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2 **Supplemental Figure 1. Successful generation of double-negative (DN) engineered**

3 **lymphoid progenitors in vitro.** (A) Scheme of the mouse CD19 CAR construct m1928z1 and

4 the configuration of signaling moieties. (B) Time line for in vitro differentiation and culture of

5 engineered lymphoid progenitors. Lineage⁻c-kit⁺ and Sca-1⁺ hematopoietic stem and progenitor

6 cells (LSK) were sorted from bone marrow and transduced with lentiviral supernatant on day -

7 3. OP9-DL1 and LSK co-culture in the presence of IL-7, FLT3-L and Doxycycline started on

8 day 0 and lymphoid progenitors were transferred to new OP9-DL1 cells every 3-4 days. After

9 a sorting step on day 10, Tom⁺ cells were frozen on day 13. For adoptive transfer experiments,

10 engineered lymphoid progenitors were thawed and re-cultured on OP9-DL1 with cytokines for

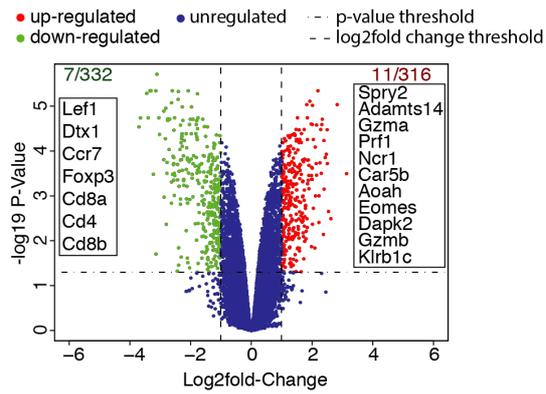
11 another 7 days. (C) Representative FACS plots of dTomato expression (left), CD4 and CD8

12 expression (right) on im1928z1 CAR-engineered lymphoid progenitors on day 20 of in vitro

13 culture. (D) Gating strategy for FACS analysis of transgene-positive CAR lymphoid

14 progenitors and their progenies.

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17 **Supplemental Figure 2. CARiK cells derived from im1928z1-expressing lymphoid**

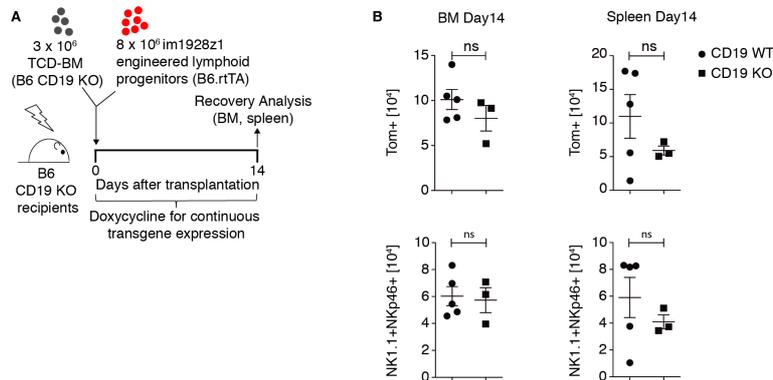
18 **progenitors exhibited a NK cell-like transcriptional program.** Volcano plot for comparison

19 of differently regulated transcripts in either im1928z1 or iTom lymphoid progenitor-derived

20 progeny (harvested from spleens 28 days after transplantation). Gene symbols in the boxes

21 indicate selected transcripts found to be significantly down-regulated (green) or up-regulated

22 (red) by at least twofold in im1928z1 progenies when compared to iTom progenies.



