic LCMV
G
% of Max
CTV
Control D4
CD8+ OT-I TREST SN D4
CD8+ OT-I TREST SN D1
H
% Life cells
Control
CD8+ OT-I TREST SN

Supplemental Figure 1| Impact of memory CD8 T cells on HSC quiescence, maintenance and survival.

A) Frequency of Ki-67^{+} cells within the HSC population in WT, TCRα$^{-/-}$ and Rag2$^{-/-}$-OT-I mice (*n* = 6 mice). **B,C)** Absolute cell numbers of different populations generated in the assay when HSCs are co-cultured with control medium (lacking T cells but including cytokines), BM CD4^{+} or CD8^{+} T cells. **D)** Representative FACS plot showing the gating strategy and the subsets of BM CD8^{+} T cells. **E)** Absolute cell numbers of LSKs and HSCs generated in the assay when HSCs are co-cultured with chronic BM LCMV-specific CD8^{+} T cells isolated based on recognition of $GP_{33–41}$, $GP_{276–286}$ and $NP_{396–404}$ epitopes on day 27 post infection. **F)** Absolute cell numbers of different populations generated in the assay when HSCs are cultured with supernatant (SN) derived from chronic BM LCMV-specific CD8^{+} T cells isolated based on recognition of $GP_{33–41}$, $GP_{276–286}$ and $NP_{396–404}$ epitopes on day 27 post infection. **G)** Flow cytometry analysis of the dilution of the dye Cell Trace Violet (CTV) by HSCs after 4 days of culture with control medium or *in vitro* generated resting antigen-experienced CD8^{+} OT-I T cells. Initial intensity is provided (D1= day 1). **H)** Frequency of PI^{-} LSK and HSC after 3 days of culture with control medium or CD8^{+} OT-I T_{REST} SN. Graphs show Mean ± SD of each tested condition (*n* = 5-14 wells), representative of 2-3 independent experiments.

A



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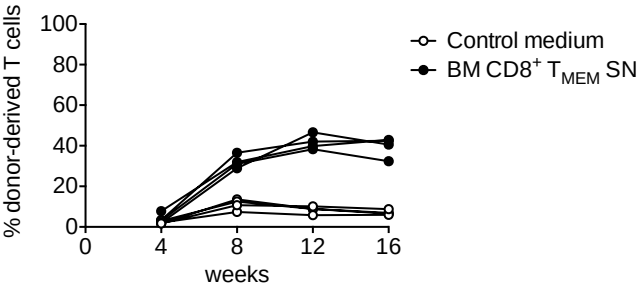

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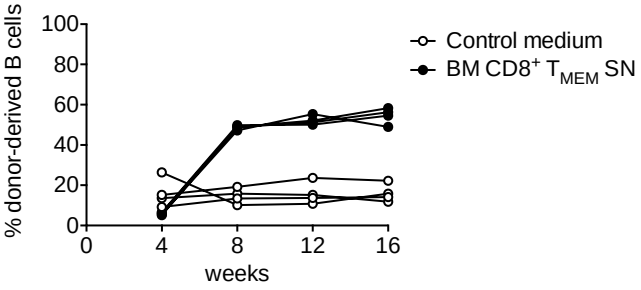

Supplemental Figure 2| HSCs cultured with BM memory CD8⁺ T cell supernatant have long-term, multi-lineage repopulation capacity.

A-C) Relative contribution of HSCs primed with control or BM CD8⁺ T_{MEM} SN to myeloid, T cell and B cell output (*n* = 4 mice).