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24 **Supplemental Figure 3. im1928z1-lymphoid progenitors demonstrated similar**

25 **development in wildtype and *Cd19* KO mice. (A)** Irradiated B6 CD19 KO recipients received

26 3 x 10⁶ B6 *Cd19* knockout TCD-BM and 8 x 10⁶ im1928z1-lymphoid progenitors. **(B)** Single

27 cell suspensions were generated from harvested BM and spleens 14 days after AT. Numbers of

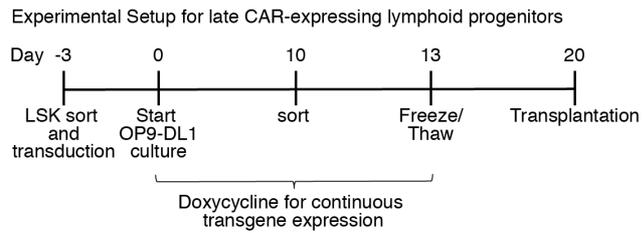
28 Tom⁺ and NK1.1⁺NKp46⁺ progenies in BM and spleens in either B6 CD19 KO (n= 3) or B6

29 CD19 WT recipients (n= 5) mice are depicted. Gating was done on the Tom⁺ population.

30 Statistics were performed by using the two-tailed Students t-test. Data represent means ± s.e.m.

31 No significant differences were detected. ns, not significant.

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34 **Supplemental Figure 4. Experimental setup for the generation of lymphoid progenitors**

35 **with delayed im1928z1 expression.** Time line for in vitro generation of engineered lymphoid

36 progenitors for late CAR expression experiments. LSK cells were sorted from bone marrow

37 and consecutively transduced with lentiviral supernatant on day -3. OP9-DL1/LSK co-culture

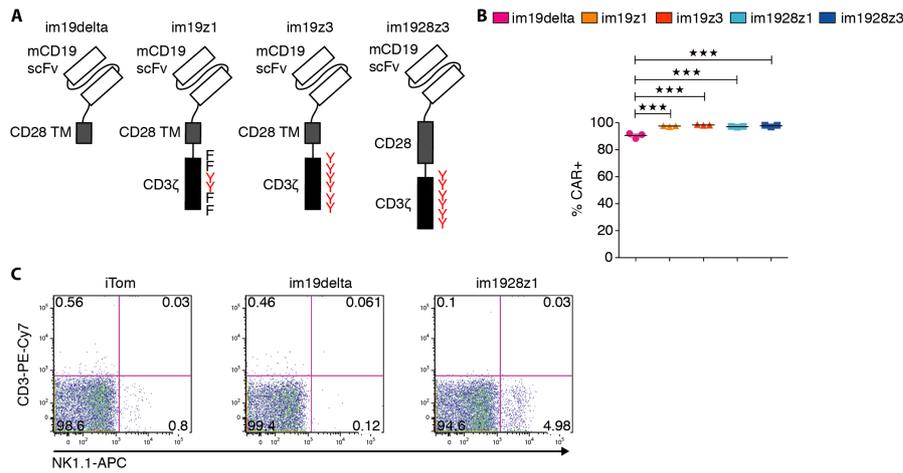
38 in the presence of IL-7 and FLT3-L and Doxycycline started on day 0 in order to allow a sorting

39 step on day 10. Tom⁺ cells were frozen on day 13. For AT experiments, engineered lymphoid

40 progenitors were thawed and re-cultured on OP9-DL1 at this point omitting Doxycycline before

41 co-transplantation.

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44 **Supplemental Figure 5. CAR design impacts lymphoid progenitor development. (A)**

45 Scheme of the mouse CD19 CAR constructs im19delta, im19z1, im19z3, and im1928z3 and

46 their configuration in respect of signaling moieties. (B) Frequencies of CAR⁺ lymphoid

47 progenitors within the Tom⁺ gate with indicated CAR constructs on day 20 of in vitro culture.

48 Comparisons were performed by using one-way ANOVA analysis with Tukey's post-test. Data

49 from a representative experiment measured in triplicates are shown and represent means ±

50 s.e.m; ***P < 0.001. (C) Representative FACS plots of NK1.1 and CD3 expression on iTom,

51 im19delta-, and im1928z1-engineered lymphoid progenitors.